Factors affecting the integrity of high density lipoproteins in the ultracentrifuge

Steven T. Kunitake and John P. Kane

Cardiovascular Research Institute and the Department of Medicine, University of California, San Francisco, CA 94143

Abstract Because of reported losses of apolipoproteins from high density lipoproteins during ultracentrifugation, we studied several factors that could affect the integrity of these lipoprotein complexes. Alteration of temperature, rotor configuration, and composition of the tubes had little effect on loss of apolipoprotein A-I. Interestingly, the high ionic strengths commonly used in ultracentrifugal isolation of these lipoproteins were associated with the smallest loss of apolipoprotein A-I. Losses increased substantially as the ionic strength of the medium was decreased. After repeated ultracentrifugation, apolipoprotein A-I content of high density lipoproteins approached a limiting value of approximately 65% of the original serum value, but no apolipoprotein A-II was lost. Our results imply that the binding environments of these two apolipoproteins in high density lipoproteins differ. Further, they imply that apolipoprotein A-I may exist in more than one type of environment or in more than one form in high density lipoproteins.—**Kunitake, S. T., and J. P. Kane.** Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. *J. Lipid Res.* 1982. **23:** 936–940.

Supplementary key words high density lipoproteins • apoA-I dissociation • apoA-II dissociation • ionic strength

Preparative ultracentrifugational flotation (1, 2) has long been the standard method for isolating lipoproteins. Compositional analyses of the lipoprotein classes are predicated upon this form of separation, and most current metabolic and structural studies employ lipoproteins isolated by ultracentrifugation.

Ultracentrifugation, however, has been reported to cause structural changes in lipoproteins, specifically, the loss of apolipoproteins. In studies of both rat and human lipoproteins, a portion of the serum content of apolipoproteins A-I and E (apoA-I and apoE) were found at density greater than 1.21 g/ml after ultracentrifugation (3–6). Furthermore, the loss of apoA-I from high density lipoproteins (HDL) during ultracentrifugal isolation is greater than the loss with other methods (7, 8), reaching as much as 50% (3).

This study was undertaken to identify the ultracentrifugational factors that are responsible for the loss of apolipoprotein from human HDL. The effects of rotor configuration, centrifuge tube type, ionic strength, and temperature were studied. We found that none of these factors influence the loss of apoA-I from HDL during ultracentrifugational isolation. Our observations further suggest that the interaction of apoA-I with the HDL complex may depend strongly on hydrophobic interactions, and that there are at least two binding environments for apoA-I in HDL.

MATERIALS AND METHODS

Serum samples

Blood was obtained from normal fasting male donors and allowed to clot. The serum was recovered after centrifugation at 15×10^3 g_{av}-min. EDTA, sodium azide, and gentamycin were added to final concentrations of 0.001 M, 0.2 μg/ml, and 5 μg/ml, respectively. The serum was used in the isolation procedure immediately after recovery.

Variation of ionic strength of ultracentrifugation medium

Four different density media were used to adjust the serum samples for ultracentrifugation. Each medium had a different final ionic strength (μ). The four systems were: *1)* H_2O with KBr, μ at $1.21\rho = 2.5M$; *2)* D_2O with KBr, μ at $1.21\rho = 1.3M$; *3)* D_2O with CsCl, μ at $1.21\rho = 0.8M$; *4)* D_2O in 0.15M NaCl with metrizimide [2 - (3 acetamido - 5 - N - methylacetamido - 2,4,6 - tridobenzamido)-2-deoxy-D-glucose; Gallard-Schlesinger], μ at $1.21\rho = 0.15M$. All media were tested simultaneously.

Ultracentrifugation in the 40.3 rotor

Aliquots of serum (2.8 ml) were adjusted to a density of 1.063 g/ml by the addition of a stock solution. The

Abbreviations: HDL, high density lipoproteins; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; EDTA, ethylene diamine tetraacetic acid.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

serum was then ultracentrifuged at 38 × 10^3 rpm for 24 hr in a Beckman model L3-50 ultracentrifuge. After ultracentrifugation, the top 3 ml (1.063T) were removed and 3 ml of a second adjusting solution was added to increase the density of the infranatant solution to 1.21 g/ml. Ultracentrifugation was resumed for 48 hr at 38 × 10^3 rpm. The HDL was removed in the top 2 ml, leaving the infranatant 4 ml (1.21-1-B). The HDL was mixed with 4 ml of a 1.21 g/ml density solution, recentrifuged for 48 hr, and again recovered in the top 2 ml (1.21 T), leaving 4 ml of infranatant solution (1.21-2-B) (**Fig. 1**).

Ultracentrifugation in the SW 50.1 rotor

To minimize contact of lipoprotein with tube walls, we studied recovery of apoA-I in the SW 50.1 swinging bucket rotor. The protocol for this ultracentrifugation paralleled that in the 40.3 rotor, except that total volumes were 5.5 ml in SW 50.1 tubes instead of 6.0 ml as they were in the 40.3 tubes.

Ultracentrifugation temperature

Ultracentrifugational isolations were performed in parallel at 5°C and 24°C in both rotor systems.

Ultracentrifugation using tubes composed of different material

To determine whether interaction with different tube materials affects the loss of apoA-I from HDL, tubes composed of cellulose nitrate and polyallomer were used. The centrifugation was performed in a 40.3 rotor as described above.

Ultracentrifugation of overlayered samples

In an attempt to shorten ultracentrifugation time, a technique of overlayering adjusted solutions with isopycnic buffers was used. A 3-ml aliquot of 1.065 g/ml adjusted serum was overlayered with 2.5 ml of 1.063 g/ml solution, and the ultracentrifugation time was shortened to 18 hr. After centrifugation, the top of 2.5 ml was removed, the infranatant solution was adjusted to 1.22 g/ml, and 1 ml of 1.21 g/ml solution was overlayered. After 24 hr of centrifugation, the HDL was recovered in the top 1 ml. All centrifugations were performed in an SW 50 rotor at 24°C, and speeds of 38 × 10^3 rpm or 50 × 10^3 rpm were used. These experiments were done at all four of the ionic strengths above.

Repeated ultracentrifugation of HDL

Serum (2.8 ml) was adjusted to a density of 1.063 g/ml by the addition of 3.2 ml of stock KBr solution. The mixture was ultracentrifuged at 38 × 10^3 rpm for 24 hr at 5°C in a Beckman 40.3 rotor. After ultracentrifugation, the top 3 ml was discarded and the bottom 3 ml

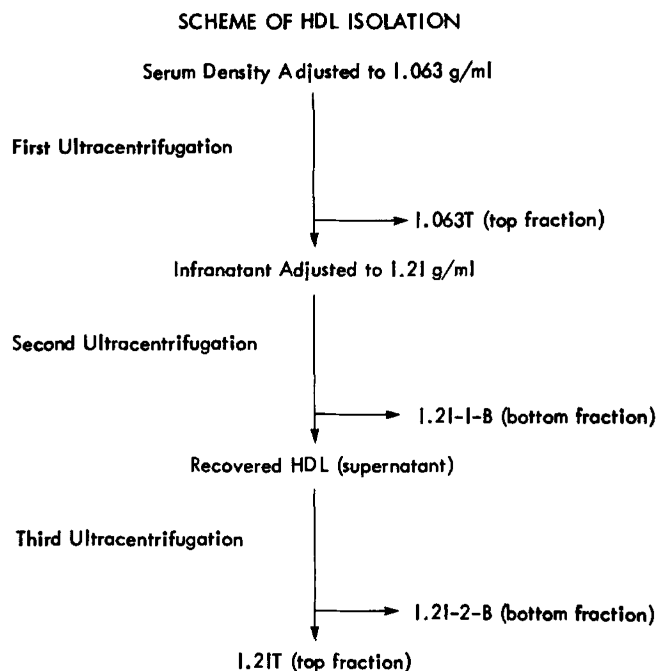

Fig. 1. The fractions obtained from the ultracentrifugation procedure are shown diagrammatically.

was adjusted to 1.21 g/ml by the addition of KBr solution. The ultracentrifugation was resumed for 48 hr. The top 2 ml (HDL fraction) was transferred to another centrifuge tube and 4 ml of 1.21 g/ml KBr solution was added. The HDL was recentrifuged for 48 hr. The last step was repeated three more times with fresh ultracentrifugal medium at a density of 1.21 g/ml.

Analysis of density fractions

The contents of apoprotein A-I and A-II in each fraction were determined by radioimmunoassay after delipidation with sodium decyl sulfate (9). Cholesterol content was measured by enzymatic fluorescence assay (1). The non-protein solvent densities of ultracentrifugal media were measured by pycnometry.

RESULTS

Effects of various factors of ultracentrifugation on the loss of apoA-I from HDL

In a typical distribution of apoA-I and cholesterol (**Table 1**) among the ultracentrifugal fractions, the bulk of the apoA-I was found in the 1.21 T. There was an insignificant amount of apoA-I in the 1.063 T. However, large amounts of apoA-I were found in the 1.21-1-B and 1.21-2-B fractions. The 1.21-1-B fraction contained a small but significant amount of cholesterol. We have not determined whether this sterol is bound to protein; it is predominantly unesterified, however. Insignificant

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

TABLE 1. Recovery of apoA-I and cholesterol in the ultracentrifuge fractions obtained from media of different ionic strengths[a]

Fraction	Recovery		
	H_2O-KBr	D_2O-KBr	D_2O-CsCl
		%	
1.063 T			
A-I[b]	0.4	0.4	0.4
Chol[c]			
1.21-1-B			
A-I	8.1	16.1	17.1
Chol	2.0	2.0	2.0
1.21-2-B			
A-I	6.9	7.2	13.2
Chol	0.2	0.5	0.5
1.21 T			
A-I	83.7	71.6	58.9
Chol	17.0	16.0	19.0

[a] Data are from one preparation but are typical recoveries of 20 other isolations.
[b] Percentage apoA-I based on serum value.
[c] Cholesterol recovery as % of total serum cholesterol.

amounts of cholesterol were found in the 1.21-2-B fraction. The total recoveries of apoA-I were between 100 and 90 percent of the serum value, indicating that apoA-I lost from 1.21 T was recovered in the 1.21-1-B and 1.21-2-B.

The densities of the 1.21 T fractions were measured directly to make sure the 1.21 T fractions had maintained a density of 1.21 g/ml throughout the various ultracentrifugations. The fractions measured fulfilled this requirement with two exceptions: *1*) During high speed isolation, 50×10^3 rpm in the SW 50.1, salt gradients form causing incomplete isolation of HDL. Thus, high speed should be avoided. *2*) Metrizimide, the non-ionic medium, forms steep density gradients, even at lower speeds of centrifugation. In order to obtain a density of 1.21 g/ml in the 1.21 T fraction, a solution of 1.50 g/ml density was used to isolate the HDL. (In this case, 54% of serum apoA-I was found in the 1.21 T.)

Increased ionic strength unexpectedly stabilized the HDL complex. When any rotor or temperature was used, the loss of apoA-I decreased with increasing ionic strength of the density medium (**Table 2**). Increased loss of apoA-I from the 1.21 T fraction with decreased ionic strength occurred concurrently with increased recovery of apoA-I in the 1.21-1-B and 1.21-2-B fractions (Table 1). The cholesterol content of the 1.21 T fractions remained similar, indicating apoA-I was lost from the rest of the HDL complex.

Neither change of temperature from 5°C to 24°C, nor change in rotor configuration from 40.3 to SW 50.1, had a significant impact on the loss of apoA-I from HDL

TABLE 2. Recovery of apoA-I in HDL prepared under different conditions of centrifugation and at different ionic strengths[a]

Rotor Configuration	Temperature	Recovery		
		H_2O-KBr	D_2O-KBr	D_2O-CsCl
	°C		%	
40.3	5	84.5 ± 3.8[b]	72.9 ± 6.8	57.8 ± 4.1
40.3	24	82.6 ± 5.8	76.5 ± 8.8	53.2 ± 2.7
SW 50.1	5	81.5 ± 6.2	62.3 ± 10.2	50.0 ± 2.0
SW 50.1	24	90.0 ± 1.0	58.6 ± 4.0	50.0 ± 1.0

[a] Each value is mean and SD of at least three measurements.
[b] Percent apoA-I based on serum value.

(Table 2). There were slightly greater losses of apoA-I in the SW 50.1 rotor, which may be due to the longer fluid column in that rotor.

The loss of apoA-I from HDL was nearly identical when either cellulose nitrate or polyallomer centrifuge tubes were used (**Table 3**). Because these tube materials differ radically in their surface properties, it is unlikely that interaction of HDL with tube wall material accounts for loss of apoA-I during ultracentrifugation.

The losses of apoA-I from HDL when the overlayer protocol was used were the same as occurred under the normal procedure after one centrifugation at a density of 1.21 g/ml. Thus, the overlayer protocol had no great advantage over the normal procedure.

Effects of repeated ultracentrifugation on HDL

There was a progressive loss of apoA-I with repeated ultracentrifugation of HDL at a density of 1.21 g/ml, which approached an apparent asymptotic value of 35% (**Fig. 2**). In contrast, no loss of apoA-II was observed.

DISCUSSION

Investigation of the effects of conditions of ultracentrifugation on dissociation of apolipoprotein A-I from HDL

The loss of apoA-I from HDL during conventional ultracentrifugal isolation has been well documented (3–6). The observation (7, 8) that losses of apoA-I from

TABLE 3. Recovery of apoA-I in cellulose nitrate and polyallomer ultracentrifuge tubes

Tube Type	Recovery	
	H_2O-KBr	D_2O-CsCl
	%	
Cellulose nitrate	85[a]	63
Polyallomer	85	66

[a] Percent of total serum apoA-I content.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

HDL by other isolation methods, such as precipitation and gel permeation chromatography, are smaller than losses due to ultracentrifugation suggests that some specific aspect of ultracentrifugation accounts for this loss. Factors unique to ultracentrifugation that could facilitate the loss of apoprotein A-I from HDL are wall effects (rotor configuration and tube type), high ionic strength of ultracentrifugation medium, low temperature of ultracentrifugation, and ultracentrifugal force.

Wall effects occur when floating or sedimenting particles contact the centrifuge tube while traveling along their radial paths. When this happens, the impact or chemical interaction along the tube surface may cause loss of apoA-I from HDL. In our experiments comparing isolation of HDL in a 40.3 fixed-angle rotor, where extensive wall interaction occurs, with isolation in an SW 50.1 swinging-bucket rotor, where there is minimal interaction, we found little difference in the loss of apoA-I from HDL. Likewise, in comparing the loss of apoA-I with cellulose nitrate (hydrophilic) and polyallomer (hydrophobic) centrifuge tube types, no difference was found in apoA-I lost from HDL. Empirically, these results suggest that wall effects were also not a major factor in the loss of apoA-I from HDL.

Conventional systems of isolation of lipoproteins by ultracentrifugation involve the addition of inorganic salt to the ultracentrifugal solvent to achieve discriminating background densities. This addition of salt greatly increases the ionic strength of the solution. High ionic strength could be responsible for the loss of apoA-I from HDL. By using density media of widely varying ionic strengths to achieve background densities of 1.21 g/ml, we found the opposite: lowering the ionic strength actually increased the loss of apoA-I from HDL. This finding was true for all the conditions of centrifugation that we tested. Thus we may conclude that high ionic strength is not responsible for the dissociation of apoA-I from HDL during ultracentrifugation.

When lipoproteins are cooled, as in the ultracentrifuge, the lipid components can undergo phase changes (11). The structure of the lipoprotein can change and thus could cause a loss of apoA-I from HDL during ultracentrifugation. We did not find any difference in the loss of apoA-I from HDL ultracentrifuged at 5°C or 24°C.

The centrifugal force acting upon a lipoprotein particle can best be interpreted by the velocity at which a lipoprotein travels when subjected to that force. Schumaker[1] has determined that the velocity of HDL when centrifuged at 38×10^3 rpm in a 40.3 rotor is of the same magnitude as the velocity of HDL moving about in solution due to random thermal motion at room temperature. Furthermore, the velocity of a lipoprotein due to centrifugal force is several orders of magnitude slower than the velocity of blood in the arterial system. These arguments imply that centrifugal force is not sufficient to induce shear denaturation of HDL.

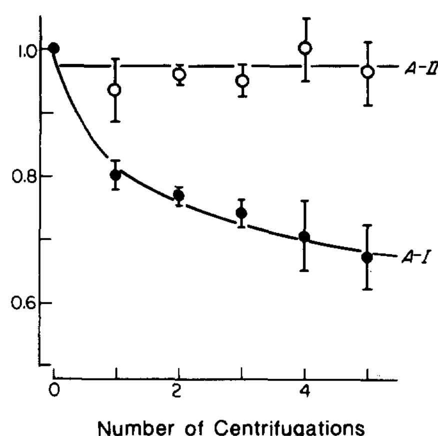

Fig. 2. The relative amounts of apoA-I (●) and apoA-II (○) recovered in HDL after repeated centrifugation were summarized for the mean values ± SD of five experiments.

Our results indicate that none of the variables in ultracentrifugation that we explored, including temperature, interaction with tube wall, and high ionic strength, account for the loss of apoA-I observed. Thus, the primary factor is still unidentified. One possible factor, unique to ultracentrifugation, is the high pressure generated in the fluid column by centrifugal force. Shen and Scanu (12) have shown that apoA-I at an air–water interface can be driven into aqueous solution by increasing surface tension. Since increasing the hydrostatic pressure acting on HDL particles may be equivalent to increasing the surface tension on the surface monolayer, this may be the ultracentrifugal factor responsible for the loss of apoA-I. Other researchers have shown that apoA-I, once free in solution, will self associate, and that self-associated apoA-I is slower to reassociate with lipid (13, 14). This would tend to enhance the separation of apoA-I from HDL.

Implications on the nature of binding of apolipoproteins to HDL complexes

The binding of apoA-I to HDL complexes can be viewed as an equilibrium state normally having a very low concentration of unassociated apoA-I. In the centrifuge, however, a state of disequilibrium exists because the sedimenting, unassociated apoA-I is not able to reassociate with the floating HDL complexes. Thus, any factor that increases the tendency toward dissociation will increase the loss of apoA-I from HDL.

[1] Schumaker, V. N. Unpublished communication.

Downloaded from www.jlr.org by guest, on June 19, 2012
ASBMB JOURNAL OF LIPID RESEARCH

Decreasing the ionic strength of the density medium increases the loss of apoA-I. Thus, the association of apoA-I with the complex must be weakened by removal of salt. The possible interpretations of this observation are: *1*) apoA-I has strong hydrophobic interaction with the HDL complex; *2*) strong electrostatic repulsions exist between apoA-I and phospholipid, which are unmasked at low ionic strength; or *3*) the surface tension of the HDL monolayer is dependent upon ionic strength.

Our observation that the content of apoA-I remaining with the HDL complexes after repeated ultracentrifugation approached a limiting value of approximately 65% suggests that the apoA-I in the complexes may exist in two types of binding environments, one of which involves very high affinity. In contrast, apoA-II did not dissociate under these conditions, suggesting that it has extremely high affinity for all the HDL complexes in which it appears. This is in keeping with the observations of Lagocki and Scanu (15) that human apoA-II apparently has higher affinity for the canine HDL complex than does canine apoA-I. It is likely that the more stable of the two environments for apoA-I involves interaction with apoA-II since apoA-I and apoA-II form mixed association products (16) and the affinity of apoA-I for lipid is increased by apoA-II (17). Other factors that could contribute to the appearance of multiple binding environments for apoA-I might include different molecular species of apoA-I and the existence of subspecies of HDL with varied composition or architecture.

The technical assistance of Leila Kotite, Kitty Tang, and Bess Fung is greatly appreciated. This work was supported in part by National Institutes of Health grants HL 14237 Arteriosclerosis SCOR and HL 24696. Steven T. Kunitake is a Bank of America-Giannini Foundation Fellow.

Manuscript received 2 October 1981 and in revised form 25 January 1982.

REFERENCES

1. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
2. De Lalla, O. F., and J. W. Gofman. 1954. Ultracentrifugal analysis of serum lipoproteins. *Methods Biochem. Anal.* **1:** 459–478.
3. Fainaru, M., M. C. Glangeaud, and S. Eisenberg. 1975. Radioimmunoassay of human high density lipoprotein apoprotein A-I. *Biochim. Biophys. Acta.* **386:** 432–443.
4. Fainaru, M., R. J. Havel, and T. E. Felker. 1976. Radioimmunoassay of apolipoprotein A-I of rat serum. *Biochim. Biophys. Acta.* **446:** 56–68.
5. Curry, M. D., P. Alaupovic, and C. A. Suenram. 1976. Determination of apolipoprotein A and its constitutive A-I and A-II polypeptides by separate electroimmunoassays. *Clin. Chem.* **22:** 315–322.
6. Mahley, R. W., and K. S. Holcombe. 1977. Alterations of the plasma lipoproteins and apoproteins following cholesterol feeding in the rat. *J. Lipid Res.* **18:** 314–324.
7. Rooke, J. A., and E. R. Skinner. 1979. The dissociation of apolipoproteins from rat plasma during isolation by precipitation with polyanions. *Int. J. Biochem.* **10:** 329–335.
8. Jahani, M., R. B. Huttash, and A. G. Lacko. 1980. A novel chromatographic method for the preparation of high density lipoproteins. *Prep. Biochem.* **10:** 431–444.
9. Vigne, J-L., and R. J. Havel. 1981. Metabolism of apolipoprotein A-I of chylomicrons in rats and humans. *Can. J. Biochem.* **59:** 613–618.
10. Huang, H., J. W. Kuan, and G. G. Guilbault. 1975. Fluorometric enzymatic determination of total cholesterol in serum. *Clin. Chem.* **21:** 1605–1608.
11. Small, D. M., D. L. Puppione, M. L. Phillips, D. Atkinson, J. A. Hamilton, and V. N. Schumaker. 1980. Crystallization of a metastable lipoprotein. Massive change of lipoprotein properties during routine preparation. *Circulation.* **62, III:** 118.
12. Shen, B. W., and A. M. Scanu. 1980. Properties of human apolipoprotein A-I at the air–water interface. *Biochemistry.* **19:** 3643–3650.
13. Massey, M. B., A. M. Gotto, and H. J. Pownall. 1981. Human plasma high density apolipoprotein A-I: effect of protein-protein interactions on the spontaneous formation of a lipid-protein recombinant. *Biochem. Biophys. Res. Commun.* **99:** 466–474.
14. Osborne, J. C., Jr., and H. B. Brewer, Jr. 1977. The plasma lipoproteins. *Adv. Protein Chem.* **31:** 253–337.
15. Lagocki, P. A., and A. M. Scanu. 1980. In vitro modulation of the apolipoprotein composition of high density lipoprotein. *J. Biol. Chem.* **255:** 3701–3706.
16. Osborne, J. C., Jr., G. M. Powell, and H. B. Brewer, Jr. 1980. Analysis of the mixed association between human apolipoproteins A-I and A-II in aqueous solution. *Biochim. Biophys. Acta.* **619:** 559–571.
17. Assmann, G., and H. B. Brewer, Jr. 1974. Lipid-protein interactions in high density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **71:** 989–993.

Downloaded from www.jlr.org by guest, on June 19, 2012