Dextran Sulfate-Mg$^{2+}$ Precipitation Procedure for Quantitation of High-Density-Lipoprotein Cholesterol

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Introduction

Recent recognition of the importance of HDL\textsuperscript{1} cholesterol as a strong inverse risk factor for coronary artery disease (1–4) has led to substantial demand for this assay by clinical and research laboratories. Various methods, including ultracentrifugation, electrophoresis, and specific precipitation, have been used to separate the lipoproteins. Although ultracentrifugation techniques are useful for reference (5), they are expensive and require equipment and a level of technical sophistication not available in many routine laboratories. The precipitation methods have, in general, been most suitable for routine quantitation of lipoproteins. Several such methods have been described in the recent literature, most of them based on earlier work of Burstein et al. (reviewed in 6). The method used most extensively in major population studies involves use of heparin in conjunction with Mn$^{2+}$ to precipitate VLDL and LDL (7–9). HDL is then quantitated in terms of the amount of cholesterol remaining in the supernatant solution. This method has been extensively studied (10, 11) and modifications have been described (11, 12). The procedure has its drawbacks, of which the major one is the reported interference of the Mn$^{2+}$ with the enzymic assays for cholesterol (13). Also, heparin is relatively expensive and may vary with respect to this characteristic, depending on its source and the method of preparation.

Therefore, with the objective of defining a separation procedure giving results comparable with those by the heparin–Mn$^{2+}$ method but without the associated interference and other problems, we have considered various alternatives. In previous experiments, lipoprotein precipitation by the combination of Mg$^{2+}$ and dextran sulfate, a synthetic heparin analogue, demonstrated good correlation with precipitation by heparin and Mn$^{2+}$. Precipitation with dextran sulfate and Mg$^{2+}$ was also highly reproducible (14). Therefore, we evaluated published methods with dextran sulfate. In agreement with previous investigators (6), we observed that lipoprotein precipitation was a function of the molecular size of the dextran sulfate: the larger the dextran sulfate molecule, the greater the tendency for lipoprotein to precipitate. Separation methods (15, 16) in which 500 000 g/mol preparations of dextran sulfate were used underestimated HDL cholesterol, in comparison with the heparin–Mn$^{2+}$ and ultracentrifugation methods (14). Another standard dextran sulfate preparation (M, 15 000) used for lipoprotein separation under similar conditions (6) did not completely precipitate LDL/VLDL in our experience. We therefore predicted that a material of intermediate molecular mass might give separations with Mg$^{2+}$ comparable with those obtained with heparin and Mn$^{2+}$. After comparing dextran sulfate preparations of various molecular masses, either synthesized in this laboratory or obtained from commercial sources, a dextran sulfate of 50 000 daltons used with Mg$^{2+}$ gave separations most comparable with those obtained with heparin and Mn$^{2+}$. We selected Mg$^{2+}$ as the cation because of its apparent lack of interference with enzymic cholesterol assays. We did not use Ca$^{2+}$, which is similar to Mg$^{2+}$ in lipoprotein precipitation, because it reportedly is less effective in separating hypertriglyceridemic specimens (6). With dextran sulfate of 50 000 g/mol, lipoprotein separation was relatively insensitive to the dextran sulfate concentration and less sensitive to Mg$^{2+}$ concentration than when we used the other dextran sulfate preparations. To obtain appropriate specificity in lipoprotein separation, we adjusted the Mg$^{2+}$ concentration for optimum precipitation of LDL and VLDL without excessive precipitation of HDL.

The combination of phosphotungstate and Mg$^{2+}$ (6, 17, 18) was not chosen, because our preliminary experiments indicated that the method gave variable results. Lipoprotein separations were quite sensitive to reagent concentrations and temperature, which resulted in poorer precision than with the heparin–Mn$^{2+}$ and dextran sulfate–Mg$^{2+}$ procedures (14). The use of polyethylene glycol (19) was rejected because the method readily precipitated HDL and other plasma proteins at concentrations only slightly higher than were necessary for precipitation of LDL (14). Therefore, if one considers the

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\textsuperscript{1} Nonstandard abbreviations: HDL, LDL, VLDL, high-, low-, and very-low-density lipoproteins, respectively; apoA-I, apolipoprotein A-I; the major protein of HDL; apoB, apolipoprotein B, the major protein of LDL and VLDL; EDTA, disodium ethylenediaminetetraacetate; CDC, Centers for Disease Control; PIPES, 1,4-piperazineinedethanesulfonic acid; LRC, Lipid Research Clinics.
range in concentrations of lipoproteins and other proteins in specimens, the polyethylene glycol method might be less specific.

**Principle**

The specific mechanisms of lipoprotein precipitation by polyanions and divalent cations have not been established with certainty. However, interaction between negatively charged groups on the polyanions and positively charged groups on the protein moieties of the lipoproteins are probably important. Divalent metal ions interact with negatively charged groups (such as phospholipids) on the lipoproteins to facilitate formation of insoluble complexes (20). The larger, lipid-rich lipoproteins, VLDL and LDL, form insoluble complexes more readily than do the smaller, protein-rich HDL. The insoluble complexes can be sedimented by low-speed centrifugation, provided their density is sufficiently greater than that of the solution. In the presence of high concentrations of triglyceride-rich lipoproteins, the insoluble complexes may either remain suspended in the solution or float to the surface. On the other hand, other plasma proteins may coprecipitate with the lipoproteins, thereby increasing the density of the insoluble complex and facilitating sedimentation even in specimens with high triglyceride concentrations. The extent of protein coprecipitation is a function of the method and conditions of precipitation. Cholesterol remaining in the supernatant solution can be considered to represent HDL, if sedimentation of LDL/VLDL is complete and no HDL has precipitated.

Colorimetric methods involving either strong acid or enzymic reagents have generally been used to quantitate cholesterol (reviewed in 27). Methods may be direct, i.e., applied directly to specimens without pretreatment, or may include a preliminary solvent extraction of the lipids from other interfering constituents. Because approximately 80% of cholesterol in HDL from humans is in form of the esters, a preliminary hydrolysis step may be used to cleave the ester linkage. With enzymic assays hydrolysis is necessary, and may be accomplished by adding a cholesterol ester hydrolase to the reaction mixture. The 3β-hydroxy group of free cholesterol is then oxidized by cholesterol oxidase with liberation of hydrogen peroxide, which can be coupled to a colorimetric reaction.

**Materials and Methods**

**Precipitation Reagents**

1. **Dextran sulfate.** Material (M, 50 000 ± 5000) with acceptable quality can be obtained from SOCHIBO, SA, Boulogne, France, 92100. Store preferably in a refrigerated desiccator. Prepare a stock solution as follows: Add 2.0 g of dextran sulfate to 80 mL of de-ionized water in a beaker, and adjust the pH if necessary to 7.0 with dilute HCl. Transfer quantitatively to a volumetric flask and adjust the final volume to 100 mL to give a concentration of 20 g/L. Store at 4°C. A preservative solution containing, per liter, 50 g of NaN₃ (J.T. Baker Chemical Co., Phillipsburg, NJ 08865), 1.0 g of chlor amphenicol, and 0.5 g of gentamicin sulfate (both from Sigma Chemical Co., St. Louis, MO 63178) can be added to this solution at the rate of 10 mL/L before final volume adjustment.

2. **Stock Mg²⁺ solution.** Reagent-grade MgCl₂·6H₂O, which is hydroscopic, should be stored tightly closed or in a desiccator to minimize water uptake. Prepare a stock solution of 1.0 mol/L by dissolving 20.3 g of MgCl₂·6H₂O in 80 mL of de-ionized water in a beaker; adjust the pH to 7.0 with a dilute NaOH solution. Transfer quantitatively to a 100-mL volumetric flask and adjust the final volume to 100 mL. Mg²⁺ concentration can be verified by atomic absorption analysis. Store at 4°C.

3. **Combined working reagent.** Mix equal volumes of the dextran sulfate and MgCl₂ stock solutions to produce a solution with dextran sulfate concentration of 10 g/L and MgCl₂ concentration of 500 mmol/L. Alternatively, prepare a combined reagent by dissolving both constituents, dextran sulfate and MgCl₂, in de-ionized water. Adjust pH to 7.0 and the final volume to give, per liter, 10 g of dextran sulfate and 500 mmol of MgCl₂. This reagent prepared by either method when added to specimens will give final concentrations of dextran sulfate of 0.9 g/L and Mg²⁺ of 45 mmol/L.

**Note:** Combined solutions prepared with dextran sulfate stock solutions containing the preservative have been stored for as long as four months at 4°C without apparent change in their ability to precipitate lipoproteins.

4. **Control materials.**

**Note:** Precision and accuracy in the precipitation procedure can be monitored by analyzing control materials that are similar to patients’ specimens in analyte and matrix properties. Many of the materials available commercially do not meet these specifications.

Prepare control materials by pooling fresh specimens (plasma with EDTA as anticoagulant or serum) from donors or that remaining after laboratory analysis (22). Exclude turbid or hypertriglyceridemic specimens so that the total triglyceride value is less than 1 g/L. The use of two pools, one with HDL cholesterol in the low normal (200–400 mg/L) range and one in the mid (500 mg/L) range, is recommended. Plasma may be treated with thrombin to minimize subsequent fibrin formation. Transfer aliquots to vials and seal. Quick-freeze the contents and store in a freezer with a stable temperature, preferably at or below −60°C. To allow verification of method accuracy, obtain a target value by requesting analysis of aliquots from an experienced laboratory such as those of the Lipid Research Clinics Program. Alternatively, a commercial material (Lipid Fraction Control Serum, Hyland Diagnostics, Division of Travenol Laboratories, Inc., Deerfield, IL 60015) is currently available that has an accurate reference value for HDL cholesterol.

**Reagents for Enzymic Cholesterol Analysis**

**Note:** The precipitation procedure described here appears to be compatible with any of the available cholesterol assays, provided their accuracy and reproducibility are adequate. We have analyzed cholesterol in supernates by using Liebermann–Burchard reagent according to the Lipid Research Clinics method (9, 23); this is both accurate and precise but may not be practical for many clinical laboratories. We have also used with good results a manual enzymic assay as reported by Cooper et al. (24), which we describe below in detail, because the published procedure may not be available to some readers.

Microbial cholesterol ester hydrolase (EC 3.1.1.13) was obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN 46250, and microbial cholesterol oxidase (EC 1.1.3.6) was obtained from Beckman Microbes, Carlsbad, CA 92005. PIPES buffer (1,4-piperazinediethanesulphonate) was obtained from Research Organics, Cleveland, OH 44125. Horseradish peroxidase, Type VI (EC 1.11.1.7), 4-aminoantipyrine, and sodium cholate were purchased from Sigma Chemical Co.; phenol was obtained from J.T. Baker Chemical Co., and Triton X-100 from Rohm and Haas, Philadelphia, PA 19105. The enzyme solutions were stored at 4°C.

Prepare the following reagents:

1. **PIPES buffer, 50 mmol/L, pH 6.9.** Into a 1000-mL glass beaker containing approximately 800 mL of distilled water, add 17.7 g of PIPES buffer and stir for approximately 10 min.
until the solution clears. Equilibrate the solution to 37 °C and adjust, if necessary, to pH 6.9. Quantitatively transfer the solution to a 1000-mL volumetric flask; after cooling to ambient temperature, adjust the volume to 1000 mL.

2. **Stock mixed reagent.** The following reagents are dissolved in 400 mL of PIPES buffer in a 500-mL volumetric flask: 4-aminopyrrole, 0.203 g; sodium cholate, 1.292 g; potassium chloride, 7.46 g; and Triton X-100, 1.0 mL. Adjust the final volume to 500-mL with PIPES buffer.

   **Note:** This solution is stable for at least one month at 4 °C.

3. **Stock phenol reagent:** Prepare from phenol stored in a desiccator. Carefully but quickly weigh 3.8 g of phenol crystals and dissolve in PIPES buffer in a 500-mL volumetric flask; bring to volume with the buffer.

   **Note:** This reagent has been stored successfully for as long as a month at 4 °C in a tightly closed glass container.

4. **Working reagent.** Mix 50 mL of the stock mixed reagent and 50 mL of the stock phenol reagent. Add 25 U of cholesterol oxidase, 25 U of cholesterol esterase, and 1250 U of peroxidase (based on the respective specific enzyme activities), either from concentrated solutions of the enzymes or by weighing the dry enzyme preparations.

   Alternatively, the enzymes can be added to the stock mixed reagent at the time of initial preparation. In this instance, however, the stock mixed reagent should be prepared fresh daily. Volumes can be adjusted, depending on the number of specimens to be analyzed.

5. **Calibrators**

   **Note:** Calibration of enzymic cholesterol methods with primary standards of cholesterol in water or alcohol solution may give incorrect cholesterol values for specimens for the following reasons. Incomplete hydrolysis of esterified cholesterol in specimens by the cholesterol ester hydrolyase reaction, which has been reported (25–27), would result in low results because the remaining esterified cholesterol is not a suitable substrate for the oxidation reaction. Also, the alcohol or detergent in aqueous primary standards may inhibit the enzymes (28) and give different reaction rates in the standards than in specimens. Therefore, we recommend the use of secondary-separate-based calibration standards with analyte and matrix properties similar to those of the specimens. However, the secondary standard must have an accurate target value determined by an accepted reference technique.

   We prepared a suitable secondary standard as follows: Reconstitute Lipid Fraction Control Serum (lot no. 4610 Y002A; Hyland Diagnostics), according to the manufacturer’s instructions. Dilute with an equal volume of 60 g/L bovine serum albumin, Cohn Fraction V (Sigma Chemical Co.), in 0.15 mol/L NaCl solution. Freeze aliquots in sealed vials for subsequent use as a secondary calibrator for assay of cholesterol in supernates. The reported target value for this pool is 1640 mg/L as determined by the modified Abell–Kendall method (29) at the Clinical Chemistry Standardization Section of CDC. The dilution yields a value of 620 mg/L, which is a more appropriate for assaying HDL specimens. Another suitable secondary calibrator is Standard Reference Material 905, a human serum pool available from the National Bureau of Standards, Washington, DC 20234. This material has a target value for cholesterol assigned by an assay involving isotope dilution–mass spectrometry, which is a candidate Definitive Method for cholesterol (30). Alternatively, an in-house pool of human donor or pooled serum or plasma can be prepared as described previously (22); dilute with albumin solution (60 g/L) to obtain a total cholesterol value in the 800–1000 mg/L range. A target value can be established by a suitable reference method.

**Collection of Specimens**

Collect blood from the antecubital vein of seated subjects who have fasted for 12–14 h. Apply a tourniquet to locate the vein, but remove it during blood collection. To obtain EDTA-treated plasma, collect blood into tubes containing EDTA (final concentration, 1.5 g/L); mix thoroughly and cool immediately. For serum, collect blood without anticoagulant and allow it to stand 30 min at room temperature for clotting. Separate cells by centrifugation. Complete lipoprotein separations the same day.

**Notes:** The necessity of fasting before specimen collection for HDL cholesterol measurement has not been established with certainty. In one study (31) values did not differ significantly between specimens collected after a meal and specimens taken after an overnight fast. However, other studies demonstrate appreciable changes in HDL cholesterol after a meal high in fat (32) or carbohydrate (unpublished observation). Therefore, where practical, it is advisable to draw a fasting (12–14 h) specimen. EDTA-treated plasma is the preferred medium for lipoprotein quantitation because the stability of the lipoprotein constituents is enhanced. Heparin and citrate anticoagulants can produce severe interference with lipoprotein precipitation. Serum is also commonly used for separation, although HDL results are not exactly equivalent to those obtained with EDTA-treated plasma.

HDL separations should be made as quickly as possible after blood collection. Various changes occur during storage, including enzymic and nonenzymic transfers of lipids among lipoproteins, pH shifts, bacterial action, and production of metabolites, which can substantially change lipoprotein composition and their separation characteristics (33, 34). If separations cannot be made on the day of collection, specimens can be frozen at −15 °C for several weeks and at −60 °C for as long as two years without substantial change.

**Procedure**

**Precipitation step**

1. Allow specimens, control materials, and precipitation reagents to equilibrate to room temperature.

2. Using a manual pipette, transfer 1.0-mL aliquots of plasma and control material into appropriately labeled tubes. Culture tubes (10 × 75 mm) are suitable. Specimen volumes can be adjusted so long as a proportionate volume of precipitant reagent is added.

3. Add 100 μL of the combined dextran sulfate–MgCl2 working solution to each tube. Immediately after addition of this reagent, mix the contents of each tube in sequence for at least 3 s, with a vortex-type mixer.

   **Note:** Alternatively, 50 μL each of the separate dextran sulfate and MgCl2 stock solutions can be added sequentially to the 1.0-mL specimens, with thorough mixing after each addition.

4. Allow the tubes to stand at room temperature for 10 min before sedimenting the insoluble lipoproteins by centrifugation.

   **Note:** Centrifugation for 30 min with a refrigerated centrifuge (4 °C) attaining 1500 × g is preferred. However, results by this method are nearly the same as those obtained with centrifugation in an unrefrigerated benchtop centrifuge at 1000 × g for 15 min.

5. Remove tubes from the centrifuge and inspect supernates for turbidity. Obtain an aliquot of the clear supernates for cholesterol analysis, or transfer the supernatant solution with a Pasteur pipette to a second labeled vial for later analysis.

   **Note:** Any turbidity or cloudiness in the supernates indicates incomplete sedimentation of LDL/VLDL and consequent contamination and overestimation of HDL. This is usually observed in specimens with high triglyceride values.

6. Turbid supernates can be conveniently cleared by one of the following methods:

   (a) Without separating the turbid supernate from the precipitate, add to the separation tube 1.0 mL of 0.15 mol/L NaCl solution and another 100 μL of combined precipitant reagent. Mix thoroughly with a vortex-type mixer, then cen-
Enzymic cholesterol assay

1. Turn on the spectrophotometer and adjust the wavelength to 500 nm.
2. Label tubes for water blank, calibrators, control materials, and specimens.
3. Dispense 2.0 mL of enzymic cholesterol reagent into each of the tubes and place them in an ice bath.
4. Add 100 μL of water, calibrator, control material, or supernate to the appropriate tubes and mix thoroughly.

Note: Mix specimens thoroughly to assure homogeneity before pipetting.
5. Transfer tubes to a water bath at 37 °C for 20 min.
6. Cool tubes to room temperature and within 15 min read absorbance after adjusting spectrophotometer to zero with the water blank.
7. Calculate cholesterol in specimens and control materials in relation to the calibrator.

\[
\text{Cholesterol unknown} = \frac{\text{absorbance of unknown}}{\text{absorbance of calibrator}} \times \text{cholesterol calibrator}
\]

Note: Depending on precision requirements of the particular application and the precision of the pipettes and spectrophotometer, the specimen and (or) calibrators may be analyzed in duplicate.

Calculation

Cholesterol in supernates must be corrected for the dilution incurred in adding the precipitant reagents. Multiply the assayed cholesterol value by 1.1. When necessary, correct by the appropriate factor for dilution of turbid supernatant solutions as described above.

**Results and Discussion**

The precipitation method with \( M_1 \) 50 000 dextran sulfate and \( M_2 \) as described here gives results highly correlated with those of the heparin-Mn2+ procedure. Final reagent concentrations were established to obtain results for supernatant cholesterol approximately 10–20 mg/L lower than results by the LRC heparin-Mn2+ method with Mn2+ at 48 mmol/L final concentration (Table 1). The LRC method is reasonably accurate in relation to an ultracentrifugation reference procedure (10, 11), but has been previously demonstrated to overestimate HDL slightly (by 10–20 mg/L) in EDTA-treated plasma specimens because of incomplete precipitation of the apoB-containing lipoproteins (11, 35). The systematic difference in supernatant cholesterol observed between the heparin-Mn2+ and the present dextran sulfate-Mg2+ method is quite consistent for normolipidemic men and women as well as for hypercholesterolemic subjects (Table 1). The bias was smaller for hypertriglyceridemic subjects, with their concomitant low HDL values. A larger average difference was observed in specimens in which both cholesterol and triglyceride were above normal. However, this difference was attributable to a single specimen that had supernatant cholesterol of 532 mg/L by the heparin-Mn2+ procedure vs 385 mg/L by the dextran sulfate-Mg2+ method, both values being consistent in duplicate separations. The duplicate heparin-Mn2+ supernates of this specimen averaged 96 mg of apoB-associated cholesterol per liter, indicative of incomplete LDL/VLDL precipitation, while the dextran sulfate-Mg2+ precipitates averaged 126 mg of apoA-I per liter, suggesting substantial precipitation of HDL. Except for this one specimen, between-method cholesterol differences were less than 70 mg/L.

ApoB is the major protein constituent of LDL and VLDL. Its measurement in supernates allows detection of incomplete precipitation of these lipoproteins. On the other hand, apoA-I, although present in small amounts in VLDL, is the major protein constituent of HDL. Therefore, high apoA-I values in the precipitate fractions can indicate excessive precipitation of HDL. Consideration of apoB concentrations in supernates (Table 1) suggests that this dextran sulfate-Mg2+ procedure more specifically precipitates VLDL and LDL without excessive HDL precipitation than does the heparin-Mn2+ method. Heparin-Mn2+ supernates contained, on the average, 10.3 mg of apoB-associated cholesterol per liter, while dextran

### Table 1. Lipoprotein Precipitation by Dextran Sulfate and Mg2+ (DSM) Compared with Heparin and Mn2+ (HM)

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Conc, mg/L, in supernate</th>
<th>Cholesterol (LRC)</th>
<th>ApoB-associated cholesterol *</th>
<th>Precipitate ApoB-L, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc, mg/L, total plasma, mean ± SD</td>
<td>HM</td>
<td>DSM</td>
<td>(HM – DSM)</td>
</tr>
<tr>
<td>Overall</td>
<td>48</td>
<td>2497 ± 564</td>
<td>1797 ± 1768</td>
<td>493.7</td>
</tr>
<tr>
<td>Normolipidemic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>20</td>
<td>2184 ± 387</td>
<td>1132 ± 482</td>
<td>488.8</td>
</tr>
<tr>
<td>Females</td>
<td>9</td>
<td>2266 ± 284</td>
<td>800 ± 212</td>
<td>671.7</td>
</tr>
<tr>
<td>Hypercholesteremic</td>
<td>9</td>
<td>3098 ± 268</td>
<td>1472 ± 518</td>
<td>460.7</td>
</tr>
<tr>
<td>Hypertriglyceridemic</td>
<td>4</td>
<td>3510 ± 396</td>
<td>5692 ± 3598</td>
<td>324.1</td>
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</tbody>
</table>

* Plasma cholesterol range, 1340 to 3820 mg/L. * Plasma triglyceride range, 450 to 10 750 mg/L. * Separations were performed in duplicate. Values are the means for duplicate determinations. * Paired SD 21.3, Student’s paired t-test 5.09, significant at p < 0.001. * Quantitated by radial immunodiffusion assay (11, 12, 36). * Quantitated by radial immunodiffusion assay (11, 12, 37). * ApoA-I not measured in some lipemic specimens because of losses associated with filtration of turbid supernates.
sulfate–Mg2+ supernates contained <1.0 mg/L. On the other hand, the dextran sulfate precipitates contained somewhat more apoA-I (average, 47.4 mg/L), suggesting there was slightly more HDL precipitation by this method than with heparin–Mn2+ precipitation, in which precipitate fractions averaged 31.2 mg/L. The 16 mg/L difference in apoA-I represents approximately 5 mg of HDL cholesterol per liter.

The LRC Liebermann–Burchard cholesterol method gives results in excellent agreement with the reference method of Abell–Kendall as applied at the Clinical Chemistry Standardization Section of the CDC (29). In this laboratory, results are generally within 10 mg/L of the CDC value and CVs of approximately 1% are obtained for control pools. The enzymic cholesterol assay described here, calibrated with Lipid Fraction Control Serum, was in good agreement with the LRC method. The dextran sulfate–Mg2+ supernates analyzed by the enzymic assay averaged 464.9 mg/L vs 475.8 mg/L by the LRC cholesterol assay. The average difference was 10.9 mg/L, with a paired standard deviation of 16.7 mg/L. The agreement (Figure 1) between the two assay methods was good, as indicated by the parameters of the linear regression: slope = 0.958, y-intercept = 9.2 mg/L, and correlation coefficient = 0.989.

Results for control materials (Table 2) demonstrate similar relationships between the two precipitation methods as well as between the two cholesterol assays. With cholesterol assay by the LRC method, the dextran sulfate–Mg2+ supernates averaged 23 mg/L lower than the heparin–Mn2+ supernates for both serum (AQ 6) and plasma control materials. Results for dextran sulfate–Mg2+ supernates obtained by the enzymic assay were slightly lower than those obtained by the LRC assay on the three pools.

Between-day precision for cholesterol analysis alone was reflected in a CV of 1.2% by the LRC assay and 2.2% by the enzymic assay on the low-total-cholesterol (MQ 3) pool. This is consistent with the CVs for precipitation and cholesterol assay of 3.9 and 3.6% for the two precipitated pools by the enzymic assay, compared with 2.4 and 2.9% for the same pools by the LRC assay. Precision in the precipitation step and with LRC cholesterol analysis was slightly better by the dextran sulfate–Mg2+ procedure than by the heparin–Mn2+ procedure. CVs were 2.4 and 2.9% for the two pools by the former method and 3.9 and 3.0% by the latter procedure.

The dextran sulfate–Mg2+ procedure was compared with the LRC heparin–Mn2+ method on 199 routine specimens submitted to our laboratory for lipoprotein quantitation. Many of the patients from whom these specimens were drawn were hyperlipidemic, as indicated by the lipid distributions (mean ±SD, and range, in mg/L): total cholesterol 2533 ± 664, 1240–5880; total triglycerides 2213 ± 2982, 100–24 000. HDL cholesterol results obtained by the two precipitation methods are illustrated in Figure 2. The correlation coefficient of 0.980, slope of 0.955, and the y-intercept of 8.91 mg/L indicate the relationship between the two methods. Cholesterol in the dextran sulfate–Mg2+ supernates averaged 450.4 mg/L, as compared with 463.5 mg/L in heparin–Mn2+ supernates, an average difference of 13.1 mg/L with a paired SD of 27.2 mg/L, which indicates a relationship similar to that presented in Table 1. Of 199 heparin–Mn2+ supernates, 100 (50%) contained measurable apoB (average, 6.8 mg of apoB-associated cholesterol per liter), whereas 41 (21%) of the dextran sulfate–Mg2+ supernates contained apoB-associated cholesterol (average 1.3 mg/L). The AQ6 control pool, precipitated in duplicate on 25 days with these specimens, exhibited a between-run CV of 2.7% vs 3.8% for the heparin–Mn2+ procedure under the same conditions.

Of 170 of these specimens, which were initially precipitated from whole plasma, 68 (40%) had turbid supernates by the heparin–Mn2+ procedure. The mean plasma triglyceride value in specimens that exhibited turbid heparin–Mn2+ supernates was 4253 mg/L. With dextran sulfate–Mg2+ precipitation, 35 specimens (20.6%) exhibited supernate turbidity; the plasma triglyceride values in these specimens averaged 5227 mg/L. Therefore, the dextran sulfate procedure gave better sedimentation of the complexed VLDL/LDL lipoproteins than did the standard heparin–Mn2+ procedure.

Turbidity in supernates results from incomplete sedimentation of the insoluble lipoprotein complex. Generally associated with hypertriglyceridemic specimens, it occurs when the density of the lipoprotein complex is near that of the

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**Table 2. Analytical Performance on Control Materials**

<table>
<thead>
<tr>
<th>Cholesterol concn, mean ± SD, mg/L (and CV, %)</th>
<th>LRC cholesterol</th>
<th>Enzymic cholesterol</th>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>519 ± 6.4 (1.2)</td>
<td>517 ± 11 (2.2)</td>
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<tr>
<td>HDL cholesterol</td>
<td></td>
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<tr>
<td>Separated with</td>
<td></td>
<td></td>
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<tr>
<td>In serum (AQ 6)</td>
<td>437 ± 17 (3.9)</td>
<td>414 ± 10 (2.4)</td>
</tr>
<tr>
<td>In plasma (in-house)</td>
<td>562 ± 17 (3.0)</td>
<td>539 ± 16 (2.9)</td>
</tr>
</tbody>
</table>

* LRC, Lipid Research Clinics assay with Liebermann–Burchard reagent (5, 23); enzymic, the enzymic assay of Cooper et al. (24) as described in Procedure: 15 determinations each in separate analytical runs performed over a three-month period. * Determined in low total-cholesterol pooled serum (MQ3).
solution; this precludes sedimentation under the usual conditions of low-speed centrifugation. In samples with very high triglyceride values the insoluble complex may actually form a layer at the top of the solution. Cholesterol analysis in a turbid supernate would substantially overestimate HDL because of the presence of LDL/VLDL. The nonsettling material in a turbid supernate can be conveniently removed by an ultrafiltration procedure previously described for heparin–Mn2+ supernates (12).

To test this clearing procedure on dextran sulfate–Mg2+ supernates, 10 normolipidemic specimens (3.0 mL each) were treated with 0.3 mL of the combined dextran sulfate–Mg2+ solution described above. Half of each treated specimen was subjected to centrifugation at 1500 × g for 30 min at 4 °C to produce a clear supernate. The other half was subjected to ultrafiltration. Cholesterol in the filtrates averaged 456 mg/L and in the supernates 473 mg/L. All filtrates were free of apoB, indicating complete removal of LDL/VLDL. As a further test of the method, routine hypertriglyceridemic specimens that gave turbid supernates with dextran sulfate and Mg2+ were subjected to ultrafiltration. Of 25 turbid supernates so treated, filtrate cholesterol values averaged 370 g/L. The same specimens were also precipitated by the heparin–Mn2+ method with filtration or dilution of turbid supernates as necessary; heparin–Mn2+ values averaged 382 mg/L.

Alternatively, dextran sulfate–Mg2+ supernates that are turbid after the initial centrifugation can be cleared by dilution. Without separating the turbid supernate from any sediments material, simply add a volume of 0.15 mol/L NaCl solution equal to that of the initial sample and an additional proportionate volume of the combined dextran sulfate–Mg2+ solution, as described in Procedure. After thorough vortexmixing followed by centrifugation, the supernate is usually clear. To test this method, we added to each of 10 turbid dextran sulfate–Mg2+ supernates 1.0 mL of 0.15 mol/L NaCl and 0.1 mL of combined dextran sulfate–Mg2+ solution. Cholesterol values (after dilution correction) in the clear supernates averaged 381 mg/L, compared with 402 mg/L in the corresponding heparin–Mn2+ supernates. Therefore, this method also appears to give acceptable results.

Hypertriglyceridemic specimens that give turbid supernates can also be precipitated after removal of the VLDL and chylomicrons, if present, by ultracentrifugation. We subjected 26 specimens to ultracentrifugation for 20 h at d 1.006 (9). The infranatant fraction, free of VLDL, was subjected to precipitation by the heparin–Mn2+ and dextran sulfate–Mg2+ methods. The dextran sulfate–Mg2+ supernates averaged 428 mg/L, compared with 438 mg/L for the heparin–Mn2+ supernates.

The choice of reagents and separation conditions for the dextran sulfate–Mg2+ method were based on results of a series of experiments to determine the effect of dextran sulfate molecular mass and concentration and Mg2+ concentration on lipoprotein precipitation. In Figure 3 lipoprotein precipitation, as a function of molecular size and concentrations of the dextran sulfate and Mg2+ concentration, is compared with precipitation by heparin and Mn2+. In general, the larger the dextran sulfate and the higher the concentrations of dextran sulfate and (or) Mg2+, the more lipoprotein was precipitated. The titration curves for lipoprotein precipitation with dextran sulfate of M, 50 000 at concentrations of either 0.45 or 0.90 g/L were similar to that with heparin and Mn2+. By contrast, dextran sulfate of M, 15 000 produced less lipoprotein precipitation at all concentrations, whereas dextran sulfate of M, 500 000 precipitated more lipoprotein at equivalent concentrations.

Experiments were performed to determine the effects of solution ionic strength and pH on lipoprotein solubility with the dextran sulfate method. Three each of normolipidemic, hypercholesterolemic, and hypertriglyceridemic specimens were precipitated by dextran sulfate (M, 50 000) and Mg2+ at the same final concentrations as those given in Procedure but with combined reagent solution pH ranging from 6.0 to 8.0. Results suggested that within the pH range 6.5–7.0 lipo-

Fig. 2. Cholesterol as measured by the Lipid Research Clinics assay in heparin–Mn2+ (x) and dextran sulfate–Mg2+ (y) supernates of 199 routine specimens. The dotted line indicates y = x; the solid line illustrates the relationship between two precipitation methods by linear regression: slope 0.955, y-intercept 8.9 mg/L, r = 0.980.

Fig. 3. Lipoprotein precipitation by dextran sulfate and Mg2+ as a function of molecular mass and reagent concentration, as compared with precipitation by heparin and Mn2+ (O), heparin at 1.3 g/L (x) final concentration with Mg2+ concentration as indicated. Dextran sulfate of M, 50 000 at 0.45 g/L (A), 0.91 g/L (B), and 4.55 g/L (C); dextran sulfate of M, 15 000 at 0.91 g/L (A); and dextran sulfate of M, 500 000 at 0.91 g/L (C), all with Mg2+ at the indicated concentrations.
protein precipitation was constant, with mean cholesterol values between 414 and 416 mg/L. At higher or lower pH, lipoprotein precipitation was slightly less; mean cholesterol values were 427 mg/L at pH 6.0, 425 mg/L at pH 7.5, and 432 mg/L at pH 8.0. Virtually all of this pH dependence was observed in the three hypertriglyceridemic specimens, however. In the normolipidemic and hypercholesterolemic specimens, supernatant cholesterol values did not vary significantly over pH 6.0–8.0. On the basis of these results we recommend adjusting the precipitant reagent to neutral pH.

The ionic strength of the precipitant reagent solution influenced lipoprotein precipitation. The higher the ionic strength, the less the tendency for lipoprotein precipitation. When the dextran sulfate solution was prepared in 0.15 mol/L NaCl solution rather than water, supernatant cholesterol values averaged approximately 15 mg/L (3%) higher. Therefore, the solutions should be prepared in high-quality water to obtain the indicated level of accuracy under these conditions. Also, reagent-grade MgCl₂·6 H₂O and dextran sulfate of high quality should be used.

Lipoprotein separation by some precipitation methods is reportedly a function of the temperature before and during centrifugation. This does not appear to be the case with the dextran sulfate–Mg²⁺ method. Values for supernatant cholesterol were not significantly different (≤6 mg/L) when the incubation and centrifugation steps were done at either 4 or 23 °C. These results suggest the incubation step can be conveniently performed at ambient temperatures, and centrifugation need not be performed in a refrigerated centrifuge. However, where possible, centrifugation at a consistent temperature in a refrigerated centrifuge would be preferable.

The stability of the combined precipitant reagent was evaluated over a period of four months. Combined solutions prepared at weekly intervals and containing preservative as described in the Materials and Methods section were accumulated over this period and stored in a refrigerator at 4 °C. Each of the solutions was then used to prepare normolipidemic, hypercholesterolemic, and hypertriglyceridemic specimens. Supernatant cholesterol results did not exhibit any trends that would indicate deterioration of the solutions over the four-month period. The means of duplicate determinations on the three pools were all within 20 mg/L.

Mn²⁺ produces a positive interference with many of the enzymic cholesterol assays (13) but does not interfere with Liebermann–Burchard methods. We performed experiments to discover whether the dextran sulfate and Mg²⁺ reagents described here produced interference with either the LRC Liebermann–Burchard or the enzymic cholesterol assay. Plasma, freed of LDL and VLDL by ultracentrifugation at d = 1.063 g/L, was dialyzed against 0.15 mol/L NaCl. The combined dextran sulfate–Mg²⁺ precipitant solution was added in a volume of 0.1 mL/mL of specimen to give final concentrations of 0.06 g of dextran sulfate and 46 mmol of Mg²⁺ per liter. A control specimen was prepared by diluting the same LDL/VLDL-free plasma with water at 0.1 mL/mL. Cholesterol was analyzed in each solution 10 times by both the enzymic and the LRC assays as described. Cholesterol content of the HDL solution containing dextran sulfate–Mg²⁺ averaged 325 and 322 mg/L by the LRC and enzymic assays, respectively, while the control solution averaged 326 and 323 mg/L by the two assays. Therefore, the dextran sulfate and Mg²⁺ reagents described here appear not to interfere with either the LRC Liebermann–Burchard or this enzymic cholesterol assay. Dextran sulfate of Mn 500 000 has been reported, however, to interfere with another enzymic cholesterol assay (38). The interference may be due to aggregation and partial inhibition of some pancreatic cholesterol ester hydrolases by dextran sulfate (personal communication from Garry Handelman).

### Table 3. Accuracy of the Enzymic Cholesterol Assay with Calibration by Primary or Secondary Standards

<table>
<thead>
<tr>
<th>LRC b cholesterol, mg/L</th>
<th>Enzymic cholesterol, a mg/L</th>
<th>Primary c standard</th>
<th>Lipid fraction d control serum</th>
<th>Frozen plasma e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay materials (each n = 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AQ6</td>
<td>437</td>
<td>378</td>
<td>414</td>
<td>407</td>
</tr>
<tr>
<td>Plasma pool</td>
<td>523</td>
<td>490</td>
<td>538</td>
<td>526</td>
</tr>
<tr>
<td>Mean</td>
<td>567</td>
<td>505</td>
<td>553</td>
<td>543</td>
</tr>
<tr>
<td>Dextran sulfate–Mg²⁺ supernates (n = 26)</td>
<td>488</td>
<td>431</td>
<td>479</td>
<td>468</td>
</tr>
</tbody>
</table>

* Enzymic assay as described in Procedure. b Assay by Lipid Research Clinics Method with Liebermann–Burchard reagent (9, 10). Accuracy comparable with reference method (29). c Precisely 500 mg/L; Biodynamics/bmc, Indianapolis, IN 46250. d Hyland Diagnostics, Deerfield, IL 60015, diluted twofold as described in Procedure. e In-house material of donor plasma diluted fivefold as described in Procedure.

Accuracy in cholesterol quantitation is in large measure a function of the calibration technique. Table 3 illustrates accuracy results for a series of control materials analyzed by the LRC Liebermann–Burchard method and by the enzymic method, calibrated with one primary standard and two types of secondary standard. The primary standard contained unesterified cholesterol in water solution with detergent. Use of the primary standard resulted in values for specimens that were low by approximately 10%, even though rate curves demonstrated the reactions had reached equilibrium during the 20-min incubation. Calibration of the enzymic assay with a commercial lyophilized human serum (Lipid Fraction Control Serum) or human plasma (in-house) gave results similar to the LRC values. The Lipid Fraction Control Serum is commercially available with a target value established by the modified Abell–Kendall reference procedure (29). The Lipid Standardization Laboratory of the CDC kindly provided a reference target value for the in-house pool. In other experiments, not shown, the reaction rate in this enzymic assay was considerably slower for the primary standard of cholesterol with detergent in water solution than for the two secondary calibrators, which had reaction rates similar to those of dextran sulfate–Mg²⁺ supernates.

These results are consistent with reports of other investigators (25–27). The observed underestimation of specimen cholesterol has been attributed, at least in part, to incomplete hydrolysis of esterified cholesterol in specimens by cholesterol ester hydrolase in the enzymic reagent. Approximately 70–80% of the cholesterol in specimens is present in the esterified form, which, without hydrolysis, is not a suitable substrate for the cholesterol oxidase. Hence, cholesterol in specimens would be underestimated to the extent of incomplete hydrolysis in relation to a primary standard of free cholesterol, which does not require hydrolysis. Also, the alcohol or detergent required to solubilize unesterified cholesterol in a primary standard may interfere with the enzyme activity (28), producing reaction rates for the primary standards that differ from those for serum or plasma specimens.

Therefore, calibration with a secondary standard may be more appropriate with the enzymic cholesterol assays. The secondary standard should resemble specimens in the proportion of esterified and free cholesterol as well as matrix properties. In addition, a reliable target value is essential for obtaining accurate results with a secondary standard. In some applications, the use of both a primary and a secondary cali-
brator might be warranted. Provided the secondary calibrator fell within a certain accuracy range in relation to a primary standard, results for unknown specimens could be calculated in relation to the reference value for the secondary standard. This approach to calibration, for example, has resulted in excellent accuracy and precision with the LRC cholesterol method (23).

Evaluators’ Results

Evaluators E.E.B. and B.S. compared the present dextran sulfate–Mg2+ method with a commercial method involving a sodium phoshphotungstate solution buffered at pH 5.7 (DMA Isopol; Data Medical Associates, Arlington, TX 76011); enzymic cholesterol assay was with the Multistat III Micro Centrifugal Analyzer (Instrumentation Laboratory, Lexington, MA 02173). The cholesterol assay was calibrated with an aqueous standard provided with the Instrumentation Laboratory kit but diluted threefold with water. Analysis of 33 serum specimens (20 in duplicate) yielded a mean value of 518 mg/L for the dextran sulfate supernatant, compared with 514 mg/L for the DMA Isopol supernatant. The relationship by linear regression between the two methods was as follows: dextran sulfate–Mg2+ = 0.880 DMA Isopol + 65.0 mg/L (r = 0.967). Among-day CVs of 3–6% were obtained on control materials by the dextran sulfate–Mg2+ method with enzymic cholesterol assay (Table 4). Application of this method to paired serum and EDTA-plasma specimens from 20 subjects produced a mean and SD (mg/L) of 394 ± 88 for serum and 397 ± 87 for plasma specimens.

Evaluators E.J.S., D.C., M.H., and H.B.B. compared this dextran sulfate–Mg2+ procedure with a commercial method involving dextran sulfate of Mr 500 000 (Gilford Diagnostics, Cleveland, OH 44135). Cholesterol assay was by the Gilford enzymic procedure. On 50 EDTA-plasma specimens, cholesterol in supernatant by the Gilford method averaged 379 mg/L and by the present dextran sulfate method 389 mg/L. The agreement between the two methods was indicated by the linear regression relationship: dextran sulfate–Mg2+ = 1.04 Gilford – 4.68 mg/L (r = 0.991). Supernates of both methods were free of apo B as determined by radial immunodiffusion. Apo A-I in the supernates obtained by the present method with the lower Mr, dextran sulfate averaged 1104 mg/L vs 1142 mg/L in Gilford supernatant, compared with 1176 mg/L in the same specimens before precipitation. Use of the lower Mr, dextran sulfate appeared to give better separation for hypertriglyceridemic specimens.

These Evaluators also reported severe interference with the Gilford enzymic cholesterol assay by heparin–Mn2+ supernatants (13). Analysis of 20 heparin–Mn2+ (92 mmol/L) supernates gave a mean cholesterol value of 1620 mg/L by the Gilford enzymic assay, much higher than the mean value of 440 mg/L obtained by the LRC Liebermann–Burchard assay (9) of the same supernates.

Evaluators D.A.W. and J.H. compared the dextran sulfate–Mg2+ precipitation method with a tentative reference method for HDL (unpublished) that involves heparin–Mn2+ precipitation of the VLDL-free fraction of plasma obtained after ultracentrifugation. This approach eliminates the problem of incomplete sedimentation, encountered especially with hypertriglyceridemic specimens. Supernates of both methods were analyzed by the modified Abell–Kendall reference method (29) and the dextran sulfate supernatants were also analyzed by an enzymic cholesterol assay performed essentially as described in the Procedure (24). On serum control materials, cholesterol values obtained by enzymic assay of the dextran sulfate–Mg2+ supernatants averaged within 12 mg/L (Table 4) of the heparin–Mn2+ supernatants analyzed by the modified Abell–Kendall method. The dextran sulfate–Mg2+ precipitation method with enzymic cholesterol assay gave among-day CVs of 1.8 to 3.4%. By comparison, cholesterol in dextran sulfate–Mg2+ supernatants analyzed by the modified

| Table 5. Population Distributions of Plasma HDL Cholesterol, mg/L |
|----------------------|-----------------|-----------------|-----------------|-----------------|
|                     | Females c       |                  |                 |                 |
| Age, yr             | 5th | 95th | 5th | 95th |
| 5-9                 | 555 | 380 | 740 | 532 | 360 | 730 |
| 10-14               | 549 | 370 | 740 | 522 | 370 | 700 |
| 15-19               | 481 | 300 | 630 | 522 | 350 | 740 |
| 20-24               | 454 | 300 | 630 | 533 | 330 | 790 |
| 25-29               | 447 | 310 | 630 | 560 | 370 | 830 |
| 30-34               | 455 | 280 | 630 | 561 | 360 | 770 |
| 35-39               | 434 | 290 | 620 | 550 | 340 | 820 |
| 40-44               | 443 | 270 | 670 | 578 | 340 | 880 |
| 45-49               | 454 | 300 | 640 | 594 | 340 | 870 |
| 50-54               | 441 | 280 | 630 | 620 | 370 | 920 |
| 55-59               | 476 | 280 | 710 | 622 | 370 | 910 |
| 60-64               | 515 | 300 | 740 | 638 | 380 | 920 |
| 65-69               | 511 | 300 | 780 | 633 | 350 | 980 |
| 70+                 | 505 | 310 | 750 | 607 | 330 | 920 |

* Data from Lipid Research Clinics Program for 10 North American populations (40). † Total of 3546 white males. ‡ Total of 3382 white females.

| Table 4. Evaluators: Analytical Performance of Dextran Sulfate–Mg2+ Method on Control Materials |
|---------------------------------------------|-----------------|-----------------|-----------------|-----------------|
| Evaluator Pool  | Target value, mg/L | Mean | Bias | SD | CV, % |
| E.E.B. and B.S. | In house | 294 | 18 | 253 | −41 | 14.6 | 5.8 |
| CDC-65b         | 508 | 18 | 519 | 11 | 15.7 | 3.0 |
| CDC-67          | 595 | 18 | 623 | 28 | 23.0 | 3.7 |
| CDC-69          | 442 | 20 | 439 | −3 | 8.0 | 1.8 |
| D.A.W. and J.H. | CDC-AQ7 | 261 | 20 | 273 | 12 | 6.1 | 2.2 |
| CDC-35          | 508 | 20 | 496 | −12 | 9.0 | 1.8 |
| CDC-67          | 595 | 20 | 593 | −2 | 20.0 | 3.4 |

* Heparin–Mn2+ precipitation and modified Abell–Kendall (29) cholesterol assay by Clinical Chemistry Standardization Section CDC. † Commercial enzymic cholesterol assay and calibrator on Multistat III Micro Centrifugal Analyzer (Instrumentation Laboratory). ‡ Enzymic cholesterol assay with secondary serum calibrator (24).
Table 6. Relative Risk of Coronary Heart Disease as a Function of HDL Cholesterol (Average Risk = 1.00)

<table>
<thead>
<tr>
<th>HDL cholesterol, mg/L</th>
<th>Women</th>
<th>Men</th>
</tr>
</thead>
<tbody>
<tr>
<td>300</td>
<td>1.00</td>
<td>1.82</td>
</tr>
<tr>
<td>350</td>
<td>1.00</td>
<td>1.49</td>
</tr>
<tr>
<td>400</td>
<td>1.94</td>
<td>1.22</td>
</tr>
<tr>
<td>450</td>
<td>1.55</td>
<td>1.00</td>
</tr>
<tr>
<td>500</td>
<td>1.25</td>
<td>0.82</td>
</tr>
<tr>
<td>550</td>
<td>1.00</td>
<td>0.67</td>
</tr>
<tr>
<td>600</td>
<td>0.80</td>
<td>0.55</td>
</tr>
<tr>
<td>650</td>
<td>0.64</td>
<td>0.45</td>
</tr>
<tr>
<td>700</td>
<td>0.52</td>
<td>—</td>
</tr>
</tbody>
</table>

Risk estimators from Framingham Heart Study (41).

Abell-Kendall assay averaged 423, 278, 480, and 575 mg/L for pools AQT, CDC 35, CDC 67, and CDC 69, respectively, all within 28 mg/L of the values for heparin–Mn²⁺ supernates by the same cholesterol assay. A fresh plasma specimen analyzed 20 times with enzymatic assay of dextran sulfate–Mg²⁺ supernates averaged 668 mg of cholesterol per liter, compared with 727 mg/L with the modified Abell-Kendall assay of heparin–Mn²⁺ supernates.

Interpretation

The Lipid Research Clinics Program has reported population distributions of lipids and lipoproteins including HDL cholesterol for 10 North American areas (39, 40). HDL cholesterol quantitation was by the LRC Liebermann-Burchard reagent method after heparin–Mn²⁺ precipitation (9). Mean, 5th, and 95th percentile values by sex and five-year age range are presented in Table 5. The population mean for HDL cholesterol was approximately 550 mg/L in boys, decreased to approximately 450 mg/L with puberty, and remained nearly constant until age 55, after which concentrations increased slightly. In females the population means were approximately 550 mg/L through childhood and early adulthood, after which values gradually increased. HDL cholesterol values by the present dextran sulfate–Mg²⁺ method would be expected to average approximately 10 mg/L lower than those values listed in Table 5. The statistical risk of coronary heart disease associated with HDL cholesterol values was estimated in 50–80-year-olds in the Framingham Heart Study Population (41). Relative risk multipliers from this study are given in Table 6. HDL cholesterol values on patients can be reported with the appropriate reference range and the estimated level of relative risk (42).

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