Simplified Determination of the Lipid Components of Blood Serum

Harold V. Connerty, Anglis R. Briggs, and Edward H. Eaton, Jr.

A simplified scheme of analysis is presented for the determination of free and esterified cholesterol, the phospholipids, and the neutral fats (triglycerides) of blood serum. The hydroxamic acid reaction is used to determine total esterified fatty acids. The phospholipids are determined after acid digestion of a trichloroacetic acid precipitate of protein-phospholipid, and both total and free cholesterol are determined by a modified ferric ammonium sulfate reagent. Complete details of methods and calculations are described and discussed.

Interest in the determination of lipids in blood has been stimulated by the suspected relationship between abnormal lipid metabolism and atherosclerosis (13, 20, 21). The important lipids to be considered in this regard are cholesterol (free and esterified), the phospholipids, and the neutral fats (triglycerides) (16). Only 5 percent of the total plasma fatty acids are not esterified; these acids are bound to albumin and furnish a very rapidly metabolized source of lipid energy (7). The sum of all these components constitutes the "total lipids."

The methods for the determination of total lipids, far from ideal, are inaccurate and present technical difficulties. The various methods make use of direct weighing of the isolated lipids (22, 23), nephelom-
etry, or chromic acid oxidation of the isolated lipids according to the techinics of Bang and Bloor (2, 4, 5). The latter method is non-specific and inaccurate since many organic compounds, including any impurities accompanying the lipids, are capable of being oxidized by the chromic acid. It is also necessary to make a correction for cholesterol, which has a different oxidation factor.

The estimation of total fatty acids by the method of Stoddard and Drury offers no simplification of techinie (25). The separation and washing of only a few milligrams of fatty acid by filtration through a specially prepared Gooch crucible is particularly troublesome. Separating the liberated fatty acids by solvent partition obviates the tedious filtration, but several additional steps are introduced.

In the scheme of analysis proposed, the total fatty acids are determined as their esters, the total esterified fatty acids (T.E.F.A.) by means of the hydroxamic acid reaction. Originally introduced as a spot test by Feigl (8), this reaction has been applied by several investigators in the industrial and biologic fields to the quantitative determination of carboxylic acid esters (9, 11). The fatty acids in blood, which are present in the neutral fats or triglycerides, in the phospholipids, and in the cholesterol esters, are determined, since all these substances are in reality esters (3, 19, 24).

Study of the literature shows that the reagents and the techinie of performing the hydroxamic acid reaction recommended by the various authors, as well as the results obtained, vary considerably. In the course of our work we have made an extensive study of most of the variables that enter into this reaction. As a result we have been able to simplify the procedure so that optimal sensitivity and reproducibility result.

The phospholipids in serum are determined by precipitating them in conjunction with the proteins from 0.2 ml. of serum by means of 5% trichloroacetic acid. Following centrifugation, the supernatant fluid, which contains the serum inorganic phosphorus, is discarded. The protein-phospholipid precipitate is then digested and the phosphorus therein determined colorimetrically (27). Since phospholipids contain about 4 per cent by weight of phosphorus, multiplication of the numerical value of the phospholipid phosphorus by a factor of 25 gives the actual weight of the phospholipids present.

Both total and free cholesterol are determined. An improved modification of the Killani-Zlatkis-Zak procedure (14, 28), which was sug-
gested to us by study of the recent article by Leffler (15), is offered. No evaporation or special apparatus is required.

By use of the appropriate gravimetric factors, one can calculate the amount of fatty acid combined with the phospholipids and with the cholesterol esters. Subtracting this figure from that for the T.E.F.A. gives a measure of the quantity of neutral fat (triglycerides) present.

**Determination of T.E.F.A.**

**Reagents**

99% *Isopropyl Alcohol, Reagent Grade.* This reagent must be free of esters. Some grades of isopropanol are contaminated with esters. These can be removed by adding solid KOH and distilling.

3N *Methanolic Sodium Hydroxide.* Place 80 ml. of methanol in a 100-ml. glass-stoppered graduate, and add 20 ml. of a 50% solution of sodium hydroxide (Baker). Mix until solution is complete and check by titration against standard acid. The solution may be refrigerated, but will keep for at least 4 wk. at room temperature.

8% *Hydroxylamine Hydrochloride Solution.* Dissolve 8 gm. of hydroxylamine hydrochloride in 100 ml. of anhydrous reagent methanol. Slight warming may be necessary to effect solution.

*Alkaline Hydroxylamine Reagent.* Mix equal parts of 3N methanolic sodium hydroxide and 8% methanolic solution of hydroxylamine hydrochloride; then filter. The reagent is usable for 1 wk. if stored in a glass-stoppered Pyrex container.

3N *Hydrochloric Acid.* Dilute 250 ml. of concentrated HCl to 1 L. with distilled water. Check by titration against standard alkali and adjust.

5% *Ferric Perchlorate Stock Solution.* Dissolve 5 gm. of non-yellow ferric perchlorate† in 100 ml. of absolute ethyl or methyl alcohol.

*Ferric Perchlorate Reagent.* Place about 60 ml. of absolute methanol in a 100-ml. glass-stoppered graduate and add 10 ml. of 5% ferric perchlorate stock solution and 7 ml. of 70% perchloric acid. Then add absolute methyl alcohol to 100 ml. Two milliliters of this

*Diazo methane, used in the early phases of this work, was found unnecessary. It may be used, however, in conjunction with the hydroxamic acid reaction to quantitate the nonesterified fatty acids in serum.
†Obtainable from G. Frederick Smith Chemical Co., Columbus, Ohio.
reagent (acidity 0.85 ± 0.05N) when mixed with 1 ml. of the alkaline hydroxylamine reagent should require 0.3–0.4 ml. of 1N NaOH to neutralize the excess acidity. Methyl red or ammonium thiocyanate is used as the indicator.

**Blank Solution.** Place 4 ml. of water in a 100-ml. volumetric flask and fill to the mark with isopropanol.

**Olive Oil Standard.** First prepare a 200 mg.% w/v solution of pure, unadulterated Olive Oil by accurately weighing out 200 mg. of Olive Oil in a counterbalanced 10-ml. beaker. Add 7 ml. of warm isopropyl alcohol and transfer quantitatively to a 100-ml. volumetric flask by means of repeated rinses with the solvent. Make up to the mark with 99% isopropanol. The stability of this standard is 1 mo. under refrigeration. To prepare the actual working standard which contains 0.16 mg. of Olive Oil glycerides per milliliter, place 8 ml. of the 0.2% solution in a dry 100-ml. volumetric flask, add 4 ml. of distilled water, and then dilute to the mark with 99% isopropanol. This solution is stable for 1 wk. at room temperature, for longer periods under refrigeration. Warm the chilled standard to room temperature before use.

**Procedure**

Place 20 ml. of isopropanol into a perfectly dry 25-ml. volumetric flask and add 1 ml. of serum slowly, drop by drop, with constant shaking. Place the flask in a 56°C water bath for 10 min., remove and allow to cool to room temperature. Then dilute to the 25-ml. mark with isopropanol and mix well.

A very fine protein precipitate results, and the lipids are completely dissolved. Transfer about 18 ml. to a 15 × 150 mm. test tube, insert a stopper, and centrifuge. Transfer 5 ml. of the clear supernatant fluid to a glass-stoppered (Pyrex No. 99650 or 98550) test tube, and add 1 ml. of alkaline hydroxylamine reagent. Insert the stopper and mix. Place in a 37°C water bath for 15 min., and then add 2 ml. of ferric perchlorate reagent, and mix. Allow to stand 10 min. and determine the optical density at 520 mμ against a reagent blank. The latter consists of 5 ml. of the blank solution plus 1 ml. of alkaline hydroxylamine reagent, treated like the test solution.

The standard is developed by treating 5 ml. of the Olive Oil working standard (1 ml. equalling 0.16 mg.) in exactly the same manner as the unknown. Since the standard contains 0.8 mg. of esters and is
compared with 0.2 ml. of serum, it represents a concentration of 400 mg. of T.E.F.A. per 100 ml. of serum. Thus,

\[
\text{Optical density of unknown} \times 400 = \text{milligrams T.E.F.A./100 ml. serum.}
\]

**Determination of Cholesterol**

**Reagents**

1% *Digitonin Solution.* 1 gm. of digitonin in 50 ml. of ethanol (95% U.S.P.), and add 50 ml. of distilled water.

*Cholesterol Stock Standard (1 mg./ml.).* Dissolve 10 gm. of Pfanstiel cholesterol in 100 ml. of boiling absolute ethanol. Cool the solution with a mixture of ice cubes and water until crystallization is complete. Collect the crystals on a Hirsch filter and discard the filtrate. Recrystallize two more times, but use only enough hot ethanol to just dissolve the cholesterol. Dry the purified sterol in a vacuum desiccator to constant weight. Dissolve 100 mg. in a sufficient quantity of isopropanol to make 100 ml.

*Cholesterol Working Standard.* Transfer 8 ml. of stock standard to a 100 ml. volumetric flask, add 4 ml. of water, and dilute to the mark with isopropanol. One milliliter contains 80 µg. of cholesterol and is equivalent to 200 mg. of cholesterol per 100 ml. of serum.

*Acetate Buffer, pH 5.5.* This consists of 50 ml. of 50% w/v solution of sodium acetate, and 2 ml. of glacial acetic acid.

50% w/v *Solution of Sodium Acetate.* Dissolve 50 gm. of sodium acetate in water and dilute to 100 ml.

0.5% *Sodium Metasilicate Solution.* Dissolve 0.5 gm. Na$_2$SiO$_3$·9H$_2$O in 100 ml. of distilled water.

*Acetone, Reagent Grade.*

*Stock Ferric Ammonium Sulfate Solution.* Dissolve 10 gm. of ferric ammonium sulfate in 100 ml. of distilled water (1 ml. equaling 11.6 mg. of Fe$^{+++}$).

*Cholesterol Color Reagent.* Mix 50 ml. concentrated H$_2$SO$_4$ and 50 ml. glacial CH$_3$COOH by slowly pouring the H$_2$SO$_4$ into the CH$_3$COOH. Cool and then add 0.5 ml. of stock ferric ammonium sulfate solution. This reagent contains approximately 50 µg. of ferric iron per milliliter.

**Procedure**

The 1:25 isopropanol extract of serum used for the hydroxamic acid reaction also serves for the determination of cholesterol (15).
Free Cholesterol

Place 2 ml. of the isopropanol extract in a glass-stoppered heavy-duty 12-ml. centrifuge tube, and add 1 ml. of 1% digitonin solution and 1 drop of Acetate Buffer (pH 5.5). Mix by stirring with a platinum wire whose free end is bent into the form of a hook and whose other end is fused into a short piece of glass rod. (Rotation of this device between the fingers is very effective in stirring.) Then place the centrifuge tube in ice-cold water (stored in the refrigerator) for 30 min. Then centrifuge at 1500 rpm for 5 min. Discard the supernatant fluid and drain the remainder, being careful not to lose any of the precipitate. Wash down the sides of the tube and the precipitate with 2.5 ml. of acetone. Stir the precipitate thoroughly by rapidly rotating the platinum wire back and forth. Then add 1 drop of 0.5% sodium metasilicate solution and centrifuge again. Discard the supernatant and drain the remainder completely.

Add 1 ml. of glacial acetic acid to the precipitate and stir with the platinum wire until dispersion is complete. Add 4 ml. of color reagent, insert a glass stopper, mix thoroughly by inverting the tube 10 times. Then place in a 56° water bath for 7 min. Remove and cool the tubes in cold water (stored in the refrigerator) for 5 min. Measure the optical density at 560 m\(\mu\) against a reagent blank prepared by mixing 4 ml. of color reagent with 1 ml. of glacial acetic acid.

Standard

Place 0.5 ml. of cholesterol working standard in a glass-stoppered centrifuge tube, and add 1.5 ml. of isopropanol, 1 drop of Acetate Buffer, and 1 ml. of 1% digitonin. Allow precipitation to occur, then proceed as with the unknowns.

Total Cholesterol

Add 4 ml. of cholesterol color reagent to a glass-stoppered test tube and overlay with 1 ml. of the 1:25 isopropanol extract of serum. Insert a glass stopper and mix by inverting the tube 10 times. Place in a 56° water bath for 7 min., remove and cool at least 5 min. in cold water. After cooling the color is extremely stable, even at room temperature. Measure the optical density at 560 m\(\mu\) against a reagent blank prepared by mixing 4 ml. of Color Reagent with 1 ml. of isopropanol and then heating in the water bath at 56° along with the unknowns.
One milliliter of working standard is also treated in exactly the same manner as the unknowns.

Calculations

$$\text{Free cholesterol} = \frac{U}{S} \times 50 = \text{mg. free cholesterol per 100 ml. of serum},$$

$$\text{Total cholesterol} = \frac{U}{S} \times 200 = \text{mg. total cholesterol per 100 ml. of serum},$$

where $U$ is the optical density of the unknown and $S$ the optical density of the standard.

**Determination of Phospholipids**

**Reagents**

_Digestion Mixture._ Mix 50 ml. of distilled water, 25 ml. of concentrated sulfuric acid, and 25 ml. of 70% perchloric acid.

_2.5% Aqueous Ammonium Molybdate Solution._

_Elon Solution._ Dissolve 1 gm. of Elon (p-methylaminophenol sulfate, Eastman Kodak No. P615) in 100 ml. of a 3% solution of sodium bisulfite (NaHSO₃)

_Trichloroacetic Acid, in 100%, 10%, and 5% w/v Concentrations._

_(50%) w/v Solution of Sodium Acetate._

**Phosphorus Standard (1 mg./ml. of P)**

Dissolve 4.394 gm. of dried potassium dihydrogen phosphate (KH₂PO₄) in sufficient water to make 1 L. (Acidify with 2 ml. of concentrated sulfuric acid as a preservative before diluting to the mark.)

**Working Standard**

Dilute 1 ml. of the phosphorus standard to 250 ml. with distilled water (1 ml. = 4 μg. of P). Measure 0.2 ml. of serum into a 16 × 150-mm. glass-stoppered Pyrex test tube having a graduation mark at 10 ml. Add 5 ml. of 5% solution of trichloroacetic acid, drop by drop, mix by lateral shaking, and then centrifuge for several minutes until the precipitate is tightly packed. Decant and discard the supernatant fluid and invert the tube over filter paper until almost all the droplets of fluid adhering to the walls of the tube have drained away. Then add 1 ml. of digestion mixture and a small glass bead to the protein-phospholipid precipitate and heat until a colorless (or very faint yellow) digest is obtained. About 1/2 hr. is usually
sufficient. We use a small cast-iron skillet 8 in. in diameter, half filled with beach sand and heated on an electric hot plate. The tubes are slanted at an angle of about 30°, supported by the rim, in this procedure. After digestion is complete, allow the tubes to cool for 1–2 min., cautiously add 1 ml. of distilled water, and heat to boiling for 15 sec. to convert any pyrophosphoric acid into orthophosphoric acid. Add 1 ml. of a 50% solution of sodium acetate and distilled water up to the 10 ml. mark. Then add 1 ml. of 2.5% solution of ammonium molybdate. Mix and add 1 ml. of Elon reagent and mix again thoroughly. Allow to stand for 15 min., then measure the optical density at 700 mμ against a reagent blank prepared by mixing 0.25 ml. of concentrated sulfuric acid, 1 ml. of 50% solution of sodium acetate, 1 ml. of 2.5% solution of ammonium molybdate, 1 ml. of Elon reagent, and 8.75 ml. of distilled water.

The standard contains 20 μg. of phosphorus (5 ml. of dilute standard), 0.25 ml. of concentrated sulfuric acid, and 1 ml. of 50% solution of sodium acetate and is treated in exactly the same manner as the unknown. This technic yields a color that possesses remarkable stability for several hours. The blank remains permanently colorless and water-clear unless it is contaminated with a trace of phosphorus.

**Calculation**

The standard contains 20 μg. of phosphorus and is compared with the phospholipid-phosphorus present in 0.2 ml. of serum.

\[
\frac{U}{S} \times \frac{20}{1000} \times 500 \times 25 = \frac{U}{S} \times 250 = \text{mg. phospholipid per 100 ml. of serum},
\]

where \( U \) is optical density of the unknown and \( S \) is optical density of the standard. The factor 25 in the equation reflects the fact that phosphorus constitutes about 4 per cent of the average weight of phospholipids.

**Calculations**

Examination of Table 1 shows that equivalent molar quantities of the various lipid esters yield approximately the same amount of colored ferric hydroxamate. By expressing values for the three classes of lipids (phospholipids, cholesterol esters, and triglycerides) in milliequivalents per liter of fatty acids, the milliequivalents per
Table 1. Molar Absorptivities* Wavelength of 520 mµ of Ferric Hydroxamates Formed from Different Fatty Acid Esters

<table>
<thead>
<tr>
<th>Substance</th>
<th>Molecular wt.</th>
<th>Functional groups</th>
<th>Molar absorptivity</th>
<th>Equivalent absorptivity per ester group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl oleate</td>
<td>296.5</td>
<td>1</td>
<td>820 ± 17</td>
<td>820</td>
</tr>
<tr>
<td>Methyl linoleate</td>
<td>292.4</td>
<td>1</td>
<td>814 ± 19</td>
<td>814</td>
</tr>
<tr>
<td>Methyl palmitate</td>
<td>270.5</td>
<td>1</td>
<td>840 ± 13</td>
<td>840</td>
</tr>
<tr>
<td>Cholesterol acetate</td>
<td>428</td>
<td>1</td>
<td>882 ± 29</td>
<td>882</td>
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<tr>
<td>Cholesterol oleate</td>
<td>650</td>
<td>1</td>
<td>810 ± 25</td>
<td>810</td>
</tr>
<tr>
<td>Cholesterol palmitate</td>
<td>824</td>
<td>1</td>
<td>843 ± 39</td>
<td>843</td>
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<tr>
<td>Lecithin</td>
<td>783</td>
<td>2</td>
<td>1615 ± 45</td>
<td>807</td>
</tr>
<tr>
<td>Tripalmitin</td>
<td>807.35</td>
<td>3</td>
<td>2445 ± 71</td>
<td>815</td>
</tr>
<tr>
<td>Olive oil</td>
<td>Av. approx. 880</td>
<td>3</td>
<td>2460 ± 58</td>
<td>820</td>
</tr>
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</table>
| *Molar absorptivity is the absorbancy (optical density) of a molar solution for a 1-cm. light path: Molar absorptivity = Optical density gm. per liter mol. wt. × light path (cm.)
| Mazola oil            | Av. 869       | 3                 | 2400 ± 47          | 800                                     |
|                       |               |                   |                    |                                         |

*Molar absorptivity divided by number of ester groups.

The number of milliequivalents per liter of the fatty acids of the olive oil standard used in determining the T.E.F.A. is calculated as follows:

\[
\frac{10W \times (3)(276)}{866} = \frac{W}{28.87} \text{ mEq./L. of olive oil fatty acids,}
\]

where \(W\) is the concentration of T.E.F.A. per 100 ml. of blood serum expressed as milligrams of olive oil and \(\frac{3(276)}{866}\) is the gravimetric factor for converting \(W\) milligrams of olive oil as triglyceride per 100 ml. into the corresponding weight of fatty acids. The factor 10...
converts this to milligrams per liter. Dividing this value by 276, the milliequivalent weight, converts it into milliequivalents per liter.

Olive oil is a mixture of triglycerides containing (10) Oleic Acid (84 per cent), linoleic acid (4.5 per cent), palmitic acid (8 per cent), stearic acid (2 per cent), and arachidic acid (0.1 per cent). Multiplying the molecular weight of each component by the percentage composition and adding these products gives an average value of 276 for the molecular weight of the fatty acids in olive oil. Since each triglyceride molecule contains three fatty acids and a glycerine moiety, the average molecular weight of each olive oil molecule would be 866.

The number of milliequivalents of the phospholipids (P.P.L.) in serum is calculated as follows:

$$\frac{10 \cdot P \cdot (2 \cdot 277)}{793} = \frac{P}{39.65} = \text{mEq./L. of P.P.L. fatty acids},$$

where $P$ is milligrams per 100 ml. of P.P.L. in blood serum. Phospholipids contain two esterified fatty acids having an average molecular weight 277 per molecule. The value 793 represents the molecular weight of lecithin (26).

The calculation of the milliequivalents per liter of the cholesterol esters is somewhat different, owing to the fact that these substances are really determined as free cholesterol since the standard used in their quantitation is the free alcohol and not the ester. One millimole of cholesterol of molecular weight 386 combines with 1 mM of fatty acid of average molecular weight 277 to form cholesterol ester. Therefore

$$\frac{10 \cdot H}{386} = \frac{H}{38.6} = \text{mEq./L. of cholesterol ester fatty acid},$$

where $H$ is milligrams per 100 ml. of Cholesterol Ester (expressed as free cholesterol).

The triglycerides in serum are calculated as follows:

$$\frac{W}{28.87} - \left( \frac{P}{39.65} + \frac{H}{38.6} \right) = T = \text{mEq./L. of triglycerides},$$

where $W$ is milligrams of T.E.F.A. per 100 ml. of serum, $P$ milligrams of phospholipids per 100 ml. of serum, and $H$ milligrams of
Cholesterol Ester (as free cholesterol) per 100 ml. of serum. This formula may be simplified to

\[
T = \frac{1.04W}{30} - \frac{P + 1.04H}{40} = \text{mEq./L. of triglycerides.}
\]

28.87 \( T = 30 - T \) = mg. of triglycerides per 100 ml. of serum

Hence the total lipids in serum are calculated as the sum of the values of the triglycerides, the total cholesterol, and the phospholipids, all expressed in milligrams per 100 ml.

Table 2 shows the results of the analyses of 12 random sera by

<table>
<thead>
<tr>
<th>No.</th>
<th>T.E.F.A.*</th>
<th>Phospholipids*</th>
<th>Cholesterol esters*</th>
<th>Total cholesterol</th>
<th>Triglycerides*</th>
<th>Total lipids</th>
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<td>134</td>
<td>220</td>
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<td>11.7</td>
<td>7.6</td>
<td></td>
<td>20.5</td>
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</table>

*First (upper) of each pair of figures is in milligrams per 100 ml.; the other in milliequivalents per liter.
†Sera 36 and 37 were drawn 2 hr. after eating. The donor of Serum 36 had ingested 2 eggs and 1 oz. of Mazola oil.
The other sera including Serum 39 were taken from fasting subjects.
‡CS-1 and CS-2 are different sera concentrated by evaporation overnight of some of the water at room temperature; 50 mEq./L. of Mazola oil were added to CS-1.
the methods herein proposed. One can see that a wide range of variation of all of the lipid components of serum can be readily determined.

Discussion

T.E.F.A. Method

Our attention was called to the use of isopropanol as a lipid solvent in an article published by H. H. Leffler (15) in which he proposed the use of isopropanol as an extraction solvent for cholesterol. Simple in-vitro experiments with isopropanol demonstrate that it is also an excellent solvent for olive oil, Mazola (corn) oil, tripalmitin, and vegetable lecithin, as well as cholesterol olate and cholesterol palmitate. To check further the solubility of phospholipids in isopropanol, we have analyzed several sera in replicate, using the proposed direct method (18, 27), a 1:25 Bloor's extract, and a 1:25 isopropanol extract, and have found that the phospholipid values agree very closely. These comparison studies are shown in Table 3. Bloor's ethanol-ether extract of serum may be substituted for the isopropanol extract of serum with very little modification of these tests. However, Bloor's solvent suffers from the disadvantages resulting from the volatility and high vapor pressure of ether.

Carboxylic acid esters react with hydroxylamine in alkaline solution to form hydroxamic acids, which produce a red-to-violet color with ferric salts in weak acid solution (9, 11).

\[
R-C-OEt + NH_2OH \xrightarrow{NaOH} R-C-NHOH + EtOH
\]

\[
R-C-N-O-H + Fe^{+++} \xrightarrow{\text{O} \rightarrow \text{Fe}^{3+}} R-C-N-O + H^+
\]

Since 95 per cent of the fatty acids in blood serum is in esterified form, this reaction offers a simple, accurate method for the determination of these lipid components. Only 5 per cent escape detection. The reaction occurs in two stages. During the first stage the hydroxamic acid is formed under strongly alkaline conditions, while the colored ferric hydroxamate is developed during the second stage under mildly acidic conditions. The following variables influence the reaction.

1. Water Concentration. The presence of more than 5 per cent of water in the first stage greatly decreases the sensitivity of the
Table 3. Solubility of Serum Phospholipids in Bloor's Solvent* and in Isopropanol†

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Isopropanol extract (mg./100 ml.)</th>
<th>Bloor's extract (mg./100 ml.)</th>
<th>Modified Zilversmit (mg./100 ml.)</th>
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*Ethanol 3 parts, ether 1 part.
†Reference values were obtained by means of the modified Zilversmit method described in the text. Conventional 1:25 serum-solvent extracts were prepared. Six 5-ml. replicates of each respective extract were evaporated to dryness at room temperature using the apparatus described by Leffler (15). The residues were then digested with the sulfuric-perchloric acid digestion mixture described in the text and the various phospholipid values determined. Results are expressed as milligrams of phospholipids per 100 ml. of serum.

reaction. Four per cent water is added to the standard and to the blank to compensate for that present in the serum extract.

2. Concentration of Sodium Hydroxide and of Hydroxylamine Hydrochloride. It has been found as a result of numerous trials that our recommended proportions and concentrations of these reagents yield the highest molar absorptivities in isopropanol. Methanol is a much better solvent for both sodium hydroxide and hydroxylamine than ethanol. Methanolic sodium hydroxide does not become discolored; it is quite stable.

3. Reaction Temperature and Reaction Time. The optimal reaction temperature and reaction time have been found to be 15 min. at 37°. These conditions give an average molar absorptivity of 820 for methyl oleate, which differs by only 15 per cent from that obtained by Goddu et al. (9) under much more drastic conditions (reaction mixture refluxed for 5 min.).

4. Concentration of Ferric Perchlorate and Perchloric Acid in the Color Reagent. A considerable excess of ferrie salt must be present since hydroxylamine reduces ferric iron rather quickly but the ferric hydroxamate resists reduction. The color is stable for at least ½ hr., but the blank becomes decolorized within 5 min. When 2 ml. of the ferric perchlorate reagent are mixed with 1 ml. of the alkaline hydroxylamine reagent, the excess acidity should require 0.3–0.4 ml. of N NaOH for neutralization. The presence of too much perchloric acid decreases the intensity of the color and results in a great loss of sensitivity.
The question naturally arises: Do equivalent quantities of all esters, when converted to hydroxamic acids under the same conditions, produce the same amount of color with ferric iron? The answer: Not exactly, but very nearly. We have determined the molar absorptivities of the ferric hydroxamates of the methyl esters of several different fatty acids and of the triglyceride tripalmitin, of cholesteryl acetate, cholesteryl oleate, and lecithin, and of Olive Oil and Mazola oil. The results are shown in Table 1.

It will be noted that the molar absorptivities of the diester lecithin and of the triester tripalmitin are approximately two and three times, respectively, those of the monoesters. This finding, also reported by Goddu et al. (9), is as it should be, since two or three reactive ester groups, as the case may be, are present.

The slight differences in reactivity of the various esters that occur in blood do not constitute an objection to the quantitative use of the method, since all the esters contribute some characteristic component share to the final result (optical density). Each contribution need not be absolutely proportional in a rigorous mathematical sense since it is only necessary to establish a range of normal values for the fatty acid esters in blood determined by the hydroxamic acid reaction. Any abnormal increase or decrease will thus be readily revealed or detected. Whereas it would be possible (but expensive) to prepare a composite standard consisting of a mixture of the various esterified lipid components in the proportions in which they occur normally in blood, we feel that the use of a semiautomatic standard such as olive oil or Mazola oil is justified and convenient. Olive oil (10) consists of triolein (84 per cent) and other glycerides (15 per cent), while 98.6 per cent of Mazola oil consists of glycerides—12.6 per cent saturated fatty acids, chiefly palmitic (10 per cent) and 86 per cent saturated fatty acids consisting of linoleic—56 per cent—and oleic—30 per cent (1). The average molecular weight of the fatty acids in olive oil is very close to the accepted average molecular weight of fatty acids in serum (276 for olive oil vs. 277 for serum) (17).

**Cholesterol Method**

The original cholesterol method of Kiliani-Zlatkis-Zak (14, 28) (KZZ) has been modified so that a single color reagent is used, making possible development of the color under controlled temperature. It has also been found unnecessary to evaporate any of
the serum extracts. In the original KZZ method, the serum extract was evaporated to dryness and the residue was then dissolved in 3 ml. of glacial acetic acid containing ferric chloride. Then 2 ml. of concentrated sulfuric acid was added so as to form a separate lower layer. When these two acids were mixed by inversion, a considerable amount of heat was developed and the color was produced. When this technic was applied to the analysis of replicates, it was found that very poor agreement was obtained. It was also noticed that the shades of color obtained during the summer were more brownish than those obtained during the winter. These results were interpreted to mean that more rigid temperature control than that obtained by haphazard mixing is necessary for the accurate reproducibility of this reaction. The ferric iron concentration has also been reduced to approximately 50 μg./ml. This was done in order to make the reaction more specific for cholesterol and also to moderate the reaction rate for the cholesterol in the standard (in pure solvent). It appears that the phospholipids in the serum extract slow the reaction rate to some extent, thus allowing the cholesterol in the standard to react at a slightly faster rate. The use of this minute amount of iron also gives superposable absorption curves (Fig. 1)

![Spectral absorption curves for color complex produced by treating (A) 1 ml. of a 1:25 isopropanol extract of serum and (B) 80 μg. of pure cholesterol in 1 ml. of 96% isopropanol with 4 ml. of cholesterol color reagent under the conditions described in the text.](image)
over the wave length range 380–625 m\(\mu\) for the cholesterol in the serum extract and the cholesterol in the standard. This shows that this proposed reaction is quite specific for cholesterol under these conditions.

In the precipitation of the cholesterol digitonide, the addition of buffered sodium acetate and the chilling of the mixture result in a well-flocculated quantitative precipitation in less than 30 min. The addition of 1 drop of 0.5% sodium metasilicate solution to the acetone wash solution binds the precipitate together and prevents any loss when the acetone is decanted following centrifugation. We have made comparison studies of our proposed method with the Carr-Drekter method (6, 12). These results are shown in Table 4.

**Phospholipid Method**

The advantages of the proposed method for the determination of phospholipids are: (1) The analysis is done directly on serum, and

### Table 4. Cholesterol Comparison Studies

<table>
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<th>Serum No.</th>
<th>Proposed method, single color reagent (mg./100 ml.)</th>
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*Carr-Drekter (6).
thus the evaporation of a serum extract is avoided. (2) The phospholipids are precipitated with the proteins, thus separating the phospholipid phosphorus from the inorganic phosphorus salts. (3) The proposed digestion reagent digests very smoothly and gently and need not be watched continually. There are no explosive tendencies. (4) The use of 50% sodium acetate solution results in the production of an extremely stable color by means of control of the pH. The color is stable for several hours. (5) The improvised sand bath (iron skillet half filled with beach sand) used to digest the protein precipitates is a convenient innovation.

References
2. Bang, I., Biochcm. Z. 91, 86 (1918); Biochem. Z. 91, 235 (1918).