CXLIX. A MICROMETHOD FOR THE DETERMINATION OF THE FATS AND LIPINS OF BLOOD.

By JOHN ALEXANDER MILROY.

*From the J. C. White Department of Biochemistry,
The Queen's University, Belfast.*

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THE chief micromethods which have been employed for the estimation of the fats and lipins are the oxidation method of Bang [1918] and Bang and Blix [1927], the nephelometric method [Bloor *et al.*, 1922], and the microtitration method [Stewart and White, 1925]. Bang's method has been recently modified by Bloor [1928]. A large number of papers has been published dealing with modifications of Bang's method. The chief references to the literature are given by Leites [1927].

The technique adopted in the following method is based on the principle of saponifying all compounds in the blood, which contain fatty acids, and of subsequently determining the amount of fatty acids isolated from the products of saponification. The advantages of this procedure are discussed fully by Leathes and Raper [1925], who also give references to the literature.

The original object of the following investigation was to apply Strebinger's [1919] method of oxidation to the estimation of the fats and lipins of blood. Strebinger has proved that the method gives accurate results with chemically pure substances; but, in the course of my own work, the method was found to be a laborious and exacting one when applied to the estimation of the fats and lipins in blood. All possibility of accidental contamination with any organic material such as fibres of filter paper must be excluded. Washed asbestos, or Jena glass tubes with filtering discs of fritted glass of porosity < 7 had to be used instead of paper. The method is therefore not well adapted for routine work.

A colorimetric method was therefore tried. This method is based on the formation of a coloured salt of the isolated fatty acids of the blood with the base of a dye-stuff. A number of basic dyes belonging to the triphenylmethane series was tried for this purpose. The base of malachite green has the advantage of being colourless in solution, while its salts are green, so that there is only one colour change involved in salt formation; but the interaction of the base with fatty acids is a slow process even when hastened by heat. Ultimately the base of Nile-blue hydrochloride, first used by Lorrain Smith [1908, 1911] for staining neutral fats and fatty acids, was found to give the best results. The colour change due to salt formation with fatty acid occurs rapidly.

TECHNIQUE OF METHOD.

The Nile-blue hydrochloride used was extracted with ether and with xylene for 12 hours at room temperature. These solvents acquired only a faint red colour with yellow fluorescence; the oxazine dye therefore contained mere traces of the oxazone [Thorpe, 1907]. The 0.16 g. of Nile-blue hydrochloride was then dissolved in alcohol in a 500 cc. volumetric flask. Excess of silver oxide precipitated from 0.25 g. of silver nitrate by the addition of caustic alkali was washed alkali-free with water, dehydrated with absolute alcohol, and finally added as a suspension in alcohol to the solution of the dye-stuff. The solution was then made up to 500 cc. with redistilled absolute alcohol, thoroughly shaken and allowed to stand in the dark at room temperature until all the Nile-blue hydrochloride had been decomposed, and the solution had acquired the pure red colour of the free base. The solution was then filtered from silver chloride and excess of silver oxide through a Jena glass filter tube with fritted glass disc of porosity 5-7. When filtered through paper part of the dye is adsorbed, forming the blue coloured salt. The solution had a pure red colour, and no yellow fluorescence could be detected. A solution of suitable concentration to serve as an indicator for the estimation of the fatty acids isolated from about 0.1 cc. of blood was obtained by diluting part of this filtrate 1 in 10 with pure absolute alcohol. The diluted solution was stored in a brown bottle in the dark, and was found to retain its colour unaltered for several months, no yellow fluorescence being detectable. Since the molecular weight of Nile-blue A ($C_{20}H_{20}ON_3Cl$) is 353, the diluted alcoholic solution of the dye base is less than 0.0001 *M*.

Estimation of total fatty acid content of blood.

I. Bloor, Pelkan and Allen's method [1922] was adopted for the extraction of the fats and lipins of blood. 2 cc. portions of the alcohol-ether filtrate (= 0.1 cc. blood) were measured from a 10 cc. burette graduated in 0.02 cc. into test-tubes of about $\frac{3}{4}$ inch diameter. 0.5 cc. of 0.1 *N* alcoholic potash was added to each test-tube, the test-tubes were immersed in a beaker of water heated on an electric hot plate and the contents evaporated to dryness. The tubes were then placed in a drying oven at 80-85° for half an hour to remove traces of alcohol. The dry saponified residue was rendered acid with 0.1 cc. *M* phosphoric acid, care being taken in delivering the acid from a microburette that no drops were left adherent to the upper parts of the test-tubes. The tubes were then rotated so that the phosphoric acid was brought into contact with the whole surface of the dry residue, and were placed in a drying oven at 80-85° until the solution in each had been reduced in volume to a drop. 10 cc. of pure light petroleum of boiling-point 50-60° (redistilled from 30 % aqueous caustic potash) were added to extract the fatty acids and the solution was heated to the boiling-point, allowed to cool, and filtered through fat-free filter paper of 5.5 cm. diameter into test-tubes of accurately measured bore.

The tubes used were of resistance glass, and of 17 mm. bore as measured by a graduated cone reading to 0.1 mm. The acid residue was washed thrice with about 2 cc. portions of light petroleum, and the washings were filtered into these test-tubes. Care must be taken that none of the aqueous solution of mineral acid is transferred. The tubes containing the filtrates were then immersed in a beaker of water heated on an electric hot plate. The initial temperature of the bath was about 55° and was allowed to rise gradually to 75° in order to prevent loss of solution through bumping. When the greater part of the light petroleum had been driven off, the test-tubes were placed in a drying oven at 80° for about 30 minutes to remove the last traces of solvent. The tubes were then allowed to cool, and 10 cc. (measured from a microburette graduated in 0.02 cc.) of the dilute solution of the base of Nile-blue were added to each. The solutions were thoroughly shaken, and the tubes stoppered with corks previously washed with redistilled alcohol.

Preparation of standards for comparison. Pure palmitic acid (Kahlbaum), m.p. 62.5° (uncorrected), was recrystallised from light petroleum. 0.0641 g. of the recrystallised acid was placed in a 250 cc. flask, dissolved in light petroleum and made up to 250 cc., the solution being 0.001 *M*. Part of this solution was placed in a microburette with stoppered reservoir. The burettes used were either 2 cc., graduated in 0.01 cc., or 10 cc., graduated in 0.02 cc. Seven numbered test-tubes of uniform bore (17 mm.) were placed in a stand, 0.48 cc. of 0.001 *M* palmitic acid was measured into test-tube (1), and then successively (2) 0.58, (3) 0.69, (4) 0.83, (5) 1.0, (6) 1.20, (7) 1.44 cc. into the other test-tubes, the quantities being arranged in a geometrical progression with a common ratio of 1.20. Palmitic acid being a weak acid only converts a fraction of the dye base into the blue coloured cations of the salt. If necessary, the series of tubes may be extended to nine with a progressive increase in the amount of the blue coloured fraction of the dye. The solutions were evaporated to dryness in the water-bath, the last traces of light petroleum being removed by heating the tubes in a drying oven at 80°. 10 cc. of the alcoholic solution of the Nile-blue base were next added to each tube. The test-tubes were then closed with corks previously washed with redistilled alcohol, and sealed with paraffin wax. Their contents were thoroughly mixed and the solutions allowed to stand for ten minutes. If kept in the dark the standard solutions retain their colours unaltered for several days. The colours of the solutions in the test-tubes containing the fatty acids isolated from blood were then compared with those of the standard series. The following concrete example may be given. The solution of the fatty acids isolated from 0.1 cc. of blood was found to have a colour intermediate between tubes (4) and (5) of the standard series. 0.1 cc. blood therefore contained more than 0.00083 and less than 0.001 millimol. fatty acid. An approximate estimate of the fatty acid in the blood was thus obtained with an error of less than 20 %. A more accurate result may be obtained by preparing another standard series of tubes with quantities of palmitic acid in a geometrical progression with a smaller common ratio such

as 1.05 covering the range between 0.00083 and 0.001 millimol. A more convenient but somewhat less accurate method is to add the alcoholic solution of Nile-blue base from a 10 cc. microburette to the tube containing the fatty acid from blood until the colour of the solution in it matches with no. 4 of the standard series. If y be the total volume in cc. of the diluted solution, then the quantity of fatty acid in it is $\frac{0.00083 \times y}{10}$ millimol. The results of the analyses given later are stated as millimol. fatty acid per 100 cc. or, in the case of those obtained by Bang's procedure, per 100 g. blood. The results may also be stated in terms of any of the higher fatty acids, palmitic, oleic, or stearic, by multiplying the result in millimols. by the factor 0.256, 0.282 or 0.284. The total fatty acid content of 100 cc. of defibrinated ox blood was thus found to be 0.83 (4 estimations), 0.82 (3 estimations), 0.80 (2 estimations) millimol. of fatty acid. When Bloor's method is applied to whole blood, Bloor [1928] states that the extraction of fats and lipins may not be quite complete. Hence the foregoing results are possibly too low, but are in good agreement with one another in view of the small quantities of blood analysed.

II. In a second series of experiments Bang's method of weighing the blood for analysis was adopted. About 100 mg. (98.5 to 126) of defibrinated ox or sheep blood were placed on a weighed slip of fat-free absorbent paper, and the weight of blood determined by difference, a torsion balance reading to 1.0 mg. being used. The pieces of paper with absorbed blood were then dried in the air, placed in numbered test-tubes and stored in an evacuated desiccator over calcium chloride.

1. *Determination of the total fatty acid content of the blood.*

This estimation, which gives the total fatty acids present as neutral fats, soaps and lipins, was carried out in the following way. The weighed slips of paper with dried blood were extracted with 10 cc. of boiling absolute alcohol. The tubes containing the alcoholic extracts were allowed to stand overnight. The alcoholic solutions were again boiled, cooled and transferred to a second set of test-tubes. The paper slips with blood were then washed once with alcohol and twice with ethyl ether, and the washings transferred to the second series of tubes. 0.5 cc. 0.1 *N* alcoholic potash was added to each, the tubes were immersed in water in a beaker, heated on an electric hot plate, and their contents evaporated to dryness. The saponified material was then heated in an electric drying oven at 85° for at least 30 minutes to remove the residual alcohol. The higher fatty acids were set free by the addition of 0.1 cc. of *M* phosphoric acid to the saponified residue. The other details of the procedure were the same as those already described in the method for analysing the alcohol-ether filtrate used in Bloor's method.

In order to ensure that all solvents and papers used were free from fats, a blank experiment was carried out with a negative result, the red colour of the alcoholic solution of the Nile-blue base remaining unaltered.

2. *Estimation of fatty acids derived from neutral fats.*

The neutral fats were extracted from the dried blood with light petroleum. Bang [1918] obtained low values for the neutral fat by the oxidation method, varying from 0.01 to 0.05 % in human blood of normal individuals on a mixed diet. For this reason larger amounts of blood were used. The first estimation was carried out with 0.211 g. of ox blood absorbed on two slips of fat-free paper. For the two estimations of neutral fat in sheep's blood, the quantities taken for analysis were 0.363 g. and 0.337 g., the blood samples being absorbed on three slips of paper. The dried blood was boiled in each case with 10 cc. of light petroleum, and the extracts were allowed to stand at room temperature in stoppered tubes for at least 12 hours. The extracts were filtered, and evaporated to dryness. The residues were then saponified with 0.5 cc. of 0.1 *N* alcoholic caustic potash, and 3 cc. of alcohol. The remaining details of the technique were the same as those already described for the estimation of the total fatty acids. Since the concentration of fatty acids isolated from the neutral fat of blood is normally much lower than that of any of the standard solutions of palmitic acid, the quantity *y* cc. of the dilute alcoholic solution of Nile-blue base finally added to obtain matching of the colours was always much less than 10 cc.

In view of the possibility that the low values for fatty acid of neutral fat might be due to incomplete extraction by the light petroleum, the following method of successive extraction with alcohol and light petroleum was tried. The samples of dried blood were first extracted with absolute alcohol, and the residues left after evaporation of the alcohol were extracted with light petroleum. The light petroleum extract was then filtered, and treated in the way already described. The values given in the table are much higher than those obtained by direct extraction with light petroleum. A preliminary extraction with alcohol thus appears to set free part of the neutral fat, or to render it accessible to the solvent action of light petroleum.

A few experiments were also carried out with acetone as solvent. The acetone was allowed to act on the dried blood at room temperature for 24 hours.

Some of the values obtained by the foregoing method are recorded in the following table, the same sample of ox or sheep blood being used for all the determinations.

Millimol. fatty acid per 100 g. blood.

Blood	Alcoholic extract	Light petroleum (direct extraction)	Alcohol and light petroleum extraction	Acetone
Ox	0.83	0.07	0.42	0.49
	0.81	—	0.43	—
	0.82	—	—	—
Sheep	0.88	0.12	0.45	0.52
	0.90	0.12	0.43	0.53
	0.91	—	0.44	0.50
	0.88	—	0.43	—

Purification of solvents.

All solvents must be completely freed from acids and fats. Light petroleum (B.P. 50–60°) ("analytical reagent and free from aromatic hydrocarbons") was thoroughly washed with a concentrated aqueous solution of potassium hydroxide, and distilled from this solution in an all-glass still. The absolute alcohol was redistilled from calcium oxide. The ethyl ether was washed with an aqueous solution of caustic alkali, freed from aldehyde and acids with solid potassium hydroxide and finely powdered potassium permanganate, decanted into a second bottle and dehydrated with calcium chloride. It was finally distilled. The acetone was washed for several days with a saturated solution of potassium carbonate, and distilled. All distillations were carried out in all-glass stills, with ground in glass connections.

Each solvent was then tested in the following way. About 20 cc. were evaporated to dryness in a test-tube, and 10 cc. of the alcoholic solution of Nile-blue base were added. The red colour of the solution of the base remained unaltered. This simple method enables one to ascertain whether any given solvent can be used with safety.

All glassware was washed with a solution of chromic acid in concentrated sulphuric acid. Washing with potassium permanganate solution (about 2 %), followed by the removal of any adherent manganese dioxide with hydrochloric acid, was found later to be an equally effective and more convenient method [Horst, 1921].

DISCUSSION.

The present communication deals only with the technical details of the method. Any discussion of the significance of the experimental figures would be premature, until a much larger number of comparative analyses has been completed. The writer intends to apply the method to the investigation of human blood.

SUMMARY.

1. A method for the estimation of the total fatty acids in small quantities of blood has been described.
2. By selective extraction of the dried blood with different solvents, the fatty acid content of some of the components in the mixture of substances extractable by alcohol can be determined.

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