GASOMETRIC MICRODETERMINATION OF LIPIDS IN PLASMA, BLOOD CELLS, AND TISSUES

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Outline of Analyses

The lipids are extracted from plasma with alcohol-ether mixture by Bloor's (1914) method as somewhat modified by Man and Gildie (1932-33). A portion of the alcohol-ether extract is saponified and used for total cholesterol precipitation by Windaus' (1909) digitonin method. The digitonide precipitate is measured by its carbon content, which is determined by the manometric microcombustion method of Van Slyke, Page, and Kirk (1933).

In estimation of the other lipid fractions the alcohol-ether extract is freed of non-lipid substances by driving off the solvent at a temperature not exceeding 60° and redissolving the lipids in the residue with petroleum ether. Aliquots of the petroleum ether solution are analyzed as follows: The total lipids are estimated by determination of the total non-volatile carbon. The free cholesterol is determined by digitonin precipitation without saponification. Lipid amino nitrogen (of cephalin and perhaps other amino lipids) is estimated by driving off the solvent, emulsifying the residue in water, and determining the aliphatic amino nitrogen by the micromanometric nitrous acid procedure of Van Slyke (1929). The total phosphatides are estimated from the phosphorus content of the lipid mixture. For this purpose the organic matter is destroyed and the phosphoric acid in the residue is determined by the procedure of Kirk (1934), in which strychnine phosphomolybdate is precipitated and its amount is estimated by manometric determination of the carbon in the precipitate. We have found this procedure more exact than methods based on precipitation of the phosphatides with acetone plus magnesium chloride (Bloor, 1929).
Gasometric Microlipids

or calcium chloride (Katsura et al., 1933). Like Boyd (1931), we have been unable to find conditions for quantitative precipitation by these procedures of such minute amounts of phosphatides as we have desired to determine. We have, however, been able to obtain nearly quantitative results by precipitating larger samples of phosphatides, such as were employed by Bloor (1929). A full discussion of the acetone-MgCl₂ precipitation of the phosphatides will be given later, together with a procedure for gasometric estimation of the precipitated phosphatides.

In the carbon combustions by the method of Van Slyke, Page, and Kirk (1933), a combustion period of 2.5 minutes was found to be sufficient for all determinations described in this paper. Blanks must be run on the volatile organic solvents in which the lipid samples are dissolved. 10 cc. of solvent should not leave enough organic non-volatile residue to give a measurable pressure at 2 cc. volume.

All pieces of apparatus which come into contact with the lipid material are initially cleaned in chromic-sulfuric acid. If the combustion tube at the end of each analysis is immediately protected from contamination with dust, rinsing with distilled water suffices to clean the tube for the next analysis. The detached tube may conveniently be placed in a 400 cc. beaker, which is covered by a watch-glass or a crystallizing dish or may be kept inverted in a metal rack stand in contact with a clean towel. The filter tube, used for washing the strychnine phosphomolybdate precipitate, is washed with water after each analysis to remove traces of acetone. It requires no cleaning with chromic-sulfuric acid.

The small amount of material (0.2 to 0.6 mg. of carbon) required for carbon determination, exact to 1 part in 200, makes it possible to obtain accurate results for total lipids with the extract from 0.05 cc. of blood, and for cholesterol with the extract from 0.15 cc. 3 cc. of plasma suffice for determination of all the lipid fractions mentioned above, with duplicate analyses in each case.

Reagents for Lipid Extraction

Alcohol-ether 3:1. 3 volumes of redistilled 95 per cent alcohol and 1 volume of redistilled ether, as used by Bloor.

Petroleum ether. Commercial petroleum ether which distils at between 30-60° is washed by shaking with concentrated sulfuric
acid (200 cc. of sulfuric acid for 1500 cc. of petroleum ether). This can be conveniently done in large glass-stoppered bottles. The petroleum ether should be left in contact with the sulfuric acid for at least 2 days, during which period the bottle is frequently shaken. The petroleum ether is then decanted off and distilled.

Sand, purified from organic material by ignition.
Fat-free filter paper (extracted with ether).

Reagents for Cholesterol Saponification and Precipitation

Saturated solution of sodium hydroxide in 95 per cent alcohol. This solution is kept in a glass bottle, stoppered by a vaselined-greased glass stopper (to prevent precipitation of carbonates). The bottle is kept in the dark. The solution should be discarded if it turns yellow.
0.04 per cent aqueous filtered solution of phenol red.
Approximately 1 N hydrochloric acid.
1 per cent solution of Merck's digitonin in 50 per cent redistilled alcohol. In preparing this reagent it is usually necessary to heat the solution slightly.
Ether, redistilled.

Reagents for Lipid Nitrogen

For micro-Kjeldahl digestion. To 10 volumes of the 3:1 sulfuric-phosphoric acid mixture of Van Slyke (1926-27) is added 1 volume of a 1 per cent solution of metallic selenium in concentrated sulfuric acid. The selenium catalyzes the digestion of the otherwise somewhat resistant fatty acids.

For lipid amino nitrogen. The reagents are those for the manometric amino nitrogen method described by Van Slyke (1929) and by Peters and Van Slyke (1932).

Reagents for Lipoid Phosphorus

The reagents are described in the accompanying paper by Kirk (1934).

Special Apparatus Required

Apparatus for Microdetermination of Total Lipids Only

Two Jena sintered glass filters, No. 4-G-4, 2 cm. in diameter, with ground joints.
Two 25 cc. volumetric flasks with ground joints to fit the sintered glass filters.

*Apparatus for Complete Lipid Analyses*

For Extraction of Lipids
Two 100 cc. volumetric flasks with ground joints.
Two water-cooled Graham condensers with ground joints to fit 100 cc. flasks.

For Preparation of Petroleum Ether Extract
Several 1000 cc. Pyrex beakers.
Two Jena sintered glass filters, No. 17-G-4, 7 cm. in diameter, with ground joints (Fig. 5).
Two 100 cc. volumetric suction flasks with ground joints to fit the sintered glass filters.

For Storing of Petroleum Ether Extract
Several 125 cc. glass-stoppered bottles.

For Estimation of Total Lipid, Cholesterol, and Phosphorus (Strychnine Molybdate) by Carbon Combustion
Several combustion tubes (described by Van Slyke, Page, and Kirk (1933)).

For Saponification and Estimation of Total Cholesterol
Several ordinary Pyrex test-tubes 175 X 25 mm.
One transfer tube sealed into glass stopper (Fig. 1).
One 25 cc. volumetric flask with ground joint to fit transfer tube stopper.

For Washing of Cholesterol Digitonide
Several filter sticks with detachable alumnum filter disks (Fig. 3).

For Digestion, Precipitation, and Washing of Lipoid Phosphorus
Several 200 X 25 mm. Pyrex digestion tubes with constriction at the 25 cc. mark.
Several standard Pyrex glass beads (ordinary glass does not suffice).
Several 15 cc. centrifuge tubes for precipitation.
One Pregl asbestos filter tube with ground joint (Fig. 1, Kirk, 1934).
One 250 cc. Erlenmeyer flask with ground joint.

For Lipid Total Nitrogen—Several 250 X 25 mm. Pyrex digestion tubes for micro-Kjeldahl digestion.
For Lipid Amino Nitrogen—Several 150 cc. beakers.

One Van Slyke-Neill manometric apparatus serves for the final determination of all fractions.

Preparation of Plasma Extracts

Drawing Blood for Analysis—Heparin is used instead of oxalate as anticoagulant because oxalate may combine with some of the phosphatides (MacLean and MacLean, 1927). According to Shope (1928) both oxalate and citrate may cause partial saponification of the cholesterol esters. Man and Gildea (1932–33) also found distinctly lower values for total fatty acids in oxalated plasma than in heparinized plasma and serum. Heparinized plasma is preferred to serum, because of the possibility that cephalin might be influenced by the process of coagulation. Furthermore, the use of plasma permits the immediate separation of cells, so that little time is given for phosphorus compounds of the cells to enter the plasma. To determine whether heparin offered the same protection as citrate against disintegration of the platelets (and consequent contamination of the plasma by cephalin contained in the platelets), the lipid amino nitrogen content of a heparinized plasma sample was compared with that of a citrated sample of the same plasma. Practically identical values were found in the two samples. Heparin is also preferred to citrate as anticoagulant in order to avoid changes in the water content of the plasma due to the salt effect: in the comparative analysis of the heparinized and citrated plasma mentioned above 13 per cent higher values for total fat were found in the heparinized than in the citrated sample.

When a complete analysis including all the lipid fractions described below is to be made, enough blood is drawn to yield 3 cc. of clear plasma. This will provide material for duplicate analyses. If only a part of the fractions described is to be determined, the blood sample may be correspondingly diminished. The volumes of normal plasma represented in the amounts of ether-alcohol extract used for single determinations are as follows: total lipids, 0.045 cc.; total cholesterol, 0.06 cc. (the equivalent of 0.15 cc. is used for saponification); free cholesterol, 0.1125 cc.; total phosphatides, 0.054 cc. (the equivalent of 0.225 cc. of plasma is used for each digestion); total lipid nitrogen, 0.225 cc.; lipid amino nitrogen, 0.45 cc.
Gasometric Microlipids

**Separation of Cells and Plasma**  As soon as possible after it is drawn, the blood sample is centrifuged in a 15 cc. centrifuge tube. 45 minutes centrifugation at 2000 r.p.m. are sufficient to leave in suspension in the plasma less than 1 per cent of the original number of platelets. The separation of platelets from the plasma must be complete or too high values for plasma lipid amino nitrogen will result. Also, in pipetting off the supernatant plasma care is taken to prevent elements from the buffy layer over the red cells from contaminating the sample.

**Extraction of Lipids from Plasma**

For the complete analysis 3 cc. of plasma are added drop by drop to 75 cc. of redistilled alcohol-ether mixture (3:1) in a volumetric flask, which is calibrated at 100 cc. volume and provided with a neck ground to fit a water-cooled condenser. During the addition of the plasma the mixture is agitated thoroughly to obtain a finely divided precipitate. Two clean glass beads are added to produce even boiling. The alcohol and ether are then allowed to reflux for 1 hour. After this period the condenser is rinsed twice with alcohol-ether and the flask is cooled to room temperature. The content is made up to volume with alcohol-ether, and, after thorough mixing, is quickly filtered through a fat-free filter paper into a glass-stoppered clean bottle. Approximately 90 cc. of filtrate are obtained. This filtrate, each cc. of which represents 0.03 cc. of plasma, will be termed A-E Extract A.

If only total lipids are to be determined, 0.2 cc. of plasma may be extracted with 10 cc. of alcohol-ether in a 25 cc. volumetric flask and made up to 25 cc. for filtration. The extraction is most conveniently made by immersing the volumetric flask into a beaker with boiling water and keeping the flask immersed for a few seconds after the contents have started boiling. The addition of a glass bead prevents bumping. The filtrate, each cc. of which represents 0.008 cc. of plasma, will be termed A-E Extract B. Values for total plasma lipids obtained by this microprocedure agree within 2 per cent with values obtained by analysis of A-E Extract A.

**Extraction of Lipids from Blood Corpuscles**

After centrifugation of the blood sample and syphoning off of the plasma, the buffy coat and the upper 3 mm. of the compact
layer of red blood cells are removed by suction. By means of a pipette with a wide opening 3 to 3.5 cc. of cells are transferred to a 20 cc. beaker which has been previously weighed to within 1 mg. As much of the sample is used as will leave the pipette without difficulty; the exact size of the sample is estimated by renewed weighing of the beaker. 3 cc. of sand, purified from organic material by ignition, are added to the cells in the beaker and the whole is thoroughly mixed by stirring with a glass rod. A semiliquid mixture results which can easily be poured into the extraction flask with the help of the stirring rod. The small amount of residue left in the beaker is transferred to the extraction flask with two successive portions of 4 cc. of water, the water also being used to rinse the glass rod. When the transfer is completed, the contents of the extraction flask are mixed by vigorous shaking to insure complete laking of the cells. The alcohol-ether extraction fluid is then added slowly with frequent shaking. A glass stopper is inserted and the contents of the flask again vigorously shaken for half a minute to insure a finely divided precipitate, after which the mixture is boiled under a reflux condenser.

After the reflux extraction is completed, the contents are as usual made up to 100 cc. volume, after which an extra 3 cc. of the alcohol-ether mixture is measured into the flask to compensate for the 3 cc. of sand used.

**Extraction of Lipids from Tissues**

The instructions given by Bloor (1929, p. 278) for weighing and grinding of the sample are followed. 3 cc. of ignited sand are mixed with the sample. 300 mg. of liver, kidney, pancreas, or heart are used for reflux extraction with 75 cc. of alcohol-ether, making a final volume of 103 cc.

**Total Cholesterol in Alcohol-Ether Plasma Extract**

*Saponification of Cholesterol Esters—*5 cc. of A-E Extract A are pipetted into a Pyrex test-tube (175 × 22 mm.) and 2 cc. of a saturated solution of sodium hydroxide in redistilled 95 per cent alcohol are added. The test-tube is partly immersed for 2½ hours in water at 85°, or, more conveniently, placed in an electric oven at 85°. When the contents of the tube have been reduced to about 3 cc., a cork stopper covered with tin-foil is firmly inserted
into the opening of the tube. The stopper is provided with a small furrow to allow escape of alcohol vapors.

The total cholesterol could also be determined in the petroleum ether solution of purified lipids, instead of in the total alcohol-ether extract, but there are advantages in using the latter. It permits one to begin saponification as soon as the plasma is extracted. In consequence the saponified mixture for total cholesterol is ready for digitonin precipitation at about the same time that the Petrol Extract A (see below) is ready for free cholesterol precipitation, so that both cholesterol determinations can then be carried through together. Also, the alcohol-ether extract mixes homogeneously with the alcoholic sodium hydroxide, whereas petroleum ether does not; and the conditions of saponification are more readily controlled with the alcohol-ether extract. The exact control of cholesterol ester saponification has required more care than any other step of the analysis. If treatment with alkali is not severe enough, which is likely to occur if the temperature during the saponification falls much below 85°, saponification is incomplete and the total cholesterol yield is low. But if the treatment is too severe, some cholesterol is destroyed and the results are again too low. The latter may occur if the volume of the saponification mixture becomes too much reduced towards the end of the saponification. This is more difficult to avoid if the petroleum ether solution is used.

Neutralization of Alkali—After 2½ hours the tube is cooled and 2 drops of a 0.04 per cent aqueous phenol red solution added. The contents of the tube are then neutralized with 1 N hydrochloric acid.

Extraction of Cholesterol with Petroleum Ether—From the saponified solution the total cholesterol is extracted by means of five successive 5 cc. portions of petroleum ether, and is transferred to a 25 cc. calibrated volumetric flask. The contents of the tube are carefully heated with each portion of petroleum ether by immersion in a beaker of hot water, and agitated to insure thorough mixing. Part of the petroleum ether evaporates; the rest is drawn as completely as possible into the 25 cc. flask, as shown in Fig. 1. Care must be taken to prevent drops of the water-alcohol solution from being drawn over into the volumetric flask. So much petroleum ether evaporates that the five portions do not fill the 25 cc. flask.
After rinsing the capillary of the transferring tube below the ground stem with petroleum ether, the contents of the flask are made up to 25 cc. with petroleum ether. A clean ground glass stopper is inserted into the flask and the petroleum ether solution is mixed. If the plasma is normal, a 10 cc. sample of petroleum ether, representing 0.06 cc. of plasma, is pipetted into a combustion tube for digitonin precipitation. If the plasma is lipemic, only 5 cc. of the petroleum ether solution are used, representing 0.03 cc. of plasma.

Note on Washing of Volumetric Flask and Pipette—The volumetric flask and the pipette for measuring out the sample are cleaned before each analysis with petroleum ether. The pipette is afterwards dried in a current of air. The petroleum ether left over in the volumetric flask after pipetting off the sample may be used over again after distillation.
Precipitation of Cholesterol Digitonide—To the petroleum ether sample in the combustion tube is added 0.2 cc. of a 1 per cent digitonin solution (in 50 per cent redistilled alcohol). The contents of the tube are allowed to evaporate at not over 60°. The best precipitate is obtained if the evaporation is made very slowly (3 to 4 hours or overnight). Under these conditions the precipitation of the cholesterol is complete.1 If the evaporation is performed rapidly, the petroleum ether may creep up towards the neck of the tube and escape contact with the alcoholic digitonin solution at the bottom of the tube. Suction should not be employed during the evaporation as particles of dust are likely to be drawn into the tube and would not be removed by the subsequent washings. The temperature during the evaporation must not exceed 60°, as higher temperature might render the phosphatides partially insoluble and make the washing of the cholesterol digitonide precipitate incomplete.

Washing of Cholesterol Digitonide Precipitate—5 cc. of redistilled ether are added to the residue in the combustion tube and drawn up through an immersion filter stick, the lower portion of which, containing the alundum filter disk, is detachable (see Fig. 3). Several tubes can be washed, each with a separate filter stick, at the same time. The suction must be so regulated that the flow up into the filter stick is not rapid or bubbling. A total of five successive washings with 5 cc. of ether each is made, the filter stick being removed from the tube before each new addition. When the ether washing of a series of tubes is completed, the waste bottle in the vacuum line should be emptied. If this precaution is neglected, the ether vapors in the waste bottle may expand when the later hot water washings enter the bottle, and material on the end of the filter stick may be blown off by the back pressure. If this occurs at a moment when the stick is held outside the tube, the analysis will be lost.

After the last ether washing the tubes are heated on the steam bath to allow escape of any remaining ether before the washings with hot water. If the hot water is added to the tube in the pres-

1 The Liebermann-Burchard reaction on chloroform extract of the content of the tube after this has been evaporated to dryness was found negative. Also quantitative results were uniformly obtained by analysis of cholesterol standard solution.
ence of ether, some material may be lost by expulsion with ether vapor. When completely dry, the precipitate is washed four times with 5 cc. portions of boiling distilled water, the water being drawn through the filter stick into the empty waste bottle in the vacuum line. After the washings are completed, the lower part of the filter stick is pushed off into the bottom of the combustion tube by means of a glass rod. The water in the tube is completely evaporated by heating the tube in a steam bath, and the sample is ready for combustion.\(^2\) The washing of a sample with ether and water requires about 5 minutes.\(^3\)

\(^2\) If in analysis of a sample with unusually high cholesterol content the carbon dioxide developed exceeds the amount which can be measured in the
Fig. 4. Arrangement for evaporating alcohol and ether from the alcohol-ether extract.

ordinary Van Slyke-Neill chamber, the duplicate cholesterol digitonide sample may be saved by redissolving the precipitate in hot absolute alcohol and performing the non-volatile carbon determination on an aliquot of the solution.

If instead of a detachable filter stick a non-detachable one is used (see Fig. 2), the precipitate adhering to the end of the filter and to the outside of the filter stick may be redissolved and washed into the combustion tube by means of hot absolute alcohol, 1 to 2 cc. portions of which are alternately poured into the tube of the filter stick and over the outside of the tip of the filter. The absolute alcohol is afterwards evaporated on the steam bath and the residue determined by combustion. Care should be taken not to touch the stick with the fingers within 2 inches from the tip, and, in pouring the alcohol into the filter stick, to avoid drops running down the outside of the stick and getting into contact with the fingers. The use of a filter stick with a non-detachable tip is, however, quite tedious, about 20 minutes being required for the preparation of each sample. Identical results are obtained by both procedures.
**Note on Combustion of Cholesterol Digitonide, Prepared As Described Above**—When the 1 cc. of combustion fluid is added to the tube containing the end of the detachable filter stick, care should be taken not to deliver the fluid into the open end of the filter tube, as too little fluid will remain outside the filter to insure contact with the cholesterol digitonide precipitate left on the walls of the combustion tube. To prevent breaking of the glass filter the tube is not shaken during the combustion, but complete contact between the combustion fluid and the walls of the combustion tube is secured by vigorous lowering of the mercury leveling bulb.

If this precaution is observed, one filter stick may serve for many analyses. It is advisable to test each filter on a standard cholesterol solution before use. The alundum disks in the filter sticks, when not in use, have been kept in chromic acid mixture. Probably owing to this unnecessary precaution, crumbling of the alundum has occurred after prolonged use. New alundum disks can easily be fused into the same filter sticks.

**Petroleum Ether Extraction of Residue from Alcohol-Ether Extract**

**For Determination of Total Fat, Free Cholesterol, Total Lipid Nitrogen, Lipid Amino Nitrogen, and Lipoid Phosphorus**

**Evaporation of Alcohol-Ether Extract**—75 cc. of A-E Extract A are evaporated to dryness in a 1 liter beaker, the bottom of which is immersed in water at 60° (Fig. 4). The temperature limit of 60° was found to be of the greatest importance, as the reextraction of the lipids with petroleum ether was found to be incomplete if this temperature was exceeded. When the alcohol-ether solvent was evaporated at 60° or less, the reextraction of the lipids yielded very constant results, much higher than those obtained if the beaker had been heated to 75°.

It is safe never to allow the temperature to rise above 60°. To accelerate evaporation a current of air drawn through a funnel may be used with advantage (Fig. 4). About 45 minutes are required to evaporate 75 cc. of alcohol-ether to dryness under these conditions.

**Reextraction of Alcohol-Ether Residue with Petroleum Ether**—The lipids of the residue are redissolved by repeated extractions with petroleum ether. The first extractions are made with cold, the
last ones with boiling petroleum ether. Each petroleum ether portion is filtered through a sintered glass filter (Jena, No. 17-G-4, diameter 7 cm., capacity of cup 175 cc.), ground to fit the neck of a 100 cc. calibrated volumetric flask (see Fig. 5). The filter should be of such a porosity as to allow the filtration of the residue from 75 cc. of alcohol-ether without clogging. It is usually not necessary to use suction during the filtration. If occasionally the filtration is slow, only slight suction should be used. After the extrac-

![Fig. 5. Arrangement for filtering extract through porous glass funnel.](image)

...tion with petroleum ether is complete, the filter is washed repeatedly with petroleum ether, and, after cooling, the contents of the 100 cc. flask are made up to volume. Each cc. of solution represents 0.0225 cc. of plasma. The solution will be termed Petrol Extract A.

Petroleum ether has over ether an advantage in the ease with which the former is kept anhydrous. It was also found to have the following essential properties for reextraction of the plasma lipids.

1. Exclusive solution of lipoid phosphorus. No measurable
amount of inorganic phosphorus, sodium glycerophosphate, or hexosediphosphate was dissolved by petroleum ether under the conditions of the extraction. As shown later, all the phosphorus extracted under these conditions from plasma, red blood cells, and tissues was found to be precipitable by acetone and magnesium chloride under the conditions used to precipitate phosphatides.

(2) Entire lack of solvent power for amino acids. Petroleum ether was found to dissolve no amino nitrogen from a mixture of monoamino acids from hydrolyzed casein. (3) The cholesterol digitonide was found to be more suitable for filtering and washing when it was precipitated from petroleum ether solution than when it was precipitated from ether or alcohol solution. (4) The extraction of the lipids with petroleum ether was complete. Further extraction of the residue with ether increased the redissolved carbon by only approximately 1 per cent. The Liebermann-Burchard reaction was negative in this final ether extract.

Note on Cleaning of Glass Filter—After each filtration the glass filter is cleaned with sulfuric-chromic acid cleaning mixture, and afterwards rinsed thoroughly with water. It must be completely dried before use. The drying is accomplished by filtering successive portions of alcohol, ether, and petroleum ether through the filter.

Total Lipids

In Petrol Extract A—If the fat content of the blood is normal, two 2 cc. samples of Petrol Extract A are pipetted into combustion tubes. Each sample represents 0.045 cc. of plasma. If the plasma is lipemic, however, only a 1 cc. sample, representing 0.0225 cc. of plasma, is used. Occasionally heavy lipemia may necessitate use of still smaller samples. A portion of Petrol Extract A is then diluted with petroleum ether and a suitable aliquot is taken. After the petroleum ether has been driven off from each tube, 2 drops of distilled water are added and evaporated to dryness. The vapors are finally swept out of the tube with a momentary current of air (4 to 5 seconds) and the residues are submitted to combustion.

In Petrol Extract B—Petrol Extract B is prepared from A-E Extract B. 20 cc. of the latter are evaporated to dryness in a 100 cc. beaker at not more than 60°. The residue is stirred up...
with 5 cc. portions of petroleum ether, which are passed through a sintered glass filter into a 25 cc. flask and made up to the mark. For the combustion 5 cc. portions, representing 0.032 cc. of plasma, are taken if the plasma is normal. If it is lipemic, samples of 3 or 2 cc., representing 0.0192 or 0.0128 cc. of plasma, are taken.

**Free Cholesterol**

For analyses of normal plasma two samples of 5 cc. each of Petrol Extract A, representing 0.1125 cc. of plasma, are pipetted into combustion tubes. The cholesterol is precipitated with digitonin and the precipitate is washed and subjected to combustion as described for the determination of total cholesterol.

For lipemic bloods, samples of 3 or 2 cc. of Petrol Extract A, representing 0.0675 or 0.045 cc. of plasma, may be taken.

**Total Lipid Nitrogen**

For gasometric micro-Kjeldahl analyses duplicate 10 cc. samples of Petrol Extract A are measured into 200 × 25 mm. Pyrex test-tubes. Two glass beads are added to each, and the samples are evaporated to dryness on the steam bath. The evaporation is performed slowly to prevent the petroleum ether from creeping. When the residue is dry, 1 gm. of potassium persulfate is added in substance, followed by 1 cc. of water and 1 cc. of the sulfuric-phosphoric acid-selenium digestion mixture. In adding the water and the digestion fluid, care is taken to wash down any persulfate crystals which may stick to the walls of the tube. Otherwise, error due to development of oxygen gas from undigested persulfate during the reaction with hypobromite may occur. Only gentle heating should be applied to the tube during the 1st minute of the digestion. Too intense initial heating may cause excessive foaming and deposition of material high on the inside of the tube, where contact with the digestion fluid is difficult to obtain. Even when the heating is begun carefully, it is frequently necessary to remove the tube temporarily from the flame, and, by holding the tube in an oblique position, insure contact between the digestion fluid and particles of undigested material on the walls of the tube. The digestion of the lipids was found to be complete in 20 minutes. If the selenium catalyst was omitted from the digestion mixture, digestion was slower, and nitrogen values 2 to 3 per cent too low were likely to be obtained.
Neutralization of the acid-digest is performed as described by Van Slyke (1926–27) (also Peters and Van Slyke (1932) pp. 353–358). The neutralization should be made immediately after cooling, and the tubes then closed until ready for the gasometric analysis, to prevent absorption of any ammonia from the air. For the gasometric analysis the new hypobromite reagent of Van Slyke and Kugel (1933) is used.

A blank is run with each set of reagents.

Lipid Amino Nitrogen ("Cephalin")

Emulsification of Lipids and Transfer to Manometric Chamber—
Two samples of 20 cc. each of Petrol Extract A, representing 0.45 cc. of plasma, are pipetted into 150 cc. beakers and evaporated to dryness without heating the outside of the beakers above 60°.

The residue at the bottom of each beaker is brought into emulsion by addition of 2 cc. of distilled, ammonia-free (tested with Nessler's reagent) water and by stirring with a rubber-tipped glass rod. The 2 cc. of lipid suspension are poured from the beaker into the cup of the Van Slyke apparatus and slowly admitted to the chamber, allowing complete drainage of the walls of the cup. The beaker, glass rod, and cup are washed with three successive 1 cc. portions of water, making a total volume of 5 cc. Care is taken to bring into emulsion any material that might have crept up the sides of the beaker. In each of the washings the inside of the cup is scrubbed with the rubber-tipped glass rod. Even if the suspension is not homogeneous, which is sometimes the case in samples of lipemic blood, it will become so after shaking in the chamber with acetic acid. The quantitative transfer of the emulsion is easy if the material has not been heated above 60°.

Removal of Air from Mixed Solution of Lipids and Acetic Acid—
1 cc. of glacial acetic acid is added exactly from an Ostwald pipette. The manometric chamber is evacuated and shaken for 1½ minutes and the extracted air is ejected. The extraction is once repeated, and an additional minute bubble of air is ejected. As the lipid suspension shows no tendency to foam, addition of caprylic alcohol has not been found necessary.

Decomposition of Amino Groups—The total reaction time necessary for complete decomposition of the cephalin amino groups was found to be 3 times that for the α-amino groups of amino acids at the same temperature (Table I and Fig. 6).
FIG. 6. Time required for reaction of cephalin amino group with nitrous acid.
2 cc. of sodium nitrite solution are measured into the chamber from an Ostwald pipette. The chamber is not evacuated immediately as in the ordinary amino nitrogen analysis but the solution is allowed to remain in the upper portion of the chamber during the first 2 minutes of the reaction time. This precaution is taken because it was found that otherwise part of the lipid solution would stick to the upper portion of the chamber and escape contact with the nitrite solution. After 2 minutes the chamber is evacuated till the mercury in it has fallen to 1 or 2 cm. above the 50 cc. mark, and is shaken for 15 seconds to insure complete mixing of the solutions. As in the ordinary amino nitrogen analysis, the reaction mixture is permitted to stand in this position until within 1 minute of the end of the reaction time (3 times that of ordinary \(\alpha\)-amino groups (see Fig. 6)). Two or three times during the reaction period the mixture is shaken for a few seconds to insure contact with the film of solution adhering to the walls of the middle portion of the chamber. During the last minute the mixture is shaken as usual to complete the evolution of the \(N_2\) formed. The remaining portion of the analysis is identical with that for the determination of ordinary \(\alpha\)-amino groups.

From 25 to 30 minutes are required to emulsify the lipid residue, transfer it to the manometric chamber, and determine the amino nitrogen.

**Blank**—With each series of analyses the usual blank is run with 5 cc. of water instead of the lipid emulsion. Each preparation of

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**Table I**

*Reaction Time for Amino Groups in Cephalin Suspension by Manometric Amino Nitrogen Determination of Van Slyke*

<table>
<thead>
<tr>
<th>Period No.*</th>
<th>Amino nitrogen developed</th>
<th>Per cent of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3769</td>
<td>86.0</td>
</tr>
<tr>
<td>2</td>
<td>0.4167</td>
<td>95.4</td>
</tr>
<tr>
<td>3</td>
<td>0.4340</td>
<td>99.1</td>
</tr>
</tbody>
</table>

*The temperature during the reaction was 23.6°. Each period lasted 3 minutes and 18 seconds. The gas developed in each period was collected separately.*
petroleum ether used must also be tested by treating 20 cc. as described above. It is of the greatest importance to make sure that the petroleum ether is sufficiently purified to give a blank nearly as low as that of distilled water. It was found that the residue of 20 cc. of Kahlbaum's petroleum ether "for fat determination" gave a blank of approximately 120 mm. pressure at the 0.5 cc. mark. This blank was not caused by any nitrogenous substances in the petroleum ether, but was reduced to approximately the blank of distilled water when the petroleum ether had been purified as described under "Reagents."

Cleaning of Chamber after Analysis—After each series of analyses the manometric chamber is cleaned by filling it with sulfuric-chromic acid cleaning mixture, with precautions for preventing contact with the rubber connection at the bottom of the chamber, as described by Van Slyke and Neill (1924).

Lipoid Phosphorus

Principle—The lipids are digested by Neumann's wet ashing procedure, and the phosphoric acid in the digest is determined by the gasometric method of Kirk (1934).

Evaporation—Two samples of 10 cc. each of Petrol Extract A are measured into 200 X 15 mm. Pyrex digestion tubes, which have previously been calibrated at 25 cc. Each tube is constricted to 10 to 12 mm. diameter at the calibration mark and is identical with the tubes used for digestion in the manometric total base determination of Van Slyke, Hiller, and Berthelsen (1927) (see Peters and Van Slyke, 1932, p. 403). One Pyrex glass bead is added and the petroleum ether is evaporated to dryness on the steam bath. (The ordinary type of digestion tubes used for digestion of the lipids for nitrogen determination may be used instead of the calibrated tubes. In this case tubes used for phosphorus determination should be kept carefully separated from those used in the nitrogen determination, to avoid contamination with phosphoric acid from the sulfuric-phosphoric acid digestion mixture used in the Kjeldahl analyses.)

Digestion of organic matter and neutralization of the digest is carried out as described in the accompanying paper by Kirk (1934). 1 cc. of water is added to the dry residue, followed by 1 cc. of the sulfuric-nitric acid digestion mixture. Further addition of nitric
acid to complete the digestion is usually unnecessary. 6 cc. of the neutralized digest, representing 0.054 cc. of plasma, are used for each analysis.

A blank must be run on the reagents, with 10 cc. of petroleum ether instead of the sample.

*Calculation*—The factors for calculation of the various lipid fractions are given in Table II. The factors for total phosphatides were obtained by multiplication of the phosphorus factors by 23.54.

\[
\begin{align*}
\text{Mg. carbon in sample} & = (p_1 - p_2 - c) \times f_{\text{carbon}} \\
\text{cholesterol in sample} & = (p_1 - p_2 - c) \times f_{\text{cholesterol}} \\
\text{lipo nitrogen in sample} & = (p_1 - p_2 - c) \times f_{\text{nitrogen}} \\
\text{amino nitrogen in sample} & = (p_1 - p_2 - c) \times f_{\text{amino nitrogen}} \\
\text{phosphorus in sample} & = (p_1 - p_2 - c) \times f_{\text{phosphorus}} \\
\text{phosphatide in sample} & = (p_1 - p_2 - c) \times f_{\text{phosphatide}} \\
\text{substance per 100 cc. plasma} & = (\text{mg. substance in sample}) \times (100/\text{cc. plasma represented in sample})
\end{align*}
\]

The \( c \) correction in each analysis represents the blank on the whole procedure.

The \( f \) factors are given in the original papers on manometric carbon, nitrogen, phosphorus, and \( \text{NH}_2 \) determination, but for convenience those which apply to the determinations described in this paper are collected in Table II.

For convenience in calculation of the mg. per cent values of the different lipid fractions in normal and lipemic plasma Table III is included, giving the volume of plasma represented in each sample.

*Calculation of Total Lipid*—An approximate value for the total lipids can be obtained by multiplying the total petroleum ether-soluble carbon by 1.3, on the assumption that the carbon content of the mixture is 77 per cent.

However, the lipids represented include those with carbon contents varying from 83.9 per cent in the case of cholesterol to 66.2 per cent in the case of cephalin. Hence it is obvious that the total lipid value cannot be calculated accurately by using a single calculation factor. In the publication of the manometric carbon method factors for calculation of "mixed plasma lipids" were given in Table I of that paper. It was, however, pointed out that these factors could be used only if the sample analyzed consisted of a mixture of cholesterol with either the neutral fats or with the fatty
<table>
<thead>
<tr>
<th>Temperature</th>
<th>Carbon</th>
<th>Cholesterol when the diglyceride is oxidized</th>
<th>Fatty acids</th>
<th>Neutral fat</th>
<th>Lipid phosphorus</th>
<th>Total phosphatide</th>
<th>Total lipid nitrogen by NH₃-nitrogen by nitrous acid reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a = 2.0</td>
<td>a = 2.0</td>
<td>a = 2.0</td>
<td>a = 1.0</td>
<td>a = 2.0</td>
<td>a = 2.0</td>
<td>a = 0.5</td>
</tr>
<tr>
<td></td>
<td>0.001474</td>
<td>0.000579</td>
<td>0.001941</td>
<td>0.001921</td>
<td>0.00005190</td>
<td>0.001222</td>
<td>0.000813</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>65</td>
<td>376</td>
<td>31</td>
<td>11</td>
<td>62</td>
<td>810</td>
</tr>
<tr>
<td>16</td>
<td>17</td>
<td>58</td>
<td>572</td>
<td>21</td>
<td>01</td>
<td>34</td>
<td>807</td>
</tr>
<tr>
<td>17</td>
<td>18</td>
<td>50</td>
<td>569</td>
<td>11</td>
<td>00.001890</td>
<td>06</td>
<td>804</td>
</tr>
<tr>
<td>18</td>
<td>19</td>
<td>42</td>
<td>566</td>
<td>00</td>
<td>80</td>
<td>00.0005078</td>
<td>800</td>
</tr>
<tr>
<td>20</td>
<td>22</td>
<td>12</td>
<td>554</td>
<td>61</td>
<td>41</td>
<td>00.0004972</td>
<td>759</td>
</tr>
<tr>
<td>21</td>
<td>23</td>
<td>05</td>
<td>552</td>
<td>52</td>
<td>32</td>
<td>47</td>
<td>786</td>
</tr>
<tr>
<td>22</td>
<td>24</td>
<td>35</td>
<td>563</td>
<td>00.001891</td>
<td>71</td>
<td>53</td>
<td>00.000798</td>
</tr>
<tr>
<td>23</td>
<td>25</td>
<td>27</td>
<td>560</td>
<td>51</td>
<td>00</td>
<td>47</td>
<td>779</td>
</tr>
<tr>
<td>24</td>
<td>26</td>
<td>20</td>
<td>557</td>
<td>61</td>
<td>41</td>
<td>00.0004972</td>
<td>759</td>
</tr>
<tr>
<td>25</td>
<td>27</td>
<td>12</td>
<td>554</td>
<td>61</td>
<td>41</td>
<td>00.0004972</td>
<td>759</td>
</tr>
<tr>
<td>26</td>
<td>28</td>
<td>05</td>
<td>552</td>
<td>52</td>
<td>32</td>
<td>47</td>
<td>786</td>
</tr>
<tr>
<td>27</td>
<td>29</td>
<td>35</td>
<td>563</td>
<td>00.001891</td>
<td>71</td>
<td>53</td>
<td>00.000798</td>
</tr>
<tr>
<td>28</td>
<td>30</td>
<td>27</td>
<td>560</td>
<td>51</td>
<td>00</td>
<td>47</td>
<td>779</td>
</tr>
<tr>
<td>29</td>
<td>31</td>
<td>20</td>
<td>557</td>
<td>61</td>
<td>41</td>
<td>00.0004972</td>
<td>759</td>
</tr>
<tr>
<td>30</td>
<td>32</td>
<td>12</td>
<td>554</td>
<td>61</td>
<td>41</td>
<td>00.0004972</td>
<td>759</td>
</tr>
<tr>
<td>31</td>
<td>33</td>
<td>05</td>
<td>552</td>
<td>52</td>
<td>32</td>
<td>47</td>
<td>786</td>
</tr>
<tr>
<td>32</td>
<td>34</td>
<td>35</td>
<td>563</td>
<td>00.001891</td>
<td>71</td>
<td>53</td>
<td>00.000798</td>
</tr>
<tr>
<td>33</td>
<td>35</td>
<td>27</td>
<td>560</td>
<td>51</td>
<td>00</td>
<td>47</td>
<td>779</td>
</tr>
<tr>
<td>34</td>
<td>36</td>
<td>20</td>
<td>557</td>
<td>61</td>
<td>41</td>
<td>00.0004972</td>
<td>759</td>
</tr>
</tbody>
</table>
acids obtained after saponification, as in Stoddard's method, since the factors for neutral fat and fatty acids differ by only 1 per cent. At the same time the assumption must be made (as does Bloor) that the mixture consist of 2 parts of fat to 1 part of cholesterol.

### TABLE III
**Volume of A-E Extract A and Petrol Extract A Used for Individual Lipid Analyses of Normal and Lipemic Plasma**

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Lipemic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>For saponification 5 cc. A-E.*</td>
<td>For saponification 5 cc. A-E. After saponification 10 cc. aliquots of petroleum ether solution analyzed</td>
<td>For saponification 5 cc. A-E. After saponification 10 cc. aliquots of petroleum ether solution analyzed</td>
</tr>
<tr>
<td>~ 0.06 cc. plasma</td>
<td>~ 0.03 cc. plasma</td>
<td></td>
</tr>
<tr>
<td><strong>Free cholesterol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 cc. P. E. † ~ 0.1125 cc. plasma</td>
<td>3 cc. P. E. ~ 0.0675 cc. plasma</td>
<td></td>
</tr>
<tr>
<td><strong>Total fat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 cc. P. E. ~ 0.045 cc. plasma</td>
<td>1 cc. P. E. ~ 0.025 cc. plasma</td>
<td></td>
</tr>
<tr>
<td><strong>Lipid nitrogen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 cc. P. E. ~ 0.225 cc. plasma</td>
<td>Same</td>
<td></td>
</tr>
<tr>
<td><strong>Lipoid phosphorus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 cc. P. E. used for digestion. 6 cc.</td>
<td>Same</td>
<td></td>
</tr>
<tr>
<td>sample of diluted digest analyzed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>~ 0.054 cc. plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lipid amino nitrogen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 cc. P. E. ~ 0.45 cc. plasma</td>
<td>Same</td>
<td></td>
</tr>
</tbody>
</table>

* A-E represents A-E Extract A.
† P. E. represents Petrol Extract A.

We have found it convenient in the daily analyses to report the total lipid values simply as "total lipid carbon." If instead of the total lipid carbon value the accurate total lipid value is desired, this can be calculated by summation of the individual lipid fractions determined in the separate analyses. The fraction remain-
Gasometric Microlipids

ing after the carbon values for cholesterol, cholesterol esters, and phosphatides have been subtracted from the "total lipid carbon" can be assumed to consist chiefly of neutral fats with a carbon content of 76.7 per cent. The calculation will be illustrated by an example.

<table>
<thead>
<tr>
<th>Total lipid carbon</th>
<th>Cholesterol</th>
<th>Lipoid phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg. per cent</td>
<td>mg. per cent</td>
<td>mg. per cent</td>
</tr>
<tr>
<td>1459</td>
<td>524</td>
<td>194</td>
</tr>
</tbody>
</table>

19.85 mg. lipoid phosphorus corresponds to $19.85 \times 23.54 = 468$ mg. phosphatide or $(468 \times 66.2)/100 = 310$ mg. phosphatide carbon

524 mg. cholesterol esters contain $(524 \times 82.5)/100 = 432$ mg. carbon

194 " contain $(194 \times 83.9)/100 = 163$ mg. carbon

Total lipid carbon from phosphatides, cholesterol esters, and cholesterol = 905 mg. carbon

Carbon from neutral fat = $1459 - 905 = 554$ mg.

554 mg. carbon corresponds to $(554 \times 100)/76.7 = 723$ mg. neutral fat

Total lipid, 468 (phosphatides) + 524 (cholesterol esters) + 194 (free cholesterol) + 723 (neutral fat) = 1909 mg. per cent

Total lipid calculated by approximate factor = $1.3 \times$ total carbon = $1.3 \times 1459 = 1897$ mg. per cent

EXPERIMENTAL

The results of saponification of a standard solution of cholesterol palmitate are given in Table IV. In Table V values obtained by extraction of the same plasma by different methods are presented. The reflux extraction procedure with the alcohol-ether mixture of Man and Gildea as described in this paper gave the highest yield. In Table VI the results of duplicate extractions of the same plasma with this procedure are given. The quantitative recovery of free cholesterol, cholesterol esters, lipid nitrogen, lipid amino nitrogen, and lipoid phosphorus is presented in Table VII. The lipids were dissolved in alcohol-ether and added to the extraction fluid before the reflux extraction was started. The cholesterol, cholesterol palmitate (prepared according to Page and Rudy (1930)), and a phosphatide preparation from egg yolk, freshly prepared according to MacLean and MacLean (1927), were added to separate aliquots of the same plasma.
### TABLE IV

*Saponification of Cholesterol Palmitate*

<table>
<thead>
<tr>
<th>Cholesterol in sample</th>
<th>Cholesterol found</th>
<th>Deviation from theoretical</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>per cent</td>
</tr>
<tr>
<td>0.2490</td>
<td>0.2542</td>
<td>+2.0</td>
</tr>
<tr>
<td>0.2490</td>
<td>0.2528</td>
<td>+1.5</td>
</tr>
<tr>
<td>0.2490</td>
<td>0.2440</td>
<td>-2.0</td>
</tr>
<tr>
<td>0.2490</td>
<td>0.2546</td>
<td>+2.2</td>
</tr>
<tr>
<td>0.2490</td>
<td>0.2528</td>
<td>+1.5</td>
</tr>
</tbody>
</table>

### TABLE V

*Comparison of Results Obtained by Extraction of Same Plasma by Different Methods*

Results are expressed in mg. per 100 cc. of plasma.

<table>
<thead>
<tr>
<th></th>
<th>Plasma A</th>
<th></th>
<th>Plasma B</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gasometric</td>
<td>Colorimetric</td>
<td>Gasometric</td>
<td>Colorimetric</td>
</tr>
<tr>
<td></td>
<td>Total Int.</td>
<td>Total cholesterol</td>
<td>Total Int.</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td></td>
<td>Free cholesterol</td>
<td>Total cholesterol</td>
<td>Free cholesterol</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bloor's procedure; extraction with equal volumes of absolute alcohol and chloroform</td>
<td>603 27.5 *</td>
<td>230.7</td>
<td>763 42.1 *</td>
<td>304.6</td>
</tr>
<tr>
<td>Bloor's procedure; extraction with alcohol-ether (3:1)</td>
<td>597 30.8</td>
<td>199.5</td>
<td>235.5</td>
<td>759 42.0</td>
</tr>
<tr>
<td>Mann and Gildea's reflux extraction with alcohol-ether (3:1), as described in text</td>
<td>600 31.4</td>
<td>202.0</td>
<td>251.1</td>
<td>835 35.1</td>
</tr>
</tbody>
</table>

* Values for total cholesterol were not obtainable in the chloroform-alcohol extract, probably owing to splitting of the chloroform during the saponification.
TABLE VI
Comparison of Results Obtained by Duplicate Extractions of Same Plasma by Mann and Gildea's Reflux Procedure

Values represent mg. per 100 cc. of plasma.

<table>
<thead>
<tr>
<th>Material</th>
<th>Total fat</th>
<th>Total cholesterol</th>
<th>Free cholesterol</th>
<th>Lipid nitrogen</th>
<th>Total lipid nitrogen</th>
<th>Lipid phosphorus</th>
<th>Phosphatide calculated from phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma A</td>
<td>2061</td>
<td>582</td>
<td>440</td>
<td>6.83</td>
<td>18.06</td>
<td>11.65</td>
<td>274</td>
</tr>
<tr>
<td></td>
<td>2072</td>
<td>595</td>
<td>445</td>
<td>6.44</td>
<td>17.44</td>
<td>11.51</td>
<td>271</td>
</tr>
<tr>
<td>Plasma B</td>
<td>1360</td>
<td>1342</td>
<td>1305</td>
<td>1358</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE VII
Recovery by Gasometric Procedure of Cholesterol, Cholesterol Esters, Cephalin Nitrogen, Lipid Nitrogen, and Lipoid Phosphorus Added to Plasma

Values represent mg. per 100 cc. of plasma.

<table>
<thead>
<tr>
<th></th>
<th>Free cholesterol</th>
<th>Total cholesterol</th>
<th>Lipid amino nitrogen*</th>
<th>Lipid nitrogen*</th>
<th>Lipoid phosphorus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original</td>
<td>67.5</td>
<td>162.6</td>
<td>2.64</td>
<td>12.70</td>
<td>3.51</td>
</tr>
<tr>
<td>Added</td>
<td>296.3</td>
<td>105.4†</td>
<td>3.26</td>
<td>10.97</td>
<td>25.40</td>
</tr>
<tr>
<td>Calculated</td>
<td>305.8</td>
<td>268.2</td>
<td>5.90</td>
<td>23.67</td>
<td>28.91</td>
</tr>
<tr>
<td>Found</td>
<td>296.9</td>
<td>274.1</td>
<td>6.18</td>
<td>22.84</td>
<td>28.47</td>
</tr>
<tr>
<td>Deviation, mg. per cent</td>
<td>8.9</td>
<td>+6.9</td>
<td>+0.28</td>
<td>-0.83</td>
<td>-0.44</td>
</tr>
<tr>
<td>&quot; per cent</td>
<td>-2.91</td>
<td>+2.20</td>
<td>+4.74</td>
<td>-3.50</td>
<td>-1.52</td>
</tr>
</tbody>
</table>

* The lipid amino nitrogen, total lipid nitrogen, and lipoid phosphorus were added in the form of phosphatide prepared from egg according to MacLean and MacLean (1927).
† The cholesterol ester preparation used for addition was cholesterol palmitate, prepared according to Page and Rudy (1930).

Average Percentage Errors

The average percentage errors in duplicate analyses of the same plasma extract are the following: total lipid, 0.50; free cholesterol, 0.50; total cholesterol, 2 (error chiefly incurred in saponification); total lipid nitrogen, 0.75; lipid amino nitrogen ("cephalin nitrogen"), 3; and lipoid phosphorus, 0.75.
As mentioned in the introduction to this paper it was not found possible to obtain quantitative results by applying the acetone-MgCl₂ precipitation of Bloor to the small amounts of phosphatide ordinarily used for the gasometric carbon method. Only about 85 per cent of the phosphatide carbon was recovered after precipitation of samples containing about 0.5 mg. of phosphatide. Our results in this respect agree with the observations of Boyd. When larger samples were used for precipitation, however, as described by Bloor, the precipitation of the phosphatide was found to be practically complete, with standard solutions of pure phosphatides, with mixtures of pure phosphatides and other lipids, and with extracts of plasma, red blood corpuscles, and tissues. We were thus able fully to confirm Bloor's statement that, under the conditions prescribed by him, only a small fraction of the phosphatide (in our analyses approximately 2.2 per cent) escapes precipitation. In preparation of the samples for precipitation the precaution of avoiding heating of the alcohol-ether extract residue above 60° was strictly observed. Also filtration of the petroleum ether extract through a sintered glass filter was preferred to separation of impurities by centrifugation and decantation.

It was soon found, however, in analyses of plasma extracts, that if the acetone-MgCl₂ precipitate was redissolved in moist ether, according to Bloor's directions, the amount of lipid in the final ether solution was definitely less than the amount of phosphatide calculated from the phosphorus content of the original petroleum ether extract (see Table VIII). The precipitation of the phosphatides by acetone-MgCl₂ was practically complete, but part of the precipitated phosphatide did not redissolve in moist ether, in spite of the fact that liberal time and care were used in the attempt to bring all the precipitate into solution.

The question arose whether this insoluble portion represented a definite lipid fraction. The phosphatides in the petroleum ether extract from a large volume of human blood were therefore precipitated with acetone-MgCl₂ according to Bloor. As much as possible of the precipitate was redissolved in moist ether. A residue remained and was dissolved in hot alcohol. Aliquots of the alco-
holic solution were analyzed for nitrogen and phosphorus. The N:P ratio found was 0.875 (instead of the usual ratio for cephalin and lecithin of 0.438), showing this fraction to be a diaminomonophosphatide.

**TABLE VIII**

*Comparison of Phosphatide Values of Plasma Obtained by Bloor's Isolation Method and Oxidative Procedure with Values Calculated from Gasometric Phosphorus Estimations Performed on Same Petroleum Ether Extracts*

<table>
<thead>
<tr>
<th>Hospital No.</th>
<th>Acetone-MgCl₂ precipitate, redissolved in moist ether, determined by Bloor's oxidation</th>
<th>Total precipitate calculated as gasometric phosphorus × (100/4.17)</th>
<th>Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg. per 100 cc.</td>
<td>mg. per 100 cc.</td>
<td>mg. per 100 cc.</td>
</tr>
<tr>
<td>8634</td>
<td>350</td>
<td>476</td>
<td>116</td>
</tr>
<tr>
<td>7049</td>
<td>484</td>
<td>549</td>
<td>65</td>
</tr>
<tr>
<td>8740</td>
<td>442</td>
<td>530</td>
<td>88</td>
</tr>
<tr>
<td>8520</td>
<td>137</td>
<td>197</td>
<td>60</td>
</tr>
</tbody>
</table>

**TABLE IX**

*Analyses of Egg Phosphatide Solution, Concentrated to Dryness below 60° and Redissolved in Petroleum Ether*

<table>
<thead>
<tr>
<th>Total phosphatide carbon in sample</th>
<th>Phosphatide carbon not pptd. by acetone-MgCl₂</th>
<th>Phosphatide carbon precipitated by acetone-MgCl₂</th>
<th>Phosphatide carbon accounted for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td></td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>1.331</td>
<td>0.024</td>
<td>1.80</td>
<td>1.244</td>
</tr>
<tr>
<td>1.331</td>
<td>0.027</td>
<td>2.03</td>
<td>1.234</td>
</tr>
<tr>
<td>1.331†</td>
<td>0.018</td>
<td>1.35</td>
<td>1.236</td>
</tr>
</tbody>
</table>

* Calculated from gasometric phosphorus analysis (P × (66.2/4.17)). If the supernatant fluid contained non-phosphatide impurities, the carbon would be higher than calculated from the phosphorus. Possibly error from this source causes the 5 per cent deficit in total carbon accounted for (last column).

† To this sample were added 2.1 mg. of cholesterol and 4.0 mg. of palmitic acid.
In Table IX the results of precipitation of a prepared solution of phosphatides with acetone-MgCl$_2$ are given. The phosphatide preparation was prepared from egg yolk according to MacLean and contained both cephalin and lecithin. The analyses were performed by the gasometric carbon method.

In Table X the results of analyses of extracts of plasma, red blood corpuscles, and liver tissue by acetone-MgCl$_2$ precipitation and by gasometric phosphorus determination are given. The

**Table X**

Analyses of Blood and Tissue Extracts Obtained by Refluxing with Alcohol-Ether, Concentrating Filtrate to Dryness below 60°, and Extracting Residue with Petroleum Ether

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount of material represented in extract sample</th>
<th>Phosphatide pptd. by acetone-MgCl$_2$</th>
<th>Total phosphorus in petroleum ether extract per 100 cc or gm. material</th>
<th>Ratio, mg. phosphatide to mg. P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Part soluble in moist ether</td>
<td>Residue insoluble in moist ether</td>
<td>Caron calculated as $a + b$</td>
</tr>
<tr>
<td>Human plasma</td>
<td>1.125 cc.</td>
<td>0.998 mg. C</td>
<td>0.184 mg. C</td>
<td>1.182 mg. C</td>
</tr>
<tr>
<td>&quot; red blood cells</td>
<td>0.450 mg. C</td>
<td>0.603 mg. C</td>
<td>0.051 mg. C</td>
<td>0.714 mg. C</td>
</tr>
<tr>
<td>Dog liver</td>
<td>1.105 gm.</td>
<td>2.011 mg. C</td>
<td>0.351 mg. C</td>
<td>2.362 mg. C</td>
</tr>
</tbody>
</table>

* Lipid calculated as carbon $\times$ (100/66.2).

† "Phosphatide" = (100/66.2) $\times$ carbon in precipitate obtained with acetone and MgCl$_2$; $P$ = total phosphorus in petroleum ether solution.

petroleum ether extracts were prepared as described previously in this paper.

In the analyses of Table X an average of 2.2 per cent (in no case over 3 per cent) of the total petroleum ether-soluble phosphorus was found in the supernatant fluid after the acetone-MgCl$_2$ precipitation. This observation, that the petroleum ether-soluble phosphorus in extracts of plasma, red blood corpuscles, and liver tissue is precipitated to the same extent (97.8 per cent) as standard solutions of phosphatides, is evidence that all this extract phosphorus represents phosphatides, and that the petroleum ether extract
prepared under the conditions outlined for the method does not contain other phosphorus compounds in measurable amounts.

If the above phosphatide to phosphorus ratios are corrected for the small phosphatide fraction left in the supernatant fluid, the ratios for plasma, red blood cells, and liver tissue become respectively 23.51, 24.41, and 22.70, and give an average ratio of 23.54.

In order to calculate phosphatides from the phosphorus of the petroleum ether extract the phosphorus values should therefore be multiplied by 23.5. The lipid figures obtained from the petroleum ether-soluble phosphorus are definitely higher than those obtained from the acetone-MgCl₂ precipitate redissolved in ether by the procedure of Bloor, because the total phosphorus values include the diaminophosphatide fraction which, after the precipitation, is insoluble in moist ether, and also include the small portion of phosphatide which, because of slight but measurable solubility, escapes precipitation with acetone-MgCl₂.

Our corrected phosphatide to phosphorus ratio of 23.5 found in plasma approximates the theoretical ratio, 23.98 for stearyllinolyl cephalin. Results to be reported later show in fact that the lipid amino nitrogen of human plasma is sufficiently high to indicate that nearly all the phosphatide may be cephalin.

Note on Application of Gasometric Carbon Method to Analyses of Phosphatides Precipitated with Acetone-MgCl₂ by Bloor's Procedure

Although the phosphatides are more exactly estimated from the phosphorus content of the petroleum ether extract, the fact that 98 per cent of the phosphatides can be precipitated with acetone-MgCl₂ makes the precipitation procedure also applicable, provided phosphatide samples of sufficient size are used, as described by Bloor, and provided also that the phosphatide fraction insoluble in moist ether is included in the combustion. Comparison of the carbon of the acetone-MgCl₂ precipitate with the total petroleum ether-soluble phosphorus may prove of value if knowledge of the C:P ratio in a phosphatide mixture is desired.

In determining the amount of phosphatide in the acetone-MgCl₂ precipitate we have found it advantageous to replace Bloor's dichromate oxidation and titration method by the gasometric carbon estimation. The possibility of error from reduction of some of the
dichromate by HCl liberated from MgCl₂ in the precipitate does not affect the gasometric carbon method. This source of error is avoided when, as in Bloor's method, only the phosphatides redissolved from the precipitate by moist ether are oxidized. The error might, however, attain importance if, in order to include the diaminophosphatide, the whole precipitate with its magnesium chloride were subjected to the Bloor oxidation.

The following procedure for estimation of the precipitated phosphatides by gasometric carbon determination was found satisfactory.

A sample of filtered Petrol Extract A, containing about 2 mg. of phosphatides, is measured into a combustion tube and concentrated to 2 cc. volume. The precipitation with acetone-MgCl₂ and the subsequent washing of the sample are carried out exactly as described by Bloor.

If a special manometric Van Slyke-Neill chamber calibrated at 10 cc. volume (Van Slyke, Page, and Kirk, 1933) is available, the total phosphatide precipitate can be burned as one sample. The small amount of acetone left in the combustion tube after the last washing is evaporated in the steam bath. As magnesium chloride tends to crystallize out together with some acetone, it is in this analysis especially important to observe the precaution of adding a few drops of distilled water when the contents of the tube are apparently dry, and to continue the heating in the steam bath until also the water has evaporated. The chlorine vapors which are liberated during the combustion do not interfere with the analysis. Most of the vapors are absorbed in the moisture which collects in the bulbs of the connection tube during the combustion. A combustion period of 2.5 minutes is sufficient.

If only the ordinary Van Slyke-Neill chamber, calibrated at 2 cc. volume, is available, as much of the precipitate as will dissolve in moist ether is brought into solution and the ether is transferred to a 10 cc. volumetric flask. In order to obtain an absolutely clear and homogeneous solution a few drops of absolute alcohol are added to the moist ether solution before the sample is made up to volume. 3 cc. aliquots of the moist ether solution are pipetted into combustion tubes and the solvent is evaporated. The residue in the combustion tube in which the precipitation was performed, consisting of the phosphatide fraction insoluble in moist ether, is
likewise evaporated to dryness and the carbon content determined gasometrically. The total phosphatide value of the precipitate is easily calculated from the sum of the non-volatile carbon in the moist ether solution and in the magnesium chloride residue.

SUMMARY

Microgasometric methods are described for total lipids, total and esterified cholesterol, phosphatides, lipid amino nitrogen (cephalin), and total lipid nitrogen. The principles of these methods are outlined in the introduction.

For total lipid estimation alone, 0.2 cc. of plasma suffice for duplicate analyses.

For complete estimation in duplicate of the different lipid fractions 3 cc. of plasma are required.

The practice of estimating blood phosphatides in the solution obtained by precipitating them with acetone-MgCl₂ and redissolving in moist ether, has been found to give low results, because of the presence of a diaminophosphatide which does not redissolve in moist ether. If the entire acetone-MgCl₂ precipitate is determined by carbon combustion, however, the results agree with those of the total lipid phosphorus estimation.⁴

BIBLIOGRAPHY


⁴ The special apparatus required for the methods described in this paper can be obtained from Eimer and Amend, New York.
GASOMETRIC
MICRODETERMINATION OF LIPIDS IN
PLASMA, BLOOD CELLS, AND TISSUES
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