Precipitation of Apo E-Containing Lipoproteins by Precipitation Reagents for Apolipoprotein B

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We measured the solubility of apolipoprotein E (apo E) after precipitation, with heparin-Mn2+ or dextran sulfate-Mg2+, of lipoproteins containing apo B. Data from 46 randomly selected subjects suggest that apo E is readily precipitated by dextran sulfate-Mg2+, but that heparin-Mn2+ preferentially precipitates apo E associated with apo B-containing lipoproteins while leaving the apo E-containing fraction of high-density lipoproteins (HDL) in solution. In a more detailed analysis of three subjects, we measured the lipoprotein association of apo E by column chromatography on agarose beads, before and after its precipitation from plasma. This study confirmed the preferential solubility of apo E associated with HDL lipoproteins. Using plasma from two normolipemic subjects, we maintained the heparin concentration at 1.30 g/L and varied the manganese concentration from 9.2 to 184 mmol/L. A 46 mmol/L concentration best separated apo E-containing HDL from apo B-containing lipoproteins. Thus, at these final concentrations, heparin-Mn2+ appears to precipitate the apo E associated with apo B-containing lipoproteins, leaving soluble most of the apo E associated with lipoproteins of HDL size.

Additional Keyphrase: variation, source of

There is strong epidemiological evidence that high-density lipoprotein (HDL), as measured by its cholesterol content, represents a significant negative risk factor for cardiovascular disease. Furthermore, small differences in HDL concentrations are associated with relatively large differences in risk (7). Thus, considerable effort has focused on development of methods for HDL cholesterol determination that are adaptable to routine clinical screening centers yet sufficiently precise to reflect these small differences reliably.

Both clinical and research laboratories usually quantify HDL cholesterol by precipitation with a polyvalent anion and a divalent cation that aggregate under conditions specifically chosen to precipitate the apo B-containing lipoproteins, VLDL and LDL, while leaving the major HDL apoprotein (apo AI) and its associated cholesterol in solution (2). These methods are widely used because they are rapid, inexpensive, adaptable to large sample sizes, and at least as precise as the traditional method of lipoprotein separation by ultracentrifugation (3). The reagent now used in most laboratories is a combination of heparin and manganese chloride (3); recently, however, use of dextran sulfate and magnesium chloride (4) has increased, in association with the use of enzymic methods for cholesterol determination, with which the heparin/manganese reagent produces a substantial background. Another reagent frequently used is a phosphotungstic acid/magnesium chloride combination (3).

A detailed comparison (3) of these precipitating reagents in terms of the resulting HDL measurements of cholesterol showed that, although results by all methods were highly intercorrelated, the heparin procedure (final reagent concentrations, 1.30 g of heparin and 92 mmol of manganese per liter) yielded consistently higher concentrations of HDL cholesterol than did the other two methods. Precipitation of apo B was complete, with minimal precipitation of apo AI (3). Thus, this is usually considered the reference method for HDL cholesterol determination, as recommended by the laboratories in the Lipid Research Clinic (LRC) program (5). HDL is not a homogeneous class of lipoproteins; rather, it comprises subpopulations distinguished by size (6), density (7), and specific proteins (apolipoproteins) (8). In a recent method (9), sequential precipitation with heparin-manganese and dextran sulfate differentially separated the major subclasses of HDL, HDL2, and HDL3. The effectiveness of this procedure suggests that these precipitating reagents have different affinities for some component of these HDL subclasses.

Apo E is known to maintain a dynamic relationship among the plasma lipoproteins. We have recently shown (10) that when the plasma lipoproteins are separated by gel-filtration chromatography without ultracentrifugation, apo E is associated with VLDL and chylomicrons, and with two other lipoprotein subfractions that are distinct from LDL and HDL, one of them slightly larger than LDL but smaller than VLDL, the other intermediate in size between HDL and LDL. This latter apo E-enriched subclass appears to have density characteristics of HDL2. Thus, one difference between HDL subclasses may be ascribed to apolipoprotein composition, and this difference may underlie the differential solubilities cited above.

Because apo E binds to heparin, affinity chromatography with heparin-Sepharose has been used to isolate apo E-containing subclasses of VLDL (11) and HDL (12, 13). In addition, heparin-Mn2+ precipitates an apo E-rich HDL (referred to as HDL3) that accumulates in cholesterol-fed dogs (14). The questions arise as to whether apo E-containing subclasses of normal human HDL are differentially precipitated by the commonly used HDL cholesterol reagents, and whether this could alter significantly the measurement of total HDL cholesterol. The present study addresses those questions.

Materials and Methods

Blood samples were from volunteers and patients attending the Lipoprotein Clinic at Mount Sinai Hospital. All subjects had been fasting for 12 to 14 h. Blood was drawn from an antecubital vein into tubes containing EDTA as
anticoagulant. The plasma was separated immediately at 4°C, and precipitations were performed within 24 h.

Liphepin® (sodium heparin from porcine intestinal mucosa, 5000 USP units/mL) was obtained from Riker Laboratories, Northridge, CA; "dextran sulfate 500" (5 × 10^6 Da) and sodium phosphotungstate from Sigma Chemical Co., St. Louis, MO; and Biogel A 15M (200–400 mesh) from Bio-Rad Laboratories, Richmond, CA. Reagent kits for determining cholesterol and triglyceride were from Abbott Laboratories, N. Chicago, IL, and Calbiochem–Behring Corp., La Jolla, CA, respectively. Aprotinin (Trasylol) was obtained from Mobay Chemicals, New York, NY. All other reagents were analytical grade and were from Fisher Scientific Co., Fair Lawn, NJ.

The concentrations of cholesterol and triglyceride in plasma, in supernates after precipitation of plasma, and in column-resolved fractions were measured by specific enzymic methods (15, 16) in an ABA-100 discrete analyzer (Abbott Laboratories). In measuring cholesterol in plasma supernates after heparin/manganese precipitation, we corrected the result by subtracting the value obtained with use of 0.15 mol/L NaCl instead of plasma. When we used a final concentration of 1.3 g of heparin and 92 mmol of Mn²⁺ per liter, this was equivalent to about 80 mg of cholesterol per liter.

To measure apo AI, apo B, and apo E in plasma, HDL supernates, and column fractions, we used specific radioimmunoassays, as previously described (10). We fractionated the lipoproteins in plasma and in the plasma supernates after precipitation by using beads of agarose packed in 25 × 100 cm glass columns and equilibrated with pH 7 buffer containing, per liter, 10 mmol of sodium barbitol, 150 mmol of sodium chloride, 0.1 g of EDTA, 0.2 g of sodium azide, and 50 kio-kallikrein units of aprotinin to inhibit proteolytic activity.

Precipitation Procedures

Heparin–Mn²⁺. For studies involving manganese at a final concentration of 92 mmol/L, we pre pared a 2 mol/L solution of MnCl₂. When we were testing various concentrations of manganese, we used manganese chloride solutions with Mn²⁺ concentrations ranging from 0.2 to 4.0 mol/L.

To each milliliter of test EDTA-treated plasma, add sequentially 40 μL of heparin solution (5000 USP units/mL) and 50 μL of the MnCl₂ solution, thoroughly mixing after each addition. Incubate the samples for 10 min at room temperature, then centrifuge for 30 min at 1500 × g and 4°C. Without delay, transfer the supernates into vials and store at 4°C before lipid and apolipoprotein assay. Supernates to be subjected to column chromatography are dialyzed against the pH 7 buffer (see above) before they are applied to the column.

For analysis of precipitated lipoproteins, wash the pellets, by centrifugation, three times with a wash solution containing 150 mmol of sodium chloride per liter plus heparin and manganese in the proportions used in the original precipitation. Redissolve the washed pellet by suspending it in 1 mL of 1500 × g and 4°C before lipid and apolipoprotein assay. Supernates to be subjected to column chromatography are dialyzed against the pH 7 buffer (see above) before they are applied to the column.

Because the MnCl₂ reagent creates a significant blank reading in this enzymic cholesterol method, include a separate blank for each concentration of Mn²⁺ used.

Dextran sulfate–Mg²⁺. We prepared separate solutions of dextran sulfate 500 (20 g/L) and MgCl₂ (2.0 mol/L) and combined them in equal proportions. To each milliliter of test sample, add 100 μL of this combined solution, vortex-mix, and centrifuge at 1500 × g for 30 min. Promptly remove the supernates and store them at 4°C until analysis.

To measure the precipitated apolipoproteins, redissolve the pellet as described above.

Phosphotungstate–Mg²⁺. To prepare the sodium phosphotungstate reagent, dissolve 4.0 g of phosphotungstic acid in 50 mL of water, then add 16 mL of 1.00 mol/L sodium hydroxide, and dilute with water to 100 mL. For lipoprotein precipitation, sequentially add 100 μL of the sodium phosphotungstate solution and 20 μL of the 2.0 mol/L MgCl₂ to 1 mL of EDTA-treated plasma. After mixing, incubate the samples for 30 min at 4°C, then centrifuge at 1500 × g for 30 min at 4°C. Remove and store the supernates immediately, as in the other procedures.

Results

In the first study we wanted to compare the cholesterol concentration with the proportion of the plasma apo E that remained in the HDL supernatant fraction after precipitation with the heparin–Mn²⁺ and the dextran sulfate–Mg²⁺ procedures. Results for EDTA-treated plasma containing a range of lipoprotein concentrations, when analyzed by Student's paired t-test (17), showed that cholesterol values obtained after precipitation with heparin–Mn²⁺ were significantly higher than after precipitation with dextran sulfate–Mg²⁺ (mean ± SE, 452 ± 25 mg/L and 435 ± 24 mg/L, respectively). The heparin–Mn²⁺ procedure also yielded a significantly (p < 0.001) higher proportion of soluble apo E relative to total plasma apo E (mean ± SE, 32.0 ± 3.7%) than did dextran sulfate–Mg²⁺ (13.9 ± 1.6%).

To examine these differences more closely, we grouped the 46 subjects by HDL cholesterol concentration into five sets: <300 mg/dL (n = 12); 310–400 mg/dL (n = 11); 410–500 mg/dL (n = 11); 510–600 mg/dL (n = 7); and 610–700 mg/dL (n = 6). We compared, for each group, the proportion of total plasma apo E remaining soluble after respective addition of each of the reagents. Both heparin–Mn²⁺ and dextran sulfate–Mg²⁺ precipitated a large proportion of plasma apo E in subjects whose concentrations of HDL cholesterol were 400 mg/dL or less (Figure 1). In subjects whose concentration of supernatant cholesterol exceeded 400 mg/dL, the proportion of apo E remaining in the supernatant increased progressively with either reagent, but was always greater with heparin–Mg²⁺ than with dextran sulfate–Mg²⁺. For both procedures, the amount of apo B that remained in the supernatant after precipitation represented less than 0.5% of plasma apo B (mean ± SE = 4.24 ± 0.30 and 5.28 ± 0.59 mg/L for dextran sulfate–Mg²⁺ and heparin–Mn²⁺, respectively).

Fig. 1. Percentage of plasma apo E remaining in solution after precipitation with heparin (Hep)–Mn²⁺ or dextran sulfate (DSO₄)–Mg²⁺ in 46 subjects grouped according to concentration of HDL cholesterol. Error bars indicate ± SE.
Because the fraction of apo E associated with the HDL is highly correlated with the apo AI and HDL cholesterol (10), these data suggested that heparin–Mn$^{2+}$ was specifically precipitating apo E associated with apo B-containing lipoproteins, but leaving the apo E-associated with HDL unprecipitated. To test this hypothesis, we submitted plasma and HDL supernates, after precipitation with heparin–Mn$^{2+}$ or dextran sulfate–Mg$^{2+}$, to lipoprotein fractionation on agarose columns and examined the apo E distribution across the lipoprotein spectrum by radioimmunoassay. We so studied three subjects. The results from two of them are shown in Figures 2 and 3.

The subject shown in Figure 2 had a very high HDL cholesterol value, which was higher as measured with heparin–Mn$^{2+}$ than with dextran sulfate–Mg$^{2+}$ (860 vs 770 mg/L, respectively). Of the plasma apo E, 90% was in the HDL-size fraction III (10). After precipitation with dextran sulfate–Mg$^{2+}$ only 9.9% of the plasma apo E was accounted for in the supernate. This small proportion chromatographed in the HDL region (Figure 2). In contrast, heparin–Mn$^{2+}$ left 64.0% of the plasma apo E in the supernate, all of which was associated with particles the size of large HDL (360–460 mL elution volume).

The second subject had an HDL cholesterol concentration of 388 mg/L as measured by the heparin–Mn$^{2+}$ procedure; the dextran sulfate–Mg$^{2+}$ method gave a value of 341 mg/L. As in subject 1, these two procedures precipitated markedly different amounts of apo E. The dextran sulfate–Mg$^{2+}$ procedure left only 5.8% of the plasma E in solution; the heparin–Mn$^{2+}$ procedure left 38.4%. On gel chromatography of the soluble plasma components after precipitation with dextran sulfate–Mg$^{2+}$, the apo E was found to be decreased in all subfractions. In contrast, heparin–Mn$^{2+}$ appeared to have precipitated the apo E associated with the VLDL and the intermediate-size lipoproteins, but to have preferentially left soluble most, but not all, of the apo E associated with HDL in the original plasma.

The third subject we studied gave similar results: after fractionation of whole plasma, apo E was distributed almost entirely between the apo E particles of intermediate size (fraction II = 310–360 mL elution volume) (47%) and fraction III (53%). After precipitation with dextran sulfate–Mg$^{2+}$, 18% of the apo E remained in solution, and this was uniformly distributed among the various lipoproteins. After heparin–Mn$^{2+}$ precipitation, 45% of the plasma apo E remained in the supernatant. Agarose chromatography showed that there was no apo E in the intermediate-size lipoproteins (fraction II), whereas 83% of the apo E in fraction III (relative to the original plasma) remained in solution.

An additional seven plasma samples were also studied by the phosphotungstic acid–Mg$^{2+}$ precipitation procedure. Results, however, were variable and this method was not studied in more depth.

These three chromatographic studies indicate that although heparin–Mn$^{2+}$ separates apo E-containing HDL from other apo E-containing lipoproteins, the procedure is not quantitative because apo E-containing HDL is partly precipitated. We therefore undertook studies to identify precipitation conditions under which all of the apo E-containing HDL would be soluble, while not compromising the separation of lipoproteins containing apo B and apo AI. We chose manganese chloride as the variable, because apo B precipitation appears to be more sensitive to variations in this component than to the heparin concentration (18). For this study, we submitted identical aliquots of EDTA-treated plasma from two subjects to precipitation, using reagents containing a constant final heparin concentration of 1.30 g/L and 11 manganese concentrations ranging from 9.2 to 184 mmol/L. The two subjects so studied are those for whom results are shown in Figures 2 and 3. The total mass of apo E expected to be associated with HDL was estimated from the chromatographic profiles of whole plasma. The results for these two subjects are shown in Figure 4, a and b. Values for HDL cholesterol were constant between 30 and 100 mmol of Mn$^{2+}$ per liter. In contrast, the apo E value for the supernates was very responsive to Mn$^{2+}$ concentration in both subjects. In the subject depicted in Figure 4a (and shown previously in Figure 2), the apo E supernate concentration decreased steadily between 9.2 and 92 mmol of manganese per liter, whereas the apo E in the precipitate increased in an inverse manner (data not shown). The reference value, 43.4 mg/L (dotted line, Figure 4), was determined by integrating the apo E contained in HDL-size lipoproteins (shown in Figure 2) and dividing by the volume applied. At 92 mmol of Mn$^{2+}$ per liter, the concentration used in the studies shown in Figures 1–3, only 36.9 mg of the HDL apo E per liter remained soluble, compared with the expected value of 43.4 mg/L. As a percentage of the total apo E in plasma (69%), this value for the supernate is very close to that (64%) seen in the same subject in the study shown in Figure 2, in which 92 mmol of Mn$^{2+}$ per liter was also used. At 46 mmol of Mn$^{2+}$ per liter, however, 40.5 mg of the apo E remained soluble per liter, a value much closer to the expected value of 43 mg/L. The apo B in the supernate, although higher (9.5 mg/L) than at 92 mmol/L (5.0 mg/L),
lipoprotein-bound apo E, but may precipitate a subclass of apo E-containing lipoproteins associated with apo B. We still do not know whether it is the association of apo E with apo B that leads to the precipitability or, instead, the physical form of the lipoprotein itself. It is possible, for example, that apo E-containing lipoproteins devoid of apo B might be precipitable if they are sufficiently large and rich in cholesterol ester.

The important question arises as to whether the differential precipitation of apo E by heparin–Mn\(^{2+}\) and dextran sulfate is quantitatively significant in terms of HDL cholesterol determination. The actual proportion of the total HDL cholesterol associated with apo E is not known, and it may vary in different subjects. Unpublished data from our laboratory, obtained by using immunoaffinity chromatography to isolate apo E-containing HDL from three normolipemic subjects, suggest that less than 2% of the total HDL cholesterol may be physically associated with apo E-containing lipoproteins. In the present study, changes in the manganese concentration that led to small changes in apo E precipitation had no effect on values for HDL cholesterol (Figure 4). Nevertheless, the heparin–Mn\(^{2+}\) and dextran sulfate–Mg\(^{2+}\) procedures consistently resulted in significant differences in values for HDL cholesterol, both in this study and in others (3), concomitant with major differences in the average values for apo E that remains soluble.

Our data thus indicate that the methods involving dextran sulfate–Mg\(^{2+}\) and phosphotungstate–Mg\(^{2+}\) precipitate apo E regardless of lipoprotein association, while that involving heparin–Mn\(^{2+}\) specifically precipitates apo E associated with apo B, leaving most apo E in HDL in solution. Decreasing the final manganese concentration from 92 to 46 mmol/L appears to provide the most effective means of completely solubilizing of apo E-containing HDL. Our studies have evaluated this modification in only two normolipemic subjects, however, and individual plasmas with subtle differences in the lipid composition of the lipoproteins may vary in the relative precipitability of apolipoproteins E and B. This has been shown previously to occur for apo B-containing lipoproteins (3). Specifically, these studies by Warnick et al. showed that hypertriglyceridemic plasma was more susceptible to incomplete precipitation of apo B than was plasma of normolipoproteinemic subjects. The observation that incomplete precipitation was more apt to occur at 46 mmol/L manganese than at 92 mmol/L emphasizes the need for caution when this procedure is applied to hypertriglyceridemic plasmas. Furthermore, Figure 4 shows a continuous decline in the value for apo E in the supernate with increasing concentrations of manganese, with no clear leveling off of the value. Thus, the modification we suggest must be evaluated for different lipoprotein patterns before it can be recommended for general use.

We have previously suggested—from studies utilizing immunoaffinity chromatography—that apo E-containing HDL actually consists of several subclasses with different apolipoprotein composition (20). These data confirm studies by others utilizing heparin affinity chromatography (12, 13) and raise the possibility that the partial precipitation of apo E relates to the partitioning of apo E among these subclasses. Thus, the small amount of apo E-containing HDL precipitated by higher concentrations of manganese may represent a subclass of HDL with apo E as the only or predominant apolipoprotein, whereas that remaining soluble may be associated with HDL particles containing apo AI and (or) other apolipoproteins.

In summary, the data presented here document striking differences in the extent to which the currently most widely
used precipitation methods for HDL determination co-pre-
cipitate HDL containing apo E with the apo B-containing
lipoproteins. Of particular interest is the observation that
use of heparin–Mn$^{2+}$ (final concentrations 1.30 g of heparin
and 46 mmol of Mn$^{2+}$/liter) completely precipitates apo
E associated with apo B-containing lipoproteins without co-
precipitating the HDL-associated apo E. This modification
may provide a method for rapidly determining the apo E
distribution in plasma between apo B-containing and non-
apo B-containing lipoproteins.

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