Cholesterol in Serum and Lipoprotein Fractions
Its Measurement and Stability

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The potential importance of the β-lipoproteins as contributors of the cholesterol in atherogenesis suggests that extensive use of measurements of the β-lipoprotein cholesterol (BLPC), as well as of the total cholesterol in serum is desirable. For both total and BLPC, researches involving their estimation in connection with the atherogenesis problem will commonly require statistical evaluation of "before and after" values or of comparisons between population groups, two situations demanding detailed information on the reliability of the methods.

The present paper describes methods, applicable to 0.1 ml. serum samples, for total cholesterol and for its fractionation into BLPC and the α-lipoprotein cholesterol (ALPC) by paper electrophoresis and by cold ethanol precipitation. Data are given also on the storage of samples for cholesterol measurement, on the reproducibility of the several cholesterol measurements, and on spontaneous intraindividual variations of serum cholesterol concentration.

It is shown that the BLPC separated by paper electrophoresis is equivalent to that from cold ethanol fractionation and that the total cholesterol in serum and in the electrophoretically separated fractions is stable at

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room temperature for months when it is air dried on filter paper. Finally, it is shown that the analytical errors with the present methods are smaller than the spontaneous intraindividual variability of human serum.

MATERIALS

Paper-electrophoresis apparatus. The apparatus is patterned after that built by Swahn (1, 2). A rectangular moist chamber of acrylic plastic (Plexiglas or Lucite) 230 mm. long by 210 mm. wide supports four paper strips horizontally on plastic points (at intervals of 1 to 2 cm.), so as to be midway in the vertical chamber depth of 12 to 15 mm. The ends of the strips of filter paper (Whatman No. 1), 45 x 350 mm., are bent straight down to dip into buffer solution about 45 mm. below the bed of points. A thin sponge rubber gasket assures a tight closure of the flat top plate on the edges of the side and end walls of the moist chamber.

Michaelis' veronal buffer. Five L. contain 29.428 Gm. of barbital sodium, 19.428 Gm. of sodium acetate (NaC₂H₃O₂·3H₂O) and about 15 ml. of normal HCl to make pH 8.6.

Sudan Black B solution (Swahn) (2). One Gm. of Sudan Black B is added to a mixture of 600 ml. absolute alcohol and 400 ml. water. The mixture is stirred constantly while it is heated to boiling and boiled 10 minutes. It is cooled and filtered through a large filter of Whatman No. 1 paper. If black specks appear on the dyed papers the solution is refiltered.

Aqueous KOH, 33%. Ten Gm. of KOH plus 20 Gm. of water.

Alcoholic KOH, 2%. Six ml. 33% aqueous KOH diluted to 100 ml. with absolute ethanol.

Standard cholesterol solutions. Dissolve 60.0 mg. of pure dry cholesterol in absolute alcohol and dilute to 500.0 ml. in a volumetric flask. A 2.0 ml. portion of this contains 240 μg. of cholesterol. When used as described it corresponds to a serum containing 240 μg. per 100 ml. Portions of this solution are accurately diluted twice and six times to give 120 μg. and 40 μg. standards, respectively.

Liebermann-Burchard reagent. Place 90 ml. of acetic anhydride in a glass-stoppered Erlenmeyer flask. Cool in ice to 8° or colder. Keep cold while adding, dropwise, 4.5 ml. of concentrated H₂SO₄ with constant stirring. Let stand 5–10 minutes in the ice bath, add 45 ml. of glacial acetic acid and bring to 25° before use. Use within an hour.

ACD solution. One L. contains 22.0 Gm. of trisodium citrate dihydrate, 8 Gm. of citric acid monohydrate, and 22.0 Gm. of dextrose.

Sodium acetate buffer. Two hundred ml. of 4M sodium acetate and 400 ml. of 10M acetic acid are diluted with water to make 1 L. This buffer when diluted 1:80 with distilled water should have a pH 4.00 ± 0.02 at 25°.
Alcohol-buffer. Add 2.4 ml. of the above acetate buffer to 250 ml. of 95% ethanol and dilute with water to 1 L. This should be prepared fresh daily.

CALCULATIONS

In the analysis of the results, both of duplicate measurements with the same method and of the application of different methods to the same serum, we have used the following statistics

Mean Difference

\[ \bar{\Delta} = \frac{\Sigma\Delta}{N}, \]

where \( \Delta \) is the difference (having regard to sign) between first and second measurements and \( N \) is the number of pairs of measurements.

Standard Deviation of the Differences (S.D. \( \Delta \))

\[ (S. \ D. \ \Delta)^2 = \frac{N\Sigma\Delta^2 - (\Sigma\Delta)^2}{N(N-1)} \]

Note that where there is no difference between the means of the first and second measurements this value is almost equal to \( S. \ E. \ M. \) multiplied by the square root of 2 (= 1.414), where \( S. \ E. \ M. \) is standard error of measurement.

Standard Error of Measurement (S.E.M.)

\[ (S. \ E. \ M.)^2 = \frac{\Sigma\Delta^2}{2N} \]

Standard Error of the Mean Difference

\[ S. \ E. \ \bar{\Delta} = S. \ D. \ \Delta / \sqrt{N}. \]

Percentage Mean Difference

These values are obtained by multiplying the above values by 100 and dividing by the grand mean of the measurements in the series. For example:

\[ \bar{\Delta} \% = \frac{100\bar{\Delta}}{\sqrt[2]{\bar{X}_1 + \bar{X}_2}}. \]

TOTAL CHOLESTEROL MEASUREMENT

In the present methods the final estimation of cholesterol is essentially the same for the BLPC as for the total cholesterol in 0.1 ml. serum, so it is useful to consider this first. This is an adaptation of the method of Abell et al. (3), in which cholesterol esters are hydrolyzed with alcoholic KOH, total cholesterol is extracted from the alcoholic solution by pe-
troleum ether (B.P. 60–80°), and, after evaporating the solvent, the Liebermann-Burchard reaction is utilized. Our version, which we call the K5 method, has been applied to thousands of samples in five different laboratories and the experience with this method and some details of the procedure are briefly indicated below.

Procedure

Two ml. of fresh alcoholic KOH are added to the measured sample (0.1 ml. of serum) and to standards and reagent blanks, in glass-stoppered test tubes. Hydrolysis is complete within 90 minutes at 37°. This system is stable, so the stoppered tubes may be left for at least a week in an incubator or for several weeks at room temperature before proceeding further. A 2-ml. portion of water is then added and the mix is extracted by shaking briefly (60 seconds) with 4-ml. portions of redistilled petroleum ether and transferring the supernatant extracts with a capillary (Pasteur) pipet directly to the Evelyn colorimeter tube. Two extractions, with a final rinse of the pipet, extract 98 to 99 per cent of the cholesterol. With three extractions only a very rough and hasty technic in transfer is needed for complete recovery.

After adding 1 drop of 25% acetic acid in petroleum ether to each tube, the solvent is evaporated by a stream of warm air directed down into the tube. When the tube appears dry when held to the light, the color development can be started, or this may be delayed for weeks, if need be, because the dry residue in the tube is stable.

Colorimeter Readings

In the development of color with the Liebermann-Burchard reagent we find it essential, especially when working in the field or when using varied batches of chemicals, to make serial readings on each tube to assure recording the actual maximum with a 620 mμ filter. At 20–25° this usually means starting readings at 20 minutes and repeating at 5-minute intervals until the maximum is known. With the Evelyn photoelectric colorimeter, readings at 15-second intervals are convenient, so a batch of 20 tubes at a time is suitable.

REPRODUCIBILITY

The reproducibility of this K5 method has been extensively studied and it has been repeatedly checked with digitonin precipitation methods and with the simpler Bloor extract procedure in which hydrolysis is omitted and the mixed free and ester cholesterol is extracted with Bloor's
Table 1. Reproducibility, in Four Laboratories, of the K5 Method for Total Cholesterol in 0.1 ml. of Serum from Healthy Men

There were 100 pairs of duplicate analyses in each series except in Minneapolis where the number was 118.

<table>
<thead>
<tr>
<th></th>
<th>Minneapolis</th>
<th>Naples</th>
<th>Cape Town</th>
<th>Bologna</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.E.M.* (mg./100 ml.)</td>
<td>±3.52</td>
<td>±2.96</td>
<td>±4.52</td>
<td>±3.57</td>
</tr>
<tr>
<td>S.E.M.%</td>
<td>±1.42</td>
<td>±1.64</td>
<td>±2.44</td>
<td>±1.79</td>
</tr>
<tr>
<td>r*</td>
<td>0.970</td>
<td>0.992</td>
<td>0.979</td>
<td>0.978</td>
</tr>
</tbody>
</table>

All series used 100 pairs of duplicate analyses except Minneapolis, using 118.

* Standard error of measurement.
% Percentage of the mean of the series.
* Coefficient of correlation.

alcohol-ether mixture. Data on simple replication in four laboratories are summarized in Table 1.

It will be observed that the reproducibility of results with the K5 method is such that, in general, two out of three replications will depart less than ±2 per cent from the first measurement. The poorest reproducibility we have seen was in the Cape Town series summarized in Table 1, where the work was done under great pressure of time with an unstable photoelectric colorimeter. The high coefficient of correlation in the Cape Town series reflects the wide range of cholesterol (90 to 400 mg./100 ml.) in the series.

As shown in Table 2, the K5 method tends to produce values very slightly higher than from the digitonin methods of Sperry and Webb (4) and of Foldes and Wilson (5). This might be explained on the theory that the K5 method gives a slight overestimate because the Liebermann-Burchard reaction as used in K5 is not completely specific. It seems equally plausible that the digitonin methods give slight underestimates.

Table 2. Comparison of Results from Present (K5) Method with Those from the Digitonin and the Bloor Extract Methods in Two Laboratories

<table>
<thead>
<tr>
<th></th>
<th>K5-Digitonin* (Minneapolis and Naples)</th>
<th>K5-Bloor Milano</th>
<th>Bloor-Digitonin*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>20</td>
<td>134</td>
<td>27</td>
</tr>
<tr>
<td>Δ (mg./100 ml.)</td>
<td>5.7</td>
<td>-44.0</td>
<td>-49.2</td>
</tr>
<tr>
<td>S.E. Δ (mg/100 ml.)</td>
<td>±2.0</td>
<td>±0.88</td>
<td>±4.0</td>
</tr>
<tr>
<td>Δ %</td>
<td>2.3</td>
<td>-19.6</td>
<td>-30.3</td>
</tr>
<tr>
<td>S.E. Δ %</td>
<td>±0.5</td>
<td>±0.39</td>
<td>±2.5</td>
</tr>
</tbody>
</table>

* Comparison made with method of Foldes and Wilson (5).
* Comparison made with method of Sperry and Webb (4).
because the digitonide is not absolutely insoluble in the solvents used. For most purposes the difference is negligible but in any case the digitonin result can be computed at 0.97 times the K5 result or, conversely, K5 = 1.03 × digitonin. In the series of 20 samples covering the range 100 to 292 mg./100 ml., in Table 2, the coefficient of correlation between the means of duplicates from the two methods was r = 0.9918, the mean value by K5 being 211.3 and by digitonin being 205.6 mg./100 ml.

The comparison of Bloor extract values with those from the Sperry and Webb digitonin method gives, as expected, a difference similar to that found when the K5 method is compared with the Bloor extract. On the average, the Bloor extract value is 15.9 per cent too high but the discrepancy is reasonably consistent and the coefficient of correlation between Bloor extract and digitonin values in 50 samples was r = 0.87.

The discrepancy between the K5 and the Bloor extract values is far larger and more serious. Primarily this stems from the fact that the color intensity resulting from the Liebermann-Burchard reaction acting on cholesterol esters is higher than that with free cholesterol, so the Bloor values, which are obtained from a mixture of free and ester cholesterol but computed as though all were in the free form, are erroneously high. With storage the proportion of free to ester cholesterol may be changed by the action of esterases in the serum (6). The data in Table 1 refer to serum started through analysis within 1 to 3 hours of blood drawing. For this condition the finding in Minneapolis on 134 bloods, each analyzed in duplicate by the K5 and by the Bloor extract method, is that the K5 result can be predicted by multiplying the Bloor extract value by the factor of 0.836, the standard error of estimate being ±10.24 mg./100 ml.

**PAPER ELECTROPHORESIS PROCEDURE**

A pencil line is drawn across each paper strip at a point which will be about 3 cm. from the cathode end of the bed of the moist chamber. The paper is dipped in buffer, blotted until the gloss disappears, placed in the moist chamber bending down the ends to dip in buffer in the electrode vessels, and covered for an hour or more before applying 0.1 ml. of serum in a single point at the center of the pencil line. Good results are obtained also if the serum is applied to the dry paper by spreading it uniformly along the pencil line, keeping 5 mm. away from each edge of the paper and immediately adding the buffer dropwise to the remainder of the paper.

The moist chamber is covered and a D.C. potential of 185 volts is applied for 15 hours. The strips are dried by hanging them by one end in
room air or by warming on a smooth clean surface (e.g., a photographer's print drier). As will be seen, in this dry state the paper may be stored for at least many weeks before proceeding further.

It is desirable to run, in parallel, two strips for each sample, one to be stained as a guide for cutting the other strip into \( \alpha \)- and \( \beta \)-lipoprotein parts. When the dry strips are examined before staining two brown spots are usually visible, one having migrated with the albumin about two thirds of the length of the strip, while the other has migrated only about half as far and is in the globulin region. Further, when the strip is examined under ultraviolet light, a large fluorescent spot appears, the albumin brown spot being within this. The positions of these spots, which may be outlined with pencil, are useful guides for cutting the paper so as to separate the \( \alpha \)- and \( \beta \)-lipoproteins.

The uncut strip to be dyed is immersed in Sudan Black B solution for 10–60 minutes and then is briefly washed in ethanol-water (1 to 1) and is dried. If there is any doubt that this shows where to cut the undyed strip, the pencil marks made on the two strips as noted above may be compared.

**Extraction of the Electrophoresis Paper Strips**

Each portion of paper is rolled and placed in a glass-stoppered test tube of 13–18 ml. capacity. Two milliliters of 2% alcoholic KOH are added, and the tube is inclined so as to wet the paper and is incubated at 37 to 40° for at least 90 minutes. A 4-ml. portion of petroleum ether is added, followed by a 6-ml. portion of ethanol-water (1 to 2). The tubes are shaken for 60 seconds, allowed to stand briefly and the petroleum ether layer is transferred to an Evelyn colorimeter test tube by a Pasteur pipet with a 2-ml. rubber bulb. After use the pipet is conveniently washed with petroleum ether from a polyethylene wash bottle, the washings being collected in the colorimeter tube. The petroleum ether extraction is repeated twice more, using 4-ml. portions each time. One drop of 25% glacial acetic acid in petroleum ether is added to each tube and the solvent is evaporated as in the total cholesterol analysis.

Reagent blank tubes and standard tubes are prepared by similar procedures. Reagent blanks are made with alcoholic KOH, incubated, and extracted in the same way as the serum samples. Filter paper and buffer salts are not added since they were found to contribute no color. Standards are set up by measuring 2-ml. portions of alcoholic cholesterol standard solutions into glass-stoppered tubes. A dry strip of buffer-treated paper, 120 mm. long, is placed in each of the tubes for "beta-
standards" which should contain amounts of cholesterol in the range expected for the \( \beta \)-lipoprotein samples. Two "alpha standard" tubes containing 40 \( \mu \)g. of cholesterol are given 50-mm. portions of similar paper. To each standard tube is added 0.12 ml. of 33\% aqueous KOH. The tubes are incubated and thrice extracted with petroleum ether in the same way as the other tubes. The rest of the analysis is done as in the ordinary cholesterol measurement.

**COLD ETHANOL FRACTIONATION PROCEDURE**

This is a small-scale adaptation of the first step of Cohn's Method X for blood protein fractionation (7, 8, 9). \( \alpha \)-lipoprotein cholesterol appears in the supernatant fluid after the precipitation while the \( \beta \)-lipoprotein cholesterol appears in the precipitate.

To 0.1 ml. of serum in a glass-stoppered round-bottomed centrifuge tube is added 0.03 ml. of ACD solution and the whole is cooled to \(-5^\circ\) with constant stirring by a plastic-enclosed magnetic particle. Then, slowly, 0.52 ml. of fresh alcohol-buffer is added while stirring continues. Centrifuging for 30 minutes at \(-5^\circ\) completes the sedimentation and the supernatant can be decanted and drained off (at \(-5\) to \(-10^\circ\)).

The residue is dissolved in 2 ml. of alcoholic KOH, incubated at 37\(^\circ\) for 90 minutes, treated with 2 ml. of water and then is extracted twice with 4-ml. portions of petroleum ether. The cholesterol determination is completed as described above for total serum cholesterol by the K5 method.

**RESULTS FROM DUPLICATE FRACTIONATIONS**

Replication studies on the \( \beta \)-lipoprotein cholesterol (BLPC), both from paper electrophoresis and from cold ethanol separation, are summarized in Table 3. In our experience the agreement between duplicates tends to be better in the cold ethanol method (S.E.M. = \( \pm 4.3 \) vs. \( \pm 5.8 \), the

<table>
<thead>
<tr>
<th>Comparison</th>
<th>( N )</th>
<th>( \bar{A} )</th>
<th>( \bar{A} % )</th>
<th>S.D. ( \bar{A} )</th>
<th>S.D. ( \bar{A} % )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper-paper</td>
<td>36</td>
<td>..</td>
<td>..</td>
<td>( \pm 8.1 ) mg.</td>
<td>( \pm 4.0 )</td>
</tr>
<tr>
<td>Ethanol-ethanol</td>
<td>169</td>
<td>..</td>
<td>..</td>
<td>( \pm 6.1 ) &quot;</td>
<td>( \pm 3.0 )</td>
</tr>
<tr>
<td>Paper-ethanol</td>
<td>128</td>
<td>1.0 mg.</td>
<td>0.5</td>
<td>( \pm 13.8 ) &quot;</td>
<td>( \pm 8.8 )</td>
</tr>
<tr>
<td>Sum&quot;-K5*</td>
<td>128</td>
<td>5.3 mg.</td>
<td>2.2</td>
<td>( \pm 12.2 ) &quot;</td>
<td>( \pm 5.0 )</td>
</tr>
</tbody>
</table>

* Sum of BLPC and ALPC from cold ethanol.

* Total cholesterol measured by K5 method.
difference being slight but highly significant in large series). But with both methods at least two thirds of second analyses fall within ±4 per cent of the BLPC value obtained in the first analysis.

On the average, the estimation of BLPC from paper electrophoresis agrees reasonably well with that from cold ethanol, as shown in Table 3. The correlation coefficient between results with the two methods is \( r = 0.953 \).

The comparison of the sum of the ALPC and BLPC with the total cholesterol measured directly with the K5 method indicates a small and fairly consistent discrepancy, such that ALPC plus BLPC gives an average overestimate of 2.2 per cent of the K5 result, as shown in Table 3. It should be noted that the standard deviation of the difference between the sum of the paper strips and the direct (K5) total cholesterol measurement includes the effects of three measurement errors. The standard error of measurement of \( \alpha \)-lipoprotein cholesterol (ALPC) is somewhat smaller than that for BLPC and the standard error of measurement of the sum of ALPC and BLPC was ±6.70.

The percentage of total cholesterol in the \( \beta \)-lipoprotein fraction was computed by both the paper electrophoresis and the cold ethanol methods in 128 serum samples. The mean values were 80.9 and 82.4 per cent, respectively, and the S.D. Δ between methods was ±4.0 per cent. In 36 such duplicate estimates with the paper electrophoresis method the range was 63 to 93 and the standard error of measurement ±1.1 per cent of total cholesterol. Using the cold ethanol fractionation method for BLPC and the K5 method for total serum cholesterol the percentage of total cholesterol in the beta fraction was computed in duplicate for 164 comparable cases. The range was 63 to 97 and the standard error of measurement ±2.1 per cent.

**STORAGE STUDIES**

It is well known that it is hazardous to store nonsterile blood for more than a day or two in an ordinary refrigerator if cholesterol is to be measured. But repeated studies in this laboratory have shown that the concentration of total cholesterol in nonsterile human blood serum stored at \(-20^\circ\) is stable for at least 5 years, though sampling presents difficulties because the thawed system is a mixture of phases. These results refer to cholesterol as measured by a method involving hydrolysis of the esters and subsequent application of the Liebermann-Burchard reagent; without this step variable results are obtained because of spontaneous hydrolysis and esterification in the stored serum (6).

Besides deep-freeze storage, we have found that total serum cholesterol
is well maintained for many months at room temperature in lyophilized (dry) serum. This is a convenient way to maintain a reference standard for checking analytical methods. It is not even necessary to use proper freeze-drying to get a stable product. Small quantities of serum (up to 1 ml.) may be dried without cooling in a wide-mouthed vial placed in a vacuum line close to an open supply of anhydrous magnesium perchlorate or phosphorous pentoxide for 1 hour. For example, an average of 234.5 mg./100 ml. of total cholesterol was obtained from 8 analyses on 2 fresh human serum samples. Fourteen analyses on the same serums vacuum-dried in this simple fashion and then stored for 30 days at 37° gave an average of 235.3 mg./100 ml.

Air-Dried Samples of Serum

These results led to further attempts at simplification and it was found that the total cholesterol in small samples (0.1 ml.) of serum may be preserved for at least several months at temperatures of 25-30° merely by air drying on filter paper. The serum is measured out so as to cover the maximum area (2 to 3 cm. in diameter) on Whatman No. 1 paper which is then hung up in the air for a few hours to dry.

The subsequent analysis, of course, requires extraction of the cholesterol from the dried sample of filter paper but this can be combined with the hydrolysis step so there is no great addition to the labor of analysis. The procedure described above for the analysis of paper electrophoresis strips is used, but we find that more satisfactory extraction results if the temperature is maintained at 70° for the combined hydrolysis-extraction step.

A series of 126 samples of serum drawn in Cape Town, covering a range of from 82 to 424 mg. of total cholesterol, were studied in this way. The mean for the fresh serums analyzed, each in duplicate, at Cape Town was 196.1 ± S.E. = 6.3 and for the paper-dried replicates done at Minneapolis, in duplicate, from 3 to 6 weeks later, the mean was 197.7 ± 5.5, the coefficient of correlation between the two analyses being $r = 0.986$. The average difference, without regard to sign, between the analyses done on the fresh and on the dried serums was 8.0 mg./100 ml., and the standard deviation of the differences between results on fresh and on dried serum was ±9.8 mg./100 ml.

A second test, under somewhat more favorable conditions for the measurements of cholesterol in the fresh serum, was made in Italy. Blood samples were taken from 112 clinically healthy men in Cagliari, Sardinia. After separation of the serum, 0.1-ml. portions were dried on paper and the remainder placed in screw-capped vials and stored in cold thermos
jugs at 0°C until analyzed in Naples a few days later. The dried samples on paper were stacked up, wrapped in Saran Wrap and were mailed to Minneapolis. The mean from the fresh material at Naples was insignificantly (0.2 mg./100 ml.) lower than the mean from the papers analyzed at Minneapolis, the average difference between the two methods, without regard to sign, being 5.4 mg./100 ml. The standard deviation of these differences was ±6.9 mg./100 ml.

The paper strips after paper electrophoresis may be dried similarly and analyzed at leisure many weeks later. No attention need be paid to the temperature of storage. Staining the strips to identify the lipoprotein distribution may be done at any stage between electrophoresis and final extraction and completion of the analysis.

**DISCUSSION**

**Metabolic Influences**

The bloods used in the foregoing analysis of the results with the various cholesterol methods were all from clinically healthy men. These methods have also been applied to blood from hospital patients with a variety of diseases. The agreement between duplicates analyzed for total cholesterol by the present K5 method in several series of patients was similar to that found for healthy subjects. However, somewhat greater variability between replicates in paper and cold ethanol fractionation was found among patients with disordered cholesterol and/or lipid metabolism in general (cirrhosis, nephrosis, diabetes). In a series of 11 such patients the sum of ALPC plus BLPC on paper averaged 7.5 mg./100 ml. higher than the direct measurement of the total cholesterol, the standard deviation of the differences being ±12.9 mg./100 ml. or ±5.0 per cent of the mean total cholesterol. In this same series of 11 patients the BLPC estimation from paper electrophoresis averaged 9.6 mg./100 ml. higher than the BLPC separated by cold ethanol and the standard deviation of the differences was ±25.6 mg./100 ml. Even when allowance is made for the average difference between the BLPC results with the two methods, the standard deviation of the difference was ±10.8 per cent of the grand mean as compared with ±6.9 per cent for "normal" men and this is highly significant statistically. Apparently in these very abnormal bloods there may be unidentified factors which reduce the reproducibility of the BLPC results.

**Intraindividual Variations**

The practical effect of the method error in any analytical method should be judged after due consideration of the limitations imposed by
the presence of other uncontrolled or uncontrollable variations present in the practical situations in which the method is applied. In the case of cholesterol in human serum, spontaneous, intraindividual variation is a factor to be considered whenever the problem concerns "before and after" measurements.

In order to compare the analytical error of the present methods with these true intraindividual variations, we adopted the following general scheme. With suitably selected subjects, blood samples were drawn on two occasions, A and B, and analyses 1 and 2 were performed in duplicate on each sample. The differences between analyses A1 and A2 and between B1 and B2 give a measure of the method reproducibility, while the differences between samples A1 and B1 and between A2 and B2 include both method error and the intraindividual variability. The results of this procedure in three series of men, all studied in the basal fasting state, are given in Table 4.

Variability in the method itself accounts for no more than a small fraction of the variation between occasions in any of the series. The healthy young soldiers, living under highly constant controlled conditions in the laboratory, are the most stable group but even in them the standard deviation between occasions is four times that between replicates of the same serum. The intraindividual variability of the obese young men, and particularly of the coronary patients, is greater. In the face of such spontaneous variation of patients on what is supposed to be a constant

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**Table 4. Method, Occasion, and True Intraindividual Variability of Total Cholesterol and β-Lipoprotein Cholesterol Concentrations**

<table>
<thead>
<tr>
<th>Series</th>
<th>Item</th>
<th>N</th>
<th>Grand mean</th>
<th>Variance of differences</th>
<th>Standard deviation of differences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Method                Occasion &quot;Indie.&quot;</td>
<td>Method Occasion &quot;Indie.&quot;</td>
</tr>
<tr>
<td>A</td>
<td>Total cholesterol</td>
<td>22</td>
<td>209.5</td>
<td>19.97</td>
<td>400.6</td>
</tr>
<tr>
<td>B</td>
<td>β-Lipoprotein cholesterol</td>
<td>13</td>
<td>169.8</td>
<td>11.52</td>
<td>504.8</td>
</tr>
<tr>
<td>B</td>
<td>Total cholesterol</td>
<td>13</td>
<td>214.3</td>
<td>21.16</td>
<td>558.9</td>
</tr>
<tr>
<td>C</td>
<td>β-Lipoprotein cholesterol</td>
<td>24</td>
<td>242.7</td>
<td>67.71</td>
<td>1447.8</td>
</tr>
<tr>
<td>C</td>
<td>Total cholesterol</td>
<td>24</td>
<td>273.4</td>
<td>23.46</td>
<td>900.3</td>
</tr>
</tbody>
</table>

* Series A = healthy soldiers studied on a constant regimen in the laboratory on 2 occasions one week apart. Series B = healthy obese young men studied on 2 occasions 4 days apart during a control period with diet ad lib. Series C = ambulant middle-aged men with coronary heart disease studied, while supposedly on a constant regimen of diet and exercise, on 2 occasions 3 months apart.

N = Number of men (occasions = 2N, analyses = 4N).

* All values in mg. of cholesterol per 100 ml.
regimen it is obvious that these are serious limitations to the demonstration of the effects of therapy with limited numbers of patients. It should be recognized that the values for intraindividual variation given in Table 4 will not be reduced by taking the mean values of duplicate analyses of the same bloods. For total cholesterol, a direct trial of this calculation, using the means of duplicate analyses on each occasion in Series C of Table 4, gave, as predicted, variance = 888.8 for the gross individual difference between occasions.

Testing for Effect of Drugs

Such data on replicate variances ascribable to method error and to intraindividual variability are, of course, basic guides for planning research projects. Suppose it is desired to test the effect, if any, of a drug that is reputed to reduce the serum cholesterol and, accordingly, analyses will be made on serums from patients before and after getting the drug. Assume that that method and intraindividual variances, apart from any systematic effect of the drug, will be similar in the control and drug periods. What then, can be predicted about the number of patients required to "prove", at \( p = 0.01 \), an effect of the drug amounting to an average fall of 20 mg./100 ml. in total cholesterol? Alternatively, what is the least average fall to prove the point with a given number of patients?

With single analyses on one control and one experimental occasion the values for \( p = 0.01 \) are: \( t = 3.25, 2.86, 2.76 \) for 10, 20, or 30 patients, respectively. From Table 4 it is calculated that the standard errors to be expected for the mean "occasion" difference with these numbers of patients will be \( \pm 9.49, \pm 6.71, \) and \( \pm 5.48 \) mg./100 ml., respectively; for 20 mg./100 ml. mean change the corresponding \( t \) values are 2.10, 2.98, and 3.65. Hence 20 would be a good estimate of the fewest patients to be studied.

Alternatively, with 20 patients, it is easy to estimate the least mean change that would be highly significant \( \bar{X}/6.71 = 2.86 = t \), or \( \bar{X} = 19.2 \) mg./100 ml. (for \( p = 0.01 \)).

Eliminating the method error completely by making a large number of replicate analyses on the same bloods would not significantly change the foregoing indications. With perfect analyses, the standard error of the mean difference between occasions with 20 coronary subjects would not be less than \( 29.61/\sqrt{20} = \pm 6.62 \) mg. total cholesterol per 100 ml., so to reach \( p = 0.01 \), \( \bar{X} \) would have to be 18.9 mg./100 ml.

Obviously, it is more important to reduce the "occasion" variance. Other things being equal, with single samples on each of two control and
two "drug" periods, the standard error of the mean difference between occasions would be reduced by almost one third compared with that from single occasions. With 20 coronary patients it should approach ±4.74 mg./100 ml. for total cholesterol with samples on each of two occasions.

**Addenda**

Since the foregoing was written, a paper by Oliver and Boyd (10) has been published which adds further weight to the argument for the importance of the BLPC. Their cholesterol method does not involve hydrolysis of the esters and is not suitable for use in photoelectric colorimeters requiring much more than 1 ml. of solution for final color reading.

While this paper was being processed for publication, results were obtained of a check on the present method of preserving serum dried on filter paper and subsequent cholesterol analysis by our K5 method. Dr. George S. Boyd, Department of Biochemistry, University of Edinburgh, kindly pipetted and dried on filter paper duplicate samples of 20 serums and sent these to Minneapolis for analysis without disclosing his results obtained by the Sperry-Schoenheimer procedure applied to the fresh serum. The Minneapolis results averaged 4.6 per cent lower than the Edinburgh results, the standard error of measurement being ±1.6 per cent of the mean. Most of the small average discrepancy was contributed by two serums for which the Minneapolis values were 344 and 278 mg./100 ml. while the respective Edinburgh values were 302 and 225 mg./100 ml. These findings conform with our experience that the digitonin method generally gives slightly lower values, some of which, at least, probably represent incomplete precipitation or loss of digitonide.

**SUMMARY**

1. Methods are described for the separation, by paper electrophoresis and by cold ethanol, of α- and β-lipoproteins in 0.1 ml. of serum, with subsequent analysis of cholesterol in the separated portions.

2. It is shown that both methods of separation yield separated fractions containing substantially the same amounts of cholesterol.

3. Detailed data are given on the errors of measurement for total cholesterol and for cholesterol in the separated lipoprotein fractions.

4. Studies are reported on the stability of cholesterol in stored serum and on paper electrophoresis strips. It is shown that simple drying on filter paper causes no change in cholesterol content and yields a product that is stable for many weeks at ordinary room temperature.

5. The sources of variability in human serum cholesterol values are
examined and it is shown that spontaneous intraindividual variability is a much greater source of error than the errors of measurement with these methods.

REFERENCES