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TITRATION OF SERUM LIPOPROTEINS WITH LIPOPROTEIN PRECIPITANTS.(U)
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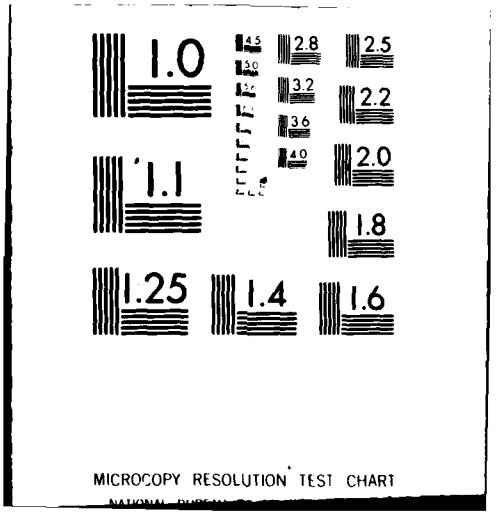
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Report SAM-TR-81-31

TITRATION OF SERUM LIPOPROTEINS WITH LIPOPROTEIN PRECIPITANTS

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Interim Report for Period September 1977 — October 1980

USAF SCHOOL OF AEROSPACE MEDICINE
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NOTICES

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This report has been reviewed by the Office of Public Affairs (PA), and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

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Serum lipoproteins were titrated by sequentially increasing the concentration of precipitant in each of a series of aliquots of a serum pool or of a single serum. After standing 2 hrs at 4°C, each tube was centrifuged and the concentration of cholesterol in the supernate was measured. The precipitants tested were dextran sulfate-$MnCl_2$ and phosphotungstate-$MgCl_2$. As the concentration of precipitant increased, the concentration of cholesterol in the supernate decreased until the very low density lipoproteins (VLDL) and most of the low density lipoproteins (LDL), but none (or very little) of the high density lipoproteins (HDL), had

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20. ABSTRACT (Continued)

precipitated. Further increases in the volume of added precipitant usually resulted in little or no decrease in the cholesterol level in the supernate until some of the HDL had precipitated. However, serum specimens differed in their response to the titrants. Titration alone sometimes gave equivocal indications of the correct HDL cholesterol level when our dextran sulfate reagent (not a commercial product) was used. Monitoring the titrations with lipoprotein electrophoresis permitted unequivocal identification of the HDL cholesterol level in most of the dextran sulfate titrations and in all our phosphotungstate titrations. These lipoprotein titrations were highly useful in establishing the optimal volume of precipitant solution and in monitoring the routine HDL cholesterol determinations.

TITRATION OF SERUM LIPOPROTEINS WITH LIPOPROTEIN PRECIPITANTS

INTRODUCTION

Recent years have seen a growing recognition of the importance of the negative correlation between cholesterol associated with high density lipoproteins (HDL) and the risk of coronary artery disease (1). This risk was reported to be independent of the risk associated with the low density lipoprotein (LDL) cholesterol level (2). Since the latter association is positively correlated, it is not surprising that the ratio of the total cholesterol to the HDL cholesterol in serum is an even better indicator of risk of coronary artery disease than either level alone (3). Consequently, measurements of HDL cholesterol levels are required to provide the best serum-based estimate of cardiovascular disease risk.

Serum HDL cholesterol levels are measured by several methods; the most popular is to precipitate the very low density lipoproteins (VLDL) and the LDL with a combination of polyanions and divalent cations and to measure the cholesterol in the supernate, which contains the HDL and associated cholesterol. Three polyanion-cation combinations are often used: heparin-$MnCl_2$, dextran sulfate-$MgCl_2$, and phosphotungstate-$MgCl_2$. Each combination has advantages and limitations. When applied to the same serum samples, these methods give similar average values, but the differences, though small, are statistically significant (4).

In an attempt to assess the correct HDL cholesterol level, we titrated serum samples with increasing volumes of lipoprotein precipitants and measured the cholesterol level in the resulting supernates. We postulated that the cholesterol level in the supernates would decrease with increasing amounts of precipitant until all the VLDL + LDL were precipitated, leaving the cholesterol level unchanged (a plateau on the titration curve) until sufficient precipitant was added to begin precipitating the HDL. As the following report shows, some serum samples titrated with some precipitants fit this hypothesis, but some combinations do not.

MATERIALS AND METHODS

The dextran sulfate-$MnCl_2$ precipitant solution (5) was prepared by adding 5.0 g dextran sulfate 500, sodium salt (Pharmacia Fine Chemicals*), to approximately 70 ml normal saline (0.8% NaCl in H_2O), then adding saline to reach a final volume of 100 ml, and mixing well. We added 1.0 ml of this solution to 10.0 ml of 1 M $MnCl_2$ (19.79 g $MnCl_2 \cdot 4H_2O$, ACS reagent grade, in H_2O and made

*Use of chemicals and equipment from named companies does not imply that the products are superior to other products of comparable grade from other suppliers nor does it constitute endorsement of the products by the United States Air Force.

to a final volume of 100 ml), then mixed well. The solution can be stored in a dark plastic bottle at 4-8°C, but should be prepared fresh once every week.

The phosphotungstate-$MgCl_2$ precipitant solutions A and B (5) were prepared by:

1. (Solution A) Mixing 16.0 ml of 1 M NaOH and 84 ml distilled H_2O, then adding 1.0 g phosphotungstic acid (ACS reagent grade, Mallinckrodt Chemical Co.) and mixing well. We added small volumes of HCl (5 to 10N) to adjust the pH to a value near neutrality but no higher than 7.4 (5,6). When stored in a dark plastic bottle at 20-25°C, this solution was stable at least 3 months.

2. (Solution B) Dissolving 20.33 g $MgCl_2 \cdot 6H_2O$ (ACS reagent grade, Fisher Scientific Co.) in distilled H_2O, diluting to 100 ml, and mixing well. When stored in a dark plastic bottle at 20-25°C, this solution also was stable at least 3 months.

The supernates were prepared by precipitating the lipoproteins in small test tubes (5 ml) into which 1.0 ml serum was pipetted, followed by specified volume(s) of precipitant(s). In the phosphotungstate-$MgCl_2$ procedure, specified volumes of solutions A and B (see Tables 1 and 2) were added separately to the serum, with mixing after each addition. These solutions were used in the proportions of A:B::4:1. After being capped and thoroughly mixed, the serum-precipitant solution stood at 4°C for 2 hours before being centrifuged 10 minutes at 4°C at 6000 G (10,000 rpm in a 40.3 rotor in a Spinco Model L preparative ultracentrifuge). Since centrifugation at 1400 G for 30 minutes was adequate (7), the procedure was changed, and tubes were centrifuged in an ordinary laboratory refrigerated centrifuge at 4°C for 30 minutes at 2600 rpm, using a head with a radius slightly more than 7 inches (180 mm) measured from the center of rotation to the level of the centrifuged solution. After centrifugation, the clear supernates were decanted with great care to avoid transferring any particulate matter.

Cholesterol concentrations of both serum and supernates were measured with an Abbott Bichromatic Analyzer, Model ABA-100, using Boehringer-Mannheim enzymatic cholesterol reagents. Laboratory quality control procedures maintained agreement of levels measured in control solutions to within ± 2% of levels measured in the same controls using the standard Abell-Kendall method for cholesterol determinations (8). As appropriate, the levels measured in the supernates were corrected for dilution by the precipitant solutions. Only corrected values are reported below.

Lipoproteins were separated electrophoretically on agarose gel, stained with Oil Red O, and quantitated by densitometry. The procedures used were adapted from the quantitative method of Hulley et al. (9), based on the work of Noble et al. (10). The chief differences were that the agarose gel plates were prepared by spreading the agarose gel on microscope slides and that 0.025 M, instead of 0.05 M, barbital buffer was used to prepare the agarose gel and to fill the electrophoresis cells. Sample application differed in that 0.2 ml of sample solution was mixed with 0.2 ml of ion agarose, and an aliquot of 20 µl of this mixture was placed in the sample well on the slide for electrophoresis. Samples were electrophoresed in batches of 8 slides per cell at 40 mA (5 mA/slide) for 35 minutes. Subsequent staining and densitometry were

similar to that described by Hulley et al. (9) and by Noble et al. (10), but a different densitometer was used.

Each titration experiment used a series of 1.0-ml aliquots of an adequately large individual human serum sample or of a well-mixed pool of human serum. In each titration, the smallest volume of precipitant was added to the serum in the first tube in the series. The volume of precipitant was increased by a constant increment for each successive tube in the series. Precipitation of lipoproteins, preparation of the supernate, and analysis of the cholesterol levels were then carried out with each tube in the series, as described above. Each experiment also included analysis of the undiluted serum (no precipitant added).

EXPERIMENTAL RESULTS

Results of two serum titrations using dextran sulfate are plotted in Figure 1. To help determine when the correct HDL cholesterol level had been reached (all lipoproteins except the HDL precipitated), the change in cholesterol level (Δ-chol) in successive tubes was plotted (lower part of Fig. 1). If the basic hypothesis were true, the values for Δ-chol would become zero when sufficient precipitant had been added to precipitate all lipoproteins except the HDL, and the cholesterol level in that supernate would be the correct HDL cholesterol level. However, with pooled serum and 10% dextran sulfate (Fig. 1, serum A), the close agreement between duplicate tubes in the series showed that there was no plateau on the titration curve; Δ-chol apparently did not reach zero, but may have become more negative with the largest volume of precipitant.

The 10% concentration of dextran sulfate may have been strong enough to precipitate some HDL immediately after or even before the other lipoproteins were completely precipitated. Therefore, in subsequent titrations 5% dextran sulfate was used, and the range of volumes of precipitant added was extended. The data in Figure 1, serum B, show a plateau over the precipitant volume range of 110-160 µl, with a possible slightly lower plateau at 170-200 µl. The true HDL cholesterol level in this serum therefore appeared to be 22 mg/dl. Since the blood donor was a healthy young man, albeit with a high normal cholesterol level, the accuracy of this low level for HDL cholesterol was questioned.

The possibility that some HDL had been precipitated from serum A raised the same possibility with serum B, since precipitation of some HDL would produce an erroneously low estimate of the HDL cholesterol level. This possibility was checked using lipoprotein electrophoresis of selected supernates from a subsequent titration experiment. Some decrease in the density of the alpha HDL lipoprotein band was seen with the larger volumes of precipitant.

Further titrations were therefore conducted, including quantitative lipoprotein electrophoresis of all supernates as part of the experimental protocol. Results of a representative titration of pooled serum are summarized in Figure 2. Duplicate tubes were prepared and analyzed for precipitant volumes of 50-160 µl. The results show that the cholesterol levels in the supernates decreased 1 to 2 mg/dl with each 10-µl increment in precipitant

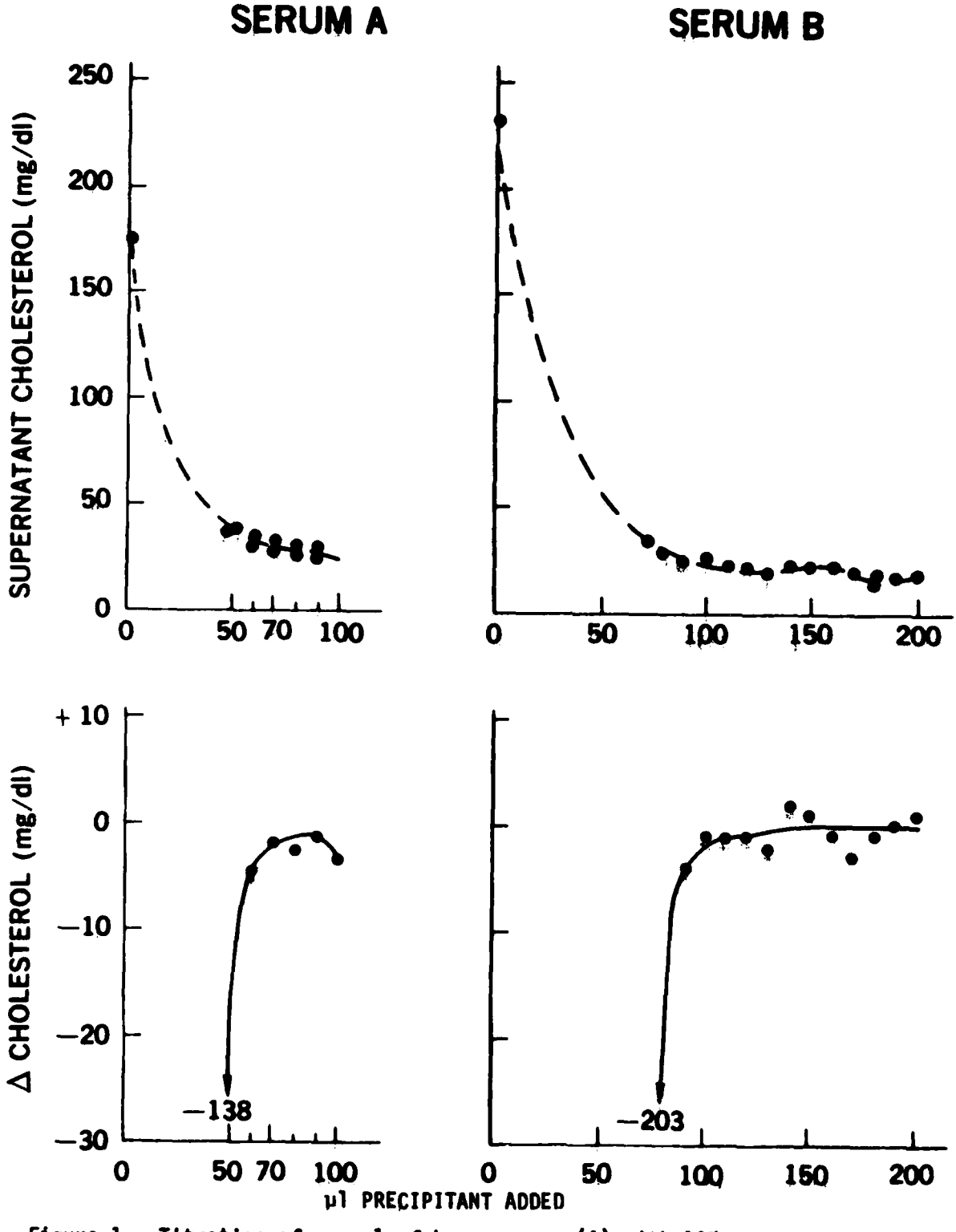

Figure 1. Titration of a pool of human serum (A) with 10% dextran sulfate-$MnCl_2$ and of an individual serum (B) with 5% dextran sulfate-$MnCl_2$. On the curve of serum A, duplicate tubes were prepared for each volume of precipitant.

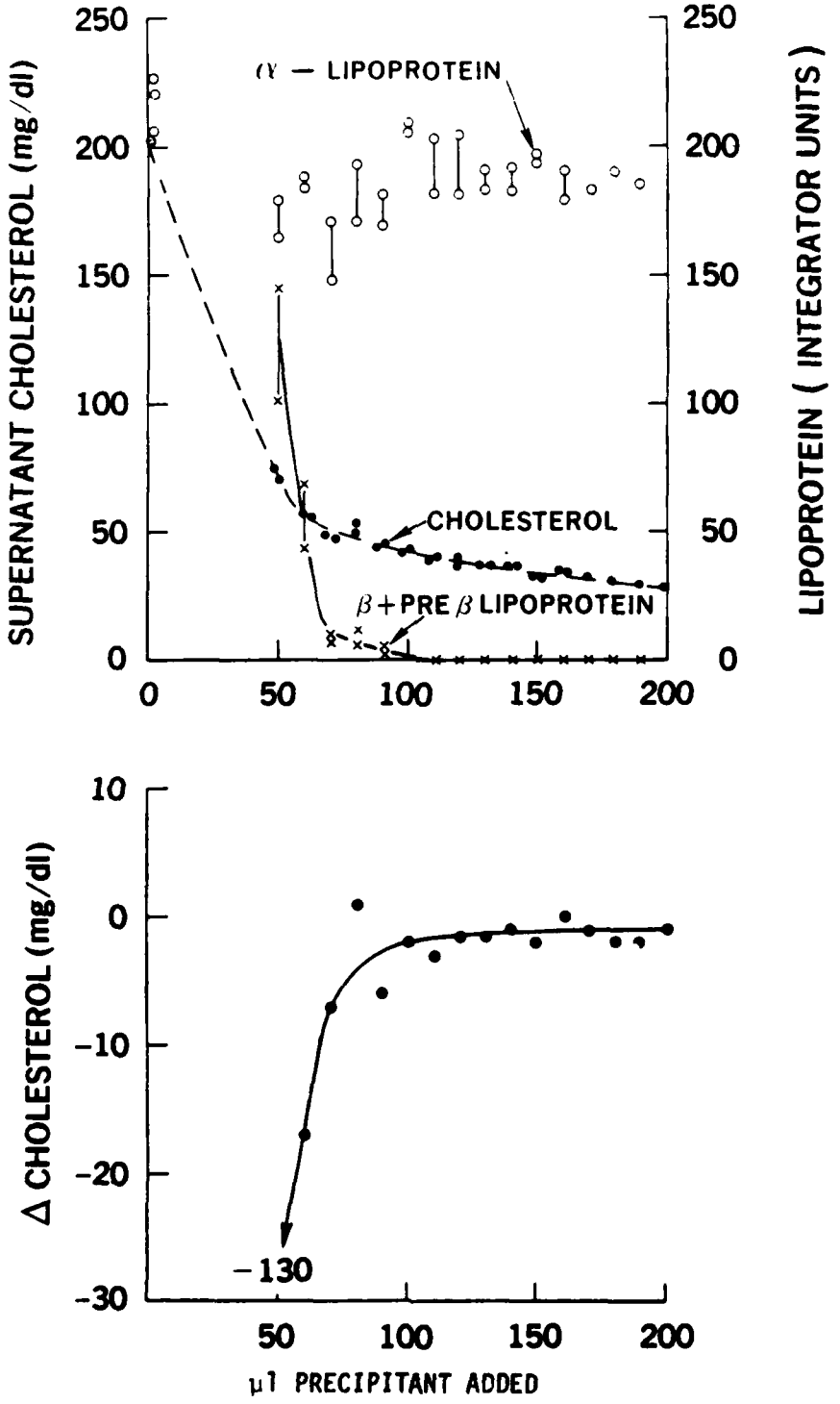

Figure 2. Titration (in duplicate) of a pool of human serum with 5% dextran sulfate followed by lipoprotein electrophoresis of supernates.

volume over the range of 100 to 200 µl. All the beta and prebeta (LDL and VLDL) lipoproteins were precipitated by volumes of precipitant larger than 100 µl, but the alpha (HDL) band appeared to be unchanged throughout the titration series. The reason for the failure of Δ-chol to reach zero is unclear. However, the variability of the HDL electrophoresis data could conceal losses of HDL of the small size required to account for the observed decreases in the cholesterol level seen when the larger volumes of precipitant were added.

Additional titrations showed that when the cholesterol level in the supernate was plotted against the volume of precipitant added, various serum samples gave a plateau that varied from broad, as with Serum B in Figure 1, to narrow or nonexistent, as with the serum in Figure 2. Because of this problem, other lipoprotein precipitants were explored. In preliminary experiments with heparin-$MnCl_2$, difficulties were encountered in separating a clear supernate in titration tubes containing suboptimal volumes of precipitant. Attention was therefore concentrated on using phosphotungstate-$MgCl_2$ as a precipitant. Table 1 lists the results obtained when a serum sample was titrated both with phosphotungstate and with dextran sulfate. With the phosphotungstate, the cholesterol level of the supernates showed a wide plateau with volumes of precipitant between 85 and 145 µl. The electrophoresis data showed the absence of beta and prebeta bands, while the alpha (HDL) band persisted across the entire titration range without significant change in intensity.

With the dextran sulfate (Table 1), the level of cholesterol in the supernate decreased with increasing volumes of precipitant until all of the prebeta and most of the beta band had disappeared. In this titration, it is uncertain whether the apparent narrow plateau of 38 mg/dl is real or is an artifact of the complexity of the experimental procedures and the difficulty of measuring low cholesterol levels with an accuracy with ±1 mg/dl. With this serum, the alpha band appeared to have the same integrated intensity in all the supernates irrespective of the volume of dextran sulfate added.

A fortunate choice of serum for another titration experiment comparing phosphotungstate and dextran sulfate as precipitating agents provided evidence for an explanation for variability of the breadth of the plateau in the titration curves of various serum samples titrated with dextran sulfate. Relevant data are listed in Table 2. The key fact is that the alpha (HDL) band in the electropherogram of this serum had two components that were sufficiently separated for their respective intensities to be estimated. The data for these bands, $alpha_1$ and $alpha_2$, and for the prebeta and beta bands are listed along with the corresponding precipitant volumes and cholesterol levels in the supernates. With the phosphotungstate-$MgCl_2$ titration, the level of cholesterol in the supernate plateaued at 43 mg/dl after the beta and prebeta lipoproteins had been precipitated. The intensities of the two alpha band components, however, were apparently unchanged by any of the volumes of precipitant. With the dextran sulfate, the cholesterol levels showed no reliable plateau. As the volumes of precipitant were first increased, the cholesterol levels in the supernates decreased and the beta band was reduced to a trace. Further increments in precipitant volume were accompanied by smaller decreases in the supernate cholesterol level, loss of some of the $alpha_2$ band, and eventual disappearance of the beta band from the supernate. In this serum, therefore, the continuing decreases in supernate cholesterol level as the volumes

TABLE 1. TITRATION OF SERUM WITH PHOSPHOTUNGSTATE-$MgCl_2$ OR DEXTRAN SULFATE-$MnCl_2$ REAGENTS

Phosphotungstate-$MgCl_2$

Precipitant volume µl	Supernate		
	Cholesterol conc. mg/dl	Electrophoresis units	
		Alpha	Beta + Prebeta
0	244	60	0
68/17[a]	36	64	0
72/18	34	61	0
76/19	35	70	0
80/20	35	59	0
92/23	35	63	0
100/25	37	73	0
116/29	35	63	0

Dextran Sulfate-$MnCl_2$

Precipitant volume µl	Supernate			
	Cholesterol conc. mg/dl	Electrophoresis units		
		Alpha	Prebeta	Beta
0	244	77	99	220
70	75	63	0	85
80	43	70	0	20
85	41	71	0	13
90	38	76	0	11
95	38	65	0	2
100	34	70	0	0
110	33	70	0	0

[a]Volume of phosphotungstate/volume of $MgCl_2$

of precipitant were increased could be attributed to loss of some of the alpha (HDL) lipoproteins.

DISCUSSION

The purpose of these experiments was to find a way to assess the accuracy of an estimate of the HDL cholesterol level in human serum without resorting to the ultracentrifuge. Both the identities and the formulations of reagents used were based on the work of Burstein et al. (5). On the basis of findings such as reported for serum B in Figure 1, a method for measuring HDL cholesterol levels in human serum was established using the 5% dextran sulfate-$MnCl_2$ reagent described. The method used 100 µl of the precipitant per ml serum.

TABLE 2. SERUM ALPHA LIPOPROTEINS TITRATED WITH PHOSPHOTUNGSTATE-$MgCl_2$ OR DEXTRAN SULFATE-$MnCl_2$ REAGENTS

Phosphotungstate-$MgCl_2$

Precipitant volume µl	Supernate				
	Cholesterol	Electrophoresis[a]			
	conc. mg/dl	$Alpha_1$	$Alpha_2$	Prebeta	Beta
0	192	10	10	10	10
48/12[b]	47	10	10	2	2
52/13	44	10	10	0	1
56/14	43	10	10	0	0
60/15	43	10	10	0	0
64/16	42	10	10	0	0
68/17	42	10	10	0	0
76/19	44	10	10	0	0

Dextran Sulfate-$MnCl_2$

Precipitant volume µl	Supernate				
	Cholesterol	Electrophoresis[a]			
	conc. mg/dl	$Alpha_1$	$Alpha_2$	Prebeta	Beta
0	191	10	10	10	10
70	45	10	10	0	2
80	32	10	10	0	1
85	27	10	9	0	1
90	25	10	8	0	1
95	24	9	8	0	1
100	22	10	7	0	0
110	19	10	7	0	0

[a]Relative intensities: 10 = initial intensity with no precipitant, 2 = faint band, 1 = trace.
[b]Volume of phosphotungstate/volume of $MgCl_2$.

However, the volume of precipitant was increased or decreased as periodic titration experiments identified slightly different volumes at the middle of the plateau on the titration curve. Lipoprotein electrophoresis later showed (Fig. 2) that with some sera, the titration curve alone did not clearly identify the correct HDL-cholesterol level. This ambiguity, coupled with the incomplete precipitation of the VLDL and LDL frequently encountered with moderately lipemic sera, prompted the investigation of other lipoprotein precipitants.

The dextran sulfate-$MnCl_2$ precipitant used in the above experiments is not the same as any dextran sulfate kit available commercially. We tested a commercial HDL cholesterol kit that uses dextran sulfate. The dextran sulfate, the bivalent cation, and the characteristics of the titration curve differed significantly from the reagents and results reported above. The HDL cholesterol levels measured with the commercial dextran sulfate kit we tested were, in fact, in remarkably close agreement with the results obtained with our phosphotungstate-$MgCl_2$ procedure.

The phosphotungstate-$MgCl_2$ method was chosen because of the wide plateau of the titration curve (cf. data in Tables 1 and 2). The rationale for this choice is as follows: Consider the goal of precipitating all of the VLDL and LDL, but none of the HDL, in a group of sera in which the lipoprotein concentrations would be expected to vary over a 2-fold range. The same volume of precipitant will be added to 1 ml of each serum, so the range between the volume of precipitant that completely precipitates the VLDL + LDL and the volume that begins precipitating the HDL should be wide, preferably at least 2-fold. The extent of departure from this ideal determines the degree of accuracy of the values obtained because it determines the extent of failure to precipitate all the LDL in some samples and the extent of precipitation of HDL in other samples.

In the laboratory, incomplete precipitation of VLDL + LDL is accompanied by telltale signs such as a cloudy supernate, a poorly solidified precipitate, or floating greasy materials in the supernate. However, if some of the HDL is precipitated with the VLDL + LDL (overprecipitation), the technician does not know that the HDL cholesterol level measured is lower than the true value. Overprecipitation is therefore a real possibility as a cause for inaccurate values. Since the phosphotungstate procedure had a wider range of volumes between incomplete precipitation and overprecipitation, it was adopted for measuring HDL cholesterol levels in this laboratory.

With the phosphotungstate procedure, the true HDL cholesterol level was identified unequivocally in all the sera tested by titration monitored with lipoprotein electrophoresis. The same was usually true with the dextran sulfate precipitant. For example, in Table 1, if the HDL cholesterol level in the dextran sulfate titration is taken as the level in the supernate from which all beta and prebeta lipoprotein was absent (100 µl precipitant), the value is 34 mg/dl. Agreement with the average value of 35 mg/dl found in the phosphotungstate titration is excellent.

Table 2, however, presents a serum in which identifying the HDL cholesterol level with dextran sulfate titration would require an uneasy choice between a supernate that included a trace of LDL or a supernate that excluded some of the HDL. The phosphotungstate titration showed that including some of the LDL would have been the "better" (but illogical) choice. Without that information, the value of 32 mg/dl would have been taken as the HDL cholesterol level in the dextran sulfate titration, and that value would have been erroneously low.

In this particular serum (Table 2), some of the HDL was precipitated by the dextran sulfate reagents before all the VLDL + LDL had been precipitated. This is the only serum in which we documented such a situation unequivocally,

but two observations suggest that it is not uncommon. The first is that in the dextran sulfate titrations, at least a fourth of the sera did not show a clear plateau in the cholesterol level of the supernate (Δ-chol = 0). The second observation is that in a comparison of the HDL cholesterol levels estimated with our phosphotungstate and our dextran sulfate procedures, the values usually agreed reasonably well. With approximately 10% of sera, however, the dextran sulfate procedure gave definitely lower values for the HDL cholesterol level than the phosphotungstate procedure did. Presumably these sera contained significant amounts of alpha$_2$ lipoproteins, which were partially precipitated by the dextran sulfate. The estimates of the HDL cholesterol levels were therefore erroneously low in those sera.

Control of the pH of the phosphotungstate reagent is very important (6)! In this laboratory, use of the reagent when the pH was uncontrolled resulted in many incomplete precipitations and in undesirably wide variance in repeat determinations. Perhaps this detail accounts for the relatively high variability found in the extensive comparison study by Warnick et al. (11). However, our procedures are difficult to relate to theirs because they used EDTA-treated plasma whereas we used serum, and they added a 25% larger volume of phosphotungstate reagent than our titrations indicated to be optimal for serum.

The data presented here indicate that titration of serum with lipoprotein precipitants, monitored by electrophoresis, is a useful tool for assessing the optimal volume of precipitant to be used in routine laboratory measurements of HDL cholesterol and for occasional monitoring of those measurements.

CONCLUSIONS

1. Titration of serum lipoproteins with lipoprotein precipitants is a guide to the serum HDL cholesterol level, but may give equivocal results.

2. Some individual serum samples respond differently to titration with different lipoprotein precipitants.

3. Titration of serum with the dextran sulfate-$MnCl_2$ reagent described herein sometimes resulted in uncertainty about the correct estimate of the HDL cholesterol level because of factors peculiar to the serum tested. The dextran sulfate precipitant we used, however, is to be distinguished from the dextran sulfate reagent in a commercial kit that we tested and found to give HDL cholesterol values in close agreement with our phosphotungstate method.

4. Titration of serum with a phosphotungstate-$MgCl_2$ precipitant, coupled with quantitative lipoprotein electrophoresis of the supernates, revealed the effects of the precipitant and permitted an unequivocal estimate of the HDL cholesterol concentration in all the sera tested this way.

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