Micro Methods for the Determination of Chylomycin Counts, Fatty Esters, Lipid Phosphorus, and Cholesterol in Blood Serum

Mary M. Clayton,* Patricia A. Adams, Gisela B. Mahoney, Shirley W. Randall, and Eleanore T. Schwartz

In the fall of 1953, a study was begun at the Maine Agricultural Experiment Station on the utilization of fats by normal and over-weight women subjects. Since this study necessitated the taking of a fasting blood sample and 4 more hourly samples following a test breakfast, it seemed desirable to use micro methods which would permit the use of finger tip blood. From published macro methods, micro procedures were developed for the determination of chylomycin counts, fatty esters, lipid phosphorus, and cholesterol. These methods made it possible to determine chylomycin counts and one of the other lipid constituents on approximately 100 μl of blood serum.

Because of the present great interest in lipid studies these methods are given in detail for the use of other workers who may wish to use micro technics.

In general, the technics used were those of Bessey and Lowry (1). These were described in detail in the Techniques Bulletin (2), a publication of the Northeastern Regional Nutritional Status Project. Certain special technics were developed in connection with each of the micro methods; these will be described with the individual methods.

*Present address: 87 Park Street, Essex Junction, Vt.

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MICRO METHOD FOR MAKING CHYLOMICRON COUNTS IN BLOOD SERUM

Chylomicron counts were determined by a modification of the method of Becker, Meyer, and Necheles (3).

EQUIPMENT

1. Microscope with dark field illumination, 20 X eyepieces, and a micrometer disc with 49 squares. (A. H. Thomas Co., Disc No. 6838.)
2. Micro slides, dark field.
3. Micro cover glasses, squares, No. 1, 22 mm.
4. Micro slide drying pads, S & S No. 556-N.
5. Lens tissue.
6. Iron wire, 36 gauge, with loop on one end for mixing serum. Several thicknesses of wire make handle.
7. Alcohol lamp.
8. Melting point capillary tubes, 100 mm. long, 1.5-2 mm. outside diameter, open at both ends.
10. Lang-Levy constriction pipette, approximately 5 µl.; may be uncalibrated.
11. Lang-Levy constriction pipettes, 5, 10, and 20 µl., calibrated.
12. Serological tubes, 6 \times 50 \text{ mm.}, and rubber stoppers. (Small ends of sleeve stoppers which are cut in half crosswise. A. H. Thomas Co., No. 2319-B.)

REAGENTS

1. Absolute ethyl alcohol.
2. Absolute ether.
3. Xylene.
4. Pyseal cement (Fisher).
5. Immersion oil.

PROCEDURE

1. Collect 2 full melting point tubes of the fasting blood sample. This amount will provide enough serum for the fasting count and for dilution of samples taken following a test meal. Two-thirds of a tube of blood is sufficient for the count only.
2. Seal both ends of tubes with Pyseal cement. Soften cement in flame of an alcohol lamp.
3. Allow clot to form. (Let tube stand about 20 minutes.)
4. Centrifuge 3 minutes at about 2000 rpm, using multiple carriers.
5. Scratch tubes with diamond point pencil about one-half inch above serum and also at interface of serum and red cells. Break tube at these points.

6. Mix serum by running the fine wire with a loop on the end into tube about 10 times. Hold tube horizontally during the mixing.

7. Transfer approximately 5 μl of the serum to a clean dry slide using a constriction pipette. Immediately put a clean, dry cover slip in place.

8. Press down cover slip. Cover with piece of lens paper and roll finger across the cover slip 4 times. This absorbs the excess serum. Do not touch cover slip with bare finger.

9. Put a drop of immersion oil on the dark field condenser. Examine slide first with low power and then with oil immersion objective (97 X).

10. Do not change the focus while making a count. Since the chylomicrons show Brownian movement, the count must be made rapidly. Include all of those definitely visible. Count chylomicrons in all 49 squares of the micrometer disc. If there are too many to count, make dilutions according to the directions given below. Count 3 fields on each of 2 slides for each sample. If the variation between 2 counts on a single slide is greater than 15 per cent, prepare a new slide. To clean the micrometer disc dust lightly with a camel’s hair brush.

DILUTIONS

1. Make necessary dilutions of high count serum with fasting serum, which ordinarily has a low count.

2. Measure 10 or 20 μl of the fasting serum into serological tubes and cap with rubber stoppers. It is possible to anticipate the need and to measure these aliquots before counting the chylomicrons in the fasting sample. This saves time. The fasting serum aliquots may be kept as long as 4 hours at room temperature.

3. Measure 5 μl of the serum to be diluted and add to the appropriate tube containing fasting serum. Mix thoroughly by tapping with the finger.

CLEANING SLIDES AND COVER SLIPS

CALCULATIONS

1. Average the 6 counts (2 slides).
2. If the serum has been diluted with fasting serum, correct the count for the number of chylomicrons in the diluting serum. Then multiply the corrected count by the dilution factor. For example:

Count on fasting serum: 3
Count on diluted serum: 33
Volume of high count serum before dilution: 5.0 μl.
Volume of fasting serum used as diluent: 20.0 μl.
Count on diluted serum due to fasting serum:
\[
\frac{20.0}{20.0 + 5.0} \times 3 = 2.4
\]
Corrected count on diluted serum: \(33 - 2.4 = 30.6\)

Dilution factor: \(\frac{20.0 + 5.0}{5.0} = 5\)

Count for undiluted, high-count serum:
\(30.6 \times 5.0 = 153\)

MICRO METHOD FOR THE DETERMINATION OF FATTY ESTERS IN BLOOD SERUM

The micro method for determining fatty esters is an adaptation of the methods of Hill (4, 5) and Bauer and Hirsch (6). The method involves the complete extraction of the fatty esters from a measured amount of blood serum with an alcohol-ether mixture, the formation of hydroxamic acid from the fatty esters by the use of hydroxylamine hydrochloride in an alkaline medium, and the formation of a lavender-colored complex of ferric hydroxamate on the addition of an acidified solution of alcoholic ferric perchlorate.

EQUIPMENT

1. Beckman spectrophotometer, model DU, with 1 cm. Corex glass cells.
2. Constant temperature water bath with rack to hold 600 ml. beakers. Electric stirrer in one corner.
3. Capillary tubes, made from Pyrex glass tubing, 10 cm. long and 2 to 2.5 mm. inside diameter. Open at both ends.
4. Cello-Grease (Fisher).
5. Rubber stoppers. (Small ends of sleeve stoppers. A. H. Thomas Co., No. 2319-B.)
6. Iron wire, 36 gauge, with loop on one end to mix serum. Several thicknesses of wire make handle.
7. Diamond point pencil.
8. Adhesive tape.
9. Extraction tubes, 10 cm. long, and 4 mm. inside diameter, made from Pyrex glass tubing. Tubing is cut into 10 cm. lengths, cleaned, and sealed in a blast lamp flame.
10. Widemouthed Erlenmeyer flasks, 250 ml. Widemouthed flasks are needed to permit evaporation of the ether used in the test.
11. Beakers, 600 ml.
12. Lang-Levy constriction pipette, 75 μl., with tip approximately 10 cm. long and short bend at end, calibrated.
15. Transfer pipettes, 1, 2, and 3 ml., certified.

REAGENTS
1. Sodium hydroxide: 2.5% in 95% ethanol. Store in glass-stoppered bottle in refrigerator. Make fresh every 2 weeks.
2. Hydroxylamine hydrochloride: 2.5 Gm. HONH₂HCl (reagent grade) made to 100 ml. with 95% ethanol. Store in glass-stoppered bottle in refrigerator. Make fresh every 2 weeks.
3. Stock reagent A: Dissolve 1.936 Gm. FeCl₃·6H₂O in 20 ml. HNO₃ (Sp. Gr. 1.2) in a 200 ml. beaker. The HNO₃ (Sp. Gr. 1.2) is made by adding 33 ml. concentrated HNO₃ (Sp. Gr. 1.35) to 67 ml. distilled H₂O. Add 20 ml. 70% HClO₄ and heat on hot plate until white fumes are given off. Volume will decrease to about one-half. Cool. Add 50 ml. distilled H₂O. Transfer to a 100 ml. volumetric flask. Rinse beaker several times with small portions of 70% HClO₄. Make to volume with 70% HClO₄. Store in glass-stoppered bottle in refrigerator. Will keep indefinitely.
4. Dilute reagent A: Pipette 2 ml. stock reagent A into a 200 ml. volumetric flask. Add 0.2 ml. concentrated HNO₃ and make to volume with 95% ethanol. Make fresh daily.
5. Ethyl ether (reagent grade), anhydrous, redistilled. It is neces-
sary to redistill the ether at 34.5° to secure low blanks. (Blanks should give optical density readings of .050 or less.)

6. Alcohol-ether mixture: Three volumes 95% ethanol and 1 volume redistilled anhydrous ethyl ether (see 5). Make up fresh daily.

7. Standard: 20 mg. C.P. tripalmitin made to 100 ml. with redistilled anhydrous ether (see 5). (1 ml. = 0.2 mg.) Make fresh before use.

COLLECTION OF BLOOD SAMPLES

1. Collect two-thirds capillary tube of blood.

2. Grease lower end (clean end) of tube with Cello-Grease and stopper with the small end of a rubber sleeve stopper. Stopper upper end of tube but use no grease. (Stoppers are put over the outside of the tubes.)

3. Tape on bottom stopper with adhesive tape.

4. Allow clot to form (15 minutes).

5. Centrifuge 3 minutes at 3000 rpm.

6. Remove top stopper.

7. Scratch tube with diamond point pencil at interface of serum and red cells. Break tube at this point.

8. Mix serum by inserting iron wire with loop on end 15 times.

EXTRACTION OF LIPIDS FROM BLOOD SERUM

1. Pipette 75 μl. serum into an extraction tube. Add 200 μl. alcohol-ether mixture. When doing a series of blood samples, clean the 75 μl. pipette between each sample. If desired, the serum aliquots may be stored at −30° prior to analysis.

2. Mix 20 seconds, using the Handee grinder. Heat in a constant temperature water bath at 50° for 2 minutes. Mix 40 seconds, cover with paraflim, and centrifuge for 2 minutes. Transfer extract to a 250 ml. widemouthed Erlenmeyer flask with a 200 μl. constriction pipette with long, straight tip. Use a different pipette for each sample. These pipettes need not be cleaned between extractions, since they are used only for extracts from the same sample.

3. Add 200 μl. alcohol-ether mixture to the precipitate in the extraction tube and repeat step 2, placing extract in the same flask as previously. This extraction procedure should be carried out 3 times to extract the lipids completely. Discard tubes after using.

PROCEDURE

1. Begin preparation of 3 blanks and 3 standards (2 ml. = 0.4
mg.) by adding 600 μl. alcohol-ether mixture to widemouthed Erlenmeyer flasks.

2. Add 3 ml. anhydrous redistilled ether to blanks and samples, and 1 ml. to standards.

3. Evaporate samples, blanks, and standards to dryness in a constant temperature water bath set at 60°. Place flasks in 600 ml. beakers containing 250 ml. water at 60°. The beakers stand in water on the rack of the water bath. The water in the bath should come slightly above the level of that in the beakers and should be stirred well. Three or 4 minutes' evaporation should be sufficient. Rotate flasks as ether evaporates.

4. Add approximately 25 ml. anhydrous redistilled ether to each flask, using a pipette with enlarged tip and rubber bulb.

5. Add 200 μl. hydroxylamine solution. Swirl to mix.


7. Evaporate to dryness in beakers, as in step 3. Heat 1 minute after flasks appear dry.

8. Cool on a wire rack over a water bath at 25°.

9. Add 10 ml. dilute reagent A, permitting the reagent to wash down sides of flask. Swirl to dissolve salts. Replace on rack over water bath. Let stand 20 minutes.

10. Read optical density in Beckman spectrophotometer at 500 mμ, using a 0.02 mm. slit width. Rinse Beckman cells with 95% ethanol and suck dry between samples. 95% ethanol is used for the reference solution for reading blanks, standards, and samples and for making cell corrections.

STANDARD CURVE

1. Pipette 1, 2, and 3 ml. standard tripalmitin solution into 250 ml. widemouthed Erlenmeyer flasks.

2. Add enough anhydrous redistilled ether to first 2 standards to make all volumes 3 ml.

3. Carry standards and blanks through the above procedure starting at step 3.

4. Subtract cell correction and average of blank readings from each of the standard readings and plot against tripalmitin concentration. Repeat standard determinations several times and use averages for final curve. In routine work, only the 2 ml. (0.4 mg.) standard is run along with the unknowns and blanks. A standard is necessary as a check on the color development in the test samples.
CALCULATIONS

1. Subtract cell corrections and average blank reading from density readings of unknown samples.

2. Multiply mg. tripalmitin from curve reading of sample by appropriate factor to obtain mg. per 100 ml. of fatty ester in blood serum (expressed as tripalmitin).

CLEANING OF GLASS TUBES AND RUBBER STOPPERS

The following methods were used. Place the 10 cm. lengths of open glass tubing in a beaker tall enough that a watch glass may be placed over the top. Cover the tubes with 50% c.p. nitric acid and heat to boiling on a hot plate. Cool and drain. Rinse 6 times with distilled water. Shake and invert the beaker several times during each rinse, using the watch glass cover. Boil with distilled water, cool, and rinse 5 more times with distilled water. Dry in an oven at 100°.

Wash the small ends of sleeve stoppers in soap and water, rubbing a few stoppers at a time between the hands. Rinse with distilled water. Place in a beaker and cover with distilled water, adding a pinch of baking soda. Boil and rinse 5 times with distilled water. Remove any water from the inside of each stopper and dry on filter paper.

CLEANING OF ERLENMEYER FLASKS AND VOLUMETRIC FLASKS

Very careful cleaning of flasks is necessary to secure normal development of color in the samples. The following procedure has been found satisfactory.

1. Rinse 3 times with hot tap water.
2. Clean with detergent solution and brush.
3. Rinse 8 times with hot tap water.
4. Have ready on hot plates two 400 ml. Pyrex beakers, one-third full of hot 50% c.p. nitric acid. Pour hot acid from 1 beaker into flask. Transfer to another flask and repeat until 6 flasks have had the acid. Continue on 6 other flasks with hot acid from the second beaker.
5. Rinse each flask 8 times with hot tap water (inside and outside).
6. Rinse 8 times with distilled water.
7. Drain and dry on drying rack.

REPRODUCIBILITY OF THE METHOD

In tests on 6 aliquots of the same serum sample, all of the values obtained came within 6.2% of the highest value.
TEST FOR THE RECOVERY OF TRIPALMITIN ADDED TO BLOOD SERUM

As a means of checking the accuracy of the fatty ester method, a recovery test was carried out (Table 1). A sample of serum was tested with no addition of fatty ester and also with 1 ml. (0.2 mg.) and 2 ml. (0.4 mg.) of the standard solution of tripalmitin added to the alcohol-ether extracts of aliquots of the same serum. The results

<table>
<thead>
<tr>
<th>Table 1. Recovery of Tripalmitin Added to Blood Serum</th>
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<tbody>
<tr>
<td>Density readings*</td>
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<tr>
<td>Density readings minus cell corrections</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Blanks</td>
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<tr>
<td>(Cell 1)</td>
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<td></td>
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<tr>
<td>Average</td>
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<tr>
<td>Standards</td>
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<tr>
<td>0.2 mg.</td>
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<tr>
<td>Tripalmitin</td>
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<tr>
<td>(Cell 2)</td>
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<tr>
<td>Average</td>
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<tr>
<td>0.4 mg.</td>
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<tr>
<td>Tripalmitin</td>
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<tr>
<td>(Cell 2)</td>
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<tr>
<td>Average</td>
</tr>
<tr>
<td>Serum</td>
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<tr>
<td>(No addition)</td>
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<tr>
<td>(Cell 1)</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Average</td>
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<tr>
<td>Serum +0.2 mg.</td>
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<tr>
<td>Tripalmitin</td>
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<tr>
<td>(Cell 2)</td>
</tr>
<tr>
<td>Average</td>
</tr>
<tr>
<td>Serum +0.4 mg.</td>
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<tr>
<td>Tripalmitin</td>
</tr>
<tr>
<td>(Cell 3)</td>
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<tr>
<td>Average</td>
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</tbody>
</table>

*Beckman readings at 500 mp and .02 mm. slit width.
Reference solution: 95% ethanol.
Cell corrections: cell 1, .007; cell 2, .003; cell 3, .007.
showed 94.8% recovery with 0.2 mg. of the tripalmitin added and 100.6% with 0.4 mg.

VALUES FOR NORMAL FASTING SERUM

The average fasting value obtained for 12 normal weight college women was 414 mg. per 100 ml., expressed as tripalmitin. (Range between 365 and 486 mg. per 100 ml. Standard deviation 32.7.)

COMMENTS ON THE METHOD

Success with the method depends largely on the care used in pipetting, in extracting the serum, and in cleaning the glassware. Also, the use of freshly redistilled ether is necessary.

MICRO METHOD FOR THE DETERMINATION OF LIPID PHOSPHORUS IN BLOOD SERUM

The micro method for lipid phosphorus is an adaptation of the macro method described by Hawk, Oser, and Summerson (7). The extracted lipids are oxidized with sulphuric acid and hydrogen peroxide, and the phosphate present determined by the Fiske and Subbarow method (8). In this method the solution containing phosphate is treated with molybdic acid. Phosphomolybdic acid is formed from inorganic phosphate present. On the addition of 1-amino-2-naphthol-4-sulfonic acid as a reducing agent, a blue color is developed, the intensity of which is proportional to the amount of phosphate present.

EQUIPMENT

(See fatty ester method for additional equipment needed to prepare the alcohol-ether extract of blood serum.)

1. Beckman spectrophotometer, model DU, with 1 cm. Corex glass cells.
2. Electric hot plates with solid metal tops.
3. Wire test tube rack with lower tube support 1 and 1/2 inches above the level of the hot plate on which it is to be used. The rack should be the same size and shape as the hot plate.
4. Pyrex test tubes, 100 × 18 mm. with lip, graduated at 5 ml.
5. Wire rack for holding test tubes.
6. Lang-Levy constriction pipettes, 75 μl. with tip approximately 10 cm. long and short bend at end, 200 μl., 500 μl., and 600 μl., calibrated.
7. Lang-Levy constriction pipettes, 2 ml. (approximate), for use in transferring samples to Beckman cells.
8. Glass beads.
REAGENTS
1. Alcohol-ether mixture. Three volumes 95% ethanol and 1 volume redistilled anhydrous ethyl ether. Make up fresh each day.
2. Sulfuric acid, 5N.
3. Hydrogen peroxide, 30%. Keep in refrigerator.
4. Ammonium molybdate (reagent grade), 2.5%. As soon as any appreciable amount of sediment forms, the solution should be discarded. Keep in refrigerator.
5. 1-Amino-2-naphthol-4-sulfonic acid reagent. Place 195 ml. of 15% sodium bisulphite solution in a glass-stoppered cylinder. Add 0.5 Gm. of 1-amino-2-naphthol-4-sulfonic acid (Eastman). Add 5 ml. of 20% sodium sulfite. Stopper and shake until dissolved. If solution is not complete add more sodium sulfite, 1 ml. at a time, with shaking, but avoid excess. Transfer the solution to a brown glass bottle and refrigerate. Keeps about 4 weeks.
6. Sodium bisulfite (reagent grade), 15%. Keep carefully stoppered and filter if turbid.
7. Sodium sulfite (reagent grade), 20%. Keep carefully stoppered and filter if turbid.
8. Stock standard phosphate solution. Dissolve 35.1 mg. pure dry monopotassium phosphate in distilled water in a 200 ml. volumetric flask. Add 4 ml. 5N H₂SO₄. Dilute to mark with distilled water and mix. (1 ml. = .04 mg. P.) Do not keep longer than 3 days.

PROCEDURE
1. Extract 75 μl. blood serum 3 times with 200 μl. alcohol-ether mixture as in the fatty ester method.
2. Transfer extract to widemouthed Pyrex test tubes (100 × 18 mm. with lip) graduated at 5 ml.
3. Evaporate to dryness in water bath at 100°.
4. Add 1 ml. 5N H₂SO₄ and 2 glass beads to minimize bumping.
5. Suspend in a wire basket about 1 and ½ inches above an electric hot plate and heat. Change the position of the tubes as required for uniform heating. Continue heating until each sample tube containing organic matter shows the same black ashy residue, with no further change.
6. Remove the tube, cool slightly, and add a drop of 30% hydrogen peroxide, allowing the drop to fall directly into the digestion mixture.
7. Replace the tube in wire basket and continue heating until contents become colorless. (It may be necessary to add a second drop of peroxide.) When colorless, heat 15 minutes longer. Cool tube, add a few ml. distilled H₂O and heat to boiling momentarily over a Bunsen burner flame to remove excess peroxide.

8. Cool tube to room temperature.

9. Add 1 ml. 2.5% ammonium molybdate and 0.5 ml. aminonaphtholsulfonic acid reagent. When testing a number of samples, add the molybdate to all tubes and the sulfonic acid reagent to only 3 tubes at a time.

10. Dilute to 5 ml. mark with distilled water and mix. Allow to stand 5 minutes and read in Beckman spectrophotometer at 720 mμ, using a 0.05 mm. slit width. Use water for the reference solution for reading blanks, standards, and samples and for making cell corrections.

11. For blanks and standards, add 600 μl. alcohol-ether mixture to test tubes and continue from step 3 above. Three blanks and 3 standards (2 μg.) are ordinarily used.

STANDARD CURVE

1. Set up tubes containing 2, 4, and 6 μg. P.
2. Add 600 μl. alcohol-ether mixture to each.
3. Carry standards and blanks through the above procedure, starting at step 3.
4. Subtract cell correction and average of blank readings from each of the standard readings and plot against P concentration. Repeat standard determinations several times and use averages for final curve. In routine work it is necessary to run 1 concentration of standard, as a check on the color development in the test samples.

CALCULATIONS

1. Subtract cell corrections and average of blank readings from density readings of unknown samples.
2. Multiply μg. P from curve reading of sample by appropriate factor to obtain mg. per 100 ml. of lipid P in blood serum. Values may be expressed as lecithin by multiplying by 25.

CLEANING GLASSWARE

All the glassware must be cleaned carefully, using hot 50% HNO₃. Avoid use of detergents which may contain phosphorus.
REPRODUCIBILITY OF THE METHOD

In tests on 6 aliquots of the same serum sample, all of the values obtained came within 4.7% of the highest value.

TEST FOR THE RECOVERY OF PHOSPHORUS ADDED TO BLOOD SERUM

As a means of checking the accuracy of the lipid phosphorus method for handling varying amounts of phosphorus, a recovery test was carried out (Table 2). A sample of blood serum was used without

<table>
<thead>
<tr>
<th>Table 2. Recovery of Phosphorus Added to Blood Serum</th>
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<tbody>
<tr>
<td>Density readings (from curve)</td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>Blanks</strong></td>
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<tr>
<td>(Cell 1)</td>
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<td>(Cell 2)</td>
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<td>(Cell 3)</td>
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<tr>
<td><strong>Average</strong></td>
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<td></td>
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<tr>
<td><strong>Standards</strong></td>
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<tr>
<td>1.98 μg. P (Cell 1)</td>
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<tr>
<td>1.98 μg. P (Cell 2)</td>
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<tr>
<td>1.98 μg. P (Cell 3)</td>
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<td><strong>Average</strong></td>
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<tr>
<td><strong>Serum (No addition)</strong></td>
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<td>(Cell 1)</td>
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<td>(Cell 2)</td>
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<td>(Cell 3)</td>
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<tr>
<td><strong>Average</strong></td>
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<td></td>
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<tr>
<td><strong>Serum plus 1.98 μg. P (Cell 1)</strong></td>
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<td><strong>Average</strong></td>
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<tr>
<td><strong>Serum plus 3.81 μg. P (Cell 3)</strong></td>
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<td><strong>Average</strong></td>
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</table>

* Beckman readings made at 720 με and .05 mm. slit width.
* Reference solution: distilled water.
* Cell corrections: cell 1, .008; cell 2, -.001; cell 3, .002.
addition of phosphorus and also with 198.1 μl. (1.98 μg.) and 380.6 μl. (3.81 μg.) of phosphorus working standard added to the alcohol-ether extracts of the same serum. The results indicated that the recovery of added phosphorus was 98.9% with 1.98 μg. P added and 100% with 3.81 μg.

VALUES FOR NORMAL FASTING SERUM

The average fasting value for lipid phosphorus obtained for 8 normal weight college women was 7.8 mg. per 100 ml. (Range between 7.0 and 8.7 mg. per 100 ml. with a standard deviation of 0.51.)

COMMENTS ON THE METHOD

Success with the method depends largely on the micro technics used and on care in cleaning the glassware. Contamination with phosphorus must be avoided.

MICRO METHOD FOR THE DETERMINATION OF TOTAL CHOLESTEROL IN BLOOD SERUM

The micro method for cholesterol is an adaptation of the methods of Kibrick, et al. (9) and Abel, et al. (10). It involves complete extraction of the cholesterol from a measured amount of blood serum with an alcohol-ether mixture, the hydrolysis of the cholesterol esters with 17 per cent benzyltrimethylammonium hydroxide during the evaporation of the alcohol-ether extract, and the use of the residue directly for the development of a green color by the Liebermann-Burchard reaction (11). The method has the advantage that the residue from the benzyltrimethylammonium hydroxide does not interfere with the color development, making it unnecessary to extract the cholesterol from the residue as in the method of Abel, et al. (10).

EQUIPMENT

(See fatty ester method for additional equipment needed to prepare the alcohol-ether extract of blood serum.)
1. Beckman spectrophotometer, model DU, with 1 cm. Corex glass cells with ground glass stoppers. (Cells from Pyrocell Mfg. Co., 207 East 84th St., New York 28, N. Y.)
2. Pyrex test tubes, 100 × 18 mm. with lip.
3. Lang-Levy constriction pipettes: 75 μl. with tip approximately 10 cm. long and short bend at end, 200 μl., 500 μl., 600 μl., calibrated.
4. Lang-Levy constriction pipette 2 ml. (approximate), for use in transferring samples to Beckman cells.

5. Water bath at 100°. (Beakers containing water on an electric hot plate are satisfactory.)

6. Water bath at about 25°. A deep, round pan containing water is satisfactory. Beakers containing water are put into the pan and the sample tubes put into the beakers.

7. Black cloth. Used to cover samples in round pan.

8. Electric hot plates with solid metal tops.

9. Wire rack for holding test tubes.

REAGENTS

1. 17% benzyltrimethylammonium hydroxide (BTA) in methyl alcohol, prepared from a 35 to 40% solution in methyl alcohol. (From Mid-west Laboratories, 1952 West Irving Park Rd., Chicago 13, Ill.) Keep in refrigerator inside covered tin can.

2. Standard cholesterol solution. Recrystallize cholesteryl acetate (Eastman) from boiling acetone which has been redistilled over CaCl₂. Keep crystals in a vacuum desiccator over P₂O₅. Weigh 22.2 mg. of the pure dry crystals. Place in a 100 ml. volumetric flask, make up to volume with alcohol-ether, and mix. One ml. = 0.2 mg. pure cholesterol. Make up a fresh solution just before using.

3. Alcohol-ether mixture (3:1). Three volumes of 95% ethyl alcohol and 1 volume redistilled ethyl ether. Make up fresh each day.

4. Modified Lieberman-Burchard reagent ("cold reagent") (9). Chill 20 volumes of acetic anhydride to a temperature lower than 10° in a glass-stoppered container. Add 1 volume of concentrated sulphuric acid. Shake well and keep cold for 9 minutes. Add 10 volumes of glacial acetic acid and warm to room temperature. Use within 1 hour.

PROCEDURE

1. Extract 75 µl. blood serum 3 times with 200 µl. alcohol-ether mixture as in the fatty ester method.

2. Transfer extract to test tubes, 100 × 18 mm., with lip.

3. Add 1.5 ml. alcohol-ether mixture to each tube containing serum extract.

4. Prepare 3 blanks. Add 600 µl. plus 1.5 ml. alcohol-ether mixture to 100 × 18 mm. tubes and continue with step 6.

5. Set up standard tubes containing 0.1 mg. cholesterol. Use a 500 µl. constriction pipette to measure the standard cholesteryl ace-
state solution. Add 1 ml. plus 600 µl. alcohol-ether mixture. Continue with step 6.

6. Add 500 µl. 17% BTA in methyl alcohol to samples, blanks, and standards. Shake slightly.

7. Evaporate to a thick, yellowish liquid in a water bath (100°) under the hood.

8. To the residue add 5 ml. modified Liebermann-Burchard reagent ("cold reagent"). Mix.


10. Allow to stand for 38 minutes and read in a Beckman spectrophotometer at 660 mµ wave length (using the red-sensitive photo tube) and 0.04 mm. slit width. Use concentrated acetic acid in the reference cell. Clean cells and transfer pipette with distilled water, absolute alcohol, and absolute ether. The timing is important. Start timing from the addition of the modified Liebermann-Burchard reagent to each set of 3 tubes. Keep a record of the time when the reagent is added and the time to read the samples. Keep the number of samples small enough that the time needed for the addition of reagent does not run over 38 minutes.

**STANDARD CURVE**

1. Set up tubes containing 0.1, 0.2, and 0.3 mg. cholesterol. Use a 500 µl. constriction pipette to measure the standard cholesteryl acetate solution.

2. Make all standards to a total volume of 1.5 ml. plus 600 µl. with alcohol-ether mixture.

3. Continue with step 6 above.

4. Subtract cell correction and average of blank readings from each of the standard readings and plot against cholesterol concentration. Repeat standard determinations several times and use averages for final curve. In routine work it is necessary to run only 1 concentration of standard, as a check on the color development in the test samples.

**CALCULATIONS**

1. Subtract cell corrections and average of blank readings from density readings of unknown samples.

2. Multiply mg. cholesterol from curve reading of sample by appropriate factor to obtain mg. per cent cholesterol in blood serum.
CLAYTON ET AL.

CLEANING OF GLASSWARE

All glassware must be carefully cleaned. Hot 50% HNO₃ has been found satisfactory for cleaning the test tubes.

REPRODUCIBILITY OF THE METHOD

In tests on 6 aliquots of the same serum sample, all values obtained came within 4.3% of the highest value.

TEST FOR THE RECOVERY OF CHOLESTEROL ADDED TO BLOOD SERUM

As a means of checking the accuracy of the cholesterol method, a recovery test was carried out (Table 3). A sample of blood serum was tested without and also with the addition of 0.1 and 0.2 mg. of cholesterol (as cholesteryl acetate) added to the alcohol-ether ex-

Table 3. Recovery of Cholesterol Added to Blood Serum

<table>
<thead>
<tr>
<th></th>
<th>Density readings*</th>
<th>Average density readings</th>
<th>Theoretical amount cholesterol (from average curve)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Density readings minus cell corrections</td>
<td></td>
<td>Recovery</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>Blanks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>.000</td>
<td>.000</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>-.001</td>
<td>.000</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>-.001</td>
<td>.000</td>
<td></td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>.076</td>
<td>.076</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>.076</td>
<td>.077</td>
<td>.076 .106</td>
</tr>
<tr>
<td>3.</td>
<td>.075</td>
<td>.076</td>
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<tr>
<td>0.1 mg. Cholesterol</td>
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<td>.152</td>
<td>.154 .208</td>
</tr>
<tr>
<td>1.</td>
<td>.152</td>
<td>.152</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>.154</td>
<td>.155</td>
<td>.154 .208</td>
</tr>
<tr>
<td>3.</td>
<td>.154</td>
<td>.155</td>
<td></td>
</tr>
<tr>
<td>0.2 mg. Cholesterol</td>
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<td>.186</td>
<td>.182 .244</td>
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<tr>
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<td>.186</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>.185</td>
<td>.186</td>
<td>.244 .185</td>
</tr>
<tr>
<td>3.</td>
<td>.174</td>
<td>.175</td>
<td></td>
</tr>
<tr>
<td>Serum (No addition)</td>
<td>.263</td>
<td>.263</td>
<td>.263 .349</td>
</tr>
<tr>
<td>1.</td>
<td>.263</td>
<td>.263</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>.266</td>
<td>.267</td>
<td>.262 .348</td>
</tr>
<tr>
<td>3.</td>
<td>.254</td>
<td>.255</td>
<td></td>
</tr>
<tr>
<td>Serum plus 0.1 mg. Cholesterol</td>
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<td>.255</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>.263</td>
<td>.263</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>.266</td>
<td>.267</td>
<td>.262 .348</td>
</tr>
<tr>
<td>3.</td>
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<td>.255</td>
<td></td>
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<tr>
<td>Serum plus 0.2 mg. Cholesterol</td>
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<td>.255</td>
<td></td>
</tr>
<tr>
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<td>.263</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>.266</td>
<td>.267</td>
<td>.262 .348</td>
</tr>
<tr>
<td>3.</td>
<td>.254</td>
<td>.255</td>
<td></td>
</tr>
</tbody>
</table>

* Beckman readings made at 660 mμ and .04 mm. slit width. Reference solution: glacial acetic acid. Cell corrections: cell 1, .000; cell 2, -.001; cell 3, -.001.
tracts of the same serum. The results showed a recovery of 98.4% with 0.1 mg. cholesterol added, and 99.7% with 0.2 mg.

VALUES FOR NORMAL FASTING SERUM

The average fasting cholesterol value obtained for 16 normal weight college women was 154.8 mg. per 100 ml. (Range 106.8 to 195.3 mg. per 100 ml. with a standard deviation of 24.65.) Average values for six normal weight older women (ages 37-63) tended to increase with age, although one subject 63 years of age showed the same average value as the one 37 years old. The average value obtained for these normal weight older women was 257.2 mg. per 100 ml. (Range 222.1 to 304.0 mg. per 100 ml. with a standard deviation of 42.80.)

COMMENTS ON THE METHOD

Success with the method depends largely on the micro technics used, careful timing, care in cleaning the glassware, and the use of recrystallized and dried cholesteryl acetate as the standard. Tests made with equivalent amounts of recrystallized and dried cholesteryl acetate and recrystallized and dried cholesterol gave identical results, showing that hydrolysis with benzyltrimethylammonium hydroxide was complete. Cholesteryl acetate is used as the standard, since it is easier to purify than cholesterol.

Results with the modified Liebermann-Burchard reagent ("cold reagent") were slightly higher than those obtained when the acetic acid, acetic anhydride, and sulphuric acid were added separately. This was partly due to the lower blank readings obtained with the "cold reagent."

SUMMARY

The procedures described make it possible to determine chylomircon counts and one of the other lipid constituents on approximately 100 μl. of blood serum. In general, the technics used are those of Bessey and Lowry. Chylomircon counts are determined by a modification of the method of Becker, Meyer, and Necheles. Dilutions of high count serum are made with fasting serum and a correction made for the chylomircons in the diluting serum.

In each of the chemical methods, 75 μl. of blood serum is used and the lipids completely extracted from the serum with alcohol-ether mixture. The method for fatty esters is an adaptation of the methods of Hill, and Bauer and Hirsch and involves the formation of a
lavender-colored complex of ferric hydroxamate on the addition of an acidified solution of alcoholic ferric perchlorate. The method for lipid phosphorus is an adaptation of the macro method described by Hawk, Oser, and Summerson. The extracted lipids are oxidized with sulphuric acid and hydrogen peroxide and the phosphate present determined by the Fiske and Subbarow method. The method for cholesterol is an adaptation of the methods of Kibrick, et al. and Abel, et al. It involves the hydrolysis of cholesterol esters with 17% benzyl trimethylammonium hydroxide during the evaporation of the alcohol-ether extract and the use of the residue directly for the development of a green color by the Liebermann-Burchard reaction.

The average fasting value for fatty esters obtained for 12 normal weight college women was 414 mg. per 100 ml., expressed as tripalmitin. For lipid phosphorus the average fasting value obtained for 8 normal weight college women was 7.8 mg. per 100 ml. The average fasting cholesterol value obtained for 16 normal weight college women was 154.8 mg. per 100 ml. The average value for 6 normal weight older women (ages 37-63) was 257.2 mg. per 100 ml.

REFERENCES