

# Determination of high density lipoprotein-cholesterol in stored human plasma

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**Abstract** This study was performed to determine the effect of frozen and unfrozen storage on plasma high density lipoprotein (HDL) cholesterol analysis. HDL-cholesterol was determined, following removal of the other lipoproteins by precipitation with heparin and MnCl<sub>2</sub>, in fresh plasma samples and in aliquots of the samples that had been stored 1) unfrozen (4°C), up to 14 days, 2) frozen (-20°C), up to 14 days, and 3) frozen (-20°C), 5-6 years. There were progressive changes in measured HDL-cholesterol values under all conditions of storage. The changes were significantly and most highly correlated with HDL-cholesterol level and they reached about 4 mg/dl for each 10 mg/dl initial HDL-cholesterol concentration. Samples with low HDL-cholesterol concentrations tended to increase with storage, and those with high HDL-cholesterol levels tended to decrease. Since the changes were in opposite directions, the group means tended to be preserved. The mean differences between fresh and stored samples were (stored-fresh): 1-14 days, 4°C, -0.10 to +4.60 mg/dl; 1 hour-14 days, -20°C, +0.80 to +1.92 mg/dl; 5-6 years -20°C, -1.92 mg/dl. Values changed most rapidly during storage at 4°C and were accompanied by changes in the precipitability of the lipoproteins; low density lipoproteins became progressively difficult to precipitate completely, and HDL became progressively easier to precipitate. The measured HDL-cholesterol level depends on which process predominates and can lead to grossly inaccurate measurements, particularly at the extremes of HDL-cholesterol concentration. —Bachorik, P. S., R. Walker, K. D. Brownell, A. J. Stunkard, and P. O. Kwiterovich. Determination of high density lipoprotein-cholesterol in stored human plasma. *J. Lipid Res.* 1980. 21: 608-616.

**Supplementary key words** plasma lipoproteins · frozen storage · unfrozen storage · heparin-manganese method

A number of studies have indicated that there is an inverse relationship between the risk for coronary heart disease and the plasma concentration of high density lipoprotein (HDL) cholesterol (1-5). Possible mechanisms by which HDL might play a direct protective role have also been studied (4, 6) as well as conditions associated with elevated (7-9) or depressed (10-12) HDL-cholesterol levels. These investigations

have led to an increased interest in the routine determination of HDL-cholesterol levels to aid in the assessment of risk for ischemic heart disease, as well as interest in further studies of the putative protective effect of HDL, and have stimulated efforts to understand better the capabilities and limitations of quantitative HDL methods (13-15).

The measured values for plasma lipids and lipoproteins are affected by a number of factors, one of which is the length of time and conditions under which samples are stored before analysis. It has been recommended that samples to be used for lipoprotein analysis be stored at 4°C and that the analyses be performed as soon as possible (16). In practice, however, delays occur for various reasons, and samples may be stored for several days, during which the lipoproteins may be altered by a number of changes related to such factors as auto-oxidation (17-19), the activity of lecithin: cholesterol acyl transferase, a plasma enzyme that catalyzes cholesterol esterification (20), the exchange of cholesteryl esters and triglycerides between HDL and other plasma lipoproteins (21-23), and even contamination by phospholipase C-producing bacteria (24, 25). While such changes might be retarded by frozen storage, freezing alters the electrophoretic and ultracentrifugal properties of the lipoproteins (26-28) and has not been recommended.

The extent to which storage-related changes might interfere with lipoprotein analyses depends on the properties of the lipoprotein in question, the lipoprotein component being measured, and the analytical method used. Several earlier studies, in which total lipoprotein mass measurements were made with the analytical ultracentrifuge, suggested that in

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

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general, the levels of all the lipoproteins, including HDL, decreased during frozen or unfrozen storage (26–28). On the other hand, in one recent study Reckless et al. (29) found that HDL-cholesterol, as measured with the commonly used heparin-manganese precipitation procedure, increased significantly in samples that were stored frozen for 6 weeks or 6 months. Miller et al. (5) found no significant change in the HDL-cholesterol values of samples stored frozen for 2 months. Although both studies examined only small number of samples, and although the results appear to be divergent, neither study found a decrease in apparent HDL-cholesterol levels during storage, suggesting that the apparent effects of storage may indeed depend on what is being measured, and on the analytical method used. Very little information is presently available about the extent to which storage affects HDL-cholesterol values determined with polyanion precipitation methods. The present study was undertaken to investigate the influence of frozen and unfrozen storage on HDL-cholesterol values obtained with the heparin-manganese chloride method (16, 30).

## MATERIALS AND METHODS

### Plasma samples

Blood from fasting subjects was collected into evacuated tubes containing solid disodium EDTA (1.5 mg/ml) and transported to the laboratory in an ice bath. The plasma was separated at 4°C within 3 hours of venipuncture and divided into several aliquots. One aliquot was analyzed immediately, and the others were stored for varying periods of time either frozen or unfrozen, in sterile vials that were sealed to prevent evaporation. The analysis scheme used is illustrated in Fig. 1.

### Unfrozen storage

Samples were obtained from 105 normal and 120 hyperlipidemic subjects. The samples from the hyperlipidemic group included 71 samples from subjects with type IIa, 18 with type IIb, and 31 with type IV lipoprotein patterns (31). All of the samples were clear or slightly turbid.<sup>3</sup> The samples were divided into two groups (Fig. 1). The first consisted of 101 samples,

<sup>3</sup> Plasma samples with triglyceride concentrations over about 300 mg/dl are usually turbid, and, when treated with heparin-Mn<sup>2+</sup>, require special handling (32) to remove completely the apoB-containing lipoprotein precipitate. Grossly turbid samples were therefore omitted from the present study, and all the samples used gave clear heparin-Mn<sup>2+</sup> supernatant solutions.

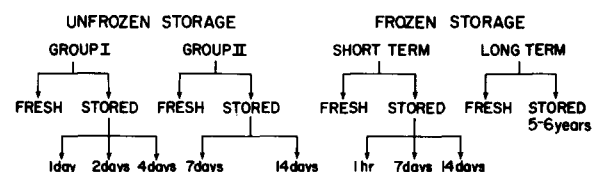


Fig. 1. Scheme for analysis of plasma samples stored under various conditions. In each case the analyses of stored samples were compared with those of fresh samples in the same group.

each of which was divided into four aliquots. The first aliquot was analyzed on the day the sample was obtained and is referred to as the “fresh sample.” The second, third, and fourth aliquots were transferred to sterile, 6-ml vials, sealed, and stored at 4–6°C for 1, 2, and 4 days, respectively, following which they were analyzed. These are referred to as the “1-day”, “2-day”, and “4-day” samples.

The second group contained 124 samples, each of which was analyzed on the day it was drawn. Aliquots of all the samples were stored under the conditions described above for 7 days and 14 days, following which they were re-analyzed. These are referred to as the “7-day” and “14-day” samples, respectively.

All the samples in each group were collected in batches of 10–20 samples over a period of 10 weeks. For most of the study, the analysis of the aliquots in each group of samples overlapped that of two other groups. This design was adopted to compensate for run-to-run analytical variability during the study.

### Short-term frozen storage

Samples were obtained from 43 normal and 61 hyperlipidemic subjects. The samples from the hyperlipidemic group included 23 samples from subjects with type IIa, 11 with type IIb, two with type III, and 25 with type IV lipoprotein patterns. All of these samples were also clear or slightly turbid. Each sample was divided into four aliquots (Fig. 1). The first was kept on ice for 1 hour and is referred to as the “fresh sample.” The second, third, and fourth aliquots were transferred to sterile 6-ml serum vials, sealed, and placed in a freezer at –18 to –23°C for 1 hour, 7 days, and 14 days, respectively. These are referred to as the “1 hour”, “7 day” and “14 day” samples. The 1 hour sample was thawed and analyzed immediately along with the fresh samples. The two remaining aliquots were thawed and analyzed on day 7 and day 14, respectively.

All of the short-term frozen storage samples were collected in groups of 10 to 12 samples per week over a period of 11 weeks. The aliquots of each group of samples were analyzed in overlapping fashion as described above.

### Long-term frozen storage

Samples were obtained from 71 normal and 21 hyperlipidemic subjects between March 1973 and March 1974. The hyperlipidemic samples included 3 with type IIa, 1 with type IIb, and 17 with type IV lipoprotein patterns. Each sample was divided into two aliquots (Fig. 1). One aliquot was analyzed immediately and the other was transferred to a sterile 6-ml serum vial, sealed, and stored at  $-18$  to  $-23^{\circ}\text{C}$  until July 1979. At that time the samples were thawed in groups of approximately 30 samples and analyzed over a period of 1 week.

### Sample preparation and analysis

Plasma total cholesterol, triglyceride, and HDL-cholesterol concentrations were determined as described previously (14, 16). Briefly, heparin sulfate ( $40\ \mu\text{l}$ ) (Riker Laboratories, Inc., Northridge, CA) and  $\text{MnCl}_2$  ( $50\ \mu\text{l}$ ) were added to  $1000\ \mu\text{l}$  plasma in final concentrations of 1.25 to 1.30 mg/ml and 0.046 M, respectively, to precipitate the apoB-containing lipoproteins. The samples were incubated in an ice bath for 30 min, and the precipitated lipoproteins removed by centrifugation at  $1500\ g$  for 30 min at  $4^{\circ}\text{C}$ . An aliquot of the clear supernatant solution was taken for analysis of HDL-cholesterol. Only samples that gave clear supernatants were used for the present studies.<sup>3</sup>

Isopropanol extracts (sample:isopropanol, 1:20 v/v) of unfractionated plasma and the HDL-containing supernatant fraction were prepared and treated with a zeolite mixture to remove interfering substances (33). All lipid analyses were performed with the AutoAnalyzer II (Technicon Instruments, Tarrytown, NY), except those performed in 1973 and 1974 when the fresh aliquots of the samples stored for 6 years were initially analyzed. The latter were performed on the AutoAnalyzer I. The triglyceride method used on both instruments was a modification of the method of Kessler and Lederer (33). The AutoAnalyzer II cholesterol procedure was based on the Liebermann-Burchard reaction, and cholesterol analyses on the AutoAnalyzer I used the  $\text{FeCl}_3\text{-H}_2\text{SO}_4$  reaction (16).

### Laboratory standardization

The laboratory was standardized according to criteria developed specifically for the Lipid Research Clinics Program (34) and remained standardized for the duration of the study. In addition, the accuracy and precision of the AutoAnalyzer II cholesterol analyses in the HDL-cholesterol concentration range was monitored continuously by use of a frozen serum pool prepared to contain a low concentration of

cholesterol. The pool was prepared and characterized by the Lipids Section, Center for Disease Control, Atlanta, GA and had a cholesterol concentration of 69 mg/dl as established by that laboratory with the reference method of Abell et al. (35). The mean AutoAnalyzer II cholesterol concentration of this pool was calculated for each of several periods during which the various phases of the present study were performed. The mean values determined for the pool ranged from 67.00 to 68.57 mg/dl; the standard deviations ranged from 0.89 to 1.56 mg/dl, and gave overall co-efficients of variation of 1.32–2.27%. The fresh aliquots of the samples that were subsequently stored for 6 years were analyzed before a low concentration serum pool was available to monitor the accuracy and precision of the AutoAnalyzer I cholesterol analyses. Instead, these parameters were estimated at the time (March 1973–March 1974) by repetitive assay of an isopropanol solution of cholesterol (50 mg/dl), equivalent in purity to National Bureau of Standards reference material (36). The mean AutoAnalyzer I cholesterol concentration of this solution was 49.27 mg/dl; the standard deviation was 2.23 mg/dl and the overall coefficient of variation was 4.5%. A serum pool similar to that described above became available shortly thereafter, and was analyzed on the AutoAnalyzer I from March–December 1974. The reference cholesterol concentration (35) was 59 mg/dl. The mean ( $\pm\text{SD}$ ) AutoAnalyzer I concentration of the pool was 59.73 ( $\pm 2.17$ ) mg/dl and the coefficient of variation was 3.6%.

LDL-Cholesterol concentrations were estimated in samples with triglyceride concentrations of less than 400 mg/dl using the empirical relationship of Friedewald, Levy, and Fredrickson (37):  $(\text{LDL-Cholesterol}) = (\text{Total Cholesterol}) - (\text{HDL-Cholesterol}) - (\text{Triglycerides}/5)$ . The values in mg/dl for total cholesterol, HDL-cholesterol, and total triglycerides used in the formula were determined by analysis of the fresh samples.

### Immunochemical studies

In one experiment, heparin- $\text{Mn}^{2+}$  supernatant fractions of stored samples were tested for completeness of precipitation of apoB-containing lipoproteins. The samples were allowed to diffuse against anti-human LDL (Miles-Yeda, Rehovot, Israel) in 2% agarose gels for 48 hr. Under the conditions of the test LDL was detected when its concentration exceeded 5 mg/dl as LDL-cholesterol. In a separate experiment, heparin- $\text{Mn}^{2+}$  precipitates of stored samples were tested for apoA-I containing lipoproteins. The heparin- $\text{Mn}^{2+}$  precipitate was resuspended in a solution containing heparin (1.24 mg/dl) and  $\text{MnCl}_2$

TABLE 1. Change in plasma HDL-cholesterol level during storage of samples at 4–6°C

	n	HDL-Chol in Fresh Samples mean ± SD (mg/dl)	Storage Time (days)	HDL-Chol in Stored Samples mean ± SD (mg/dl)	ΔHDL-Chol (stored-fresh) mean ± SD (mg/dl)	P <sup>c</sup>
Group I <sup>a</sup>	101	49.84 ± 14.98	1	49.74 ± 14.68	-0.10 ± 3.66	NS
			2	50.94 ± 14.85	+0.75 ± 5.16	NS
			4	49.20 ± 14.47	-0.64 ± 6.48	NS
Group II <sup>b</sup>	124	52.74 ± 13.14	7	51.67 ± 11.44	-1.07 ± 5.12	0.021
			14	57.35 ± 11.83	+4.60 ± 9.94	<0.001

<sup>a</sup> The mean (±SD) lipid concentrations of the samples in Group I were: total cholesterol, 253 (±59) mg/dl; triglycerides, 134 (±53) mg/dl.

<sup>b</sup> The mean (±SD) lipid concentrations of the samples in Group II were: total cholesterol, 262 (±56) mg/dl; triglycerides, 129 (±68) mg/dl.

<sup>c</sup> Paired *t* test (39).

(0.046 M), and re-sedimented at 1500 *g* for 30 min at 4°C. This washing procedure was repeated two additional times. The precipitate was then re-dissolved as described by Burstein, Scholnick, and Morfin (38), and allowed to diffuse against anti-human apoA-I (gift of Dr. John J. Albers) in 2% agarose gel as above. The sensitivity of the test was 1 mg/dl HDL-cholesterol.

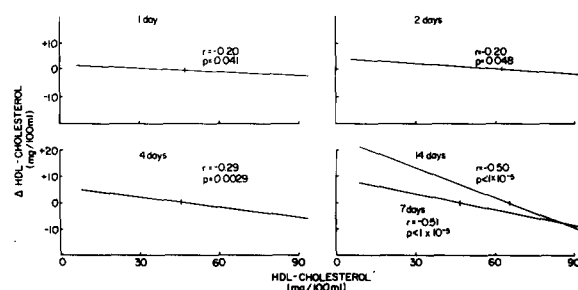
## RESULTS

The mean HDL-cholesterol levels of two groups of samples that were stored at 4–6°C for various periods are shown in **Table 1**. The mean HDL-cholesterol levels of the samples did not change significantly within the first 4 days. There was a slight but significant decrease by day 7, and by day 14 the mean level increased by 4.6 mg/dl.

The standard deviation of the change in HDL-cholesterol level increased with time. This reflected large differences in particular samples. Linear regression analyses were therefore performed to determine whether the magnitude of differences between the fresh and stored samples were related to their lipid or lipoprotein concentrations. The changes were not related to total cholesterol concentrations. There was a slight correlation with LDL-cholesterol concentrations for the 4-day samples ( $r = +0.24$ ,  $P = 0.017$ ;  $\Delta\text{HDL-cholesterol}_{(\text{frozen-fresh})} = 0.026 [\text{LDL-cho}] - 5.25$ ), but the correlation did not persist in samples that were stored for 7 or 14 days. There was also a slight correlation with triglyceride concentration for the 7-day samples ( $r = +0.20$ ,  $P = 0.02$ ;  $\Delta\text{HDL-cholesterol}_{(\text{frozen-fresh})} = 0.016 [\text{TG}] - 3.10$ ), but again, it did not persist in samples stored for 14 days. The magnitude of the change in HDL-cholesterol was significantly and inversely correlated with the fresh

plasma HDL-cholesterol level, however, even after the shortest storage time (**Fig. 2**). At all times, samples with high HDL-cholesterol levels tended to decrease, and samples with low concentrations tended to *increase*. The crossover point (the point at which concentration did not change) occurred between 40–60 mg/dl during the first 7 days (**Fig. 2**) and not much change was observed in this concentration range during this period. At 14 days, the crossover period occurred at about 70 mg/dl and accounted for the observed 4.6 mg/dl increase in the mean value for the entire group of samples (**Fig. 2** and **Table 1**).

These findings suggested that progressive changes in the precipitability of the lipoproteins with heparin-MnCl<sub>2</sub> occur with time and this was confirmed by immunochemical studies in which the heparin-MnCl<sub>2</sub> supernatants were examined for the presence of LDL, and the heparin-MnCl<sub>2</sub> precipitates for the presence of apoA-I. The heparin-MnCl<sub>2</sub> supernatants of 49



**Fig. 2.** Linear regression analysis relating the magnitude of the change in apparent HDL-cholesterol level with fresh sample HDL-cholesterol concentration, for samples stored at 4–6°C for the indicated times.  $\Delta\text{HDL-cholesterol}$  is expressed as stored sample value – fresh sample value. Regression equations: 1 day,  $\Delta\text{HDL-cho} = 0.050 (\text{HDL-cho}_{\text{fresh}}) + 2.38$ ; 2 days,  $\Delta\text{HDL-cho} = -0.068 (\text{HDL-cho}_{\text{fresh}}) + 4.15$ ; 4 days,  $\Delta\text{HDL-cho} = -0.127 (\text{HDL-cho}_{\text{fresh}}) + 5.69$ ; 7 days,  $\Delta\text{HDL-cho} = -0.197 (\text{HDL-cho}_{\text{fresh}}) + 9.32$ ; 14 days,  $\Delta\text{HDL-cho} = -0.381 (\text{HDL-cho}_{\text{fresh}}) + 24.70$ .



TABLE 2. Incidence of LDL in heparin-Mn<sup>2+</sup> supernatants of samples stored unfrozen<sup>a</sup>

n	Fresh Samples	Samples Stored	
		7 Days	14 Days
49	4	20	
48			45
% of Total	8%	41%	94%

<sup>a</sup> Samples were allowed to diffuse against anti-LDL for 48 hr at room temperature in 2% agarose gel. LDL-cholesterol was detected at concentrations of 5 mg/dl or greater. Forty-nine samples were analyzed fresh and after 7 days; 48 of the samples were analyzed after 14 days.

samples were examined by immunodiffusion against anti-LDL. LDL was detected in 8% of the supernatants of fresh samples, in almost half the samples stored for 7 days and in over 90% of the samples stored for 14 days (Table 2). Similarly, when the heparin-MnCl<sub>2</sub> precipitates of 103 samples were solubilized and allowed to diffuse against anti-AI, a precipitin line was observed in 30% of the fresh samples, in over 70% of the samples stored for 7 days, and in almost all of the samples stored for 14 days (Table 3).

The results of HDL-cholesterol analyses in a group of samples subjected to short-term frozen storage are summarized in Table 4. The HDL-cholesterol concentrations of the samples ranged from 25 to 147 mg/dl; two samples had concentrations exceeding 100 mg/dl. The mean HDL-cholesterol concentration of the frozen aliquots were significantly higher than those of the fresh samples, but the differences were less than 2 mg/dl even after 2 weeks.

Linear regression analyses were performed to determine whether the differences between fresh and frozen samples were correlated with their lipid or lipoprotein concentrations. There was no significant correlation between the magnitude of these differences and either total cholesterol or LDL-cholesterol concentration. There was a small but significant correlation between the differences for the 1-hr samples

TABLE 3. Incidence of HDL in heparin-Mn<sup>2+</sup> precipitates of samples stored unfrozen<sup>a</sup>

n	Day 0	Day 7	Day 14
103	31	72	98
% of Total	30%	72%	95%

<sup>a</sup> Immunodiffusion was performed as indicated in Legend to Table 2. The sensitivity of the assay was 1 mg/dl HDL-cholesterol.

and total triglyceride concentration ( $r = 0.23$ ,  $P = 0.021$ ;  $\Delta\text{HDL-cholesterol}_{(\text{frozen}-\text{fresh})} = -0.006 [\text{TG}] + 1.64$ ), but the correlation did not persist for samples that had been stored for 7 or 14 days.

The magnitude of the difference between the apparent HDL-cholesterol concentrations of fresh and frozen samples was, however, correlated with HDL-cholesterol concentration in fresh plasma at a high level of significance (Fig. 3). In the concentration range of 30–70 mg/dl, apparent HDL-cholesterol values increased in samples frozen 1 hr and 7 days, an effect that was more pronounced at the lower HDL-cholesterol concentrations (Fig. 3). The crossover point occurred at about 90 mg/dl for the 1-hr samples and fell to 70 mg/dl for the 7-day samples. By 14 days the values increased in samples with low HDL-cholesterol concentrations and decreased slightly in samples with high HDL-cholesterol concentrations; the crossover point was at about 60 mg/dl (Fig. 3). The magnitude of the change in the 1-hr samples was about 0.3 mg/dl for each 10 mg/dl initial HDL-cholesterol concentration, and increased slightly with storage for up to 2 weeks.

The mean ( $\pm$ SD) HDL-cholesterol level of the group of 92 samples that had been stored for 5–6 years, was 49.51 ( $\pm$ 15.06) mg/dl. When the samples were reassayed, the group mean decreased by 1.9 mg/dl to 47.59 ( $\pm$ 13.39 mg/dl) but the difference was not significant. The standard deviation of the difference between the stored and fresh samples, however, was 12 mg/dl, or 4–6 times that of samples that had been frozen for 2 weeks or less (Table 4). This finding

TABLE 4. Change in plasma HDL-cholesterol level during short-term storage at  $-20^{\circ}\text{C}$

n	HDL-Chol in Fresh Samples <sup>a</sup> mean $\pm$ SD	Storage Time	HDL-Chol in Stored Samples mean $\pm$ SD	$\Delta$ HDL-Chol (Stored-fresh) mean $\pm$ SD	$P^b$
	(mg/dl)		(mg/dl)	(mg/dl)	
104	46.04 $\pm$ 16.04	1 hr	46.85 $\pm$ 15.63	+0.81 $\pm$ 2.11	<0.001
		7 days	47.96 $\pm$ 15.22	+1.92 $\pm$ 3.06	<0.001
		14 days	46.84 $\pm$ 15.46	+0.80 $\pm$ 2.56	<0.003

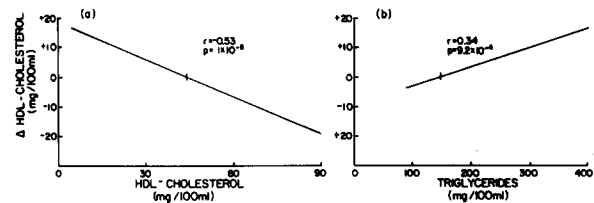
<sup>a</sup> The mean ( $\pm$ SD) lipid concentrations of the samples were: total cholesterol 250 ( $\pm$ 62.91) mg/dl; triglycerides 151 ( $\pm$ 87) mg/dl.

<sup>b</sup> Paired  $t$  test (39).

reflected rather large changes that had occurred in particular samples.

Linear regression analyses revealed no significant correlation between the observed differences and either total cholesterol or LDL-cholesterol concentration. The magnitude of the differences was most highly and most significantly correlated with HDL-cholesterol concentration ( $r = 0.53$ ;  $P = 1 \times 10^{-5}$ ) (Fig. 4a). HDL-cholesterol levels tended to increase in samples with initial levels less than 44 mg/dl and tended to decrease in samples with initial values above 44 mg/dl. The slope of the regression line indicates that the HDL-cholesterol levels of the samples tended to change by about 4 mg/dl for each 10 mg/dl increment in initial HDL-cholesterol concentration. The magnitude of this change was only slightly greater than that observed in samples stored at 4–6°C for 14 days (3.8 mg/dl per 10 mg/dl initial HDL-cholesterol concentration, compare Figs. 2 and 4a).

The magnitude of the changes that occurred after 6 years frozen storage was also correlated with total triglyceride concentration, although the correlation was lower than with HDL-cholesterol level, and considerably less significant ( $r = 0.34$ ;  $P = 2 \times 10^{-4}$ ) (Fig. 4b). HDL-cholesterol levels tended to decrease at triglyceride values below about 145 mg/dl and tended to increase at higher triglyceride levels. The slope of the regression line indicates that the HDL-cholesterol concentration changed by about 6.5 mg/dl for each 100 mg/dl change in triglyceride concentration. Inasmuch as an inverse correlation exists between circulating triglyceride and HDL-cholesterol concentrations (40), partial correlation co-efficients were calculated for both of the linear regressions in Fig. 4 in order to adjust for this relationship. When the effect of triglyceride level was accounted for, the cor-

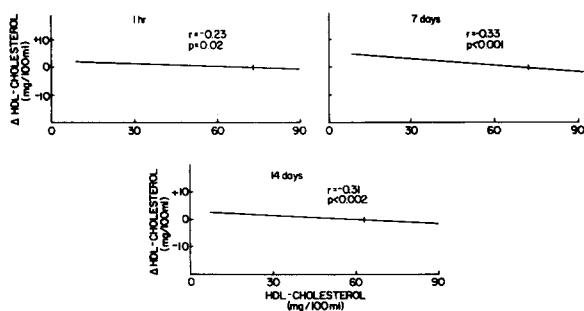


**Fig. 4.** (a) Linear regression analysis relating the change in apparent HDL-cholesterol level with fresh sample HDL-cholesterol concentration for samples stored at  $-18$  to  $-23^{\circ}\text{C}$  for 5–6 years.  $\Delta\text{HDL-cholesterol}$  is expressed as stored sample value – fresh sample value. Regression equation:  $\Delta\text{HDL-cholesterol} = -0.422 (\text{HDL-cholesterol}_{\text{fresh}}) + 18.98$ . (b) Linear regression analysis relating the change in apparent HDL-cholesterol level with fresh sample triglyceride concentration for the samples in (a). Regression equation:  $\Delta\text{HDL-cholesterol} = 0.065 [\text{TG}_{\text{fresh}}] - 9.43$ .

relation with initial HDL-cholesterol concentration was reduced from  $-0.53$  ( $P = 1 \times 10^{-5}$ ) to  $-0.46$  ( $P < 1 \times 10^{-4}$ ). On the other hand, when the effect of HDL-cholesterol was eliminated, the correlation with triglyceride concentration decreased from  $0.34$  ( $P = 9.2 \times 10^{-4}$ ) to  $0.18$  ( $P = 0.047$ ). Thus, while there appeared to be a slight and marginally significant independent effect of triglyceride level, the storage-related changes were clearly most highly correlated with initial HDL-cholesterol concentration.

## DISCUSSION

The mean HDL-cholesterol concentrations of all the groups of samples tested were in the mid-range of normal HDL-cholesterol concentration, and changed very little regardless of the period or conditions of storage. Individual samples, however, most notably those at the high and low ends of the normal concentration range, tended to change substantially during storage, but in opposite directions; this effect tended to preserve the group means. These changes occurred more rapidly in samples stored at  $4^{\circ}\text{C}$ , than in those that were stored frozen, but in both cases the changes were correlated with the initial HDL-cholesterol concentration. The correlations increased and became progressively more significant with the length of storage, suggesting that progressive changes occurred in the precipitability of the plasma lipoproteins under the conditions of the assay. This conclusion is supported by the immunochemical findings in samples stored at  $4^{\circ}\text{C}$ ; it was progressively more difficult to precipitate LDL completely, but progressively easier to precipitate HDL, or an HDL subfraction. The less efficient precipitation of LDL would tend to increase measured HDL-cholesterol levels, while an inappropriate precipitation of HDL would



**Fig. 3.** Linear regression analysis relating the magnitude of the change in apparent HDL-cholesterol level with fresh sample HDL-cholesterol concentration for samples stored at  $-18$  to  $-23^{\circ}\text{C}$  for the times indicated.  $\Delta\text{HDL-cholesterol}$  is expressed as stored sample value – fresh sample value. Regression equations: 1 hour,  $\Delta\text{HDL-cholesterol} = -0.033 (\text{HDL-cholesterol}_{\text{fresh}}) + 2.37$ ; 7 days,  $\Delta\text{HDL-cholesterol} = -0.075 (\text{HDL-cholesterol}_{\text{fresh}}) + 5.36$ ; 14 days,  $\Delta\text{HDL-cholesterol} = -0.049 (\text{HDL-cholesterol}_{\text{fresh}}) + 3.07$ .

tend to lower them; cholesterol in the supernatant fraction would therefore reflect the net result of both processes.

The changes that were apparent during short-term frozen storage apparently continued and after 5–6 years the crossover point occurred at an HDL-cholesterol concentration of 44 mg/100 ml. This seemed to continue the trend toward lower concentrations that was observed in the samples stored at  $-20^{\circ}\text{C}$  for up to 2 weeks. This conclusion was based on the assumption that the  $\text{FeCl}_3\text{-H}_2\text{SO}_4$  cholesterol method used for the initial analysis of this group of samples gave results equivalent to those obtained with the Liebermann-Burchard method used 6 years later. Although the two methods were probably not completely equivalent, the quality control data suggested that they agreed rather closely in the HDL-cholesterol concentration range. The absolute comparability of the methods would affect the group mean HDL-cholesterol value, the magnitude of  $\Delta\text{HDL-cholesterol}$ , and the position of the crossover point of the regression line. It would not, however, influence the slope of the regression line or alter the overall impression conveyed by the short-term and long-term sample data that frozen storage results in progressive alterations in the behavior in the lipoproteins with the heparin- $\text{MnCl}_2$  procedure. In this regard it is noteworthy that unprecipitated LDL was observed in all of the heparin- $\text{MnCl}_2$  supernatants of the samples that were frozen for 5–6 years, and that apoA-I was detected in all of the precipitates.

In view of the significant correlation between  $\Delta\text{HDL-cholesterol}$  and initial HDL-cholesterol concentration, it is necessary to consider the possibility that part of the correlation results from regression to the mean. This is a statistical phenomenon in which subsequent analyses of a subgroup of samples, selected from cutoff limits based on the initial analyses of its parent group of samples, tend toward the parent group mean. The effect would be due to the behavior of the laboratory methodology and the re-analysis of only a subgroup of the original population. Regression to the mean accounts for little if any of the correlations observed in the present study for several reasons. First, the repeat analyses were performed not on selected subgroups of the original populations, but on all of the samples in the original populations. Second, with the exception of the use of the  $\text{FeCl}_3\text{-H}_2\text{SO}_4$  cholesterol method mentioned above, the laboratory methods remained the same for the duration of the study. Regression to the mean would, therefore, be no greater on one occasion than on another; i.e., the slopes of the regression lines would not change progressively because of it. Progressive

changes did occur with both frozen and unfrozen samples, however, and occurred faster with unfrozen samples. Finally, the immunochemical findings provided independent confirmation of the occurrence of progressive changes during storage up to two weeks at  $4^{\circ}\text{C}$ , as well as during a 5–6 year period of frozen storage.

Several groups of workers have measured lipoprotein levels in frozen and unfrozen samples that have been stored at different temperatures for varying periods of time. The measurements originally made with the analytical ultracentrifuge generally suggested that the levels of all the lipoproteins, including HDL, decrease with storage (26–28). The more recent studies, in which HDL-cholesterol measurements were made in frozen samples with the heparin- $\text{MnCl}_2$  precipitation method, have produced somewhat variable results. Miller et al. (5) found no significant change in the mean value of a group of six samples from normolipidemic subjects after 2 months storage at  $-20^{\circ}$ . Reckless et al. (29) detected *increases* of 3% and 17% in the mean HDL-cholesterol levels of 19 samples stored for 6 weeks and 6 months, respectively, Helgeland et al. (41) analyzed 33 control samples that were stored for 4 years at  $-20^{\circ}\text{C}$  and obtained values that were 38% lower than those of fresh samples subsequently obtained from the same subjects. These findings are more difficult to evaluate, however, because the fresh and frozen samples were obtained at different times.

The studies mentioned above suggest that the effects of storage on the quantitative determination of HDL are related to the methods used. For example; the generally lower lipoprotein levels determined using ultracentrifugal techniques indicate that the lipoproteins were lost from the density range below 1.21 g/ml. The loss was greater with VLDL and LDL, than with HDL (26–28), and could have resulted either from aggregation and complete removal from solution, or from a shift to higher densities; these possibilities were not distinguished. It is clear from other studies, however, that the total cholesterol concentration of frozen samples remains stable for at least several years (42, 43) and it is more likely that some changes in lipoprotein density occurred during storage. One such change might result from a partial loss of lipid on freezing or during storage. The ease with which lipoproteins are precipitated by polyanion-metal ion procedures decreases as the lipid content decreases (44). Therefore, partial loss of lipid could result in an increase in unprecipitated apoB-containing lipoproteins in the heparin- $\text{MnCl}_2$  supernatants, which would increase the apparent HDL-cholesterol values. The increase in apoB-containing lipoproteins



in heparin-MnCl<sub>2</sub> supernatants may explain the increased triglyceride concentration reported in supernatants prepared from frozen plasma samples (29).

The specific lipoprotein structural changes responsible for the present observations were not investigated. Nonetheless, several points can be made. First, the effects of storage are not constant over the entire range of HDL-cholesterol concentration; they may be underestimated if only the change in the mean level of a group of samples is examined. Second, while the apparent changes that occurred during either unfrozen or frozen storage were related to HDL-cholesterol levels, the correlations were weak, with correlation coefficients of 0.2–0.5. It is therefore difficult to predict reliably the behavior of any particular sample; in fact, the effect would probably be missed altogether in a small group of samples. Third, the magnitude of the effect will probably depend to some extent on the methodology used. For example, while part of the change in the cholesterol content of the heparin-MnCl<sub>2</sub> supernatant may result from the actual movement of lipoproteins from one fraction to another, it might also be influenced by the exchange of cholesterol between lipoproteins during storage. If this is so, methods based on the measurement of the major protein moiety of HDL, apoAI, for example, might reveal smaller changes than those based on measurement of HDL-cholesterol. Fourth, it is difficult to generalize about the usefulness of stored samples for HDL-cholesterol analyses. Under certain conditions the increase of apparent HDL-cholesterol levels in samples with low HDL-cholesterol concentrations could lead to a significant underestimation of cardiovascular risk, when the analyses are performed in stored samples. Conversely, risk could be overestimated in patients with high HDL-cholesterol levels. The possible effects of storage therefore warrant consideration whether the analyses are performed for clinical or epidemiological purposes. Finally, the safest course of action is to perform HDL-cholesterol analyses as soon as possible, preferably as soon as the sample is drawn. The present studies indicate, however, that if samples must be stored, short-term storage at –20°C gives smaller changes in measured HDL-cholesterol levels than storage at 4°C when the measurements are made with the heparin-MnCl<sub>2</sub> procedure. ■

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