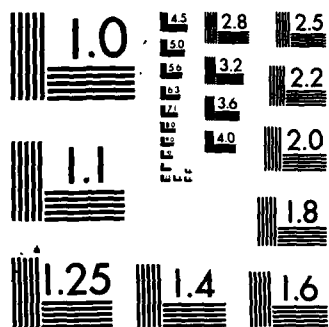END

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# LIPOPROTEIN AGAROSE GEL ELECTROPHORESIS: APPLICATION IN HDL-CHOLESTEROL METHODOLOGY

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Dale A. Clark, Ph.D.

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NOTICES

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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nations.

This report has been reviewed and is approved for publication.

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We perform agarose gel lipoprotein electrophoresis (AGLE) in this laboratory using glass microscope slides to support the agarose gel and 0.025 M barbital buffer for the electrophoresis. This report describes details, including special apparatus used to facilitate making and handling agarose gel slides.

To test correlations between serum lipid levels and the densities of the corresponding lipoprotein bands separated by electrophoresis, we electrophoresed eight individual sera and measured the densities of the resulting lipoprotein bands. Serum levels of total cholesterol, triglycerides, and HDL-cholesterol were correlated with the measured densities of the beta, prebeta, and alpha lipoprotein bands respectively. Coefficients for correlation between total cholesterol and beta lipoprotein, triglyceride and prebeta lipoprotein, and HDL-cholesterol and alpha lipoprotein were 0.638, 0.914, and 0.920 respectively. Densitometric data from 24 replicate electrophoresis slides showed that the coefficient of variance for the

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   Phosphotungstate-MgCl$_2$ method for HDL-cholesterol


19.  Abstract (continued)

percentage of dye bound to the beta, prebeta, and alpha lipoproteins was 1.9%, 2.6%, and 4.5% respectively.

We used AGLE to monitor the separation of high-density lipoprotein (HDL) from the combined low-density lipoproteins (LDL) and very low density lipoproteins (VLDL) during the titration of serum lipoproteins with increasing amounts of lipoprotein precipitant.  Results demonstrated which levels of precipitant removed all detectable VLDL + LDL and which levels of precipitant removed a significant amount of HDL.  These data, together with the levels of cholesterol in the supernate from the various tubes, identified the optimum precipitant volumes to be used in routine determinations of serum HDL-cholesterol levels.

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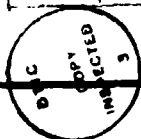
# LIPOPROTEIN AGAROSE GEL ELECTROPHORESIS: APPLICATION
# IN HDL-CHOLESTEROL METHODOLOGY

## INTRODUCTION

In recent years the interest in high-density-lipoprotein cholesterol (HDL-C) as a strong inverse risk factor for coronary artery disease (1,2) prompted the development of methodology for measuring the level of HDL-C in serum. This level is commonly measured in the supernate obtained after adding a precipitating reagent (3,4,5) to an aliquot of serum to remove all lipoproteins except the HDL. Quantitative measurement of cholesterol in the supernate, however, does not establish that the cholesterol was provided only by HDL. Lipoprotein electrophoresis can be used for this purpose. In addition, when used in conjunction with determinations of cholesterol levels in supernates obtained after addition of various amounts of precipitants, lipoprotein electrophoresis can help to establish the optimal amounts of precipitating reagent needed for accurate determination of serum HDL-C levels. This technique has been used in this laboratory for several years. Our procedures for lipoprotein electrophoresis include several adaptations of published methods, so this report will describe the method in detail and its application in determining the optimum volume of precipitating reagent for use in measuring levels of serum HDL-C.

## MATERIALS AND METHODS

**Materials:** The sources of materials are listed in Table 1.

**Specimens:** Blood was drawn from human subjects after an overnight fast. After a clot formed, the blood was centrifuged and the serum was collected and stored at 4°C. Electrophoresis was performed within the next 48 h.

**Lipoprotein electrophoresis.** The lipoprotein agarose gel electrophoresis method described herein is a modification of methods reported by others (6,7,8). The major modification is that we used glass microscope slides for agarose gel support; we also made small changes in the preparation of the Oil Red O stain and in densitometry.

**Preparation of the Agarose-Ionagar-Albumin solution.** The concentrations of agarose-ionagar-albumin are those of Noble (6), except that the barbital buffer (7) used to dilute the agarose and the ionagar was 0.025 M, pH 8.6. The following directions apply. **Albumin solution:** Using a 30% human albumin solution obtained from a commercial source, prepare a 25% albumin solution (6) by adjusting the pH of 41.5 ml with 1 M Tris buffer to pH 8.6, and then diluting to 50 ml with 0.9% NaCl solution. **Agarose and Ionagar solutions:** Weigh 200 mg agarose and 90 mg ionagar into separate 125-ml Erlenmeyer flasks and add 40 ml and 15 ml barbital buffer respectively. Stopper the flasks with a roll of 10 X 20 cm (4" X 8") gauze and place in a boiling water bath with occasional twirling for 5 min. Bring the solutions to a boil while twirling

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TABLE 1. SOURCES OF MATERIALS USED

Agarose Powder
  Cat.No. 162-0100                            Bio-Rad Laboratories
                                              32nd and Griffin Ave.
1% Bromphenol Blue                            Richmond, CA


Ionagar No. 2                                 Consolidated Laboratories, Inc.
  Code No. L12                                Box 234
                                              Chicago Heights, IL


30% Human Albumin                             Miles Laboratories
  Code No. 82-305-1                           P.O. Box 2000
                                              Elkhart, IN


Barbital Buffer                               Harleco
  Item No. 3948                               Gibbstown, NJ


Oil Red O Dye, Powder                         Hartmen-Leddon Co.
                                              Philadelphia, PA


ABA 100                                       Abbott Laboratories
                                              820 Mission St.
                                              South Pasadena, CA


Remote Control Pipette                        Lapine Scientific Co.
  Micro Model, Cat. No. 34 182011             Chicago, IL


20-µl Disposable Pipettes                     Fisher Scientific Co.
  Cat. No. 21-164-2D                          Pittsburgh, PA


Densitometer                                  Transidyne General Corp.
  Clinical Scanning Densitometer System       Ann Arbor, MI
  Models 2500/2510


Wicks, Filter Paper
  Spinco Part No. 300-29                      Beckman Instruments, Inc.
                                              Palo Alto, CA
Spinco Model R Durrum-type Cell

Spinco Model RD-2 Duostat Power Supply

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    NOTE: The citation of these items and their suppliers does not constitute
product endorsement by the U.S. Air Force nor does it imply superiority over
comparable items from other sources.

over an open flame.  Place the clear solutions (no granules of agarose or ionagar) in a water bath at 50°C and allow to equilibrate to that temperature.  <u>Agarose-ionagar-albumin solution</u>:  Mix together the prepared agarose, ionagar, and albumin solutions in the volumetric proportions of 40:10:1.

<u>Well-Formers</u>.  A glass microscope slide holder and well-former combination was designed (Fig. 1).  Each slide fits in a depression machined into the Plexiglas base.  The depression is slightly wider than the width and 3.2 mm (1/8") shorter than the length of a microscope slide.  The depth of the depression is approximately one half the thickness of the slide.  There are eight such depressions in the base.  At each end of the base there is a support block appropriately grooved to hold one end of a square support rod.  To that rod, eight metal well-formers (6), each 2 mm thick X 20 mm wide X 37 mm long, are attached by eight screws, each of which passes through a slot in the upper end of a well-former.  These screws are used to adjust the height of each well-former so that its lower end contacts a glass slide placed below it.  The lower end of the well-former, which contacts the glass slide, is oval shaped; its diameter is 2 mm at the thickest point.  A clean glass microscope slide, marked for identification on the back side with a diamond-point pencil, is placed in each depression; and each well-former is adjusted so that the lower end rests on its microscope slide.  After the apparatus is adjusted to level the base, the square rod with attached well-formers is removed for the application of agarose to the glass slides.

<u>Preparation of Agarose Gel Slides</u>.  Working with one slide at a time, carry out the following procedure on each slide mounted on the base.  Using a warm 5-ml pipette graduated to the tip, deliver 1.5 ml of warm (50°C± 2°) agarose solution onto the glass slide with a zigzag motion.  Use the pipette to quickly spread the agarose around the outer edges of the slide to ensure uniform coverage of the entire slide.  When each slide has been covered with agarose solution, replace the square rod with attached well-formers and let the unit sit, first at room temperature for 15 min, then in a dry heat incubator at 37°C for 15 min, followed by a return to room temperature.  Remove the square rod with its well-formers, leaving the agarose-coated slides on the base.  The sample wells produced with this combination of apparatus and procedures are located 18 mm from the end of the glass slide and have an optimum capacity of 20 µl.

<u>Dilution of Sample</u>.  Deliver 200 µl of sample near the bottom of a 12- X 75-mm test tube and place in a 50°C water bath.  Add 100 µl agarose-ionagar-albumin solution to the sample in the tube, and vortex for a few seconds.  Cap the tube and return it to the rack in the 50°C water bath until used for electrophoresis.

<u>Application of Sample and Tracking Dye</u>.  Holding a remote-control pipette unit in the left hand and an attached 20-µl disposable pipette in the right hand, remove excess fluid from each slide well.  Use the same apparatus to deliver 20 µl of sample mixture into each slide well.  Prepare two tracking-dye slides without sample wells for each electrophoresis cell.  To each of these slides, apply a 1% aqueous solution of bromphenol blue.  Using the sharp end of a broken wood applicator stick, apply this tracking-dye solution as a line perpendicular to the length of the slide, approximately 18 mm from one end of the slide.  Use a wax pencil to mark a line on the back side of the slide 35 mm from the point of dye application.
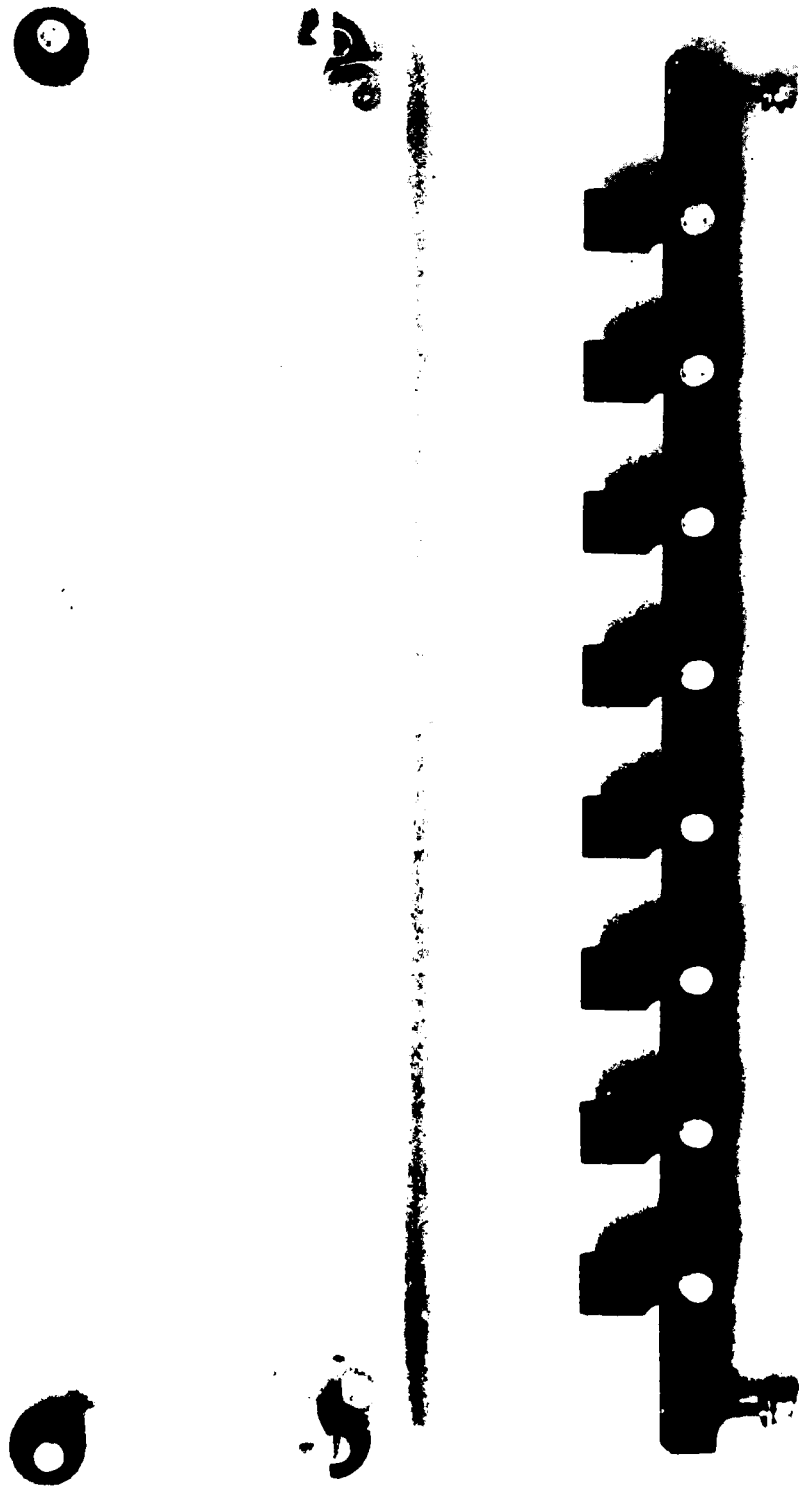
Figure 1. Slide holder and well-former combination.

Electrophoresis. A Spinco Model R Durrum-type cell (7) and a Beckman
RD-2 Duostat power supply were used for the electrophoretic separation. The
Durrum cell racks for holding paper strips were removed, but the partitions
and wick holders were left in place. Two wicks were used on each side of the
Durrum cell, but their width was reduced 6.4 mm (1/4") by cutting off a 6.4-mm
strip from one side of each wick. One wick was positioned in the wick holder
and saturated with buffer. A second wick was inserted alongside the first so
that approximately 9.5 mm (3/8") extended above the wick holder. Two wicks
were positioned similarly on the other side of the Durrum cell. After the
wicks were saturated with buffer, the 9.5-mm extension above the holder was
folded over in a 90° angle toward the center of the cell. The wick holder was
stabilized by thin O-rings of plastic tubing wedged between ends of the wick
holder and the cell. A sheet of plastic 3.2 mm X 76.2 mm X 30.5 cm (1/8"
thick X 3" wide X 12" long) was laid atop the folded-over wicks and weighted
down with a plastic block 2.54 X 4.74 X 30.5 cm (1" thick X 1-3/4" wide X 12"
long). These pieces of plastic were left in place for 24 h for saturation/
equilibration of the wicks before electrophoresis. The weighted plastic
prevented buckling of the folded-over wicks so that they would provide a flat
area for contact with the agarose gel slides subsequently placed on them.
Immediately before electrophoresis, the plastic sheet and bar were removed.
The agarose gel slides were placed, gel side down against the wick, with the
slides spanning the distance between the anode and cathode compartment wicks
and the sample or dye application point closest to the cathode. A tracking-
dye slide was placed at each end of the cell. The plastic bar was laid atop
the slides, midway between the wicks and parallel with them. Electrophoresis
was carried out at room temperature at 5-mA current per slide until the dye on
the tracking-dye slides had migrated past the wax-pencil marking 35 mm from
the starting line. The time required was usually 35 min. Following electro-
phoresis, the slides were placed in a fixing solution (9) of ethanol, glacial
acetic acid, and distilled water (60:5:35::v:v:v) for 15 min, then in another
batch of the same fixing solution for another 15 min. The slides were then
dried in a dry-heat oven at 80-85°C, usually for 45 to 60 min.

Staining and Densitometry. Oil Red O stain was prepared by the method of
Jencks (10) except that the dye mixture was stirred overnight at 45°C,
filtered at 34°C, and stored at 37°C. After being fixed as described above,
the slides were arranged in stainless-steel racks and stained in the Oil Red O
solution at 37°C for 24 h. They were then washed thoroughly in a stream
of distilled water and dried at 37°C. Densitometric scanning of the dried
stained slides was performed at 520 nm, using a Transidyne General Corporation
densitometer.

Chemical Analyses. Total cholesterol levels were measured using an
ABA-100 Bichromatic Analyzer and a commercial enzymatic reagent. HDL-C levels
were measured using the same method applied to the supernate obtained after
precipitation of LDL and VLDL by our phosphotungstate-$MgCl_2$ (11) technique.
Triglyceride levels were measured with the same ABA-100 using a commercial
enzymatic triglyceride reagent.

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RESULTS

To explore variations among individual serum samples, eight serum samples chosen at random were electrophoresed as previously described. Figure 2 shows the results obtained. The electrophoresis patterns demonstrate the separations of the alpha, prebeta, and beta lipoprotein bands separated by the methods described in this paper. The strip charts are the corresponding densitometric readouts. Because the uptake of Oil Red O dye by the lipoprotein fractions is a function of the lipids in the lipoproteins and because the serums used have various levels of cholesterol, HDL-C, and triglyceride, the densitometric data and the serum lipid levels are listed in Table 2. As expected, the densitometric data tend to vary with the relevant lipid levels. Correlations found between lipid levels and electrophoresis band densities are listed in Table 3. To evaluate the reproducibility of the agarose gel lipoprotein electrophoresis procedure, 24 aliquots of a single serum were similarly electrophoresed. The reproducibility found is summarized in Table 4. The coefficients of variation found were relatively small, in part because of the good separation between bands (cf. Fig. 2).

TABLE 2. SERUM LIPID LEVELS AND LIPOPROTEIN ELECTROPHORESIS DATA

| Serum No. | Lipids (mg/dl) | | | Densitometric electrophoresis units | | | % dye bound | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total Chol | Trig | HDL Chol | Beta | Prebeta | Alpha | Beta | Prebeta | Alpha |
| 1 | 152 | 42 | 69 | 11.03 | 2.01 | 10.86 | 46.1 | 8.4 | 45.4 |
| 2 | 204 | 64 | 76 | 12.91 | 3.38 | 10.51 | 48.1 | 12.6 | 39.2 |
| 3 | 181 | 121 | 45 | 15.05 | 4.92 | 7.42 | 54.9 | 18.0 | 27.0 |
| 4 | 344 | 294 | 36 | 16.51 | 9.56 | 3.84 | 55.2 | 31.9 | 12.8 |
| 5 | 249 | 124 | 37 | 14.17 | 2.82 | 4.23 | 66.7 | 13.3 | 19.9 |
| 6 | 173 | 82 | 45 | 15.29 | 2.15 | 6.93 | 62.7 | 8.8 | 28.4 |
| 7 | 260 | 155 | 45 | 14.58 | 5.06 | 4.53 | 60.3 | 20.9 | 18.7 |
| 8 | 167 | 112 | 46 | 13.67 | 5.65 | 7.26 | 51.4 | 21.2 | 27.3 |
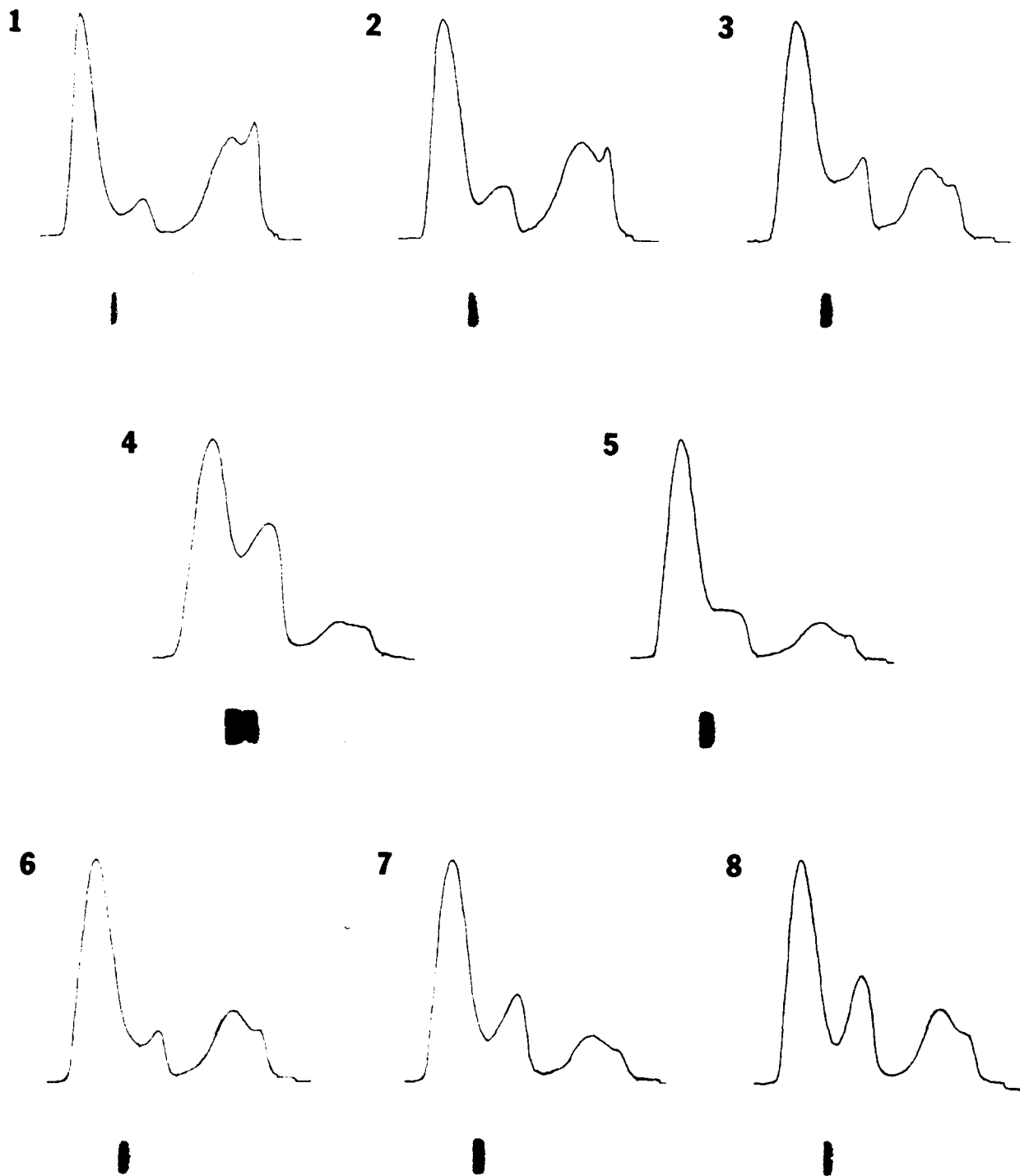
Figure 2. Electrophoresis of random serum samples. The densitometric tracing is shown above its corresponding electrophoresis slide for each of the eight serum samples.

TABLE 3. CORRELATIONS BETWEEN SERUM LIPID LEVELS AND RESULTS OF QUANTITATIVE LIPOPROTEIN ELECTROPHORESIS

| Lipoprotein | Observed Coefficients | | | Published Coefficients | | |
|---|---|---|---|---|---|---|
| | Total chol | Trig | HDL chol | Total chol | Trig | HDL chol |
| Beta | 0.638 | -- | -- | 0.673[a] | -- | -- |
| Prebeta | -- | 0.914 | -- | -- | 0.972[a] | -- |
| Alpha | -- | -- | 0.920 | -- | -- | 0.947[b] |
| N | 8 | 8 | 8 | 16 | 20 | 98 |

[a]Hulley, reference 8.

[b]Conlon, reference 13.


TABLE 4. QUANTITATIVE LIPOPROTEIN ELECTROPHORESIS: PERCENTAGE DYE BOUND TO LIPOPROTEIN FRACTIONS

| | Beta | PreBeta | Alpha |
|---|---|---|---|
| Mean % | 59.5 | 20.6 | 19.8 |
| Range % | 57.4 - 61.5 | 19.3 - 21.3 | 18.4 - 21.3 |
| N | 24 | 24 | 24 |
| 1 S.D. | 1.13 | 0.54 | 0.89 |
| CV | .0190 | .0262 | .0449 |

In lipoprotein titration experiments in this laboratory, agarose gel lipoprotein electrophoresis is routinely performed on the supernatant solutions obtained from the titration tubes (12). The results from one such experiment will illustrate the information obtained from such titrations. In this particular experiment, we added 1.0-ml aliquots of a single serum sample to each of a series of test tubes. To each tube we then added 200 µl of precipitant solution, but the composition of that solution varied systematically from tube to tube. The proportion of the 200 µl made up of phosphotungstate-$MgCl_2$ precipitating reagent increased from 0 to 100% while the proportion made up by normal saline correspondingly decreased from 100% to 0 in progressing from the first to the last tube in the series (cf. Table 5). After the serum-precipitant solution was mixed, we centrifuged the mixture at 1400 x g for 30 min at 4°C. We decanted the supernatant solution from each tube in the series into a clean test tube and took aliquots for agarose gel lipoprotein electrophoresis and for cholesterol determination. Results of this titration experiment are presented in Figure 3, which is a plot of the cholesterol content of the supernatant against the varying volumes of precipitating reagent added. Densitometric data from the agarose gel electrophoresis slides together with the cholesterol content of the corresponding tube are given in Table 5. A plateau occurred in the cholesterol level in tubes 6-9 and another in tubes 10-12. The electrophoresis data show that all the beta and prebeta lipoproteins had been precipitated in tubes 6-13 and suggest some possible loss of HDL from the alpha band in tubes 10-12.

TABLE 5.  HDL SUPERNATANT DATA

| Tube No. | Precipitant volume (µl)[a] | Chol (mg/dl) | Densitometric electrophoresis units | | | Percentage | |
|---|---|---|---|---|---|---|---|
| | | | Alpha | Beta + Prebeta | Total | Alpha | Beta + Prebeta |
| 1 | 0 | 284 | 566 | 1836 | 2402 | 24 | 76 |
| 2 | 24/6 | 278 | 521 | 1712 | 2233 | 23 | 77 |
| 3 | 40/10 | 170 | 510 | 1217 | 1727 | 30 | 70 |
| 4 | 56/14 | 60 | 800 | 139 | 939 | 85 | 15 |
| 5 | 64/16 | 52 | 691 | 74 | 765 | 90 | 10 |
| 6 | 72/18 | 50 | 691 | 22 | 713 | 97 | 3 |
| 7 | 80/20 | 50 | 796 | 14 | 810 | 98 | 2 |
| 8 | 88/22 | 50 | 570 | 31 | 601 | 95 | 5 |
| 9 | 96/24 | 50 | 569 | 46 | 615 | 92 | 8 |
| 10 | 104/26 | 48 | 537 | 36 | 573 | 94 | 6 |
| 11 | 120/30 | 47 | 544 | 31 | 575 | 95 | 5 |
| 12 | 136/34 | 47 | 516 | 58 | 574 | 90 | 10 |
| 13 | 160/40 | 41 | 450 | 42 | 492 | 92 | 8 |

[a]Volume of phosphotungstate/volume of 2M $MgCl_2$. Total volume added (phosphotungstate plus $MgCl_2$ plus saline) equals 200 µl.
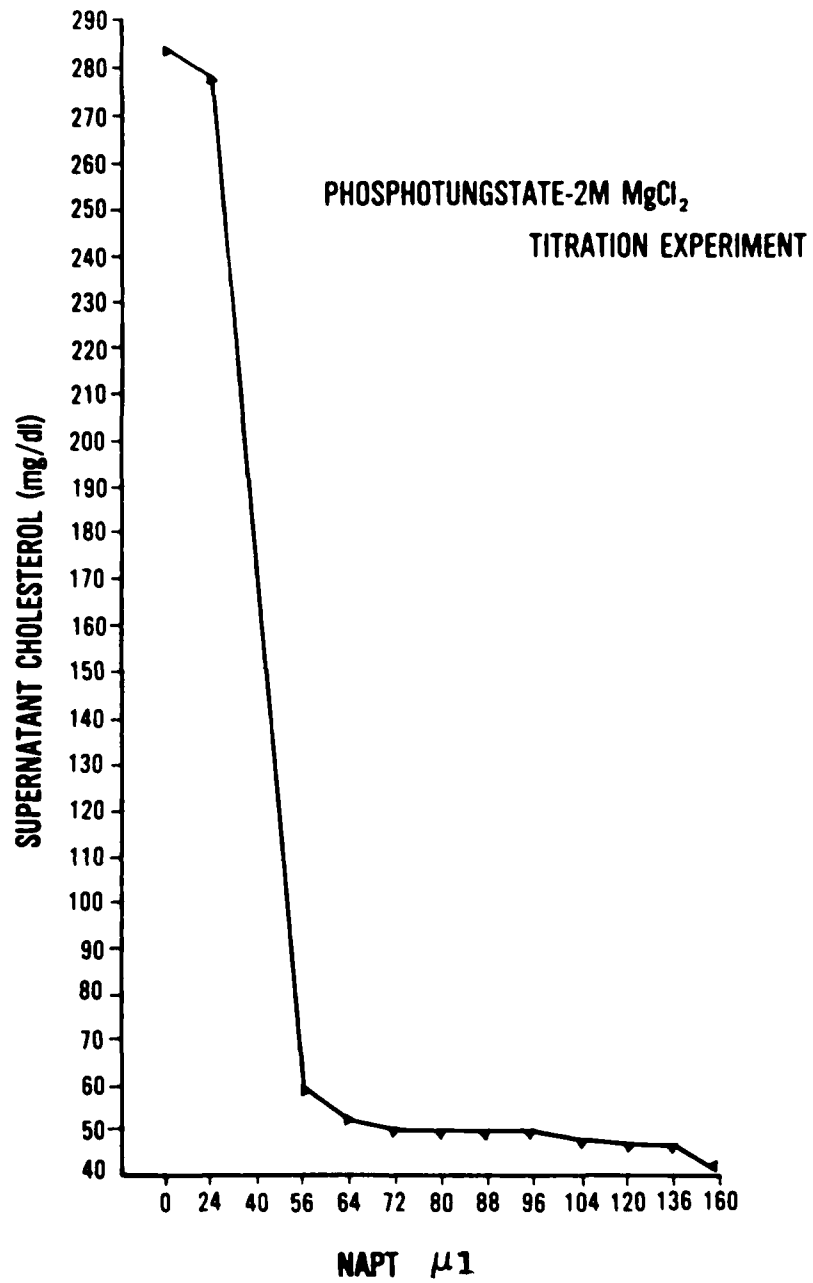
Figure 3. Cholesterol levels in the supernates are plotted against the corresponding volumes of sodium phosphotungstate-MgCl$_2$ (NAPT) precipitant added.

The closest approach to the ideal situation of precipitating all the beta (LDL) and prebeta (VLDL) lipoprotein fractions without losing any of the alpha (HDL) fraction appears to be in tubes 6 and 7, with the corresponding level of cholesterol in the supernate being 50 mg/dl. This concentration of cholesterol, therefore, is the best estimate of the true HDL-C level.

## DISCUSSION

One aspect of assessing the usefulness of agarose gel electrophoresis of serum lipoproteins is an evaluation of the qualitative relationships between visual observations of the slides (Fig. 2) and the corresponding densitometric readouts (Table 2). Good agreement between those parameters is apparent. For instance, in pattern 1 (Fig. 2) the beta and prebeta bands appear to be less than average in intensity, and the same is true for the alpha band in pattern 4. For those patterns, the values for both the densitometric electrophoretic units (DEUs) and the percentage of dye bound agreed well with the visual observations.

Such agreements are confirmed by the correlation coefficients in Table 3. Those coefficients are similar to the ones reported by Hulley (8) for beta and prebeta lipoproteins and by Conlon (13) for alpha lipoproteins. For comparing triglyceride levels with prebeta DEU scores, and HDL-C levels with alpha DEU scores, the correlation coefficients (.914 and .920 respectively) are highly significant ($P<.005$). For comparing total cholesterol with beta DEU scores, the correlation coefficient of .638 is only marginally significant ($.05<P<.1$). This fact is probably related to the finding that the total cholesterol levels are inversely correlated with the alpha DEU scores ($r = -.752$, $.025<P<.05$). When LDL-cholesterol levels (estimated as total cholesterol minus HDL-C) are compared with beta DEU scores, the correlation coefficient is somewhat larger ($r = .722$) and is statistically significant ($P<.05$). The inverse relationship between total cholesterol level and alpha DEU score may be the reason that the sum of the cholesterol and triglyceride levels does not correlate significantly with the sum of the DEU scores for all three lipoprotein bands ($r = .453$, $P>.1$). However, the ratio total cholesterol/HDL-C is highly correlated with the ratio beta DEU score/alpha DEU score ($r = .980$, $P<.001$). These correlations indicate satisfactory agreement between the quantitative serum lipid levels and the results of agarose gel lipoprotein electrophoresis.

Table 4 shows the reproducibility obtained on 24 replicate electrophoresis slides prepared from sample 7 (Fig. 2 and Table 2). Both the percentage of dye bound in each band and the associated coefficient of variation (CV) of replicate runs would probably differ with other serum samples, depending on whether the bands were well separated, or whether the edges of the bands were distinct, and whether the density of each band was sufficient for accurate densitometric measurement. The CVs of 0.019, 0.026, and 0.045 for the beta, prebeta, and alpha bands, respectively, are well within the acceptable range.

In routine clinical laboratory operations, the HDL-C level is the cholesterol level measured in the supernatant solution obtained after the addition of a specified amount of lipoprotein precipitant to a specified volume of serum. If that specified amount of precipitant is optimal, only

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HDL will remain in the supernate, all LDL and VLDL having been precipitated.
To identify the optimal volume of precipitant, we titrated serum lipoproteins
with lipoprotein precipitant and used agarose gel lipoprotein electrophoresis
to monitor the supernatant solutions to determine when the LDL and VLDL had
been precipitated or when too much precipitant had been added, causing some
HDL to precipitate. Results of one such experiment are graphed in Fig. 3 for
comparison with the corresponding quantitative data in Table 5. Ideally, as
larger quantities of precipitant were added, the cholesterol level in the
supernate should have decreased until all VLDL and LDL had been precipitated,
then plateaued at some level until enough precipitant had been added
to precipitate some of the HDL and cause the cholesterol level in the
supernate to decrease further. During this sequence, the DEU scores for the
beta-plus-prebeta fractions ideally should have decreased from a high initial
level and reached zero when the plateau in the cholesterol level was first
reached. Ideally also, the DEU scores for the alpha fraction should have been
constant for all tubes in the titration series until sufficient precipitant
had been added to precipitate some of the HDL.

In the laboratory, however, these ideals may be approached but never
realized. The data in Table 5 illustrate the practical realities. The
beta-plus-prebeta DEU scores decreased successively in progressing from tubes
1 through 5 and appeared to be roughly constant thereafter. Presumably some
lipid/lipoprotein fragment left behind by the alpha band migrating ahead of
the prebeta and beta bands accounted for the variability of the beta-plus-
prebeta DEU scores for tubes 6-9. It seems likely that the larger amounts of
precipitant in tubes 9-13 rendered some HDL marginally precipitable and in-
creased the amount of lipoprotein fragments left behind as the HDL band mi-
grated, causing the DEU scores of the slower beta and prebeta bands to be
higher. The average DEU score for the alpha band was near 560, but the values
for tubes 4-7 were higher. Possible causes for these higher scores include
insufficient sample dilution, incomplete mixing of sample with agarose, or
delivery of more than 20 µl of sample mixture into the sample well. Such
variations would increase the DEU scores but would not affect the percentage
of dye bound by the various bands. The percentage of dye bound by the alpha
fraction increased in progressing from tube 1 to tube 5, remained roughly
constant from tube 6 to tube 11, then decreased in tubes 12 and 13. The fail-
ure of the alpha band to account for 100% of dye bound in tubes 6-13 is proba-
bly caused by the trailing of lipid/lipoprotein fragments from the alpha band
referred to previously.

Taken together, the electrophoretic data indicated 1) that the LDL and
VLDL precipitation was complete in tubes 6-13, and 2) that the HDL did not
begin to precipitate until tube 10. The cholesterol concentration in the
supernates of tubes 6-9 should therefore be the best measure of the HDL-C
level. Since the cholesterol concentrations in those tubes were all 50
mg/dl, that level was the HDL-C value for that serum. All these data
supported the conclusion that the precipitant volumes used in tube 7 (which
was at the middle of the plateaus in cholesterol level and maximum percentage
of dye bound in the alpha fraction) were optimal for this serum.

Since tube 7 was at the center of the plateaus in the supernate choles-
terol concentration and in maximum percentage of dye bound in the alpha band,
the associated volumes of precipitant (80 µl phosphotungstate plus 20 µl $MgCl_2$
per ml of serum) was the dilution of choice for this serum.

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These results illustrate the essential role of agarose gel electrophoresis in establishing the optimum volume of precipitant to be used in routine determinations of serum HDL-cholesterol levels.

REFERENCES

1. Gordon, T., et al. High density lipoprotein as a protective factor against coronary heart disease. Am J Med 62(5):707 (1977).

2. Miller, N.E. The evidence for the antiatherogenicity of high density lipoprotein in man. Lipids 13(12):914 (1978).

3. Burstein, M., et al. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J Lipid Res 11:583 (1970).

4. Lopes-Virella, M.F., et al. Cholesterol determination in high-density lipoproteins separated by three different methods. Clin Chem 23(5):882 (1977).

5. Grove, T.H. Effect of reagent pH on determination of high-density lipoprotein cholesterol by precipitation with sodium phosphotungstate-magnesium. Clin Chem 25(4):560 (1979).

6. Noble, R.P. Electrophoretic separation of plasma lipoproteins in agarose gel. J Lipid Res 9:693 (1968).

7. Hatch, F.T., et al. Quantitative agarose gel electrophoresis of plasma lipoproteins: A simple technique and two methods for standardization. J Lab Clin Med 81(6):946 (1973).

8. Hulley, S.B., et al. Quantitation of serum lipoproteins by electrophoresis on agarose gel: Standardization in lipoprotein concentration units (mg/100 ml) by comparison with analytical ultracentrifugation. J Lipid Res 12:420 (1971).

9. Bio-Rad Laboratories Instruction Manual. Bio-Gram A Lipoprotein Profile. Bio-Rad Laboratories, Richmond, CA, 1971.

10. Jencks, W.P., et al. Paper electrophoresis as a quantitative method: The staining of serum lipoproteins. J Clin Invest 34:1437 (1955).

11. Clark, D.A., et al. Studies of the accuracy of measurements of serum high density lipoprotein cholesterol levels. Aviat Space Environ Med 55(10):941(1984).

12. Clark, D.A., and J. A. Wood. Titration of serum lipoproteins with lipoprotein precipitants. SAM-TR-81-31, Dec 1981.

13. Conlon, D.R., et al. Quantitative determination of high-density lipoprotein cholesterol by agarose gel electrophoresis. Clin Chem 25(11):1965 (1979).

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