Phenotyping of Lipemias by Ultrafiltration and Nephelometry of Serum Lipoproteins

Mario Werner, Carolyn K. Montgomery, Albert L. Jones, and Siegfried Nussenbaum

Nephelometry of serum before and after ultrafiltration through cellulose membranes affords a simple and rapid physical method for separately estimating pre-β-lipoproteins and chylomicrons in their native state. The method was rigorously evaluated through correlative studies with chemical analyses, electron microscopy, and electrophoresis. Together, the data indicate that this technique, combined with the determination of cholesterol as an estimate of β-lipoprotein concentration, allows the phenotyping of hyperlipemias.

Additional Keyphrases electron microscopy of lipoproteins • latex particles as size indices • sulfo-phospho-vanillin method for total lipids • cholesterol and triglycerides • methodological correlations • β- and pre-β-lipoproteins • chylomicrons • particle-size-filter-pore diameter relationship

CHEMICAL METHODS of lipid analysis usually destroy lipoprotein complexes in which lipids exist in serum. Lipoprotein analysis, therefore, is usually based on physical principles. Electrophoresis is commonly used, but differences in dye affinity among lipoprotein classes present certain difficulties to quantitation. This paper evaluates nephelometry of pre-β-lipoproteins and chylomicrons after they have been separated by ultrafiltration. Lipoproteins remain in their native state throughout the procedure.

Materials and Methods

Specimens. “Monodisperse” polystyrene latex particles (Diagnostic Products Div., Dow Chemical Co., Midland, Mich.) of 88 ± 0.8 nm, 109 ± 2.7 nm, 126 ± 4.3 nm, 176 ± 2.3 nm, 264 ± 6.0 nm, 312 ± 2.2 nm, 365 ± 7.9 nm, and 557 ± 10.8 nm diameter (mean ± SD) were suspended in distilled water. Fresh sera from fasting individuals were diluted 10-fold for filtration (1 ml of serum to 9 ml of physiologic saline). Extremely lipemic sera, however, required dilutions up to 1:500.

Filtration. Filters with an average pore diameter of 450, 300, 200, 100, or 50 nm (specified by the manufacturer) from either of two sources were used interchangeably: nitrocellulose filters from Sartorius Div., Brinkmann Instruments, Inc., Westbury, N.Y.; or mixed cellulose ester filters from Millipore Corp., Bedford, Mass. Filters of 25-mm diameter were placed in filter holders (Millipore, or Gelman Instrument Co., Santa Clara, Calif.) and a syringe containing the specimen was attached.

Turbidimetry. Two instruments were used: the Thorp Nephelometer (Particle Data, Inc., Elmhurst, Ill.) and the Amino-Bowman fluoromicrophotometer (American Instrument Co., Silver Spring, Md.). To obtain a linear signal response from the Thorp Nephelometer, conversion tables supplied by the manufacturer were used and results were expressed as light-scattering index (LSI). LSI units are arbitrarily defined by a standard supplied by the manufacturer (1). The fluoromicrophotometer was used with a red primary filter but no secondary filter. Specimens were measured in round cells, 10-mm o.d. and 75-mm long. The instrument scale was set with a suspension of latex particles (109 ± 2.7 nm diameter) of known LSI, no further conversion was required. [No generally accepted standard for turbidity measure-
ments is available at present, so an arbitrary LSI standard that is available commercially (American Instrument Co.) was used.]

Chemical methods. Total lipids were determined with the sulfo-phospho-vanillin method (2, 3), cholesterol according to Babson et al. (4), and triglyceride glycerol according to Carlson (5). Triglyceride was reported as mmol/liter, to avoid assumptions about the molecular weight of serum triglycerides. However, if one assumes an average molecular weight of 284 for fatty acids, 1 mmol of triglyceride per liter corresponds to 890 mg/liter (6). Lipoproteins were electrophoresed on thin agarose gel (barbital buffer, pH 8.8, 0.05 mol/liter, 140 V, 70 min) (7), and the electrophoretic patterns were stained with Sudan black.

Electron microscopy. Diluted native sera and filtrates were fixed in phosphate-buffered osmium tetroxide (1 g/100 ml) and shadowed for direct electron microscopic visualization by the method of Jones and Price (8). Electron micrographs were taken with a Philips EM-300 electron microscope, and particle size range was estimated from enlarged photographs.

Results

Instrument Response

On the Thorp Nephelometer, LSI correlated linearly with the concentration of suspended latex particles of 88-nm and 264-nm diameter, but for particles of 365-nm and 557-nm diameter signal response became nonlinear, and the turbidity of concentrated suspensions was overestimated (Figure 1). By contrast, signal response of the Aminco-Bowman fluoromicrophotometer was linear regardless of particle size (Figure 2). Therefore, this instrument was used for all subsequent turbidity measurements.

Filtration

Latex particle suspensions were moved through filters with different nominal pore diameters to test their filtration properties (Figure 3). Filters with 450-nm pore diameter retained all particles of 365-nm diameter and larger, and passed over 95% of particles of 126-nm diameter and smaller. Filters with 100- and 50-nm pore diameters retained all particles down to 88-nm diameter.

Electron micrographs of serum lipoproteins before and after filtration confirmed these findings (Figures 4 and 5). The size of filtered lipoproteins decreased progressively with the filter pore diameter. However, we saw some variation between filters of the same denomination, particularly those of 50-nm pore diameter, with which we could occasionally find particles with a diameter as large as 70 nm in the filtrate. Assuming molecular diameters of over 75 nm for chylomicrons (Sf > 400) and 30 to 75 nm for pre-β-lipoproteins, the 450-nm pore diameter filter should retain only large chylomicrons and various fractions of smaller chylomicrons, and filters of both the 100- and 50-nm pore size should retain all chylomicrons.

Repeated duplicate measurements of a serum pool stored at 4°C were made to test reliability (Table 1). Specimens were allowed to come to room temperature and were agitated on a mixer to disperse lipoprotein aggregates before filtration.
During the first two weeks of storage, native turbidity decreased while turbidity after filtration through 50-nm pore diameter filters remained unchanged for almost a month. The coefficient of variation between duplicate filtrates from 450-nm pore diameter filters was larger (p < 0.05) than in the native specimens, demonstrating the variation in pore size between these filters, whereas variation between duplicate filtrates from filters of 50-nm pore diameter was not significantly increased.

The turbidities of 33 sera before and after filtration through filters of 450-nm, 100-nm, or 50-nm pore diameter are shown in Figure 6. The various fractions of the total turbidity removed by each filter defined characteristic patterns. These were tentatively classified into four groups according to the turbidity removed (chylomicrons) and that remaining after the last filtration (pre-β-lipoproteins):

**Group A.** A decrease of less than 15 LSI and an LSI of less than 25 remaining.

**Group B.** LSI decreased by more than 15 but less than 25 LSI remaining.

**Group C.** LSI decreased by less than 15 but remaining LSI still greater than 25.

**Group D.** LSI decreased by more than 15 and remaining LSI still greater than 25.

### Table 1. Turbidity of a Serum Pool before and after Storage at 4°C

<table>
<thead>
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<th>Day</th>
<th>Native serum</th>
<th>450-nm pore size</th>
<th>50-nm pore size</th>
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<tr>
<td>0</td>
<td>84.0</td>
<td>43.7</td>
<td>11.5</td>
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<tr>
<td></td>
<td>85.1</td>
<td>45.3</td>
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<td>5</td>
<td>77.2</td>
<td>51.1</td>
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<td>76.6</td>
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<td>12.1</td>
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<tr>
<td>12</td>
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<td>42.7</td>
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</tbody>
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SD of difference between duplicate measurements: 0.59
CV between duplicate measurements, %: 0.98

*a Measurements made at room temperature.

**Correlation with Chemical Determinations**

Figure 7 correlates the turbidity remaining after filtration through a filter of 50-nm pore diameter, with the triglyceride, total lipid, and total cholesterol concentrations of the original specimen. Specimens assigned to the four groups similarly correlated turbidity with triglycerides and total lipids. All specimens of group A had less than 2.1 mmol of triglycerides per liter and less than 950 mg of total lipid per 100 ml. Specimens of group B had normal or moderately elevated triglycerides and total lipids. By contrast, most specimens of groups C and D had enhanced triglycerides and total lipids.

Regression lines were calculated both by taking turbidity as the dependent variable (regression of Y on X in the figures) and as the independent variable (regression of X on Y). The closeness of these two regressions, as well as the significant correlation coefficients, demonstrates the interdependence of turbidity with triglyceride and total lipid concentration. Turbidity after filtration through filters of 100-nm pore diameter was less well correlated. While the regression of turbidity on triglyceride concentration extrapolates close to the
In the native specimen the largest particle at upper left is about 100 nm in diameter. In the 50-nm filtrate all particles are less than 70 nm in diameter. Note clumping of particles, which occurs frequently, making accurate sizing difficult. × 21,000

Correlation with Electrophoresis

Figure 8 compares electrophoretic lipoprotein patterns with turbidities and cholesterol concentrations for specimens in which less than 15 LSI were removed by filtration (groups A and C). The remaining turbidity correlated with the amount of pre-β-lipoprotein seen in electrophoresis, and cholesterol correlated with β-lipoprotein. Specimens shown in Figure 9 were grossly turbid and more particles were removed by filtration (group D). On electrophoresis these nonfiltered particles appeared as a chylomicron trail between the origin and the β-lipoprotein band. In the presence of this trail the latter lost its sharp demarcation but was recognized by a more blue stain than the greyish-blue pre-β-lipoproteins and chylomicrons. Again, turbidity after filtration correlated with the amount of pre-β-lipoprotein seen in electrophoresis, and cholesterol correlated with β-lipoprotein.

Discussion

Meaningful turbidity measurements of large lipoproteins require prior separation of particles...
TRIGLYCERIDES, mM/1
TOTAL LIPIDS, mg/100ml
CHOLESTEROL, mg/100ml

Fig. 7. Correlation between turbidity remaining after filtration through filters of 50-nm pore diameter and serum triglyceride, total lipid, and cholesterol concentrations

● specimens of group A; ○, specimens of group B; ■, specimens of group C; □, specimens of group D. Regression lines of Y on X and of X on Y are plotted. Regression equations of Y on X, their error (S), and the correlation coefficients (r) are listed.

Fig. 8. Comparison of electrophoretic patterns with turbidity remaining after filtration and serum cholesterol concentration

Specimens 1-3, normal electrophoretic pattern; 4-6, enhanced β-lipoproteins; 7-9, enhanced pre-β-lipoproteins; 10-12, enhanced β- and pre-β-lipoproteins.

by size since lipoproteins of different size scatter light to a different extent. Although the size distribution of serum lipoproteins may be on a continuous spectrum, distinctive groupings make size a basis for classification (9, 10). Earlier attempts at ultrafiltration failed mostly because filters tended to clog (11). By using diluted serum, Stone and Thorp successfully separated larger fat particles from the smaller by filtration (12). However, the functionally effective pore sizes of cellulose filters differ from their nominal value, and monodisperse latex particles are not filtered in an all-or-none fashion. Consequently, fractions remain defined in operational terms.

Therefore, comparisons with electrophoretic patterns and with chemical analyses were made to show the practicability of the described simple and rapid physical methods for the separate estimation of pre-β-lipoproteins and chylomicrons.

Chylomicrons have a high ratio of light scatter to unit mass, and even marked turbidity due exclusively to chylomicrons does not reflect an excessively elevated triglyceride concentration. Particles of a size consistent with that of pre-β-lipoproteins appear generally to be the major triglyceride carriers in serum. Since the size distribution of these particles that pass a 50-nm filter is more uniform, turbidity can then be used with...
validity to estimate triglycerides. $\beta$-Lipoproteins, the principal cholesterol carriers in serum, for the most part do not scatter light.

Therefore, turbidimetry of serum before and after filtration (chylomicrons and pre-$\beta$-lipoproteins) together with cholesterol determination ($\beta$-lipoproteins) should define the three lipoprotein classes of major pathophysiologic interest for the phenotyping of hyperlipemia. However, the present classification of the hyperlipemias may prove to be too simple to do justice to the complexity of these disease states. With filters of different pore diameters the size pattern of large lipid particles may be resolved further (Figure 6), and such information may prove clinically useful. Indeed, considering the simplicity, economy, and relative reliability of the technique, it can be used even now in the smaller laboratory to obtain, rapidly and easily, either screening or follow-up information.

Rose Greenfeld, Dook Yee, and Edward Miller gave technical assistance.

References


