papers and notes on methodology

Determination of high density lipoprotein-cholesterol in human plasma stored at −70°C

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Abstract We determined the effect of storage at −70°C on the determination of plasma high density lipoprotein (HDL)-cholesterol. Plasma from 106 subjects was stored for 1, 6, and 12 months, then treated with heparin and $MnCl_2$ to remove other lipoproteins, and HDL-cholesterol was measured. The mean HDL-cholesterol level decreased by 2.9% after 1 month, and by 5.1% after 12 months. The magnitude and direction of the changes were not constant, but were correlated primarily with HDL-cholesterol concentration. After 1 month, samples with HDL-cholesterol levels below 40 mg/dl tended to increase, and those above this value tended to decrease. By 12 months, only those samples with HDL-cholesterol below 22 mg/dl tended to increase. Linear regression analysis indicated changes of 0.9–1.5 mg/dl for each 10 mg/dl initial HDL-cholesterol concentration. Storage of heparin-$MnCl_2$ supernatants, rather than unfractionated plasma, minimized these changes. The mean HDL-cholesterol of stored heparin-$MnCl_2$ supernatants was 3.3% lower after 12 months, and the change was constant regardless of lipoprotein concentration. The findings suggest the possible occurrence of changes in the heparin-$MnCl_2$ precipitability of lipoproteins during storage, which produce errors in HDL-cholesterol analyses, and indicate that samples can be stored more satisfactorily if the other lipoproteins are removed first.—**Bachorik, P. S., R. E. Walker, and P. O. Kwiterovich, Jr.** Determination of high density lipoprotein-cholesterol in human plasma stored at −70°C. *J. Lipid Res.* 1982. **23:** 1236–1242.

Supplementary key words HDL-cholesterol analysis • heparin-$MnCl_2$ method • frozen storage • plasma lipoproteins

Plasma high density lipoprotein (HDL) cholesterol levels constitute a strong inverse risk factor for the development of ischemic heart disease (1–3), and although the biochemical mechanisms underlying this association are unclear, the clinical determination of HDL-cholesterol has assumed increased importance in the evaluation of cardiovascular risk. A number of factors, however, can influence the interpretation of plasma HDL-cholesterol values. Most of the circulating cholesterol in adult man is associated with low density lipoproteins (LDL); HDL-cholesterol levels are relatively low by comparison. The analytical errors inherent in the determination of HDL-cholesterol are therefore proportionately larger than those encountered in the analysis of plasma total or LDL-cholesterol (4). Cardiovascular risk changes dramatically with relatively small changes in HDL-cholesterol concentration. Thus, the risk for premature ischemic heart disease is twice as great in men with HDL-cholesterol levels of 35–44 mg/dl than in those whose levels are 45–54 mg/dl (2). While these differences are considerable in terms of total lipoprotein mass, they can approach the limits of precision of HDL-cholesterol measurements and a relatively small error in the analysis can result in a grossly inaccurate assessment of risk. Finally, measured HDL-cholesterol levels can be influenced markedly by the analytical procedure employed and by the age and condition of the samples themselves.

We previously examined the effect of storage on plasma HDL-cholesterol values determined with the heparin-$MnCl_2$ procedure (5). ApoB-containing lipoproteins (chylomicrons, VLDL, LDL, and when present, β-VLDL and Lp(a)) are precipitated with heparin in the presence of $MnCl_2$; HDL remains soluble and HDL-cholesterol is determined by measuring the cholesterol content of the heparin-$MnCl_2$ supernatant. We found that samples stored at 4°C or −20°C underwent

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; LRC, Lipid Research Clinics Program; CDC, Centers for Disease Control.

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changes in which apoB-containing lipoproteins became progressively more difficult to precipitate, and HDL became progressively easier to precipitate under the conditions of the analysis. These changes produced errors in the measured HDL-cholesterol values, particularly in samples at the high and low extremes of the HDL-cholesterol range. In the present study, these observations have been extended to samples stored at −70°C for up to 1 year. The results indicated first, that similar changes occurred when unfractionated plasma samples were stored at −70°C, and second, that HDL-cholesterol values changed less when heparin-$MnCl_2$ precipitation was performed in fresh samples and the heparin-$MnCl_2$ supernatants were stored for 1 year at −70°C.

MATERIALS AND METHODS

Study population

Blood from fasting subjects was collected into evacuated blood collection tubes containing disodium EDTA (1.5 mg/ml) and cells were removed within 3 hr of venipuncture. Samples were obtained from 106 subjects. The plasma lipid and lipoprotein levels of the samples were as follows (mg/dl): (mean (±SD), range); total cholesterol, 243 (±86), 106–686; triglycerides, 117 (±72), 24–394; LDL-cholesterol, 171 (±82), 50–618; HDL-cholesterol 48.9 (±12.8), 9–97.

Study protocol

An aliquot was taken from each plasma sample for the determination of total cholesterol and triglycerides (see below). Four additional aliquots were taken for HDL-cholesterol analysis (**Fig. 1**). The first aliquot was treated with heparin-$MnCl_2$ to remove apoB-containing lipoproteins (see below) and HDL-cholesterol was measured in the heparin-$MnCl_2$ supernatant. This procedure was performed as soon as the plasma was prepared. Part of this heparin-$MnCl_2$ supernatant was also stored in sealed sterile vials at −70°C and analyzed 12 months later. The other three aliquots of plasma were similarly stored for 1 month, 6 months, and 12 months, after which they were thawed, heparin-$MnCl_2$ supernatants were prepared, and HDL-cholesterol was analyzed. All of the samples were collected in groups of approximately ten samples per week over a period of 11 weeks. They were analyzed initially as they were collected and were re-analyzed in the same groups following storage for the times specified. This design was adopted to compensate for run-to-run analytical variability during the study.

Lipid and lipoprotein analysis

The samples were frozen by placing the sealed vials upright in a freezer at −70°C, and were stored in groups as they were collected. Only those samples that were to be analyzed were removed from the freezer. The samples were allowed to thaw at room temperature. They were mixed several times by manual inversion and then allowed to rotate on a blood-mixing wheel for 30 min to insure complete mixing. At the end of this period the vials were unsealed and aliquots were taken for precipitation with heparin and $MnCl_2$. The heparin-$MnCl_2$ supernatants were then immediately extracted for the analysis of HDL-cholesterol. An HDL-cholesterol control pool that was also stored at −70°C was used to monitor heparin-$MnCl_2$ precipitation and HDL-cholesterol analysis during the study (see Quality control, below). Aliquots of this pool were thawed, treated with heparin-$MnCl_2$, extracted, and analyzed in parallel with each group of study samples.

Plasma total cholesterol, triglycerides, and HDL-cholesterol levels were determined as described previously (5). Briefly, heparin sulfate (40 μl) (Riker Laboratories, Inc., Northridge, CA) and $MnCl_2$ (50 μl) were added to 1000 μl of plasma (final concentration 1.25–1.30 mg/ml, 0.046 M, respectively). The samples were allowed to stand for 30 min in an ice bath, after which the precipitate was removed by centrifugation and an aliquot of the supernatant was taken for analysis of HDL-cholesterol. All of the samples used for the present study gave clear heparin-$MnCl_2$ supernatants. The LDL-cholesterol level was calculated from total cholesterol, triglyceride, and HDL-cholesterol measurements as described by Friedewald, Levy, and Fredrickson (6).

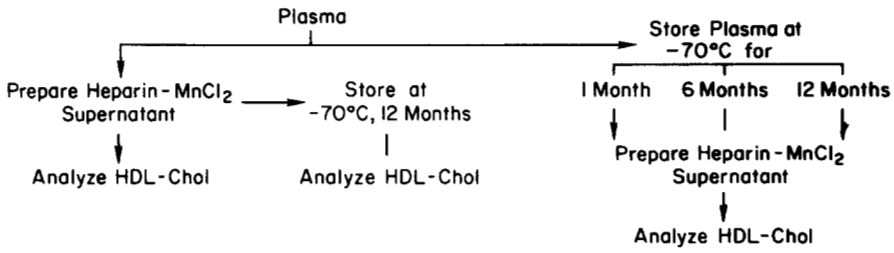

Fig. 1. Schematic representation of study protocol.

TABLE 1. Change in HDL-cholesterol level during storage of plasma at −70°C.

Samples Stored as:	HDL-Chol in Fresh Samples[a]	Storage Time	HDL-Chol in Stored Samples	HDL-Chol (Stored-Fresh)	P^b	Low Cholesterol Control Pool[c]	HDL-Cholesterol Control Pool[c]
	mean ± SD	*months*	*mean ± SD*	*mean ± SD*		*mean (±SD)*	*mean (±SD)*
Unfractionated plasma	48.9 ± 12.8					52.5 (±0.7)	42.9 (±2.4)
		1	47.5 ± 11.7	−1.4 ± 4.5	0.002	52.4 (±1.0)	42.8 (±2.0)
		6	46.6 ± 12.6	−2.3 ± 5.9	$<5 \times 10^{-4}$	52.5 (±1.5)	42.5 (±2.8)
		12	46.5 ± 12.1	−2.5 ± 3.4	$<5 \times 10^{-4}$	52.4 (±0.9)	40.9 (±1.9)
Heparin-$MnCl_2$ supernatant		12	47.3 ± 13.1	−1.6 ± 3.7	$<5 \times 10^{-4}$		

[a] One hundred and six samples were tested. The samples had the following lipid and lipoprotein concentrations (mean ± SD, mg/dl): total cholesterol, 242 (±86); triglycerides, 117 (±72); LDL-chol, 171 (±82).
[b] Paired *t* test (9).
[c] One low total cholesterol and one HDL-cholesterol control pool was used for the entire study. Each pool was analyzed in duplicate 21–23 times during each of the four periods during which the samples were analyzed.

Isopropanol extracts (sample–isopropanol 1:20 (v/v) of unfractionated plasma and the HDL-containing supernatant fraction were prepared and treated with a zeolite-containing mixture to remove interferring substances. The lipid analyses were performed with the AutoAnalyzer II (Technicon Instruments, Tarrytown, NY). The procedures used were those of the LRC Program, and have been described in detail (7).

Quality control

The HDL-cholesterol procedure was considered to consist of two distinct steps: *1*) heparin-$MnCl_2$ precipitation to remove apoB-containing lipoproteins (VLDL, LDL, Lp(a)) and *2*) analysis of cholesterol in the HDL-containing heparin-$MnCl_2$ supernatant. The cholesterol analysis step was monitored during each of the four periods in which study samples were analyzed in order to evaluate relative analytical bias between the four periods. The total cholesterol concentration of a serum pool prepared by the Clinical Chemistry Standardization Section, Centers for Disease Control (CDC), Atlanta, GA was determined in duplicate on 21–23 occasions during each of the four analytical periods of the study. This pool was prepared with a total cholesterol concentration of approximately 50 mg/dl, and was used to monitor the stability of cholesterol analyses in the HDL-cholesterol concentration range. The results are presented below.

A second serum pool, developed by CDC for the control of HDL-cholesterol analyses in the LRC Program (8), was also analyzed in duplicate simultaneously with the low total cholesterol pool. This pool contained total cholesterol and triglyceride concentrations of 159 and 71 mg/dl, respectively, and was stored at −70°C in the author's laboratory until needed. The pool was thawed, mixed, subjected to heparin-$MnCl_2$ precipitation in the usual way, and HDL-cholesterol was analyzed in the heparin-$MnCl_2$ supernatant. The results are presented below.

Statistical methods

The statistical analyses that were performed are indicated below. All calculations were performed by computer using standard packaged programs (9, 10).

RESULTS AND DISCUSSION

Storage of unfractionated plasma

Aliquots of unfractionated plasma were stored for 1, 6, or 12 months at −70°C, after which they were thawed, treated with heparin-$MnCl_2$, and HDL-cholesterol was analyzed as described in Methods. The mean (±SD) HDL-cholesterol level of the entire group of samples was 48.9 (±12.8) mg/dl when the analyses were performed on fresh samples (**Table 1**). The group mean was 1.4 mg/dl lower when the samples were stored for 1 month at −70°C before analysis, and about 2.4 mg/dl lower after storage for 6 or 12 months. These differences were statistically significant (9).

This slight but progressive decrease in the mean HDL-cholesterol concentration of plasma samples did not appear to result from analytical variability in the cholesterol measurements. The samples were initially obtained in small groups over a period of approximately 3 months. They were analyzed as they were obtained and were re-analyzed in the same order during similar periods. This procedure would minimize the effect of day-to-day analytical variability during each of the 3-month periods of analysis but would not correct for relative analytical bias between the four periods. For this reason, a quality control pool with a total cholesterol concentration that was similar to the group mean HDL-

cholesterol concentration of the samples was analyzed in duplicate on 21–23 occasions during each of the four periods in which samples were analyzed. The results are shown in Table 1. The mean cholesterol concentration of the pool for each of the four analysis periods differed from each other by no more than 0.1 mg/dl, a value that was much smaller than the observed decrease in the mean HDL-cholesterol level of the samples. In addition, there was no tendency for the cholesterol concentration of the pool either to increase or decrease during the study. We concluded that the decrease in HDL-cholesterol values of the plasma samples was not accounted for by downward drift in the cholesterol measurements.

The next question was whether the changes that occurred during storage were related to the lipid or lipoprotein concentrations of the samples, and linear regression analyses (10) were performed to examine the magnitude of the changes as a function of the levels of plasma total cholesterol, triglyceride, LDL-cholesterol, or HDL-cholesterol. There was no significant relation between Δ HDL-cholesterol concentration upon storage and either the plasma total- or LDL-cholesterol levels. This was true regardless of the length of storage. There was a significant positive correlation between Δ HDL-cholesterol level and plasma triglyceride concentration in samples that were stored for 1 month and 6 months, but the correlation did not persist in samples stored for 12 months. The linear regression equations relating Δ HDL-chol to plasma triglyceride concentration were: (n = 106); 1 month, Δ HDL-chol = 0.017 [triglycerides] −3.42; 6 months, Δ HDL-chol = 0.022 [triglycerides] −4.90. The slopes of the regression lines indicated a change of approximately 1.7 mg/dl for each 100 mg/dl change in triglyceride concentration.

The magnitude of Δ HDL-cholesterol was significantly and inversely correlated with the HDL-cholesterol concentration in fresh plasma at 1, 6, and 12 months of storage. The linear regression equations relating Δ HDL-cholesterol with HDL-cholesterol concentration were as follows: (n = 106); 1 month, Δ HDL-chol = −0.145 [HDL-chol$_{fresh}$] + 5.73; 6 months, Δ HDL-chol = −0.12 [HDL-chol$_{fresh}$] + 3.63; 12 months, Δ HDL-chol = −0.094 [HDL-chol$_{fresh}$] + 2.14. At all times of storage, samples with higher HDL-cholesterol concentrations tended to decrease and samples with lower concentrations tended to increase. This relation is illustrated in **Fig. 2** for samples that were stored for 12 months. The correlation with initial HDL-cholesterol concentration did not result from regression to the mean because it was not observed in heparin-$MnCl_2$ supernatants that were prepared from fresh samples and stored for 12 months (see below), even though the analyses of the 12-month samples and the stored heparin-

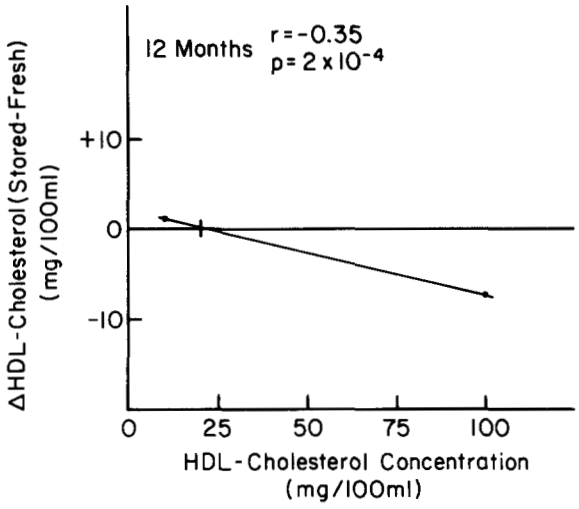

Fig. 2. Linear regression analysis relating the change in apparent HDL-cholesterol level with fresh sample HDL-cholesterol concentration in 106 plasma samples stored at −70°C for 12 months. The regression equation is given in the text.

$MnCl_2$ supernatants were performed simultaneously. The crossover point (the point at which no change in apparent HDL-cholesterol concentration was observed) occurred at 40 mg/dl in samples stored for 1 month, and at 30 and 22 mg/dl in samples that were stored for 6 months and 12 months, respectively. This effect appeared to account for the progressively lower values obtained for the mean HDL-cholesterol level of the entire group of samples after storage and also indicated that the changes that were apparent in the group means did not adequately represent the behavior of the entire group of samples. The slopes of the regression lines indicated changes of 0.9–1.5 mg/dl for each 10 mg/dl initial HDL-cholesterol concentration.

The relation between the Δ HDL-cholesterol and initial HDL-cholesterol or plasma triglyceride concentration was in opposite directions. Since the plasma HDL-cholesterol level is inversely correlated with plasma triglyceride concentration (11), partial correlation coefficients were calculated to adjust for this inverse relation. The results are shown in **Table 2.** After storage for 1 month, the correlation coefficient relating the change in HDL-cholesterol level and initial HDL-cholesterol level was −0.42. This correlation was reduced to −0.34, when corrected for the inverse relation between HDL-cholesterol and plasma triglyceride concentrations. The correlation with plasma triglyceride concentration was lower than with initial HDL-cholesterol level, and of lower significance, and was not significant after correction for initial HDL-cholesterol level (Table 2). After 6 months of storage, the correlations between the changes in apparent HDL-cholesterol concentration and both initial HDL-cholesterol level and plasma tri-

TABLE 2. Contribution of initial HDL-cholesterol and plasma triglyceride concentrations to storage-related changes in measured HDL-cholesterol concentration

Storage Time		Correlation Between Δ HDL-chol and: HDL-Chol, Uncorrected	HDL-Chol, Corrected for Triglycerides	Plasma Triglycerides, Uncorrected	Plasma Triglycerides, Corrected for HDL-Chol
1 month	r	−0.42	−0.34	0.28	0.12
	P	1×10^{-5}	5×10^{-4}	0.004	NS
6 months	r	−0.26	−0.17	0.27	0.18
	P	0.006	0.043	0.005	0.03
12 months	r	−0.35	−0.33	0.13	0.03
	P	2×10^{-4}	5×10^{-4}	NS	NS

NS, not significant.

glyceride level were about the same and equally significant. Furthermore, the magnitude and significance of the partial correlation coefficients for both variables were virtually identical. These results suggested the presence of independent, opposite, and almost equal contributions of both factors to the changes in measured HDL-cholesterol concentration that occurred after 6 months of storage at −70°C. After 12 month's storage, the correlation between the change in apparent HDL-cholesterol concentration and initial HDL-cholesterol level clearly predominated; the correlation was almost unaffected when corrected for the effect of triglyceride concentration (Table 2). As mentioned earlier, there was no significant correlation between the change in apparent HDL-cholesterol level and triglyceride concentration after 12 month's storage. The results suggested the occurrence, probably at different rates, of alterations related both to HDL and triglyceride-rich lipoproteins that contributed to the apparent changes in the HDL-cholesterol concentrations of stored samples. The independent contribution of plasma triglyceride level to Δ HDL-cholesterol was observed after only one of the three storage periods, however, and even then its significance was marginal. It is possible, therefore, that the changes in measured HDL-cholesterol concentration were influenced only by HDL-cholesterol level.

Analysis of HDL-cholesterol control pool

The HDL-cholesterol concentration of this pool was determined in duplicate on 21–23 occasions during each of the four periods in which study samples were analyzed. This pool was analyzed simultaneously with the low total cholesterol pool described above. The results are shown in Table 1. The measured HDL-cholesterol concentration of the pool was 42.9 mg/dl, and decreased slightly during the first 6 months. After 12 months, the apparent HDL-cholesterol concentration decreased by 2.0 mg/dl, and as noted above, the change did not seem to be accounted for by analytical bias in the cholesterol measurements.

This control pool was one of several serum pools that have been used for the past several years in the laboratories of the LRC program for the quality control of HDL-cholesterol analyses performed with the heparin-$MnCl_2$ procedure (8). These pools are prepared to contain triglyceride concentrations of less than 100 mg/dl, and HDL-cholesterol levels in the middle of the HDL-cholesterol concentration range (8). The pool we used in this study had been stored in our laboratory at −70°C for approximately 3 months before the study started, and before that it had been stored at −60°C at CDC for several months during its initial preparation and characterization. The pool thus differed from the samples used in the study in several ways since it was serum, considerably older than the study samples, and contained relatively low lipid concentrations. Nonetheless, to the extent that the behavior of any particular sample can be predicted from the data in Fig. 2 and Table 2 (see below), the apparent decrease in HDL-cholesterol concentration, particularly after the 12-month period of storage, agreed reasonably well with the behavior of the study samples. Pools containing HDL-cholesterol levels in the upper range were not available for study, and it remains to be determined whether such pools would undergo larger changes.

The observations in the present study were qualitatively identical to those we obtained in a previous study in which we examined HDL-cholesterol levels in plasma samples stored at 4°C and −20°C (5). In that study, we found that it became progressively more difficult to precipitate apoB-containing lipoproteins completely in stored plasma samples, and progressively easier to precipitate apoA-I-containing lipoproteins. Incomplete precipitation of apoB-containing lipoproteins would tend to increase the cholesterol content of the heparin-$MnCl_2$

supernatants, and partial precipitation of HDL would have the opposite effect; the measured values would reflect the net result of both processes. Similar alterations in lipoprotein precipitability may have been responsible for the changes that occurred in plasma stored at −70°C. It should be noted, however, that the manipulations required for the isolation of the heparin-$MnCl_2$ soluble fraction would be expected to contribute a component of variability independent of storage-related changes in the precipitability of the lipoproteins. This component of variability would arise from various sources such as uncertainties in the measurement of sample and reagent volumes and reagent preparation. While variability from these sources would be expected to be random and therefore not account for the progressive changes observed, the data do not allow an assessment of its contribution to the observed changes in HDL-cholesterol. In view of the similarity of the present results with our earlier findings (5), however, it seems likely that storage-related changes in the precipitability of the lipoproteins may have occurred and contributed significantly to the changes observed.

Storage of heparin-$MnCl_2$ supernatants prepared from fresh plasma

A portion of the heparin-$MnCl_2$ supernatant that was prepared from fresh samples was re-analyzed after it had been stored for 12 months at −70°C. The results are shown in Table 1. The group mean HDL-cholesterol concentration was 1.6 mg/dl lower in heparin-$MnCl_2$ supernatants that had been stored for 12 months. There was no significant correlation between the magnitude of the storage-related changes and the plasma levels of either plasma total cholesterol, triglyceride, LDL-cholesterol, or HDL-cholesterol concentration. **Fig. 3** illustrates the relation between the HDL-cholesterol concentration measured in freshly prepared heparin-$MnCl_2$ supernatants, and the same measurement performed after the supernatants had been stored for 12 months. The two measurements were highly correlated, and the slight negative bias was constant over the entire range of concentration.

The basis for the slight decrease in the cholesterol concentration of the stored heparin-$MnCl_2$ supernatants was unclear. Cholesterol itself appears to be quite stable during long-term frozen storage (12, 13), and although small, the change was greater than could be accounted for by the cholesterol assay procedure. One possibility is that slight losses occurred when the samples were thawed and mixed despite our efforts to insure that the samples were homogeneous before unsealing the storage vials. The change was smaller than those that occurred in unfractionated plasma, however, and was constant over the HDL-cholesterol concentration range,

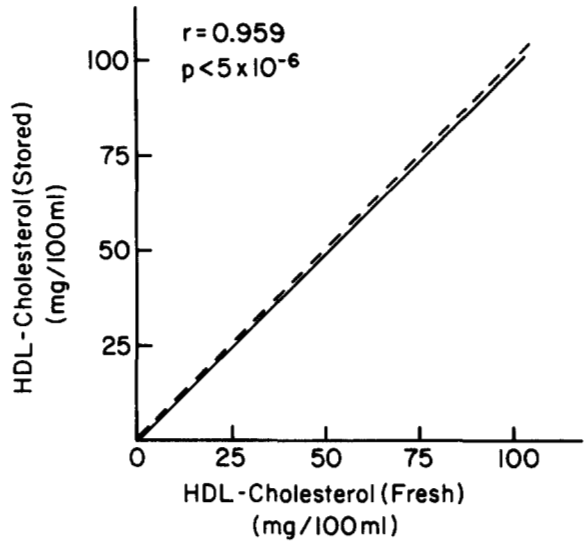

Fig. 3. Linear regression analysis relating HDL-cholesterol analyzed in freshly prepared heparin-$MnCl_2$ supernatants to that in supernatants stored for 12 months at −70°C. Regression equation (n = 106): HDL-$chol_{stored}$ = 0.977 [HDL-$chol_{fresh}$] − 0.48. The dashed line represents the line of identity.

and in this sense the HDL-cholesterol values in stored heparin-$MnCl_2$ supernatants were more stable than those in unfractionated plasma. While it would be most desirable when using the heparin-$MnCl_2$ procedure to perform the analyses in fresh samples, the findings suggest that samples may be stored satisfactorily at −70°C for at least 1 year if the apoB-containing lipoproteins are removed first.

The major findings of this study can be summarized as follows: *1*) the measured concentration of HDL-cholesterol, as determined with the heparin-$MnCl_2$ method, changed significantly when plasma was stored at −70°C; *2*) the magnitude and direction of the changes depended primarily on the HDL-cholesterol concentrations of the samples; and *3*) the changes were smaller and independent of lipid and lipoprotein concentration when the heparin-$MnCl_2$ supernatants, rather than unfractionated plasma was stored. The findings may have broad implications and several points warrant consideration. First, the present observations were made using the heparin-$MnCl_2$ method and reflect only cholesterol that did not precipitate under the conditions of the assay. As discussed earlier (5), measurements made with other methods may lead to different conclusions. The heparin-$MnCl_2$ method is widely used, however, and much of the epidemiological data relating HDL cholesterol levels with coronary risk has been developed with this procedure (1). Second, while it was expected that plasma samples stored at −70°C would undergo little change, the observed changes in samples with high or low HDL-cholesterol concentrations were appreciable. The correlations between changes in the measured HDL-cho-

lesterol concentration and HDL-cholesterol level were weak, however, with correlation coefficients below 0.5. The behavior of individual samples, therefore, could not be predicted reliably. As noted previously (5), various investigators have reported increased (14), unchanged (15), or decreased (16) mean HDL-cholesterol values for relatively small numbers of samples that were stored at −20°C for varying periods.

Finally, the observations bear on the development of quality control materials for HDL-cholesterol analysis. The present findings in plasma samples suggest that if quality control pools frozen at low temperature are to be used for HDL-cholesterol analyses, pools containing HDL-cholesterol concentrations in the range 30–50 mg/dl might undergo relatively little change in apparent HDL-cholesterol concentration over a period of at least 1 year, compared with changes that might be expected at higher HDL-cholesterol levels.

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