CXCVII. A MODIFICATION OF THE METHOD OF STEWART AND WHITE FOR THE DETERMINATION OF BLOOD-FAT, WITH OBSERVATIONS ON SEVERAL SPECIES IN POSTABSORPTIVE CONDITIONS.

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THE present report contains a study of the method of Stewart and White [1925] for the determination of blood-fat, and full details are given concerning a modification which, in our hands, has proved more practicable than their original procedure. Substances interfering with the determination have been examined and the error of the method has been determined. The data also include observations on the fluctuations occurring in a given animal and the variations in the level of blood-fat of a series of animals of several species in the postabsorptive state.

PROCEDURE.

Preparation. 5 cc. samples of oxalated blood are centrifuged for half an hour and 1 cc. of the plasma thus obtained is added to 35 cc. of an alcohol-ether mixture (3 parts 95 % alcohol—1 part ordinary ether). By adding the plasma drop by drop while constantly rotating the flask, a finely divided precipitate is obtained.

Extraction. The flask is plunged in a bath of boiling water. The mixture is boiled for a few seconds, cooled to room temperature and filtered into a 50 cc. volumetric flask. If the fat content of the blood is high, the accuracy is increased by making the alcohol-ether extract up to 100 cc. instead of 50 cc.

Saponification. Aliquot portions (15 cc.) of the filtrate are measured into 50 cc. Erlenmeyer flasks and controls are made on the same volume of alcohol-ether mixture. The mixture is evaporated to about 1 cc., then 5 cc. of 0.1 *N* NaOH are added from a burette and the mixture is again evaporated to approximately 1 cc. In order to complete the saponification, 5 cc. of absolute alcohol are added, and evaporation is continued until 2 cc. of the solution remain. A hot plate is always used for evaporation.

¹ Some of the data of this paper are taken from an essay presented by Hilda Friedman in candidacy for the degree of Master of Science, Yale University, 1928.

Acidification. Liberation of fatty acids. 5 cc. of 0.1 *N* HCl are added. This should exactly neutralise the alkali previously combined and liberate the fatty acids. The mixture is then boiled down to half its volume in order to expel any absorbed CO₂.

Titration. After adding 10 cc. of absolute alcohol, the solution is heated and titrated while hot with 0.02 *N* NaOH, using phenolphthalein as indicator.

Calculation. If 1 cc. of plasma is dissolved in 50 cc. alcohol-ether mixture and 15 cc. of the extract are used, the final aliquot corresponds to $1/50 \times 15$ or 0.3 cc. of plasma. With a final titration value for 0.02 *N* NaOH of *X*, the amount of fat present in 100 cc. of plasma is equal to $X \times 100/0.3 \times 5.38$; 5.38 being the factor when calculating in terms of tripalmitin. In a determination of fat, the titration value of the control is subtracted from that of the unknown and the difference is multiplied by the factor 1793 in order to determine the fat present in the blood sample in mg./100 cc. The result so obtained is then corrected for any change in concentration.

Recovery of the higher fatty acids. Accurately weighed amounts of pure palmitic and oleic acids were dissolved in definite volumes of the alcohol-ether mixture and determinations were made on portions of these solutions. The procedure for determining these samples was similar to directions given above for the treatment of plasma. The recovery of these fatty acids is given in Table I.

Table I. *Recovery of known amounts of fatty acids.*

Fatty acid						Amount present mg./100 cc.	Amount recovered mg./100 cc.	% recovered	Deviation of recovery
Palmitic	...	...	...	...	...	340	337.9	99.4	-0.6
Palmitic	...	...	...	...	...	888	900.0	101.0	+0.1
Palmitic	...	...	...	...	...	622	611.0	98.2	-1.8
Oleic	...	...	...	...	...	340	315.8	92.5	-7.5
Oleic	...	...	...	...	...	240	236.0	98.3	-1.7
Mixture, oleic + palmitic	...	...	...	...	...	410	406.0	98.0	-2.0
Mixture, plasma extract + oleic + palmitic	...	...	...	...	...	376	400.0	106.0	+6.0
Average									±2.52

Interfering substances. A number of observations were made to determine whether lactic or butyric acid, or their sodium salts, or acetone acted as interfering substances in the estimation of fat in the blood. 1 cc. of solution containing 1.2 mg. of lactic acid was dissolved in the alcohol-ether mixture and made up to 50 cc. 1 cc. of solution containing 0.96 mg. butyric acid was similarly treated. Determinations were made on 15 cc. aliquots of these solutions using the same procedure as in the treatment of plasma. Similar determinations were made using sodium lactate, and butyrate, and acetone. None of these substances could be titrated by this method.

Constancy of recovery. It seemed advisable to estimate the change that might occur in blood drawn at short intervals of 2 minutes, and then again at intervals of 2 hours. Results have shown that blood obtained from dogs anaesthetised with amytal and in the postabsorptive state at intervals of

2 minutes and determined separately remained practically unchanged, the average deviation of a single determination being $\pm 0.4\%$. The values of fat in blood taken at 2-hourly intervals for 8 hours, in the case of three such dogs, have shown that there is an average deviation of $\pm 2.2\%$. In the case of unanaesthetised dogs the average deviation of a single determination was $\pm 3.5\%$. Four of these determinations were corrected for total solids and yielded an average deviation of $\pm 3.7\%$ after the correction was made (average percentage deviation = ± 3.6). Repeated observations of the same animals over a period of 6 months are presented in Table II.

Table II. *Repeated observations on the same animal.*

Dog	Number of observations	Average fat value mg./100 cc.	Average % deviation
I	7	663	± 15.30
II	7	692	± 15.00
III	4	720	± 20.60
IV	4	656	± 13.00
V	2	767	± 13.25
VI	2	675	± 32.00
VII	2	691	± 2.91

Values under postabsorptive conditions. The average fat values for normal resting animals under postabsorptive conditions have been calculated from a large number of determinations and are as follows: dog 673, cat 611, monkey 501, rabbit 498, man 481 mg./100 cc. respectively.

DISCUSSION.

The changes from the technique of Stewart and White. The method described above differs in some respects from that originally presented by Stewart and White [1925]. In the first place, we have found it possible to get satisfactory results using 1 cc. of whole blood or 1 cc. of blood-plasma instead of 2 cc. as indicated by Stewart and White [1925]; thus less blood is required. Determinations have been made of the fat content of 1 cc. of whole blood and 1 cc. of plasma from the same blood sample. In some instances there has been a difference between the fat content of the whole blood and that of the plasma. Therefore, blood-plasma instead of whole blood was used whenever possible, since the red blood corpuscles may vary in the course of an experiment and thus yield an erroneous result.

According to Stewart and White [1925], the alcohol-ether extract of plasma is brought to the boil and made up to volume. In order to withdraw the supernatant fluid, the mixture is allowed to stand so that the precipitate may settle. There are several objections to this procedure. Frequently the supernatant fluid is not entirely clear. Again, the fluid withdrawal may not be uncontaminated with the precipitate. Finally, the amount of filtrate obtained is limited by this method of withdrawal. For these reasons, filtration was substituted for decantation as described above. The extraction of lipins with filtering was practically complete because plasma was used. To quote Bloor

[1928], "the statement appearing in the literature and attributed to the author that the alcohol-ether extraction is not complete by this procedure (filtering) applies only to whole blood or corpuscles and not to plasma." A hot plate is preferable to a steam-bath for boiling down the various mixtures after each step in the process.

In the final step, according to Stewart and White [1925], 2 cc. of the final solution (composed of fatty acids and 10 cc. absolute alcohol) is equivalent to 1/10 of the original sample and is transferred and titrated with 0.1 *N* NaOH from a Rehberg burette. In the present modification of the method the total final solution is used for the titration. This avoids any error of transference.

In our analyses a micro-burette, with a very fine tip, and a capacity of 1.5 cc. is used. This burette is graduated in 0.01 cc. divisions, so that it is possible to interpolate to 0.001 cc. Checks within 0.005 cc. are frequently obtained.

It is found that on titrating in the manner suggested by Stewart and White [1925], with CO₂-free alkali, the CO₂ in the air interferes to such an extent as to give uncertain end points. The titration was therefore conducted at the boiling-point as described above. Furthermore, the absolute alcohol itself is so acid as to require almost as much of the alkali for its neutralisation as is necessary for an equal volume of fatty acid solution with the small quantities measured by Stewart and White [1925]. Attempts to rid the alcohol of its acidity by distilling over lime and caustic alkali were unsuccessful. The error due to alcohol is eliminated by the use of control determinations on the reagents.

As larger amounts of the more dilute alkali are used, the error of titration may be lessened. Therefore, 0.1 *N* NaOH was replaced by 0.02 *N* NaOH.

It became necessary to make a correction for changes in concentration of successive samples of plasma from the same animal, for the fatty acids determined by this method are present in the plasma as esters and chiefly in suspension in the form of triglycerides, only a small portion existing in solution as phospholipins [Bloor, 1916, 1, 2; Boyd, 1931]. This correction was made by drying a 1 cc. portion of each sample of plasma to constant weight at 100°. Thus it was possible to estimate any change in the water content of the plasma.

Table I shows the percentage recovery of the original amount of palmitic acid to be 99.5 %, of oleic acid 95.9 %, of a mixture of palmitic and oleic acids 98 %, and of a mixture of plasma extract and palmitic and oleic acids 106 %. The average deviation is ± 2.52 %, and all results are within 5 % of the theoretical values.

Error of method. In our estimations triplicate portions were used to determine the fatty acid content of the blood-plasma sample, and the best two of the three titrations were used for calculation. In this manner, the error of the method, taking the average of 266 triplicate determinations, was found

to be ± 11 mg./100 cc. This value is equal to a deviation of ± 1.6 % from the average for resting dogs (673 mg./100 cc.). For the maximum and minimum values the error is ± 1.1 % and ± 2.8 % respectively.

The average values varied with the species. Those found for man by Wilson and Gildea¹ (481 mg./100 cc.), using the present method are not so different from those of Nicholls and Perlzweig [1927-28] who determined the fat content of human blood using a modification of the method of Stewart and White [1925] and found it to be 426 mg./100 cc. Stoddard and Drury [1929], using their own method, also worked on human beings and reported an average fatty acid content of 294 mg./100 cc. in a series of 10 blood samples. Bloor *et al.* [1922] employed his nephelometric method and found an average value of 590 mg./100 cc. for dogs, while with his newer oxidation method [1928] he obtained slightly higher values. All these investigators used methods based on that of Kumagawa and Suto [1908] who employed a solvent for fatty acids to extract them from the blood, the subsequent steps, however, differing according to the various modifications.

The constancy of recovery obtained by our method has been shown above.

Results obtained by other methods yield the same general implications as to the deviations in blood-fat. The average deviation of a series of animals observed by Terroine [1914-15] at weekly intervals was ± 4.5 %, but the widest differences were ± 30 %. Bloor [1914] found the greatest variation of any animal to be ± 12 %, while the average was ± 6 %. Patterson [1927] noted that the fasting fat content for given individuals over a considerable period of time exhibited but slight variations, the widest being ± 6.5 % with an average deviation of ± 3 %.

Observations of a series of animals under postabsorptive conditions. In a large number (266) of observations on various animals, the minimum fat value for a normal resting dog was found to be 428 mg./100 cc., while the maximum was 906 mg./100 cc. This indicates a variation of 111 % for different individuals. Terroine's [1914-15] results were not unlike ours, as he found the extreme values to be 314 mg./100 cc. and 669 mg./100 cc. respectively, the variation being, therefore, 113 %. According to Bloor [1914], the average deviation of any animal in his series was ± 7 %, the greatest being ± 14 %, while Nicholls and Perlzweig [1927-28] give the blood values as varying between 333 and 492 mg./100 cc. in human subjects.

SUMMARY.

A modification of the method of Stewart and White [1925] for the determination of blood-fat is presented. The experimental error of this method is ± 11 mg./100 cc., which represents a percentage deviation of ± 1.6 , since 673 mg./100 cc. is the average value for dogs in the postabsorptive state. The level of blood-fat varied by ± 3.6 % in the course of an observation period

¹ Personal communication from Doctors E. F. Gildea and W. R. Wilson, who obtained six and three results respectively on normal human subjects.

of 8 hours, and therefore a difference of 40 mg./100 cc. has been considered significant. It was found that the values for blood-fat in postabsorptive conditions not only varied widely (111 %) in a given species (dog), but that the average values differed in various species. From an examination of the interfering substances it has been revealed that the method does not estimate the fatty acids which exist in the blood as soaps, but only those in ester combination.

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