An Ultramicro Method for the Estimation of Plasma Cholesterol

G. S. Duboff and W. W. Stevenson*

A procedure is outlined for the ultramicro determination of total cholesterol that gives results in excellent agreement with classic methods. The determination requires 40 μl of plasma obtained from a finger-tip puncture and special handling of precipitated proteins.

By the use of the described procedure plasma cholesterol levels have been estimated 4 times daily over a period of 8 weeks in young men undergoing rigorous physical training and 3 times daily in women during the menstrual cycle.

A striking fall in total plasma cholesterol was observed in men following physical conditioning in contrast to controls, and a similar decrease was observed in women with normal menstrual cycles in contrast to women with an anovulatory menses.

It is suggested that the total cholesterol decrease in the female coincides with the phase in the menstrual cycle when estrogen activity is maximal and that the lowering of total cholesterol in men, following physical conditioning, may be due to an increased production of endogenous androgens with a consequent increase in conversion of these steroids to estrogens.

The following procedure for the determination of plasma cholesterol was used in order to be able to measure the sterol in microliter aliquots of blood plasma obtained from the finger-tip in studies requiring very frequent collection of samples. Studied were plasma cholesterol changes occurring with physical conditioning (1), and cyclic kinetic and biochemical changes in the blood associated with the rhythmicity of the menstrual cycle (2).

These studies required, in the case of physical conditioning in males, collection of blood 4 times daily for several weeks, and in the case of the menstrual cycle in women of child-bearing age, collection of blood

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3 times daily at frequent intervals between menses. Details of the experimental design and findings for both have been published elsewhere.

Before applying the ultramicro method in both studies, the reliability of accurately determining the plasma cholesterol on as little as a 40-μl sample was determined, as well as the validity of the quantitative results in terms of plasma from anticubital venous blood estimated by some conventional revision of the original Schoenheimer-Sperry technic—usually a macro procedure. For the latter, we chose the modification of Sperry and Webb, which, except for larger volumes of blood and extractant, was used as described (3). While it was not possible to carry out successfully this method with as little as 40 μl of plasma it was possible to apply the ultramicro procedure to both venous and finger-tip plasma.

**Determination of Plasma Cholesterol**

**Reagents**

- Concentrated sulfuric acid
- Glacial acetic acid, 99%
- Acetic anhydride

All reagents should be of good analytical grade. We have found Pfanstiehl's to be the most reliable source for cholesterol as a standard.

**Solutions**

To prepare a stock Standard solution, weigh out accurately on an analytical balance 400 mg. of cholesterol and transfer to a glass-stoppered 100-ml volumetric flask containing 40-50 ml. of warm glacial acetic acid. When the cholesterol is completely dissolved, dilute to mark with the same acid.

**Working Standard**

With a volumetric pipet transfer 10.0 ml. of stock solution to another 100-ml glass-stoppered flask and dilute to mark with glacial acetic acid. Each 0.2 ml. of acid contains 0.08 mg. of cholesterol, and when carried through the color development procedure yields a chromogen value equivalent to 200 mg. cholesterol per 100 ml. plasma.

**Calibration Solutions**

In order to establish the linearity function of technical and photometer performance prepare the following series of solutions as follows.
Into separate glass-stoppered 50-ml. volumetric flasks, accurately pipet 1.25, 2.5, 5.0, 7.5, 10.0, and 12.5 ml. of stock standard and dilute to mark with glacial acetic acid and mix. A 0.2-ml. portion from each flask is equivalent to 50, 100, 200, 300, 400, and 500 mg./100 ml. cholesterol, respectively, in color value produced by the Lieberman-Buchard reagent when treated as the working standard. The absorbance at 680 mμ is plotted against concentration.

**Equipment**

*Pipets, 1.0 ml. in 0.1, T.D. in 45 sec.* For measuring the 40 μl. of plasma and sulfuric acid Accupettes, with a 40-μl. capacity accurate to 0.5% may be used in place of the more expensive Lang-Levy pipet. In studies requiring serial estimations of cholesterol a numbered Accupette is assigned to each subject so that the same one is used throughout the study. This reduces the pipet error to a minimum.

*Cuvets, 10 × 75 mm.*

*Heparinized hematocrit tubes*

*Polyethylene micro space adaptors*

*Coleman Jr. Model 6D spectrophotometer.* Absorption measurements may be satisfactorily carried out in a Coleman Jr. Model D fitted with a polyethylene micro space adapter,* as the final volume is 0.94 ml. This model has a band width of 20 mμ. Other models of this make, such as 6A, 6B and 6C have band widths of 35 mμ and are not adequate. The cuvets used in this model are 10 × 75-mm. test tubes.

*Test tube vibrator.* Precipitation and mixing are critical for such small volumes, making the use of mechanical test tube mixers essential. Accurate reproducible results are not attainable by simply gently tapping the plasma and glacial acetic acid as suggested in earlier methods. It is necessary to break up the precipitated protein into as fine particles as possible. Good extraction of the cholesterol cannot be accomplished from such a small volume of plasma unless this is done. The same applies to ‘‘mixing’’ the added acetic anhydride and sulfuric acid.

*International Microhematocrit.* Centrifuge Model MB with combination head is most satisfactory and should be limited to 5000-7000 rpm. At full speed tube breakage is excessive. Test Tubes used are 7 × 70 mm.

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* Distributed by Scientific Products Inc., Evanston, Ill.

† The type distributed by Scientific Industries Inc., Springfield, Mass. (Model K-500-4 for multiple tubes or K-500-1 for singles), or the type distributed by Scientific Products Inc.
Principle
The principle underlying the technic is as follows. Glacial acetic acid deconjugates the globulin-bound cholesterol, freeing it to react with the Lieberman-Buchard reagent, acetic anhydride-sulfuric acid, with production of a color possessing excellent linearity for concentration-absorbance determinations.

Procedure
Into a 7 × 70-mm. test tube pipet 0.1 ml. glacial acetic acid and with an Accupette brought as close to the acid level as possible introduce 40 µl. of plasma. Mix tube contents in a mechanical mixer for 1 min., allow to stand for 5 min., add 0.5 ml. of acetic anhydride, and then repeat mechanical mixing. Let stand another 5 min., add another 0.5 ml. of acetic anhydride, and mix well, this time by hand. Centrifuge in a microhematocrit centrifuge for 2 min. at 5000-7000 rpm. Transfer 0.8 ml. of supernatant to 10 × 75-mm. cuvets. Transfer 0.2 ml. of working standard to a similar cuvet and 0.2 ml. glacial acetic acid to another as “reagent blank.” Add 0.8 ml. of acetic anhydride to the standard and blank cuvets and 0.1 ml. to the unknown cuvet. Add 40 µl. of concentrated sulfuric acid to each cuvet and mix well mechanically. Allow to stand for 15 min. with the polyethylene micro spacers in place. At exactly 15 min., begin reading in photometer at 680 mµ, with the reagent blank set at zero.

Calculate the amount of cholesterol in the plasma sample by applying the conventional Beer-Lambert formula:

\[
\frac{ODU_{680}}{ODS_{680}} \times 0.08 \times 2500 = \text{mg./100 ml. cholesterol in unknown}
\]

where \(ODU\) is absorbance of unknown; \(ODS\) the absorbance of the standard; 0.08 the concentration of standard; and 2500, the dilution factor for plasma.

Discussion
The results of our use of the procedure described above are summarized in Tables 1-3.

The procedure described enabled us to determine total cholesterol in human blood plasma using 40 µl. of plasma from finger-tip blood. The values determined paralleled quite satisfactorily those obtained from venous blood plasma. It is essential that technical details described be strictly adhered to. In both studies it was necessary to collect blood several times daily while the subjects were engaged in their normal
Table 1. Plasma Cholesterol Values of Venous and Finger-tip Blood* in Young Adult Males Estimated with Ultra Micromethod

<table>
<thead>
<tr>
<th>Subject</th>
<th>Venous blood (mg./100 ml.)</th>
<th>Finger-tip blood (mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Average</td>
</tr>
<tr>
<td>H.B.</td>
<td>202-206</td>
<td>204</td>
</tr>
<tr>
<td>A.H.</td>
<td>188-195</td>
<td>192</td>
</tr>
<tr>
<td>B.W.</td>
<td>209-214</td>
<td>211</td>
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</table>

*Samples for four daily determinations were collected from all subjects at the same time.

Table 2. Plasma Cholesterol Values of Venous Blood* Estimated with Macromethod (3) and Finger-Blood* Estimated with Ultra Micromethod

<table>
<thead>
<tr>
<th>Subject</th>
<th>Venous blood† (mg./100 ml.)</th>
<th>Finger-tip blood† (mg./100 ml.)</th>
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<tr>
<td>H.B.</td>
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<td>206</td>
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<tr>
<td>A.H.</td>
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<td>B.W.</td>
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†Last of four specimens collected from antecubital vein.

‡Last specimen collected for the day.

activity. Sufficient blood can be obtained by means of capillary hematocrit tubes to be able to do duplicate total cholesterol assays or one total and one esterified (hence the free cholesterol, by difference).

It is quite likely that the combination of frequency of collections plus the ultramicro method procedure applied enabled us, in the case of the physical conditioning experiment, to elicit the results obtained whereas others have failed to do so (4-7). Briefly, the results, which are graphically depicted in Fig. 1 and summarized in Table 4, adequately demonstrate that a period of physical conditioning (running 2 mi. per day over a period of weeks) lowers the plasma cholesterol significantly in individuals on the same dietary regimen as the controls.

Definite striking cyclic changes in plasma cholesterol were observed in a number of women with normal ovulating menses and without dietary restrictions. The striking midcycle fall in cholesterol level has been shown by others. Moynihan (8) showed that there is a rise before, and a fall during menstruation; similar findings were made by Okey and Boyden (9). Oliver and Boyd (10) studied the cholesterol fractions, phospholipid, and the cholesterol-phospholipid pattern of the blood during the menstrual cycle. In the earlier studies the methods employed were some modification of the classical Bloor technic.
Table 3. Plasma cholesterol levels of venous blood estimated by the macromethod (†) and finger-tip blood estimated by the ultra micromethod*  

<table>
<thead>
<tr>
<th>Subject</th>
<th>Day of cycle</th>
<th>Venous blood† (mg./100 ml.)</th>
<th>Finger-tip blood‡ (mg./100 ml.)</th>
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<th>Average</th>
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<td></td>
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<td>200-204</td>
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*All samples collected simultaneously from women of child-bearing age.
†One determination.
‡Three determinations per day.

Fig. 1. Plasma cholesterol curves in young men during physical training and detraining periods.
Table 4. Comparison of Mean Cholesterol Level of the Experimental Group for Pre-
Training and Post-Training Periods

<table>
<thead>
<tr>
<th>Phase</th>
<th>Mean (mg./100 ml)</th>
<th>Difference</th>
<th>S.E. difference</th>
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<td>206</td>
<td>29</td>
<td>1.45</td>
<td>12.021*</td>
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<tr>
<td>Post-training</td>
<td>177</td>
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*Significant at the 1% level of probability.

Oliver and Boyd employed the Schoenheimer-Sperry procedure on a macro scale.

The observed shift in cholesterol values from a preovulatory rise to a decrease followed by another rise during the cycle, may be directly or indirectly due to influences by certain hormones, most probably endogenous estrogens.

As shown in Fig. 2 the drop in cholesterol level parallels the increase in estrogen secretion, as judged by vaginal smear evidence of ovulation, a characteristic basal body temperature curve (BBT) associated with it, and correspondingly elevated urinary estrogen excretion values. Figure 3 however, records the plasma cholesterol curve typified by an anovulatory menses, the "midcycle" fall in cholesterol level being conspicuously absent because there is no midcycle elevated estrogen secretion.

![Fig. 2. Plasma cholesterol curve characteristic of changes in the menstrual cycle associated with evidence of estrus and ovulation. BBT, basal body temperature; SVS, serial vaginal smears.](image-url)
It is not unreasonable to suppose, in the case of male subjects undergoing physical conditioning during a period of organized exercise, that increased secretion of endogenous estrogen hormone by the testes occurs as a result of conversion of androgens (C19 steroids) to estrogens (C18 steroids) from increased secretion of testosterone and androstenedione by the testes. It is well established that a variety of human tissues possess the ability to convert androgens to estrogens (11–16).

References