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THE EFFECTS OF FREEZE-THAWING AND STORAGE ON THE ULTRACENTRIFUGAL PROPERTIES OF HUMAN SERUM LIPOPROTEINS

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ABSTRACT

The effects of freeze-thawing and of storage on the ultracentrifugal characteristics of human serum lipoproteins have been studied. Two different rates of freezing and thawing have been used. Storage of both sera and lipoproteins in salt solutions has been evaluated at three different temperatures: between -30 and -26°C, between -5 and 0°C, and between 0 and +4°C. Adequate preservation of lipoproteins stored as serum at a temperature between -5 and 0°C was maintained for a period of 4 weeks, between 0 and +4°C for a period of two weeks, and between -30 and -26°C for only a few days.

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INTRODUCTION

Several investigations on the effects of freezing on human serum lipoproteins have been reported in the literature. In one investigation a comparison of the extractability of lipis with ether was made between liquid and frozen lipoprotein solutions.[1] In other studies, the extent of lipoprotein change due to freezing and storage was investigated by noting the alteration in solubility.[2,3] The effects of freeze-thawing and storage on the ultracentrifugal properties of lipoproteins have also been reported.[4,5] However, these observations were limited to the study of only one lipoprotein group and were carried out over only relatively short periods of time. This paper reports (a) the effects of freeze-thawing and (b) the effects of freezing, prolonged storage, and thawing on the ultracentrifugal properties of all the major classes of human serum lipoproteins.

EXPERIMENTAL PROCEDURE

Ultracentrifugal Isolation

Isolation of the serum lipoproteins was accomplished by using one of two procedures; A[6] and B.[7] Each of these procedures allowed complete study of all the major classes of serum lipoproteins. The advantage of Procedure B, developed during the experiment, was its comparative simplicity and modest serum requirements.

Procedure A

For isolation of low-density lipoproteins, serum was diluted with an equal volume of NaCl solution ($\rho_{20/4^{\circ}}$ = 1.1172 g/ml), yielding a protein and lipoprotein-fr

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solution (background) of $\rho_{20/4}o = 1.06$ g/ml.

For isolation of intermediate high-density lipoproteins (HDL-2), serum was diluted with an equal volume of $D_2O - NaNO_3$ solution of $\rho_{20/4}o = 1.2429$ g/ml, resulting a background solution of $\rho_{20/4}o = 1.125$ g/ml.

For isolation of total high-density lipoproteins (HDL-2 and 3), serum was diluted with an equal volume of $D_2O - NaNO_3$ solution of $\rho_{20/4}o = 1.3895$ g/ml, yielding a background solution of $\rho_{20/4} = 1.20$ g/ml.

Procedure B

For isolation of the total serum lipoproteins, 2 ml serum, 0.94 ml of NaCl solution of $\rho_{20/4}o = 1.0060$ g/ml, and 3 ml of NaBr solution of $\rho_{20/4}o = 1.3895$ g/ml were mixed together, yielding a background solution of $\rho_{20/4}o = 1.205$ g/ml.

For both procedures, the above serum solutions were ultracentrifuged for 24 hr at 40,000 in a Spinco Model-L ultracentrifuge using a 40.2 rotor. Rotor temperature was maintained between 18 and 20°C. After ultracentrifugation the respective lipoprotein fractions, concentrated in the top of the 6-ml preparative tube (free of the serum proteins), were quantitatively removed with a capillary pipette into a 1-ml volumetric tube.

Ultracentrifugal Analysis

Determinations of flotation rates and concentrations were carried out in a Spinco Model-E analytical ultracentrifuge. Lipoprotein classes were classified according to their flotation rates in the media used in the above procedures. In Procedure A, the lipoprotein classes, measured according to the established low-density classification (with flotation rate versus concentration and Johnston-Ogston correction) were: $S^0_{f(1.06)}$ 100 to 400, 20 to 100, 12 to 20, 0 to 12, HDL-2, and HDL-2 and 3. In Procedure B, the corresponding lipoprotein classes were, respectively: $S_{f(1.20)}$ 185 to 485; 61 to 185; 44 to 61; 16 to 44 and 0 to 6. In Procedure A, the low-density lipoprotein classes are distributed between $S^0_{f(1.06)}$ 0 and $S^0_{f(1.06)}$ 400, which in Procedure B correspond to lipoprotein classes between $S_{f(1.20)}$ 16 and $S_{f(1.20)}$ 485. Similarly, the sum of both HDL-2 and HDL-3 evaluated in Procedure A correspond to lipoproteins of $S_{f(1.20)}$ 0 to 6 measured in Procedure B.

In Procedure A, the standard errors of the measurement for the classes of lipoproteins S^0_f 0 to 12, S^0_f 12 to 20, S^0_f 20 to 100, S^0_f 100 to 400, HDL-2, and HDL-2 and 3, are 19, 16, 22, 34, 3.6, and 10, respectively. A correlation coefficient of 0.60 has been demonstrated between the mean of the samples of 252 duplicate determinations versus the absolute [illegible] samples in the S^0_f 20 to 400 class.[8] This would [illegible] coefficient to [illegible]

measured. Therefore it is reasonable to express the above standard errors as percentage errors by dividing them by their respective means. They are 7%, 3%, 10%, 12%, 6% and 4%, respectively.

In Procedure B, duplicates have been run, and the results are given as averages together with average deviations.

Materials

Two-milliliter aliquots of serum and 1-ml aliquots of lipoprotein solutions isolated by Procedure A were pipetted into 10-ml and 5-ml screw-cap vials, respectively, which were carefully sealed under nitrogen.

Freezing Processes

For rapid freezing (r.f.), the vials containing serum were dipped into dry ice and acetone for 50 sec. Vials containing lipoprotein solutions were dipped for 60 sec. For slow freezing (s.f.), the vials were set in a deep freeze (-28°C) for 1 hr. In both processes, a final temperature of -28 ± 1°C was reached, as checked with a copper-constantan thermocouple.

Thawing Processes

For rapid thawing (r.th.), the vials containing serum were placed in a water bath at +37°C for 120 sec and those containing the lipoprotein solutions for 60 sec. For these conditions each solution reached a temperature of 24 ± 1°C. For slow thawing (s.th.), the vials were left at room temperature (24 ± 2°C) for 1 hr, at which time temperature equilibrium had usually been reached.

Storage

The effects of storage were studied at three temperatures; (1) between -30 and -26°C; (2) between -5 and +0°C; and (3) between 0 and +4°C. Solution densities were determined with a 1-ml pycnometer and pH was measured with a Model H-2 Beckman pH meter.

RESULTS

Serum

Table I presents the results of freeze-thawing of serum. Repeated cycles (from 1 to 3 times) of either rapid or slow freeze-thawing of serum produce no significant changes in either the concentration or in the flotation rates of the major classes of serum lipoproteins, with the exception of the HDL-2 class. Frequently, after such treatment, small amounts of insoluble material appeared which may have consisted of denatured serum proteins, chylomicrons, or both. However, this material did not represent degradation of any of the measured classes of serum lipoproteins.

Once it was established that minimal changes resulted from a single freeze-

Table I[a]

Serum		Treatment	Concentration (mg %) $S^0_{f(1.06)}$ 100 to 400	20 to 100	12 to 20	0 to 12	Total 0 to 400	HDL-2	HDL-283	lipo
			Freeze-thawing of serum							
A	1	orig	50	64	66	303	483	37	350	
	2	rf rth(1x)[b]	46	70	63	115	494	55	342	
D	1	orig	314	261	49	318	942	80	258	
	2	sf sth(1x)	334	269	58	328	989	--	--	
	3	rf rth(1x)	342	281	49	308	980	118	287	
	4	rf rth(3x)	302	253	49	313	917	--	--	
D_1	1[c]	orig	369	300	65	335	1069	--	341	
	2[c]	sf sth(1x)	358	300	64	326	1047	--	307	
	3[c]	rf rth(3x)	350	288	54	330	1021	--	342	
E	1	orig	163	159	55	323	700	60	243	
	2	sf sth(1x)	151	136	55	302	644	84	257	
	3	sf sth(3x)	149	131	53	305	638	45	245	
	4	rf rth(3x)	154	159	65	300	678	--	--	
E_1	1[c]	orig	110	143	49	291	593	--	--	
	2[d]	rf rth(2x)	124	148	53	314	639	--	--	
	3[c]	rf rth(3x)	140	151	50	317	658	--	--	

[a] In this and following tables the dash (-) means that no run is available. For the consideration of error see Experimental Section.
[b] The number in parentheses indicates the number of freeze-thawing processes.
[c] Average of two lipoprotein determinations.
[d] Average of four lipoprotein determinations.

thawing process, the effects of storage in a deep-freeze at temperatures between -30 and -26°C were studied in combination with each of the freezing and thawing processes (rapid and slow). Daily analyses of samples (not presented) revealed no lipoprotein concentration changes until after 7 days of storage. Table II presents the results of storage from 7 days to as long as 1 year. Although some lipoprotein changes were observed, no significant differences in degradation were observed to result from different rates of freezing and thawing. In general, over a period of study there was a significant decrease in most of the lipoprotein classes. However, a consistent but transient elevation in concentration of HDL-2 was observed after 7 days of storage.

Tables III, IV, and V present the results of a comparative study of storage of three sera F, G, and H, using Procedure B, at temperatures between -30 and -26°, between -5 and 0°C, and between 0 and +4°C, respectively.

At temperatures between -30 and -26°C, lipoproteins with flotation rates above $S_{f(1.20)}61$ were more rapidly degraded and showed more extensive degradation over comparable periods of storage than lipoproteins with flotation rates less than $S_{f(1.20)}$ 61. In contrast, the $S_{f(1.20)}16$ to 61 as well as the $S_{f(1.20)}0$ to 6 lipoprotein classes showed relative stability to storage between these temperatures. After 6 months, the change observed in the $S_{f(1.20)}0$ to 6 class was a decrease in concentration amounting at most to 30%.

At temperatures between -5 and 0°C, all lipoprotein classes appear to be stable for a period of at least 28 days. Thereafter, the $S_{f(1.20)}16$ to 61 and $S_{f(1.20)}0$ to 6 lipoprotein classes appear to be more resistant to degradation than the other lipoprotein classes. After 21 to 28 days of storage, the $S_{f(1.20)}0$ to 6 lipoprotein class showed some qualitative changes not evident in Table IV. These changes were a broadening of the lipoprotein distribution with an increase in the flotation rate of the major peak.

At temperatures between 0 and +4°C, all lipoprotein classes appear to be stable for a period of at least 14 days. Thereafter, there is no consistent pattern of degradation. However, after 7 to 14 days of storage, the $S_{f(1.20)}0$ to 6 lipoprotein class showed a broadening of the lipoprotein distribution with an increase in the flotation rate of the major peak.

Lipoprotein Solutions

Tables VI and VII present the results of freeze-thawing of lipoprotein solutions. Lipoproteins in salt solution appear to be as stable as lipoproteins in serum after

Table II

Storage of Serum at a temperature between -30° and -26°C

Serum		Treatment[a]	Serum concentration (mg %) $S^0_{f(1.06)}$ 100 to 400	20 to 100	12 to 20	0 to 12	Total 0 to 400	HDL-2	HDL-2 & 3	Tot Lipop
A	1	orig	50	64	66	303	483	37	350	83[illegible]
	2	rf-7 rth	69	104	67	278	518	47	334	[illegible]
	3	rt-90 rth	49	83	51	226	409	37	310	719
B	1	orig	127	248	82	315	772	40	256	10[illegible]
	2	sf-7 sth	166	192	67	270	695	73	290	98[illegible]
	3	sf-200 sth	16	62	44	231	353	27	226	[illegible]
	4	sf-385 sth	traces	traces	27	230	257	--	191	[illegible]
C	1	orig	234	200	39	121	594	58	258	85[illegible]
	2	rf-7 rth	168	160	34	110	472	69	238	[illegible]
	3	rf-187 rth	83	99	54	198	434	76	249	[illegible]
	4	rf-369 rth	7	10	27	220	264	--	219	[illegible]
D	1	orig	314	261	49	318	942	80	258	1100
	2	rf-7 rth	256	143	39	285	723	119	272	995
	3	rf-180 rth	241	159	35	236	671	87	278	9[illegible]
	4	rf-343 rth	95	80	39	220	434	82	186	620
D	1	orig	146	141	51	313	651	57	237	888
	2	sf-7 sth	135	121	30	287	573	61	225	798
	3	sf-7 rth	107	133	40	260	540	35	272	81[illegible]
	4	sf-180 sth	127	74	50	273	524	84	180	704
	5	sf-343-sth	traces	traces	35	218	243	--	187	430

[a] The numbers between rf or sf and rth or sth indicate days of storage.

Table III

Serum		Treatment[a]	Storage of serum at a temperature between -30° and -26°C						
			Serum concentration (mg %)						
			$S_{f(1,20)}$						
			185 to 485	61 to 185	44 to 61	16 to 44	Total 16 to 485	0 to 6	[illegible]
F	1	orig	132 ± 5	515 ± 25	56 ± 1	295 ± 9	998 ± 20	268 ± 8	1[illegible]
	2	7	159 ± 3	152 ± 3	20 ± 3	227 ± 7	558 ± 9	265 ± 6	[illegible]
	3	14	183 ± 8	225 ± 5	23 ± 5	235 ± 5	666 ± 12	260 ± 7	[illegible]
	4	28	145 ± 5	140 ± 7	25 ± 3	240 ± 9	550 ± 13	267 ± 9	[illegible]
	5	109	56 ± 11	85 ± 8	18 ± 5	231 ± 7	390 ± 16	256 ± 4	[illegible]
	6	20 1	88 ± 8	128 ± 8	30 ± 3	262 ± 8	508 ± 14	205 ± 10	[illegible]
G	1	orig	455 ± 24	308 ± 9	36 ± 2	205 ± 7	1004 ± 27	189 ± 4	[illegible]
	2	7	279 ± 14	150 ± 7	30 ± 1	216 ± 5	675 ± 16	205 ± 11	[illegible]
	3	21	179 ± 8	172 ± 17	28 ± 1	189 ± 11	568 ± 22	196 ± 3	[illegible]
	4	28	248 ± 9	105 ± 6	23 ± 2	195 ± 9	576 ± 14	195 ± 4	[illegible]
	5	102	140 ± 6	89 ± 4	28 ± 4	208 ± 10	405 ± 13	212 ± 8	[illegible]
	6	178	139 ± 10	77 ± 3	24 ± 1	169 ± 8	409 ± 13	175 ± 13	[illegible]
H	1	orig	163 ± 5	245 ± 6	30 ± 1	309 ± 8	747 ± 11	239 ± 6	[illegible]
	2	7	268 ± 7	109 ± 6	23 ± 2	252 ± 8	652 ± 12	238 ± 7	[illegible]
	3	21	139 ± 7	110 ± 10	24 ± 3	260 ± 5	533 ± 14	242 ± 12	[illegible]
	4	28	168	128	26	307	629	248	877
	5	102	91	68	15	262	436	240	676
	6	178	67 ± 10	55 ± 5	17 ± 3	250 ± 10	389 ± 15	218 ± 10	[illegible]

[a] The number indicates the number of days of storage. The samples have been frozen and thawed slowly.

Table IV

Storage of serum at temperatures between -5°C and 0°C

Serum		Treat-ment	Serum concentration (mg %)						
			$S_{f(1.20)}$						
			185 to 485	61 to 185	44 to 61	16 to 44	Total 16 to 485	0 to 6	[illegible] 0 [illegible]
F	1	orig	132 ± 5	515 ± 25	56 ± 1	295 ± 9	998 ± 20	268 ± 8	126[illegible]
	2	7	142 ± 6	493 ± 20	55 ± 1	303 ± 6	993 ± 25	263 ± 6	1256 [illegible]
	3	14	134 ± 4	501 ± 21	54 ± 2	318 ± 7	1007 ± 23	263 ± 5	1270 [illegible]
	4	28	136 ± 4	506 ± 20	56 ± 1	296 ± 6	994 ± 22	256 ± 4	1250 [illegible]
	5	109	195 ± 15	349 ± 19	48 ± 3	283 ± 5	875 ± 25	206 ± 5	108[illegible]
	6	201	148	183	44	261	636	205	84[illegible]
G	1	orig	455 ± 24	308 ± 9	36 ± 2	205 ± 7	1004 ± 27	189 ± 4	119[illegible]
	2	7	461 ± 17	318 ± 11	33 ± 2	223 ± 7	1035 ± 22	179 ± 6	1214 [illegible]
	3	21	484 ± 19	309 ± 7	31 ± 1	219 ± 4	1043 ± 24	172 ± 7	121[illegible]
	4	28	497 ± 17	307 ± 7	35 ± 3	229 ± 10	1068 ± 21	199 ± 5	126[illegible]
	5	102	343 ± 19	216 ± 6	33 ± 3	218 ± 7	810 ± 21	142 ± 6	95[illegible]
	6	178	219 ± 16	128 ± 3	28 ± 2	150 ± 5	525 ± 17	120 ± 5	645 [illegible]
H	1	orig	163 ± 5	245 ± 6	30 ± 1	309 ± 8	747 ± 11	239 ± 6	986 [illegible]
	2	7	171 ± 6	279 ± 3	30 ± 2	289 ± 7	769 ± 10	238 ± 6	1007 [illegible]
	3	21	159 ± 5	246 ± 4	29 ± 3	283 ± 3	717 ± 8	239 ± 6	956 [illegible]
	4	28	168	237	31	328	764	216	980
	5	102	138	179	25	308	650	190	840
	6	178	87 ± 8	110 ± 5	25 ± 3	280 ± 6	502 ± 12	172 ± 7	674 [illegible]

Table V

Storage of serum at a temperatures between 0° and +4°C

Serum		Treat-ment	Serum concentration (mg %) $S_{f(1.20)}$ 185 to 485	61 to 185	44 to 61	16 to 44	Total 16 to 485	0 to 6	Tot[illegible] 0 to [illegible]
F	1	orig	132 ± 5	515 ± 25	56 ± 1	295 ± 9	998 ± 20	268 ± 8	1266 [illegible]
	2	7	139 ± 6	479 ± 19	57 ± 2	290 ± 8	965 ± 22	256 ± 8	12[illegible]
	3	14	125 ± 5	486 ± 14	63 ± 2	312 ± 10	986 ± 18	247 ± 9	1233 [illegible]
	4	28	123 ± 7	437 ± 16	59 ± 2	320 ± 12	939 ± 21	236 ± 6	117[illegible]
	5	109	132 ± 4	406 15	59 ± 2	303 ± 14	900 ± 21	160 ± 9	10[illegible]
	6	201	133 ± 5	400 14	68 ± 3	322 ± 10	923 ± 18	223 ± 9	11[illegible]
G	1	orig	455 ± 24	308 ± 9	36 ± 2	205 ± 7	1004 ± 27	189 ± 4	119[illegible]
	2	7	460 ± 11	312 ± 10	39 ± 2	238 ± 11	1049 ± 19	278 ± 6	1327 [illegible]
	3	21	420 ± 10	285 ± 10	37 ± 2	218 ± 6	960 ± 15	212 ± 5	117[illegible]
	4	28	425 ± 13	299 ± 7	37 ± 2	224 ± 3	985 ± 15	139 ± 6	112[illegible]
	5	102	75 ± 15	96 ± 16	29 ± 1	162 ± 18	362 ± 24	103 ± 6	465 [illegible]
	6	178	34 ± 16	72 ± 5	29 ± 1	164 ± 8	299 ± 19	149 ± 6	448 [illegible]
H	1	orig	163 ± 5	245 ± 6	30 ± 1	309 ± 8	747 ± 11	239 ± 6	986 [illegible]
	2	7	160 ± 4	253 ± 14	31 ± 2	307 ± 7	751 ± 16	236 ± 4	987 [illegible]
	3	21	171 ± 7	174 ± 7	37 ± 2	310 ± 10	747 ± 13	243 ± 10	990 [illegible]
	4	28	142	167	35	272	774	272	1046
	5	100	152	120	29	225	710	225	935
	6	178	158 ± 20	153 ± 70	22 ± 3	214 ± 13	753 ± 26	214 ± 13	967 [illegible]

Table VI

Freeze-thawing of lipoprotein in salt solutions

Serum		Treat-ment	Serum concentration (mg. %) S_f^0 20 to 400[a]	S_f^0 0 to 20[b]	Total LDL
C	1	orig	652 ± 30	283 ± 15	935 ± 5
	2	rf rth(1x)	612 ± 35	285 ± 17	897 ± 18
	3	sf sth(1x)	621 ± 45	280 ± 15	901 ± 30

[a] The lipoproteins of this class were in NaCl solutions ($\rho_{20/4}$ = 1.007 g/ml)
[b] The lipoproteins of this class were in NaCl solutions ($\rho_{20/4}$ = 1.063 g/ml)

Table VII

Freeze-thawing of lipoproteins in salt solutions

Serum		Treat-ment	Serum concentration (mg %) $S^0_{f(1.06)}$ 100 to 400	20 to 100	12 to 20	0 to 12	Total 0 to 400	HDL-2	HDL-2 & 3	Total Lipopro[illegible]
B	1	orig	127	248	82	315	772	40	256	1028
	2	sf sth(5x)	137	260	89	290	776	0	274	1050
	3	rf rth(10x)	166	130	24	116	436	0	266	702
B_1	1	orig	166	192	67	270	695	73	290	985
	2	sf sth(5x)	135	147	54	285	621	0	300	921
	3	rf rth(5x)	178	174	58	265	675	24	309	984

repeated processes of freeze-thawing. In order to detect any significant degrad tion, freeze thawing must be repeated from 5 to 10 times. However, once degr tion appears, the actual loss of material can be very large. The HDL-2 lipopro class appears to be more labile in a salt solution than any other lipoprotein clas when successively frozen and thawed.

Tables VIII and IX present the results of storage of lipoproteins in salt solution between -30 and -26°C. After 10 days the total concentration of $S^0_{f(1.06)}$ 0 to 400 class has decreased by approximately 20 to 30%. The $S^0_{f(1.06)}$ 20 to 400 class show an earlier degradation than the $S^0_{f(1.06)}$ 0 to 20 class. In addition, these data show a lability of the HDL-2 lipoprotein class.

When the two different rates of freezing and thawing are investigated in connection with storage, measurable differences do appear for lipoproteins in specific NaCl media ($\rho_{20/4}$ = 1.007 g/ml for $S^0_{f(1.06)}$ 20 to 400, and 1.063 g/ml for $S^0_{f(1.06)}$ 0 to 20 class). In such lipoprotein solutions, the application of rapid freezing and thawing appears to produce more extensive changes than those produced by slow freezing and thawing. Comparisons of the average values of $S_{f(1.06)}$ 20 to 400 lipoproteins in stored samples exposed to the rapid process (Samples 3, 7, and 10 in Table IX) and those samples exposed to the slow process (Samples 2, 6, and 9) with their original unfrozen values showed a 34% loss for the rapid process and a 15% loss for the slow processes. This suggests that storage of this lipoprotein group after rapid freezing provokes a faster rate of degradation than storage following a slow freezing process.

The possibility that variation in pH with storage might be responsible for the observed degradations was checked. Several measurements carried out at the beginning and throughout the experimental period in samples containing no nitrogen showed no pH changes.

DISCUSSION

Our experiments lead to a definite conclusion, important from the practical point of view, for the storage of lipoproteins in clinical investigations. The results indicate that storage of serum at a temperature between -5 and 0°C assures preservation of human serum lipoproteins for a period of at least 4 weeks.

Storage of serum at between 0 and +4°C for as long as 2 weeks also reveals no quantitative or qualitative changes in the lipoprotein pattern. However, after 2 weeks of storage at this temperature qualitative changes may be observed prior to significant

Table VIII

Storage of lipoprotein in salt solutions at a temperature between -30° and -26°C.

Serum	treatment[a]	Serum concentration (mg %)		
		S_f^0 20 to 400[b]	S_f^0 0 to 20	Total LDL
C 1	orig	625	278	903
2	sf-10 sth	478	303	711
3	rf-10 rth	386	249	635
4	rf-22-rth	460	265	725
5	rf-22-sth	479	234	713
6	sf-23 sth	516	261	777
7	rf-23 rth	481	235	716
8	sf-23 rth	588	278	866
9	sf-33 sth	610	253	863
10	rf-33 rth	418	264	682
11	rf-181 sth	286	101	387
12	sf-181 sth	331	239	570

[a] The numbers between rf or sf and rth or sth indicate days of storage.
[b] See notes beneath Table VI.

Table IX

Storage of lipoproteins in salt solutions at temperatures between -30° and -26°C.

Serum		treatment[a]	Serum Concentration (mg %) $S_{f(1.06)}$ 100 to 400	20 to 100	12 to 20	0 to 12	Total 0 to 400	HDL-2	HDL-2 & 3	Total Lipoprotein
B	1	orig	127	248	82	315	772	40	256	1[illegible]8
	2	rf-7 rth	156	208	75	270	709	0	217	926
B_1	1	orig	166	192	67	270	695	73	290	[illegible]
	2	rf-10 rth	147	136	58	219	560	0	210	770
	3	sf-10 sth	144	123	59	241	567	0	270	837
	4	sf-23 sth	105	158	44	201	508	0	246	75[illegible]
D	1	orig	314	261	49	318	942	80	257	1200
	2	sf-152-sth	189	105	34	185	513	trace	223	736
	3	rf-152-sth	97	62	10	135	304	61	225	529
	4	rf-152 sth	187	124	48	221	580	59	185	765
E	1	orig	135	121	30	287	573	61	225	798
		sf-183-sth	98	86	45	248	477	--	--	--
E_1	1	orig	107	133	40	260	540	35	272	812
	2	rf-183 rth	0	0	14	84	98	0	78	176
	3	"	51	11	25	167	254	55	208	462

[a] The numbers between rf or sf and rth or sth indicate days of storage.

concentration changes of lipoprotein classes. These qualitative changes frequently appear as concentration increases and decreases within any broad lipoprotein class, without any net concentration change of that class. In all studies conducted at temperatures above 0°C an uncontrolled factor was bacterial contamination.

Storage for even a few days at -28°C is associated with variations in lipoprotein concentrations and subsequent degradation. Quantitative studies of lipoproteins stored at this temperature would be extremely uncertain and most probably invalid.

For all conditions of storage, degradation occurs primarily in the class of large lipoproteins characterized by flotation rates of $S^0_{f(1.06)}$20 to 400 (Procedure A) or $S_{f(1.20)}$61 to 485 (Procedure B). This degradation occurred under experimental conditions in which the principal variables were storage temperatures, small cyclic temperature variations during storage, length of storage, rate of freezing and thawing, and within certain limits, salt concentration. Two of these variables, storage temperature and length of storage, in particular appear to play a prominent role in lipoprotein degradation. It is important to consider how these variables may be involved in lipoprotein degradation in order to form a basis for further investigations to determine the optimal conditions for lipoprotein storage.

Possible factors responsible for the observed effects as a function of temperature of storage and duration of storage may be listed as follows:

(a) microbiological activity

(b) crystallization of water in lipoprotein solution or serum, including

  (1) crystallization of "unbound" water

  (2) crystallization of "bound" water

  (3) development of local elevations in lipoprotein concentration

(c) molecular rearrangements within the lipoprotein.

At temperatures above 0°C in particular, we cannot exclude microbiological activity, since no antibiotic was used. In contrast, for storage at temperatures below 0°C, microbiological considerations become secondary to considerations involving the crystallization of water in a lipoprotein solution or serum.

Such factors as storage temperature, duration of storage, rate of freezing, etc., associated with the degradation of lipoproteins are also known to influence the formation, structure, and growth of ice crystals. It would thus be reasonable to suspect that there may exist some relationship between the degradation of lipoproteins and the formation and structure of ice crystals either in their aqueous environment (unbound water) or in their structural or bound water content. It is difficult from our

experiments to determine which type of water, bound or unbound, upon crystallization is more important in the degradation of lipoproteins. However, in this discussion we will consider how changes in each might cause degradation of lipoprotein molecules. Crystallization of the water might cause mechanical distortion or shearing of the lipoprotein molecules, particularly the larger ones. If this were the case we would have expected a significant degradation of these lipoprotein molecules when subjected to a single freeze-thawing process. Instead detectable degradation occurred only after several freeze-thaw processes. Hence, initial crystallization of unbound water alone may not be the crucial factor in lipoprotein degradation. This leads us to consider the crystallization of bound water as a factor in the degradation of lipoprotein molecules. The bound or structural water refers to the water of hydration; this is a prominent component of the lipoprotein molecule.[9] The transformation of this water into a crystalline form could lead to the disruption of the lipoprotein molecule and to the observed degradation. However, multiple freeze-thawing or prolonged storage is necessary to produce the harmful changes in the bound water, unbound water, or both.

In the course of the freezing process and subsequent storage, local elevations of lipoprotein concentration could develop and thus influence the stability of lipoprotein molecules. An occasional phenomenon we have come upon in some of our studies suggests that this is not a factor in degradation. In a few samples of serum stored at a temperature between -5 and 0°C, we have observed an apparent separation of two phases, one of ice crystals and the other a gelatinous sediment. After carefully separating the two phases, we have found that the ice phase had a density at 20°C between 1.0048 g/ml and 1.0056 g/ml. Direct analytical ultracentrifugation of this phase indicated the presence at low concentration of some sedimenting material of high molecular weight. The gelatinous phase was only partially soluble in NaBr solution (density 1.20 g/ml). Ultracentrifugal analysis of this solution revealed a lipoprotein spectrum qualitatively similar to the original unfrozen samples. However, there was a loss of approximately 25% in total lipoprotein content, which may be ascribed either to the difficulty in handling this gelatinous phase or to actual degradation. Yet, if local elevations in concentration were a major factor in lipoprotein degradation, we would have expected a much greater loss.

Finally, structural changes in the lipid or protein moiety of the lipoprotein molecule may be a factor in the degradation process. For the lipid moiety, at these low

temperatures, this would depend on the crystalline characteristics of the lipid structural units as well as the manner in which these units are held together in the intact lipoprotein. It is interesting to note that the least stable lipoprotein molecules are those having a high lipid (particularly glyceride) content and a low protein content.

One or a combination of some of the above factors may play a crucial role in the observed degradation of lipoproteins under the aforementioned conditions of freezing and storage. Determination of the crucial factor (or factors) in such degradation and its subsequent control would make possible prolonged storage of lipoproteins with minimal alterations of ultracentrifugal properties.

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