Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure

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Abstract Studies in recent years have suggested that measurement of high density lipoprotein (HDL) subclasses may provide significant information beyond that provided by measurement of total HDL. However, conventional methodology for separation of HDL subclasses involves various types of ultracentrifugation that are time-consuming, costly, and not suitable for many clinical or epidemiological studies. We have developed a simple precipitation method for the separation of HDL subclasses in human plasma. After precipitation of apoB-containing lipoproteins with heparin-Mn^{2+}, HDL_2 is precipitated by addition of dextran sulfate (mol wt 15,000). HDL_2 cholesterol is calculated as the difference between total HDL cholesterol (heparin-Mn^{2+} supernatant) and HDL_3 cholesterol (dextran sulfate supernatant). HDL_2 determined by this method correlated well with results obtained by preparative ultracentrifugation (n = 295, r = 0.91) and analytical ultracentrifugation (n = 17, r = 0.92). In the original method the final concentration of dextran sulfate was 0.09 g/dl; however further studies indicated that 0.13 g/dl is a more suitable concentration. The chemical compositions of HDL_2 and HDL_3 isolated by the precipitation method were very similar to those of HDL_2 and HDL_3 isolated by preparative ultracentrifugation. The concentration of HDL_2 cholesterol was 40% higher in normal women than in normal men. In men with coronary heart disease, total HDL was decreased by 28%, HDL_2 was decreased by 44%, while HDL_3 was 19% lower. A similar pattern of change was found in women with coronary heart disease. In other conditions where total HDL either increased or decreased, the change in HDL_2 was always proportionately greater than the change in total HDL. HDL_3 showed relatively less change, and in some instances its concentration was unchanged. Thus HDL_2 is the more variable component and may be a more meaningful index of altered HDL metabolism.—**Gidez, L. I., G. J. Miller, M. Burstein, S. Slagle, and H. A. Eder.** Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. *J. Lipid Res.* 1982. **23:** 1206–1223.

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The association between HDL and coronary heart disease was noted in 1951 by Barr, Russ, and Eder (1).

Interest in this relationship has been intensified during the past several years, due largely to the publication of Miller and Miller in 1975 (2). Epidemiologic studies have convincingly demonstrated an inverse correlation between the concentration of HDL cholesterol and the occurrence of coronary heart disease (3–5). Levels of HDL have been shown to be influenced by many factors including sex (6), exercise (7), diet (8, 9), and the use of various medications (10).

The fact that HDL is heterogeneous in terms of hydrated density was first recognized by DeLalla, Elliott, and Gofman (11) in 1954. By analytical ultracentrifugation they separated three fractions, HDL_1, HDL_2, and HDL_3. In normal subjects, HDL_1 was a relatively minor component; the major components were HDL_2 and HDL_3 which could be separated by centrifugation at d 1.125 g/ml. In healthy individuals, HDL_2 was 50% higher in women than in men, whereas HDL_1 and HDL_3 did not differ significantly between sexes. Using the same technique, Gofman, Young, and Tandy (12) studied the incidence of coronary heart disease in a population followed for 10 years. In the group that developed coronary heart disease, HDL_1 was unaltered, but HDL_2 was 32% lower and HDL_3 was 8% lower than in the healthy controls. Subsequent studies have shown that when HDL concentration varies it is largely due to variation in HDL_2 with less change in HDL_3. This relationship has been demonstrated in patients engaged in strenuous exercise (13), patients treated with nicotinic acid and clofibrate (8), and diabetic subjects treated

with insulin (14). These studies utilized various ultra-centrifugation techniques for measurement of HDL subclasses including analytical ultracentrifugation. It thus appears from these various studies that measurement of HDL subclasses may provide significant information beyond that provided by measurement of total HDL.

High density lipoprotein subclasses have been separated and analyzed by a variety of ultracentrifugal techniques. These include: a) analytical ultracentrifugation of HDL in the density interval 1.063–1.21 g/ml (15); b) preparative ultracentrifugation at d 1.063–1.125 g/ml for HDL_2 and d 1.125–1.21 g/ml for HDL_3 (e.g., 16, 17); c) density gradient ultracentrifugation of HDL (18, 19); and d) rate zonal ultracentrifugation (20–22). These ultracentrifugal methods require special equipment, they are time-consuming and expensive, and are not suitable for routine analysis of HDL subclasses in clinical or epidemiological investigations.

In 1970 Burstein, Scholnick, and Morfin (23) described a procedure for separating lipoproteins by precipitation with dextran sulfate (DS), mol wt 15,000, and $MnCl_2$. At low concentrations of these reagents, apoB-containing lipoproteins were precipitated from serum. HDL could be completely precipitated from the apoB-free supernatant by addition of DS and $MnCl_2$ to final concentrations of 0.65 g/dl and 0.2 M, respectively; partial precipitation of HDL occurred at respective concentrations of 0.1 g/dl and 0.1 M. These observations suggested that HDL_2 and HDL_3 might be separated by DS and $MnCl_2$ at concentrations intermediate between those that will not precipitate HDL at all, and those that will precipitate HDL maximally. Inasmuch as an insoluble lipoprotein-polyanion complex forms more readily when the ratio of protein:lipid in the lipoprotein is low (24), HDL_2, which has a lower protein:lipid ratio than HDL_3, would be expected to precipitate under conditions that do not precipitate HDL_3. On the basis of these observations we developed a method for the separation of the HDL_2 and HDL_3 subclasses. We found the concentration of DS to be critical. The optimal concentration was determined in a series of experiments in which the concentration of HDL_2 was estimated at various concentrations of DS. The procedure was validated in a series of studies in which HDL_2 levels determined by the precipitation technique at a given concentration of DS were compared to and correlated with HDL_2 levels determined by ultracentrifugation. HDL_2 cholesterol concentrations determined by the precipitation procedure were similar to those obtained by ultracentrifugation in healthy adults and in patients with coronary heart disease, hyperlipoproteinemia, and diabetes. In addition, the compositions of HDL subclasses obtained by the precipitation method were similar to those of the corresponding fractions prepared by ultracentrifugation.

MATERIALS AND METHODS

Blood samples

Blood samples were obtained from students, faculty, and employees of the Albert Einstein College of Medicine, and from patients of the Life Extension Institute in New York. Blood from hyperlipidemic and diabetic subjects was obtained from outpatients attending the Lipid and Diabetes Clinics. Plasma from patients with proven coronary vessel obstruction was obtained at the time of cardiac catheterization. All subjects had been fasting for 12–14 hr. Blood was drawn from an antecubital vein into Vacutainer tubes containing 1.0–1.4 mg of dry EDTA per ml of blood. Plasma was separated immediately.

Materials

Manganese chloride · $4H_2O$ (AR) was purchased from Mallinckrodt; Lipo-Hepin® (heparin sodium, 40,000 USP units/ml) was from Riker Laboratories, Northridge, CA; and dextran sulfate (mol wt 15,000) was obtained from Sochibo, 3–5 rue Carnot, 92100 Boulogne sur Seine, France (Telex: Sochibo, Paris 270146F). All other reagents were of analytical grade. The reagent used to precipitate apoB-containing lipoproteins consisted of 0.06 vol of the heparin sodium and 1.0 vol of 1.06 M $MnCl_2$. Solutions of dextran sulfate were made up in 0.15 M NaCl. Solutions of NaBr used in ultracentrifugation studies were made up in NaCl solution of d 1.0063 g/ml. Kits for the determination of triglycerides and free and total cholesterol were obtained from Dow Diagnostics, the Dow Chemical Company, Indianapolis.

Heparin-Mn^{2+} precipitation procedures

Lipoproteins of d < 1.063 g/ml and apoB-associated lipoproteins of d > 1.063 g/ml were precipitated from plasma by the Lipid Research Clinics procedure (25) as modified by Warnick and Albers (26). These procedures are based on the method of Burstein and Morfin (27). For determination of total HDL cholesterol, 0.1 vol of the heparin-$MnCl_2$ solution was added to 1.0 vol of plasma. This resulted in a final concentration of ca. 1.26 mg of heparin per ml and 0.091 M $MnCl_2$. After 10–20 min at room temperature, the samples were centrifuged for 1 hr at 2,700 rpm at 4°C (International Refrigerated Centrifuge, Model PR-2, #269 head).[4] Ali-

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[4] The procedure of Warnick and Albers (26) utilizes centrifugation at room temperature for 30 min; in the Lipid Research Clinics procedure (25) centrifugation is for 30 min at 4°C.

quots of the heparin-Mn^{2+} supernatant were taken for total HDL cholesterol determination and precipitation of "HDL_2". ApoB was never detected (Ouchterlony immunodiffusion) in randomly selected supernatants. Two ml of the heparin-Mn^{2+} supernatant was used for precipitation of "HDL_2", and 0.5 ml was used for determination of total HDL cholesterol.

Precipitation of "HDL_2"[5]

"HDL_2" was precipitated by addition of 0.1 vol of DS solution (usually 0.2 ml) to 1 vol (usually 2.0 ml) of the heparin-Mn^{2+} supernatant. Various final concentrations of DS (0.01–0.5 g/dl) were tested in the course of the studies (see Results). After thorough mixing, the sample was left at room temperature for 20 min, and then centrifuged for 30 min at 4°C at 2700 rpm (see above). An aliquot of the supernatant was used for cholesterol analysis ("HDL_3" cholesterol). The difference between total HDL cholesterol and "HDL_3" cholesterol represented "HDL_2" cholesterol.

Validation of the "HDL_2" precipitation procedure

Three types of ultracentrifugation studies were carried out in order to validate the precipitation procedure outlined above. Concentrations of "HDL_2" cholesterol were compared to: a) cholesterol levels in HDL_2 isolated by preparative ultracentrifugation (at d < 1.125 g/ml) of the same heparin-Mn^{2+} supernatant used to precipitate "HDL_2" (295 comparisons); b) cholesterol levels in HDL_2 isolated from plasma at d 1.063–1.125 g/ml after removal of lipoproteins by ultracentrifugation at d 1.063 g/ml (32 comparisons); and c) total HDL_2 concentration determined by analytical ultracentrifugation (17 comparisons). In a fourth study the compositions of "HDL_2" and "HDL_3" were compared to those of HDL_2 and HDL_3 (d 1.063–1.125 and d 1.125–1.216 g/ml, respectively) isolated from the same pools of plasma. All densities refer to solvent densities.

Ultracentrifugation of the heparin-Mn^{2+} supernatant

Six ml of heparin-Mn^{2+} supernatant was dialyzed for 48 hr against several changes of NaCl solution (d 1.0063 g/ml) containing 0.125% EDTA. Five ml of the dialyzed solution was then adjusted to d 1.125 g/ml with solid KBr and centrifuged in a Beckman 40.3 rotor for 40 hr at 105,000 g. The lipoproteins in the top 1.5 cm of the centrifuge tube were removed after tube slicing. Cholesterol concentration in the d < 1.125 and d

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[5] "HDL_2" refers to a subclass of HDL precipitated by dextran sulfate at low concentrations (0.09–0.13 g/dl); "HDL_3" refers to the HDL subclass not precipitated by dextran sulfate. HDL_2 and HDL_3 refer to ultracentrifugally separated HDL subclasses isolated at d 1.063–1.125 and 1.125–1.216 g/ml, respectively.

> 1.125 g/ml fractions, HDL_2 and HDL_3, respectively, were determined.

Sequential ultracentrifugation of plasma

In one study lipoproteins of the d > 1.063 g/ml fraction from 4 ml of plasma were used, instead of a heparin-Mn^{2+} supernatant, for isolation of the HDL subclasses. These ultracentrifugations were carried out in a Beckman 40.3 rotor at 105,000 g for 40 hr. Density adjustments were made by addition of solutions of NaBr (in d 1.0063 g/ml NaCl solution) of appropriate density, and densities were monitored by measurement of refractive index (28). Cholesterol was determined in the following fractions: d > 1.063 g/ml (total HDL), d 1.063–1.125 g/ml (HDL_2), and d > 1.125 g/ml (HDL_3). In this study all centrifugations were done in duplicate.

Analytical ultracentrifugation

Analytical ultracentrifugation of plasma samples was performed by Dr. Frank T. Lindgren (15) at the Donner Laboratory, University of California, Berkeley.

Composition of "HDL_2", "HDL_3", and lipoproteins of d 1.063–1.125 and d 1.125–1.216 g/ml

Lipoproteins of d 1.063–1.125 and 1.125–1.216 g/ml were isolated (usually from 32 ml of plasma) for chemical characterization and electrophoresis. All separations were carried out in Beckman Quick Seal polyallomer tubes centrifuged in a 60 Ti or 70 Ti rotor; densities were adjusted with solutions of NaBr as described above. After the HDL subclasses were washed by a second ultracentrifugation at their respective higher densities, they were dialyzed for 36–48 hr against multiple changes of 0.01% EDTA–0.025% sodium azide. The solutions were concentrated using Aquacide II-A (Calbiochem-Behring Corp.), and the volumes were adjusted to 5 or 10 ml with the EDTA-azide solution.

"HDL_2" was precipitated from two or three 10-ml volumes of heparin-Mn^{2+} supernatant (derived from 20 or 30 ml of the same plasma used for the ultracentrifugal isolation of HDL_2 and HDL_3 described above). In these studies DS was added to a final concentration of 0.13 g/dl. After thorough mixing, the samples were kept at room temperature for 20 min, and then centrifuged at 2,700 rpm at 4°C for 30 min. The supernatant (containing "HDL_3") was removed, and the surface of each pellet was washed twice by overlaying it with 3 ml of a solution of 0.083 M $MnCl_2$ and 0.13 g/dl DS in 0.15 M NaCl. The samples were centrifuged and the washes were discarded. To dissolve the "HDL_2"-dextran sulfate-Mn^{2+} complex, the pellet was dispersed, with the aid of a stirring rod, in 1–3 ml of 0.09 M

Na$_2$CO$_3$ in 0.15 M NaCl and left to stand 24 hr at 4°C with occasional gentle mixing. Insoluble material was removed by low speed centrifugation.[6] The yellow supernatant was dialyzed for 48 hr against several changes of 5% BaCl$_2$ in 0.125% EDTA and then against several changes of 1% BaCl$_2$–0.125% EDTA in 0.15 M NaCl, pH 7.4, for removal of heparin and DS (23). The precipitate that formed during dialysis was removed by centrifugation, and the clear supernatant was dialyzed for 48 hr against several changes of 0.15 M NaCl–0.125% EDTA to remove Ba^{2+} and any remaining Mn^{2+}. The density of the solution was adjusted to 1.216 g/ml with a solution of NaBr of appropriate density. The dextran sulfate supernatant, which contained "HDL$_3$", was dialyzed as described above for "HDL$_2$", and adjusted to d 1.216 g/ml with solid NaBr. The "HDL$_2$" and "HDL$_3$" were then centrifuged in a 40.3 rotor for 42–46 hr at 37,000 rpm at 15°C. The lipoproteins were washed by centrifugation at d 1.216 g/ml and dialyzed against EDTA-azide solution and concentrated as described above. Inasmuch as we wished to characterize the HDL subclass that precipitated with DS under the described conditions, the "HDL$_2$" was ultracentrifuged at d 1.216 g/ml rather than at d 1.125 g/ml, in order not to bias the results by removal of any d > 1.125 g/ml lipoproteins that could have precipitated with the "HDL$_2$".

Chemical analyses

The laboratory was standardized according to the criteria developed by the Lipid Research Clinics program. Total plasma cholesterol and triglyceride concentrations were determined with the Autoanalyzer II (25). Cholesterol analyses on HDL and HDL subclasses were carried out in duplicate or quadruplicate by the method of Abell et al. (29); absorbances were recorded and printed on a Micromedic Systems MS 2 Spectrophotometer. Analyses of control samples with cholesterol in the range of 28 to 70 mg/dl gave results that averaged 1.8% below the target values, with a range of −6.4% to +2.6%. Phospholipid in isolated HDL subclasses was determined by the method of Marinetti, Erbland, and Stotz (30) as modified by Norton and Autilio (31). Triglycerides and free and total cholesterol were determined enzymatically using kits from Dow Diagnostics; cholesteryl ester content was calculated by multiplying the esterified cholesterol (total–free) values by 1.68. Protein was determined by the method of Lowry et al. (32), using bovine serum albumin as a standard.

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[6] The "HDL$_2$" complex dissolves readily in the Na$_2$CO$_3$ solution; the insoluble material was MnCO$_3$. However, some samples also contained an insoluble protein, presumably fibrinogen, which is precipitated by dextran sulfate.

Electrophoresis

SDS gel electrophoresis was performed by the method of Swaney and Kuehl (33).

RESULTS

Optimal conditions for precipitation of "HDL$_2$"

The optimal concentration of DS required to precipitate "HDL$_2$" was determined in experiments in which the final concentration of DS in the heparin-Mn^{2+} supernatant was adjusted from 0 to 0.5 g/dl. Each precipitate of "HDL$_2$" was removed by centrifugation, and cholesterol in the supernatant was determined. As the DS concentration was increased, more lipoprotein was precipitated and the concentration of "HDL$_3$" decreased. Most of the precipitation of "HDL$_2$" occurred at a final concentration of approximately 0.1 g/dl; at higher concentrations there was little additional removal of cholesterol. Although each plasma sample yielded slightly different results, in all instances there appeared to be a break at about the same concentration of DS. On the basis of twelve such "titration curves" (data not shown),[7] a final concentration of 0.09 g/dl DS was initially selected. Similar studies were performed with two other preparations of dextran sulfate. With a high molecular weight (500,000) dextran sulfate, the slope of the precipitation curve was so great (data not shown) that very small differences in concentration of dextran sulfate could affect the results considerably, and the use of this reagent was not practical. On the other hand, dextran sulfate with a molecular weight of 3,000 was less effective in precipitating "HDL$_2$" and did not result in a well-defined precipitation curve. In all subsequent studies, only the dextran sulfate from Sochibo was used.[8]

The concentration of Mn^{2+} used with heparin was that found by Warnick and Albers (26) to be best suited for precipitation of apoB-containing lipoproteins, and lower concentrations of Mn^{2+} were not tested.[9]

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[7] Representative curves are shown in Ref. 34.

[8] Preliminary studies have suggested that dextran sulfate of molecular weight 12,000 (Accurate Chemical and Scientific Corp., 300 Shames Drive, Westbury, NY 11590) might be a suitable reagent. At the same final concentrations over the range of 0.09–0.15%, both dextran sulfate preparations (Sochibo and Accurate) resulted in similar "HDL$_3$" values when tested with the same heparin-Mn^{2+} supernatant. However, not enough comparisons were carried out to validate the use of this material.

[9] For total HDL procedures that utilize a lower (0.046 M) concentration of Mn^{2+}, appropriate amounts of MnCl$_2$ can be incorporated into the dextran sulfate solution in order to achieve the desired final concentration of the reagents. Inasmuch as 0.046 M Mn^{2+} may not precipitate all the apoB-containing lipoproteins (25), apparent HDL-cholesterol might be somewhat higher. On the assumption that the

TABLE 1. Effect of storage of plasma on concentrations of HDL and HDL subclasses

Storage Conditions	Number of Subjects	HDL	"HDL$_2$"[a]	"HDL$_3$"[a]
			cholesterol, mg/dl	
Fresh	7	39.6 ± 7.6[b]	8.8 ± 4.9	30.8 ± 4.0
4 Days, 4°C	7	40.5 ± 6.9	7.6 ± 4.8	32.9 ± 3.9
P		<0.05	<0.05	<0.025
Fresh	17	52.9 ± 16.5	14.8 ± 8.2	38.1 ± 10.1
7 Days, 4°C	17	48.5 ± 14.5	14.7 ± 8.4	33.8 ± 8.2
P		<0.001	NS	<0.001
Fresh	5	53.3 ± 7.4	20.5 ± 5.1	32.8 ± 2.9
7 Days, −20°C	5	52.3 ± 7.6	21.9 ± 6.2	30.3 ± 2.1
P		NS	NS	NS
Fresh	6	62.9 ± 19.0	20.9 ± 11.1	39.8 ± 10.5
30 Days, −20°C	6	63.5 ± 18.9	22.5 ± 11.3	39.0 ± 10.8
P		NS	NS	NS

[a] Analyses were performed at a dextran sulfate concentration of 0.09 g/dl.
[b] Mean ± SD.
NS, not significant.

Effect of storage of plasma on concentrations of cholesterol in HDL and HDL subclasses

For some clinical and epidemiological studies it may be necessary to store plasma samples until analyses can be conveniently carried out. Bachorik et al. (35) studied the effects of storage on HDL cholesterol and they reported that the concentration did not change significantly within the first 4 days of storage at 4–6°C. Curb, Overturf, and Harrist (36) have reported that there were statistically significant increases in HDL cholesterol in serum frozen at −80°C throughout a 12-month period. In the present study plasma samples were stored at 4°C for 4 or 7 days, and at −20°C for 7 or 30 days. All precipitations were carried out in duplicate or triplicate, and duplicate analyses of cholesterol were performed. The concentrations of cholesterol in HDL and HDL subclasses are shown in **Table 1.** There were small changes in plasma stored at 4°C for 4 days, but storage for 7 days resulted in highly significant decreases in total HDL cholesterol and "HDL$_3$" cholesterol. The "HDL$_2$" cholesterol was unchanged inasmuch as total HDL and "HDL$_3$" decreased to the same extent (ca. 4.4 mg/dl). However, in this group of 17 subjects, the range of differences for "HDL$_2$" was from −7.3 to +8.6 mg/dl. The mean change in concentrations of HDL, "HDL$_2$", and "HDL$_3$" in plasma stored at −20°C was 1.6 mg/dl or less (except for "HDL$_3$" in plasma stored for 7 days), but none of the changes were significant.

Linear regression analysis relating the magnitude of

change in the apparent total HDL cholesterol or "HDL$_3$" cholesterol concentration with the concentration in the fresh sample (no storage) was carried out in a manner similar to that described by Bachorik et al. (35). These investigators calculated "crossover concentrations" for HDL cholesterol. Fresh samples with low initial values tended to increase in value during storage, and samples with high initial values tended to decrease in concentration during storage. At an intermediate concentration ("crossover concentration") there was no change during storage. We observed a similar phenomenon. Crossover concentrations for HDL cholesterol in plasma stored at 4°C were 49 mg/dl and 24 mg/dl for storage for 4 days and 7 days, respectively. Corresponding concentrations calculated by Bachorik et al. (35) were 45 mg/dl and 47 mg/dl, respectively. For "HDL$_3$" cholesterol we found values similar to those for total HDL cholesterol, 51 mg/dl and 19 mg/dl for storage of plasma at 4°C for 4 and 7 days, respectively. There was an insufficient number of samples stored at −20°C for 7 or 30 days to determine crossover concentrations, but the difference in concentration between fresh and frozen samples was smaller than in samples stored at 4°C. On the basis of these data, the data in Table 1, and the results of Bachorik et al. (35), storage of plasma at −20°C for short periods is preferable to storage at 4°C when the double precipitation method is applied. In most instances we performed both precipitation steps within 3–6 hr of obtaining the blood samples; all other plasma samples were used within 24–36 hr (storage at 4°C) after the blood was drawn. Recently, Bachorik, Walker, and Kwiterovich (37) reported that changes in HDL cholesterol are minimized if heparin-Mn^{2+} supernatants, rather than unfractionated plasma, are stored
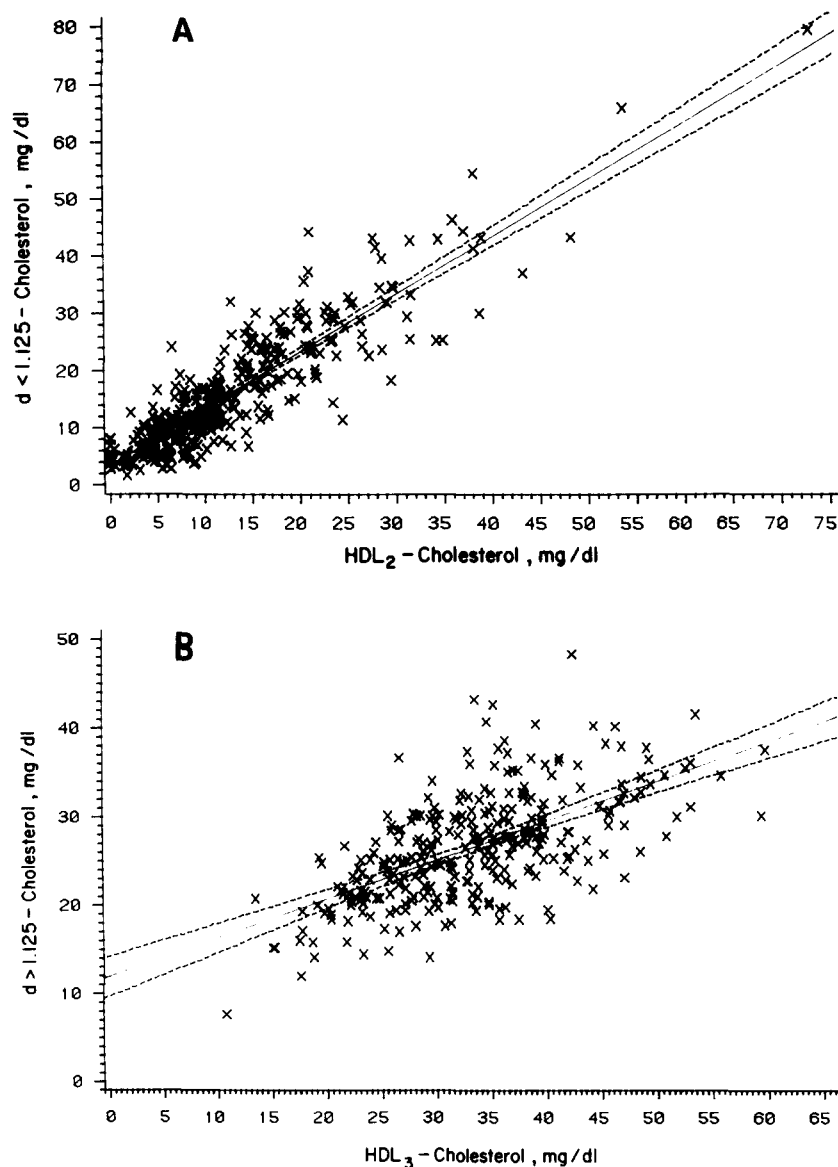
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lipoproteins containing apoB will be precipitated by the dextran sulfate, calculated "HDL$_2$" values might be slightly higher. In one series of experiments the final concentration of Mn^{2+} was 0.168 M, but this did not improve the separation of "HDL$_2$" and "HDL$_3$".

Fig. 1. Regression curves of d < 1.125 g/ml lipoprotein on "HDL$_2$" (A) and d > 1.125 g/ml lipoprotein on "HDL$_3$" (B). The dashed lines represent the 95% confidence limits of the regression lines. The ordinates are the concentration of cholesterol in the d < 1.125 (A) and d > 1.125 g/ml (B) fractions, respectively, of the heparin-Mn^{2+} supernatants. All "HDL$_2$" analyses were carried out at a final dextran sulfate concentration of 0.09 g/dl.

at −70°C. The effect of such storage of heparin-Mn^{2+} supernatants on "HDL$_2$" determinations remains to be determined.

Comparison of "HDL$_2$" concentrations determined by the precipitation procedure with those obtained by preparative ultracentrifugation

The validity of the precipitation procedure was tested in 295 plasma samples by comparing "HDL$_2$" cholesterol with the cholesterol concentration in a d < 1.125 g/ml fraction obtained by ultracentrifugation of the heparin-Mn^{2+} supernatant. Plasma was obtained from

normal males and females, ages 20–64 years, and male and female outpatients with coronary heart disease, diabetes, or type IIa or IV hyperlipoproteinemia. In this group of 295 subjects, precipitation of "HDL$_2$" was performed with a DS concentration of 0.09 g/dl.

Fig. 1A shows the computer-derived line for the regression of d < 1.125 g/ml cholesterol concentration on "HDL$_2$" cholesterol for the 295 subjects. The equation of the line is:

$$d < 1.125 \text{ g/ml chol} = 0.99 \times \text{``HDL}_2\text{'' chol} + 3.5$$

$$(r = 0.88; P < 0.001).$$

TABLE 2. Concentrations of HDL subclasses at different final dextran sulfate concentrations

Study	Subjects	HDL (Heparin-Mn²⁺ supernatant)	Dextran sulfate			
			0.09 g/dl	0.11 g/dl	0.13 g/dl	
			cholesterol, mg/dl			
			"HDL₂"			d < 1.125 g/ml
1	295	46.7 ± 16.1[a]	13.3 ± 9.8			16.7 ± 11.0[b]
1a	41	45.6 ± 14.7	12.6 ± 9.2	14.4 ± 10.3		16.7 ± 10.9[b]
2	32	48.1 ± 11.0	13.9 ± 7.4	15.4 ± 8.2	17.7 ± 7.8	19.2 ± 7.5[c,e]
			"HDL₃"			d > 1.125 g/ml
1	295		33.4 ± 8.8			26.4 ± 6.2
1a	41		32.9 ± 7.1	31.2 ± 6.5		27.7 ± 7.2[b]
2	32		34.2 ± 6.2	32.3 ± 6.0	30.4 ± 5.6	25.6 ± 4.7[d,e]

[a] Mean ± SD.
[b] Prepared from a heparin-Mn²⁺ supernatant.
[c] HDL₂ was prepared from plasma by sequential ultracentrifugation (d 1.063–1.125 g/ml).
[d] HDL₃ was prepared from plasma by sequential ultracentrifugation (d 1.125–1.216 g/ml).
[e] Total HDL cholesterol (d > 1.063 g/ml) was 49.6 ± 10.0 mg/dl.

The equations of regression lines (not shown) for males and females were, respectively:

$$d < 1.125 \text{ g/ml chol} = 0.96 \times \text{"HDL}_2\text{" chol} + 2.8$$

$$(r = 0.91; P < 0.001; N = 169);$$

$$d < 1.125 \text{ g/ml chol} = 0.94 \times \text{"HDL}_2\text{" chol} + 5.8$$

$$(r = 0.83; P < 0.001; N = 126).$$

For the regression of d > 1.125 g/ml cholesterol on "HDL₃" cholesterol (Fig. 1B), the spread of values was not as great. The equation of the line was:

$$d > 1.125 \text{ g/ml chol} = 0.43 \times \text{"HDL}_3\text{" chol} + 12.0$$

$$(r = 0.61; P < 0.001).$$

The equations of regression lines (not shown) for males and females were, respectively:

$$d > 1.125 \text{ g/ml chol} = 0.41 \times \text{"HDL}_3\text{" chol} + 12.8$$

$$(r = 0.58; P < 0.001; N = 169);$$

$$d > 1.125 \text{ g/ml chol} = 0.48 \times \text{"HDL}_3\text{" chol} + 10.1$$

$$(r = 0.62; P < 0.001; N = 126).$$

The regression line in Fig. 1A shows that "HDL₂" cholesterol is underestimated compared to the d > 1.125 g/ml cholesterol concentration. Inasmuch as the degree of precipitation of "HDL₂" is dependent on DS concentration, a series of analyses were carried out at concentrations greater than 0.09 g/dl in order to determine precipitation conditions that would result in "HDL₂" concentrations more closely matched to those obtained by ultracentrifugation. **Table 2** summarizes the results. Study 1 shows the mean values of HDL and

HDL subclasses of the 295 pairs of analyses used in Fig. 1. The data show a difference of 3.4 mg/dl between "HDL₂" and d < 1.125 g/ml cholesterol. In Study 1a, a subset of 41 of the 295 subjects, analyses were carried out at both 0.09 and 0.11 g/dl. The differences between the ultracentrifuge value and the "HDL₂" concentrations at 0.09 and 0.11 g/dl were 4.1 and 2.3 mg/dl, respectively. The "HDL₂" value obtained with DS at 0.11 g/dl (14.4 mg/dl) was significantly higher ($P < 0.001$) than the concentration at 0.09 g/dl (12.6 mg/dl).

In Studies 1 and 1a, the heparin-Mn²⁺ supernatant was used for ultracentrifugal isolation of HDL₂ in order to provide a common basis of comparison with results obtained by the precipitation method. However HDL₂ is more commonly isolated from serum or plasma by sequential ultracentrifugation. Study 2 in Table 2 shows the results of experiments in which concentrations of "HDL₂" determined at DS concentrations of 0.09, 0.11, and 0.13 g/dl were compared to concentrations of HDL₂ of d 1.063–1.125 g/ml in 32 subjects. Total HDL cholesterol (heparin-Mn²⁺ supernatant) was 48.1 ± 11.0 mg/dl compared to 49.6 ± 10.0 mg/dl for the lipoproteins of d > 1.063 g/ml isolated from plasma. "HDL₂" increased progressively as the concentration of dextran sulfate increased; its concentration at 0.13 g/dl dextran sulfate (17.7 mg/dl) most closely approximated the concentration of the d 1.063–1.125 g/ml HDL₂ (19.2 mg/dl), a difference of 1.5 mg/dl. Recoveries of the HDL subclasses, based on cholesterol determinations before and after ultracentrifugation, were 92.7 ± 7.6% from heparin-Mn²⁺ supernatants, and 90.6 ± 8.7% from plasma. The following regression equations were calculated for "HDL₂" at each of the three
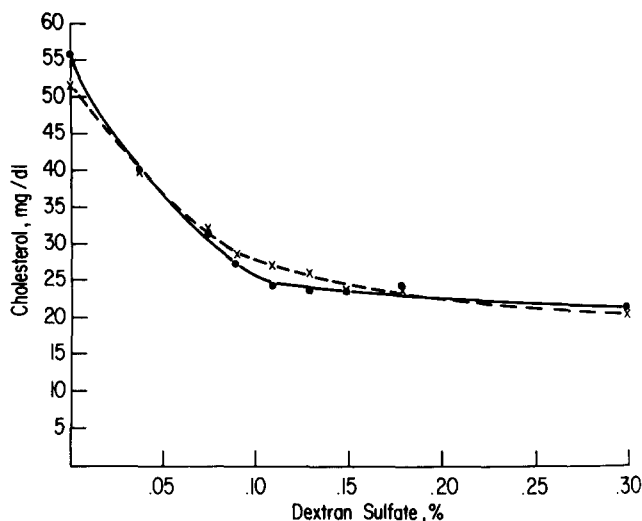
Fig. 2. Precipitation of HDL by increasing concentrations (final) of dextran sulfate in two pools of plasma. Values on the ordinate indicate the cholesterol concentration in the supernatant solution. The value at 0% dextran sulfate represents total HDL cholesterol (in the heparin-Mn^{2+} supernatant).

DS concentrations in Study 2:

d 1.063–1.125 g/ml HDL_2

$$= 0.934 \times \text{``}HDL_2\text{''}_{(0.09)} + 6.2; \quad r = 0.91;$$

$$= 0.850 \times \text{``}HDL_2\text{''}_{(0.11)} + 5.8; \quad r = 0.91;$$

$$= 0.913 \times \text{``}HDL_2\text{''}_{(0.13)} + 3.0; \quad r = 0.94.$$

It is apparent that the y intercept is lowest at 0.13 g/dl DS, suggesting that this concentration is the most suitable of the three. This was also suggested in titration experiments of plasma pools in which DS concentrations in the vicinity of 0.1 g/dl were examined in more detail than in previous similar experiments (34). The curves in **Fig. 2** suggest, indeed, that a DS concentration of 0.13 g/dl, or even 0.15 g/dl, may be optimal if one uses as an end-point a slope that approximates zero. Only small amounts of HDL cholesterol precipitated at higher concentrations of DS.

Comparison of "HDL_2" cholesterol determined by the precipitation procedure with HDL_2 determined in the analytical ultracentrifuge

Plasma samples from 17 normal male and female subjects were analyzed by analytical ultracentrifugation and by the precipitation procedure. **Fig. 3** shows regression lines for total HDL (Fig. 3a), HDL_2 (Fig. 3b), and HDL_3 (Fig. 3c), all measured in the analytical ultracentrifuge, compared to total HDL cholesterol, "HDL_2" cholesterol, and "HDL_3" cholesterol. The precipitations were carried out at 0.09 g/dl DS. The regression equations were:

$$HDL = 5.27 \times (HDL \text{ chol}) + 29.1, \quad r = 0.96;$$

$$HDL_2 = 5.54 \times (\text{``}HDL_2\text{''} \text{ chol}) + 0.32, \quad r = 0.91;$$

$$HDL_3 = 3.16 \times (\text{``}HDL_3\text{''} \text{ chol}) + 99.0, \quad r = 0.82.$$

Fig. 3c shows that "HDL_3" cholesterol and total HDL_3 cluster in a relatively narrow region.

Composition of HDL subclasses separated by either DS precipitation or ultracentrifugation

Table 3 shows the relative amounts of protein, cholesteryl esters, free cholesterol, phospholipid, and triglyceride in HDL subclasses isolated from the plasma of three normal subjects. In this study the "HDL_2" was precipitated at a final dextran sulfate concentration of 0.13 g/dl. The "HDL_2" was dissolved in 0.09 M Na_2CO_3 and treated as outlined above to remove Mn^{2+} and dextran sulfate prior to its concentration and iso-
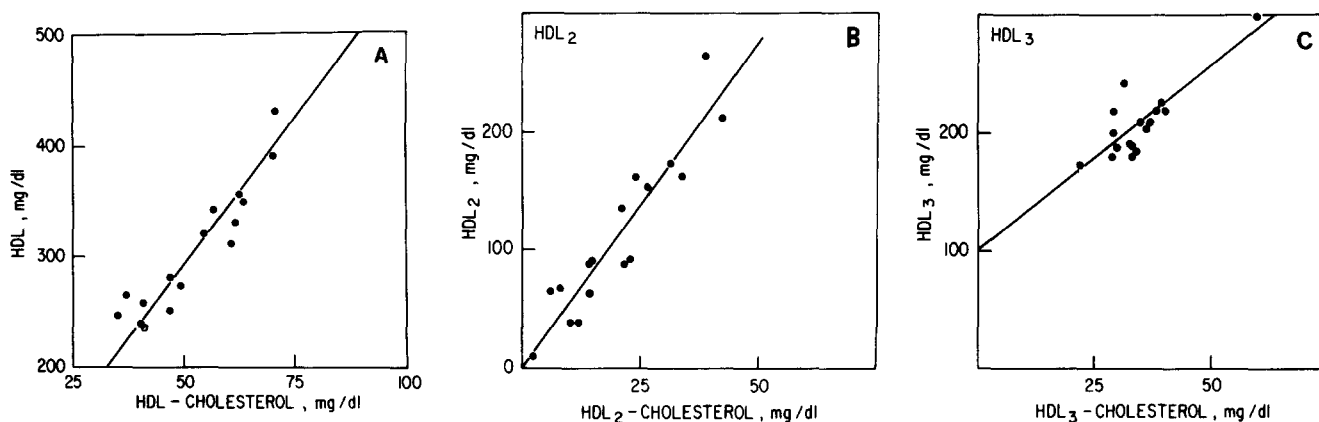

Fig. 3. Regression curves of concentrations of HDL, HDL_2, and HDL_3 determined by analytical ultracentrifugation on cholesterol concentrations in total HDL, "HDL_2", and "HDL_3", respectively, in A, B, and C. All "HDL_2" analyses were carried out at a final dextran sulfate concentration of 0.09 g/dl.

TABLE 3. Chemical composition of HDL subclasses in three normal subjects

	"HDL₂"[a]	HDL₂ 1.063–1.125 g/ml	"HDL₃"[a]	HDL₃ 1.125–1.216 g/ml
Protein	40.6 ± 2.3[b]	41.3 ± 2.4	49.8 ± 1.1	52.4 ± 1.3
Cholesteryl ester	22.5 ± 0.9	22.2 ± 0.6	19.2 ± 0.4	18.5 ± 1.1
Free cholesterol	2.8 ± 0.6	3.5 ± 0.4	2.0 ± 0.2	2.0 ± 0.2
Phospholipid	27.2 ± 1.7	26.1 ± 2.3	24.2 ± 1.2	21.6 ± 1.1
Triglyceride	6.9 ± 1.1	6.9 ± 1.6	4.7 ± 1.1	5.6 ± 1.4

	HDL_2[c]	HDL_3[c]	HDL_2[d]	HDL_3[d]	HDL_2[e]	HDL_3[e]	HDL_{2b}[f]	HDL_3[f]	HDL_2[g]	HDL_3[g]
Protein	38.0	48.8	41.0	55.0	44.4	53.0	37.0	54.5	46.2	60.9
Cholesteryl ester	22.2	17.7	16.2	11.7	18.2	16.2	14.6	17.9	18.8	14.2
Free cholesterol	5.5	2.7	5.4	2.9	6.4	3.1	6.8	3.0	3.6	2.0
Phospholipid	29.3	27.8	29.5	22.5	28.0	25.6	39.6	23.0	28.1	21.0
Triglyceride	4.7	2.9	5.7	6.1	3.1	2.0	2.0	1.6	2.5	1.9

[a] Isolated by precipitation method (0.13 g/dl dextran sulfate); purified by ultracentrifugation at d 1.216 g/ml, (N = 3).
[b] Percent ± SD.
[c] Chapman et al. (19); HDL₂, d 1.066–1.100 g/ml; HDL₃, d 1.100–1.153 g/ml.
[d] Scanu and Kruski (38).
[e] Adapted from Shepherd et al. (22); values for males and females were combined.
[f] Anderson et al. (39).
[g] Adapted from Kuksis et al. (40); values for males and females were combined.

lation by ultracentrifugation at d 1.216 g/ml. The compositions of "HDL₂" and HDL₂ (d 1.063–1.125 g/ml) were quite similar, and the compositions of "HDL₃" and HDL₃ (d 1.125–1.216 g/ml) were also similar. The latter lipoproteins had more protein and less of each of the lipid constituents than "HDL₂" or HDL₂. Also shown in Table 3 are compositions of HDL₂ and HDL₃ determined by five other groups of investigators (19, 22, 38–40). Although there were some differences among specific constituents in these five studies, there was overall agreement between these results and those obtained in the present study. In another study (data not shown) protein, cholesteryl esters, free cholesterol, and phospholipid were measured in HDL subclasses in six different pools of plasma, each pool containing plasma from two to four normal individuals. The results were similar to those in Table 3 allowing for the absence of triglyceride values. The distinct difference in composition between "HDL₂" and "HDL₃" on the one hand, and their similarity to the composition of HDL₂ and HDL₃, respectively, in these various studies is evidence for the separation of two distinct HDL subclasses by the precipitation method.

SDS gel electrophoresis of HDL subclasses

The apolipoprotein composition of HDL subclasses was determined by SDS gradient gel electrophoresis. **Fig. 4A** shows typical gel patterns of the d 1.063–1.125 g/ml HDL₂ and "HDL₂"; in Fig. 4B are gel patterns of the 1.125–1.216 g/ml HDL₃ and "HDL₃". Each lipoprotein fraction was electrophoresed in the absence or presence of 2-mercaptoethanol or dithiothreitol. The major apoprotein in all lipoprotein fractions was apoA-

I. ApoA-II was present in all the fractions, but it was less prominent in HDL₂ and "HDL₂" than in the HDL₃ fractions. In the presence of reducing agent, the apoA-II dimer was no longer present and the apoA-II monomer appeared in the vicinity of the C-apoproteins. On gels of unreduced HDL₂, HDL₃, and "HDL₃", there was a band with an apparent molecular weight > 40,000 that was eliminated or diminished in the presence of a reducing agent. A band in the position of apoE was present on all gels. This band was more prominent in patterns of the reduced HDL₂, HDL₃, and "HDL₃" than in the unreduced samples. This suggests that the band of molecular weight > 40,000 is the apo(E–A-II) complex described by Mahley and Weisgraber (41) and that it is converted to apoE and apoA-II monomer after reduction (41). The band assumed to be apoE had the same mobility as apoE in VLDL; the band assumed to be apo(E–A-II) had the same mobility as apoA-IV from rat HDL (not shown), which has a molecular weight of 46,000 (42), the same as the apo(E–A-II) complex (41). The presence of the (E–A-II) complex in "HDL₃" and not in "HDL₂" suggests that lipoproteins containing apo(E–A-II) are not precipitated by DS, whereas apoE-containing particles are largely precipitated by DS. Traces of apoB were always noted in the d 1.063–1.125 g/ml lipoprotein (Fig. 4A). Of special interest was the apparent enrichment of "HDL₂" in C-apoproteins and the relative deficiency of these apoproteins in "HDL₃". It is also to be noted in Fig. 4A that apoA-I and apoE in "HDL₂" migrated slightly more slowly than in the HDL₂ of d 1.063–1.125 g/ml. We have no explanation for the slower migration of these apoproteins. The doublet band that migrated to a position between the
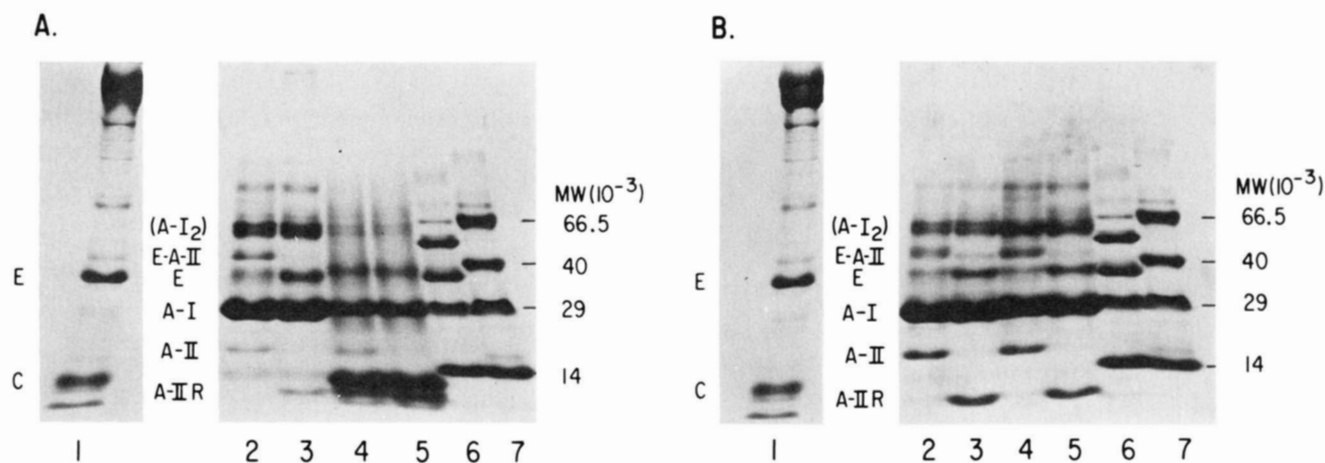
A.

B.

Fig. 4. SDS polyacrylamide gradient (3 to 27%) gels of HDL subclasses. Approximately 50 μg of protein was applied to each lane. Gel A: 1, human apoVLDL, unreduced; 2, d 1.063–1.125 g/ml; 3, d 1.063–1.125 g/ml, reduced; 4, "HDL$_2$"; 5, "HDL$_2$", reduced; 6 and 7, standards, unreduced and reduced, respectively; top to bottom: human serum albumin (mol wt 66,500); ovalbumin (mol wt 40,000); carbonic anhydrase (mol wt 29,000); and lysozyme (mol wt 14,000). Gel B: 1, same as Gel A; 2, d 1.125–1.216 g/ml; 3, d 1.125–1.216 g/ml, reduced; 4, "HDL$_3$"; 5, "HDL$_3$", reduced; 6 and 7, same as in gel A. The apoprotein designations between lanes 1 and 2 refer to bands in lanes 2–5. The designation of the A-I dimer (A-I$_2$) is tentative. The apoprotein designations to the left of lane 1 (apoVLDL) refer to bands in lanes 1–5.

unreduced and reduced albumin has an apparent molecular weight of about 55,000, and may be a dimer of apoA-I. The bands immediately above this position remain unidentified.

HDL and plasma lipid concentrations in normal males and females

In **Table 4** are listed concentrations (mean ± SD) of HDL, HDL subclasses, and total plasma triglyceride and cholesterol for normal males and females (non-sex hormone users) from age 25 to 64 years. HDL subclasses

were analyzed at 0.09 g/dl DS.[10] Values of total HDL cholesterol are similar to those reported in the Lipid Research Clinics Prevalence Study (43). In males there were no significant differences among different age groups. Among females the concentration in the 55–64 year group was greater than that in the <25 year and

[10] After plasma from approximately 200 individuals (normal subjects and patients) had been analyzed at 0.09 g/dl dextran sulfate, we concluded that a higher concentration of dextran sulfate was more suitable. Nevertheless, the remaining analyses were performed at 0.09 g/dl in order to provide a sufficiently large population.

TABLE 4. Concentrations of HDL, "HDL$_2$", "HDL$_3$", and plasma lipids in normal subjects

Age	N	HDL	"HDL$_2$"[a]	"HDL$_3$"	TG	Chol
			cholesterol, mg/dl		*mg/dl*	
Males						
<25	99	44.7 ± 9.9[b]	15.1 ± 7.9	29.6 ± 5.9	69 ± 34	164 ± 30
25–34	39	47.0 ± 12.6	13.3 ± 6.5	33.7 ± 8.5	90 ± 60	181 ± 33
35–44	50	44.9 ± 11.6	12.1 ± 7.6	32.8 ± 7.5	105 ± 55	196 ± 33
45–54	46	48.0 ± 13.3	14.8 ± 8.3	33.2 ± 8.1	96 ± 54	206 ± 29
55–64	39	46.0 ± 12.7	15.6 ± 8.0	30.4 ± 7.2	104 ± 54	223 ± 30
Total	273	45.8 ± 11.7	14.3 ± 7.8	31.5 ± 7.3	88 ± 51	188 ± 37
Females						
<25	44	52.9 ± 12.8[c]	21.8 ± 10.5[c]	31.1 ± 6.7	55 ± 20	171 ± 25
25–34	19	50.5 ± 13.5	16.1 ± 11.2	34.4 ± 5.3	72 ± 38	172 ± 23
35–44	35	53.8 ± 10.1[c]	20.5 ± 8.2[c]	33.3 ± 6.8	65 ± 30	175 ± 33
45–54	35	57.6 ± 12.9[d]	18.7 ± 9.0[c]	38.9 ± 7.5[d]	77 ± 39	205 ± 36
55–64	47	59.5 ± 17.5[c]	20.4 ± 11.4[f]	39.1 ± 9.2[c]	96 ± 41	221 ± 33
Total	180	55.5 ± 14.0[c]	20.0 ± 10.2[c]	35.5 ± 8.1[c]	74 ± 37	192 ± 37

[a] Analyses were carried out with 0.09 g/dl dextran sulfate (final concentration).
[b] Mean ± SD.
[c-f] P values for differences between males and females: [c], $P < 0.001$; [d], $P < 0.002$; [e], $P < 0.05$; [f], $P < 0.03$.

TABLE 5. Correlation between the logarithm of total triglyceride and the logarithm[a] of HDL and HDL subclasses[b]

	Female (180)[c]	Male (273)[c]
	mg/dl	
Triglycerides	4.19 ± 0.46[e]	4.34 ± 0.52
	(66.0 ± 31.5)[d]	(76.7 ± 41.7)[d]
HDL	3.98 ± 0.26	3.79 ± 0.25
	(53.5 ± 14.1)	(44.3 ± 11.2)
r =	−0.355	−0.431
P^e	<0.001	<0.001
"HDL$_2$"	2.85 ± 0.57	2.51 ± 0.59
	(17.3 ± 10.4)	(12.3 ± 7.7)
r =	−0.485	−0.415
P^e	<0.001	<0.001
"HDL$_3$"	3.54 ± 0.24	3.42 ± 0.23
	(34.5 ± 8.4)	(30.6 ± 7.1)
r =	−0.041	−0.221
P^e	<0.60	<0.001

[a] Natural logarithms.
[b] Determined at a final dextran sulfate concentration of 0.09 g/dl.
[c] Number of subjects.
[d] For values in parentheses the mean is the antilogarithm of the mean of the natural logarithm of the individual values, and the standard deviation is an approximation calculated from the antilogarithm of the log of each mean and SD.
[e] Probability that there is no correlation ($r = 0$).

25–34 year age groups ($P < 0.05$). For "HDL$_2$" there was no significant effect of age on concentrations in males or females. There were no differences in "HDL$_3$" levels among males more than 25 years of age; however concentrations in males < 25 years old were lower than in males 25–54 years old ($P < 0.05$). Concentrations of "HDL$_3$" in females older than 45 years of age were greater than those in all younger women ($P < 0.05$). Total triglyceride concentrations for both males and females were generally lower than those reported in the Lipid Research Clinics Study (43), although the same trend toward higher values with increasing age was noted. On the other hand, the total cholesterol concentrations shown in Table 4 were nearly the same as those reported by the Lipid Research Clinics (43), including the trend to higher levels with increasing age. For the total population of each sex (all ages), concentrations of HDL and HDL subclasses were higher in females than in males ($P < 0.001$). In individual age groups, values in females were higher than in males, although the differences between males and females in the 25–34 year old group were not significant. This was probably due to the relatively high HDL concentration for males and the relatively low concentration in females in this age group.

The association of HDL and HDL subclasses with total triglyceride was calculated. Inasmuch as log transformation of data results in more symmetric distribution, correlations were computed for the natural logarithm of these components. No effort was made to remove outliers in any of the groups of data. **Table 5** shows mean values (±SD) and correlation coefficients for the group of 180 normal females and 273 normal males whose nontransformed means are in Table 4. Consistent with what has been observed in many other studies (44, and references 1–7 in ref. 44), there were significant negative correlations of total HDl cholesterol with total plasma triglyceride. "HDL$_2$" levels in both males and females were both highly negatively correlated with triglyceride. The "HDL$_3$" correlations revealed a difference between males and females; while there was a strong negative correlation of "HDL$_3$" with triglyceride in males, there was no correlation in females. Davis et al. (44), in a summary of correlations of HDL cholesterol concentrations with levels of other plasma lipids and lipoproteins, showed a strong dependence of correlation on age as well as sex. Age was not considered in the statistical analyses of the data in Table 5.

Concentration of HDL subclasses in subjects with abnormal HDL levels

The double precipitation procedure was used to determine concentrations of HDL and HDL subclasses in male and female subjects with CHD. These data, and data for normal males and females, are shown in **Table 6**. Concentrations of HDL, "HDL$_2$", and "HDL$_3$" were all significantly lower in CHD subjects. In females both the absolute and relative decreases were greater than in males.

In **Table 7** are shown HDL, "HDL$_2$", "HDL$_3$", and lipid concentrations in other populations demonstrated to have altered HDL levels. The values for normal males and females are the same as those in Table 4. No effort was made to adjust any of the values according to age. All the runners ran 20–40 miles per week. Their total HDL and "HDL$_2$" concentrations were significantly ($P < 0.001$) greater than normal values; "HDL$_3$" concentrations were somewhat elevated. In both males and females with hypertriglyceridemia, total HDL was decreased approximately 40% and "HDL$_2$" was 70% lower; there were smaller decreases in "HDL$_3$". On the other hand, in hypercholesterolemic subjects with normal plasma triglyceride levels, total HDL and "HDL$_2$" were about 14% and 46% lower, respectively, than in normals; "HDL$_3$" concentrations were slightly higher.

Table 8 shows HDL and lipid concentrations in diabetic subjects. In four male patients with recently diagnosed insulin-dependent diabetes mellitus (untreated and not ketotic), HDL was reduced by 43%, "HDL$_2$"

TABLE 6. HDL concentrations in normal and CHD subjects

	N	HDL	"HDL$_2$"[a]	"HDL$_3$"	Total Chol	Total TG
		cholesterol, mg/dl			mg/dl	
Males						
Normal[b]	135	46.3 ± 12.5[c]	14.0 ± 8.1	32.2 ± 7.7	204 ± 32	101 ± 54
CHD	43	33.5 ± 12.2	7.8 ± 8.7	26.0 ± 6.0	214 ± 38	163 ± 65
P		<0.001	<0.001	<0.001	NS	<0.001
Females						
Normal[d]	82	58.7 ± 15.7	19.7 ± 10.4	39.0 ± 8.4	214 ± 35	84 ± 41
CHD	8	35.7 ± 8.9	9.2 ± 2.8	26.5 ± 7.7	215 ± 66	132 ± 54
P		<0.001	<0.001	<0.001	NS	<0.008

[a] Final concentration of dextran sulfate was 0.09 g/dl.
[b] Normal males, >34 years old; age of CHD subjects was 55 ± 10 yr.
[c] Mean ± SD.
[d] Normal females, >44 years old; age of CHD subjects was 60 ± 10 yr.

by 71%, and "HDL$_3$" by 32%. HDL and HDL subclass concentrations in both male and female non-insulin-dependent diabetic subjects (treated by diet alone) were significantly reduced and the sex difference was no longer apparent; thus the decrease in the female patients was much greater, and the greatest change was in "HDL$_2$". In men, HDL decreased by 25% and "HDL$_2$" was decreased by 41%. In women, HDL was decreased by 37% and "HDL$_2$" decreased by 56%. Thus in all subjects in whom total HDL differed from normal values, the changes in "HDL$_2$" were considerably greater than those observed for "HDL$_3$".

DISCUSSION

Our goal was to develop and validate a rapid, simple, and inexpensive method for the estimation of HDL subclasses that could be readily used in metabolic, clinical,

and epidemiological investigations. Burstein et al. (23) showed that after apoB-containing lipoproteins had been removed from serum by DS and Mn^{2+}, varying amounts of HDL could be precipitated by increasing the concentrations of the reagents. However, the conditions for the separation of two distinct HDL subclasses had not been defined. In the method described in this report we used heparin-Mn^{2+} to precipitate the apoB-containing lipoproteins to obtain a supernatant containing HDL because this methodology has been widely used and validated for determining HDL. DS at an appropriate concentration was then added to the supernatant to separate "HDL$_2$" from "HDL$_3$".

Conditions for the precipitation of "HDL$_2$"

The extent of precipitation of "HDL$_2$" depends on the concentration of Mn^{2+}, and the molecular weight of the dextran sulfate and its final concentration. The final concentration of 0.083 M Mn^{2+} is obtained when

TABLE 7. HDL and lipid concentrations in various subjects

	N	HDL	"HDL$_2$"[a]	"HDL$_3$"[a]	TG	Chol
		cholesterol, mg/dl ± SD			mg/dl ± SD	
Males						
Normal	273	45.8 ± 11.7[b]	14.3 ± 7.8	31.5 ± 7.3	88 ± 51	188 ± 37
Runners	22	60.6 ± 20.8[c]	25.4 ± 13.8	35.2 ± 9.9[d]	64 ± 22	203 ± 44
HyperTG[e]	9	26.4 ± 7.2	4.3 ± 2.8	22.1 ± 6.3	458 ± 111	232 ± 43
HyperChol[f]	12	39.1 ± 11.8	7.4 ± 4.2	31.7 ± 8.6	112 ± 34	331 ± 52
Females						
Normal	180	55.5 ± 14.0	20.0 ± 10.2	35.5 ± 8.1	74 ± 37	192 ± 37
HyperTG[e]	5	34.0 ± 8.2	5.2 ± 2.3	28.8 ± 6.8	395 ± 144	277 ± 23
HyperChol[f]	16	48.9 ± 11.7	11.2 ± 4.9	38.7 ± 9.5	143 ± 55	343 ± 48

[a] HDL subclass analyses were carried out at a final dextran sulfate concentration of 0.09 g/dl.
[b] Mean ± SD.
[c] $P < 0.001$ compared to normal males.
[d] $P < 0.04$ compared to normal males.
[e] Triglyceride concentrations were >95th percentile for particular age of each subject (44).
[f] Cholesterol concentrations were >95th percentile for particular age of each subject (44); TG was <95th percentile (44).

TABLE 8. HDL and lipid concentrations in diabetic subjects

	N	Age[a]	HDL	"HDL$_2$"[b]	"HDL$_3$"[b]	TG	CHOL
		yr		cholesterol, mg/dl ± SD		mg/dl ± SD	
Males							
Normal	19	44	51.1 ± 11.2	14.3 ± 9.1	36.8 ± 5.9	94 ± 40	205 ± 28
NIDDM[c]	13	49	38.5 ± 5.1	8.5 ± 3.4	30.0 ± 4.2	165 ± 125	208 ± 48
IDDM[d]	4	38	29.2 ± 9.4	4.2 ± 1.0	25.0 ± 10.2	196 ± 75	208 ± 66
Females							
Normal	27	51	59.3 ± 13.2	20.8 ± 7.5	38.5 ± 7.7	75 ± 26	206 ± 51
NIDDM[c]	19	52	37.4 ± 11.4	9.1 ± 7.7	28.3 ± 9.2	182 ± 89	235 ± 57

[a] Mean age of the group; the range of ages in each group was 35–65 yr except IDDM (range, 29–46 yr).
[b] Analyses were carried out at a final concentration of 0.11 g/dl dextran sulfate.
[c] Non-insulin-dependent diabetes mellitus.
[d] Insulin-dependent diabetes mellitus.

0.1 vol of 1 M MnCl$_2$-heparin solution is added to 1 vol of plasma, and 0.1 vol of DS solution is added to 1 vol of heparin-Mn^{2+} supernatant. One molar Mn^{2+} was used for the total HDL determination in order to precipitate all the apoB-containing lipoproteins, and to provide a sufficiently high Mn^{2+} concentration for the subsequent "HDL$_2$" precipitation. Dextran sulfate with a molecular weight of 15,000 has been used extensively by Burstein and his colleagues (24) for precipitation of lipoproteins. It is preferable to both the higher and lower molecular dextran sulfate preparations that were tested. Thus, the only variable that had to be considered was the final concentration of the DS. Although 0.09 g/dl DS was used in most of the studies, the validation experiments (Figs. 1 and 2, Table 2) suggested that use of a higher concentration would yield "HDL$_2$" concentrations comparable to those obtained by preparative ultracentrifugation, which was used as the standard reference method.

Validation of the precipitation method

The precipitation method was validated in two ways: *1)* concentrations of "HDL$_2$" and "HDL$_3$" were compared with values obtained by standard methods of both preparative and analytical ultracentrifugation, and *2)* the compositions of "HDL$_2$" and "HDL$_3$" obtained by the precipitation method were compared with the composition of ultracentrifugally separated subclasses.

In the validation of the precipitation method by comparing concentrations of HDL subclasses with those obtained by preparative ultracentrifugation, it is necessary to assume that the latter method provides a true reference value. However, the recovery of lipoprotein subclasses after ultracentrifugation is incomplete (92.7%), and correction for loss requires the assumption that the relative loss is the same for both HDL$_2$ (d < 1.125 g/ml) and HDL$_3$ (d > 1.125 g/ml). Such a correction would increase the concentrations of HDL$_2$ and HDL$_3$ obtained by ultracentrifugation of heparin-Mn^{2+} super-

natants by approximately 7%, yielding "corrected values" of 18.0 and 28.4 mg/dl for HDL$_2$ and HDL$_3$, respectively, in study 1 of Table 2. Using these corrected reference values, "HDL$_2$" concentration by the precipitation would be 26% too low and "HDL$_3$" concentration would be 18% too high. A similar discrepancy between the methods is found in study 1a (Table 2).

HDL$_2$ isolated by sequential centrifugation of plasma contains varying amounts of Lp(a) and other apoB-containing lipoproteins. Warnick and Albers (45) have estimated that the contribution of these additional lipoproteins is on the order of 5.6 mg/dl. Thus HDL$_2$ determined after sequential ultracentrifugation of whole plasma would have a higher cholesterol content than HDL$_2$ separated from a heparin-Mn^{2+} supernatant. Thus, in study 2 (Table 2), one must consider both incomplete recovery (90.6%) and the presence of Lp(a) and other apoB-containing lipoproteins. Correction of the ultracentrifugation value [(19.2/0.906) − 5.6] results in a new value for HDL$_2$ cholesterol of 15.6 mg/dl, and this is to be compared to values of 13.9, 15.4, and 17.9 mg/dl obtained by the precipitation method using DS concentrations of 0.09, 0.11, and 0.13 g/dl, respectively. For the HDL$_3$ fraction, the corrected value 28.3 mg/dl is to be compared with values of 34.2, 32.3, and 31.6 mg/dl obtained by precipitation at DS concentrations of 0.09, 0.11, and 0.13 g/dl, respectively. On the basis of these data it would appear that the values obtained using DS at a concentration of 0.13 g/dl yields values that most closely approximate values obtained by ultracentrifugation. The titration curves (Fig. 2) also indicate that precipitation of an HDL subclass is essentially complete at that concentration. These curves show a difference of approximately 3.5 mg/dl between "HDL$_2$" precipitated at 0.13 mg/dl and 0.09 mg/dl. In addition, 92 samples of plasma were analyzed at DS concentrations of 0.09, 0.11, and 0.13 g/dl. (Table 2 shows data for 32 of these analyses.) The data showed

that "HDL₂" values obtained with 0.13 g/dl DS were 2.8–3.5 mg/dl higher than those obtained at 0.09 mg/dl. The equations of the regression lines for HDL₂ on "HDL₂" (see Results, Study 2, Table 2) show that the y intercept (a measure of the difference in concentration) decreased with increasing concentration of DS. All of these data have led us to decide upon 0.13 g/dl as the optimal concentration of DS.

Further validation of the method was provided by comparison of values for 17 samples where HDL subclasses were determined by precipitation and analytical ultracentrifugation. The correlation between the two methods was very high, with a correlation coefficient of 0.91.

Composition of "HDL₂" and "HDL₃"

Additional evidence that DS precipitates "HDL₂" or an HDL₂-like particle was obtained in studies of the chemical composition of "HDL₂" and "HDL₃" and lipoproteins of d 1.063–1.125 and d 1.125–1.216 g/ml. The data in Table 3 show that "HDL₂" and "HDL₃" differ markedly in their protein to lipid ratios as do HDL₂ and HDL₃, and that the composition of the subclasses isolated by the precipitation method agrees closely with that of the corresponding fractions isolated by the ultracentrifugation of plasma, both in the current study and in studies by others (19, 22, 38–40).

SDS gel electrophoretic patterns of "HDL₂" and "HDL₃" were similar to those of HDL₂ and HDL₃, respectively, with respect to the major apoproteins, A-I and A-II. The ratio of A-I/A-II in "HDL₂" and HDL₂ appeared greater than in "HDL₃" and HDL₃, as judged by visual examination of the gels. This is consistent with the studies of Cheung and Albers (46), Patsch et al. (47), and Kostner et al. (48). C-apoproteins are present in both HDL₂ and HDL₃, although there are slightly higher percentages in HDL₂ (49, 50). This is also suggested in the electrophoretic patterns of HDL₂ and HDL₃ in Fig. 4. However, the very high proportion of C-apoproteins in "HDL₂" was a consistent finding that may represent specific precipitation of certain HDL families. The apo(E-A-II) complex was noted in HDL₂ (d 1.063–1.125 g/ml), but not in "HDL₂". However, the complex was found in "HDL₃". Reduced HDL₃ and "HDL₃" also contained a minor component that had a mobility corresponding to that of apoA-IV (Fig. 4B). ApoA-IV has not been demonstrated previously in human HDL, but it is present in rat HDL (42) and in human mesenteric lymph chylomicrons and free in plasma (51). However, we have no independent evidence that the apoA-IV is not a contaminant derived from the d > 1.216 g/ml fraction.

Although HDL₂ and HDL₃ are the two major subclasses of HDL, each has been separated into several subfractions by different techniques. HDL₂ has been separated into two major subclasses (HDL₂ₐ and HDL₂ᵦ) by ultracentrifugation (18, 19, 52). Cheung and Albers (46) used CsCl equilibrium density ultracentrifugation to separate HDL into seven subfractions in the density range 1.063–1.200 g/ml; five fractions were between d 1.063 and 1.118 g/ml. There were differences in apparent molecular weights and apoA-I/apoA-II ratios in these fractions. Patsch et al. (47) using zonal ultracentrifugation obtained three subfractions of HDL₂ and five subfractions of HDL₃. Discrete subfractions of "HDL₂" can be obtained by incremental changes in DS concentration,[11] and it would be of interest to compare such subfractions.

Other methods for determining HDL₂

Since the preliminary report of this method in 1979 (34), it has been used in clinical (53, 54) and epidemiological (55) studies. Other investigators have also developed methods for HDL₂ determination. Kirstein and Carlson (56) centrifuged plasma at d 1.125 g/ml to obtain HDL₃ concentration (d > 1.125 g/ml), and subtracted this value from total HDL, determined after heparin-Mn²⁺ precipitation of apoB-containing lipoproteins, in order to calculate HDL₂. Eyre, Hammett, and Miller (57) have described a method in which apoB-containing lipoproteins are precipitated with heparin-Mn²⁺ and the supernatant is centrifuged at d 1.125 g/ml in a Beckman Airfuge. HDL₂ and HDL₃ are then isolated by tube slicing.

The method described in the present report does not require any specialized apparatus, and a relatively large number of plasma samples can be processed and analyzed at a time. Furthermore, by all the criteria noted above, HDL precipitated by the dextran sulfate method as described appears to be analogous to HDL₂.

Clinical observations

There have been a number of reports of concentrations of cholesterol in HDL subclasses in normal subjects but, except for the study of Gofman et al. (58), the number of subjects in each group has been small (2–13 individuals) and the results were highly variable (Table V of reference 56). Gofman et al. (58) in 1954 analyzed HDL subclasses in 270 men and 460 women by analytical ultracentrifugation. We used factors (0.183 for HDL₂ and 0.141 for HDL₃, calculated from the analyses of d 1.063–1.125 and d 1.125–1.216 g/ml lipoproteins in Table 3) to convert their total concentration of HDL or HDL subclass to cholesterol concentrations. In men 18 to 69 years old, the mean HDL₂ concentration was 12.2 mg/dl and the mean HDL₃ concentration was 26.1

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[11] Gidez, L. I., and M. Rogers. Unpublished observations.

mg/dl. For women in the same age range, the mean concentrations of HDL$_2$ and HDL$_3$ were 19.7 and 28.4 mg/dl, respectively. These values are lower than those reported in Table 4, but the ratios of HDL$_2$-cholesterol to HDL$_3$-cholesterol for men and women are similar to ratios calculated from the data in Table 4.

The concentrations of HDL subclasses showed considerable variability, but that in "HDL$_2$" (and d < 1.125 g/ml lipoproteins) was particularly marked. The coefficients of variation for "HDL$_2$" in each age/sex group ranged from 0.40 to 0.70, whereas those in "HDL$_3$" ranged from 0.15 to 0.25 (Table 4). Greater variance in HDL$_2$ than HDL$_3$ was also noted in other studies (39, 58) where the HDL subclasses were analyzed by analytical ultracentrifugation.

The data for subjects with coronary heart disease (Table 6) confirm earlier studies of Gofman et al. (12). Although both "HDL$_2$" and "HDL$_3$" decreased, the relative decrease in "HDL$_2$" in both male and female CHD subjects was considerably greater than for either total HDL or "HDL$_3$". Moreover, the sex-related differences in HDL and HDL subclasses observed in normal subjects are nearly abolished in coronary heart disease. This suggests that measurement of "HDL$_2$" might be a more discriminating parameter for prediction of coronary heart disease than total HDL.

In long distance runners (Table 7), total HDL was elevated but the relative increase in "HDL$_2$" was considerably greater, and "HDL$_3$" was not significantly different from normal values. The range of concentrations in runners was considerable: the minimum and maximum concentrations for "HDL$_2$" were 7.8 and 73.0 mg/dl, respectively; the range for "HDL$_3$" was 21.0 to 59.5 mg/dl. The reasons for this variability are not known, but it may reflect individual variability in response to exercise.

Concentrations of HDL and HDL subclasses in patients with hyperlipoproteinemia were lower than normal (Table 7). Patients with hypertriglyceridemia (type IV) had greatly reduced levels of total HDL. Both "HDL$_2$"and "HDL$_3$" were reduced, but the greatest change was in "HDL$_2$". The inverse correlation between HDL cholesterol and plasma triglycerides has been well established (44), and this is very apparent in patients with hypertriglyceridemia in whom HDL is greatly reduced (59). In Table 5 we show a high negative correlation between triglyceride and "HDL$_2$", and it is therefore not unexpected that "HDL$_2$" is greatly reduced in these patients with hypertriglyceridemia. Patients with hypercholesterolemia had modest decreases in HDL and "HDL$_2$", but not in "HDL$_3$". This reflects the lack of correlation between HDL-cholesterol and LDL-cholesterol (43), which was also found

in a study by Schaefer et al. (59) in which plasma from a large population of patients with type II hyperlipoproteinemia was analyzed.

In four men with newly detected, untreated, insulin-deficient diabetes, HDL was decreased, but the relative decrease in "HDL$_2$" was considerably greater, and there was a small decrease in "HDL$_3$" (Table 8). Most data on the effect of insulin-dependent diabetes on HDL and HDL subclasses have been obtained from insulin-treated subjects in whom total HDL is either high or normal (60). Our studies (61) have shown that, following insulin administration, "HDL$_2$" increases to concentrations that are normal, with no change in "HDL$_3$". This is in sharp contrast to data presented by Mattock et al. (62) that showed an increase in total HDL and HDL$_3$ in insulin-treated diabetic males, with no change in HDL$_2$. However, Eckel et al. (14) have also found HDL$_2$ to be increased in insulin-treated diabetes. In patients with non-insulin-dependent diabetes, HDL was reduced in both men and women, and "HDL$_2$" was reduced to a considerably greater extent (Table 8). The subjects in both of these groups were significantly overweight and had triglyceride concentrations higher than in the control subjects. Nikkila (60) has reported that when diabetic subjects are compared with non-diabetic subjects at similar relative body weight levels, no difference in HDL cholesterol values is observed.

From our studies it appears that when total HDL is moderately decreased, i.e., by <25%, the change is due primarily to a lower "HDL$_2$" concentration; when the decrease is greater than 25%, the "HDL$_3$" concentration is also lower than normal, but the decreases in "HDL$_2$" are always greater. Whether the pattern of change is disease-specific can only be learned by making studies of larger populations in which other variables are controlled, e.g., triglyceride levels, age, diet, etc. However, in all instances "HDL$_2$" is the more variable component and changes in total HDL are primarily a reflection of changes in "HDL$_2$".▩

APPENDIX

The method for the determination of HDL subclasses is as follows. (The reagents are listed in the Materials section.) To one volume of plasma (usually 3.0 ml) is added 0.1 vol (0.3 ml) of the heparin-MnCl$_2$ solution. The sample is thoroughly mixed and left to stand at room temperature for 10–20 min. It is then centrifuged at 1500 g for 1 hr at 4°C. Aliquots of the clear heparin-Mn^{2+} supernatant are withdrawn *immediately* for "HDL$_2$" precipitation and analysis of total HDL cholesterol. To one volume (2.0 ml) of the heparin-Mn^{2+} supernatant is added 0.1 vol (0.2 ml) of DS solution (1.43 g/dl in 0.15 M NaCl). This results in a final DS concentration of 0.13 g/dl. After thorough mixing, the sample is allowed to stand at room temperature for 20 min and then centrifuged at 1500 g for 30 min at 4°C. An aliquot of the clear supernatant is removed *immediately* for cholesterol analysis ("HDL$_3$" cholesterol). "HDL$_2$" cholesterol = total HDL cholesterol minus "HDL$_3$" cholesterol.

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