CXXVI. THE ESTIMATION OF FAT IN BLOOD.

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IN view of the large number of pathological conditions in which the fat content of the blood is increased, and of our relatively meagre knowledge of fat transport and metabolism, it is eminently desirable that an accurate, and, as far as possible, non-subjective method be devised for the estimation of fatty acids in blood. The methods at present available fall into two classes: first, nephelometric, of which that of Bloor [1914] is typical; second, oxidative, of which those of Gade [1919] and Bang [1918] are typical.

Nephelometric methods are open to several objections. The personal factor is comparatively great, and the time factor is important since the suspension of fat flocculates at a measurable and varying rate [Blix, 1924]. Moreover, Bloor's method estimates both cholesterol and fatty acids, the latter being ultimately determined by difference. This last objection, of course, does not extend to the more recent method of Bloor, Pelkan and Allen [1922].

In Gade's method [1919] the fat extracted from the dried blood by ether is allowed to react for a definite time with an excess of potassium permanganate, the excess being then determined by the addition of excess of oxalic acid and back-titration with permanganate. The method is somewhat cumbersome, the reaction with permanganate must be carefully controlled with regard both to time and temperature, and an empirically determined correction has to be applied. Bang's method [1918], in which the fat is oxidised by chromic acid, the excess being determined by allowing it to act on potassium iodide and titrating the liberated iodine with thiosulphate, gave good results in the hands of Gade, as reported in his comprehensive review [1919]. As in the case of Bloor's method, however, it gives both cholesterol and fat, and the true fat content is obtained by estimating the cholesterol separately and subtracting.

Hitherto, the small amount of fatty acid obtainable from the 2 cc. of blood usually available for routine estimations has rendered it impossible to utilise, with any degree of accuracy, the hydrolytic method of estimation in which the fat is saponified by sodium hydroxide and the excess alkali titrated with acid. The new micro-burette described by Rehberg [1925], however, delivers

0.10 cc. of solution and can be used for titrations requiring fractions of that amount with at least as great accuracy as is attainable with the 10 cc. of the macro-titration. Since 0.10 cc. of $N/10$ NaOH is equivalent to 26.86 mg. of tripalmitin or 29.67 mg. of tristearin, the burette can evidently be used to titrate the fatty acids liberated by hydrolysis from the fat contained in only 1.0 cc. of normal blood.

Acting on these considerations, we attempted to estimate known amounts of synthetic tripalmitin. This was prepared from pure palmitic acid and glycerol by the method of Scheij [1899], purified by repeated extraction with alcohol, and many recrystallisations from alcohol-ether mixture, and finally from pure ether. Quantities were taken corresponding to the amounts present in 1.0 cc. of normal and lipaemic bloods (3–18 mg.). For this purpose the fat was dissolved in pure anhydrous ether and the requisite quantities of the solution were measured into 50 cc. round-bottomed flasks. After evaporation of the ether, 5 cc. of $N/10$ NaOH and 5 cc. of absolute alcohol were added, and the mixture allowed to evaporate almost to dryness on the water-bath, a process which occupied about 2 hours. We found it advisable at this stage not to attempt the direct titration of the excess of sodium hydroxide, for during the manipulation before and after hydrolysis a small amount of carbon dioxide is inevitably absorbed. Since the volumes involved in the titration are so small, and an indicator such as phenolphthalein must be used, this introduces a serious error. After hydrolysis we therefore added to the mixture 5 cc. of $N/10$ HCl. This exactly neutralised the whole of the sodium hydroxide, and liberated the fatty acids which had been produced during the hydrolysis. We then boiled down to about 1 cc. in order to drive off the absorbed carbon dioxide. The residual liquid was transferred quantitatively to a 10 cc. volumetric flask, absolute alcohol being used for the washings and for making up the volume to 10 cc. 1 cc. of this final solution, corresponding to one-tenth of the amount of fat originally taken, was titrated with $N/10$ sodium hydroxide from the Rehberg burette. The sodium hydroxide used in the titration must, of course, be free from carbonate. In our experiments, it was kept in a paraffined bottle provided with a syphon and tap, the air inlet being guarded by a soda-lime tower. In filling the burette, a short rubber tube connected the burette nozzle and the syphon. In this way the standard NaOH was prevented from coming into contact with the air. The indicator used was phenolphthalein, and a correction was applied for the amount of indicator, volume of fluid, etc. as described by Rehberg [1925].

It cannot be over-emphasised that for these estimations both reagents and apparatus must be accurately standardised. Thus, if the hydrochloric acid be slightly below $N/10$, or if slightly less than 5.0 cc. be added, the fatty acids will be incompletely liberated. Conversely, if acid be added in slight excess, it will remain to be neutralised in the titration. In either case, apparently slight variations may lead to serious errors, notwithstanding the subsequent dilution, since the volume of the titrating fluid is so small. Careful

manipulation is necessary at every stage, but granted this, as our results abundantly show (Table I), not only do duplicate titrations from the same sample agree, but duplicate samples, treated separately throughout, give results within 5 % of the theoretical. We may point out that in several of the duplicate results recorded, one of the estimations has been carried out by each of us. An important advantage of the method is that in case of necessity, as many as eight or nine titrations can be made on each sample.

Table I. *Estimation of fat in pure solutions of tripalmitin.*

Fat present g. per 100 cc.	Titre (corrected)*	Fat found g. per 100 cc.	Error %
0.300	10.3		
	10.8	0.283	5.6
	12.0		
	11.3	0.310	3.3
0.600	20.5		
	20.4	0.596	0.6
	22.8	0.618	3.0
	23.2		
0.800	29.8		
	29.8	0.800	0.0
0.900	34.0		
	34.6	0.921	2.3
1.200	43.8		
	43.6	1.174	2.2
	43.8		
	44.4	1.185	1.2
1.500	53.9		
	53.7	1.445	3.6
1.800	68.9		
	70.0	1.765	2.0
	69.6		
	69.8	1.770	1.7

* The correction derived from macro- and micro-titration of the acid and alkali used, with a standard amount of titration fluid (1 cc.) and a standard amount of indicator, was 0.7.

In extending the method to blood, we have not concerned ourselves with the selective extraction by different solvents of the various constituents of the blood-fat, but have contented ourselves with showing its applicability to the estimation of the total fatty acids of the blood irrespective of the state of combination in which they occur. 2.0 cc. of blood were added with constant shaking to about 30 cc. of alcohol-ether mixture (three parts alcohol, one part ether) in a 50 cc. flask. The mixture was brought just to boiling point, cooled, and made up to 50 cc. with alcohol-ether. After thorough mixing, it was allowed to settle and 25 cc. of the clear supernatant liquid were withdrawn for analysis. This procedure was adopted to avoid the concentration of the extract which may take place, to a varying degree, during filtration. The remainder of the procedure was exactly as described for pure solutions of fat. Table II gives some of the results we have obtained in estimating fat in blood, alone, and with added fat.

Notwithstanding the agreement of duplicate analyses, there still remains the possibility that the extract may contain, in addition to fat and fatty acids, some acidic substances soluble in aqueous alcohol-ether mixtures as well as

in water. In this category would be included such substances as acetoacetic acid, hydroxybutyric acid, etc. These, though present only in traces in normal blood, may occur to an appreciable extent in pathological specimens. They are, however, of low boiling point, and are, moreover, volatile in steam, so that they are removed by the boiling down after the addition of the hydrochloric acid. To ensure that interfering substances are absent, or are present in negligible quantities if at all, we carried out the following experiment. 50 cc. of the alcohol-ether extract from blood sample No. I (Table II), which was a pathological specimen, were evaporated to dryness on the water-bath. The residue was extracted with two 1 cc. portions of distilled water. The aqueous extract, after filtration, was boiled, and titrated with $N/10$ NaOH from the Rehberg burette. The extract was acid to phenolphthalein, indicating the absence of basic interfering substances, but was rendered distinctly alkaline by the addition of 0.004 cc. of $N/10$ NaOH. We may therefore conclude that the alcohol-ether extract contained no interfering acidic substances.

Table II.

No. of blood sample	Fat added g./100 cc.	Found g./100 cc.	Fat content of blood g./100 cc.	Mean	Error %
I. Ox blood	0.900	2.083	1.183	—	1.5
	1.500	2.698	1.198	—	—
	1.500	2.703	1.203	—	0.5
	1.500	2.706	1.206	1.198	0.7
II. Mixed specimen of human blood —pathological	—	1.845	1.845	—	0.5
	—	1.821	1.821	—	2.0
	—	1.872	1.872	—	1.0
	—	1.877	1.877	1.854	1.0
	0.300	2.130	1.830	—	1.5
	0.300	2.122	1.822	—	2.0
	1.200	3.073	1.873	—	1.0
	1.500	3.427	1.927	—	4.0
III. Human blood— normal	—	0.306	0.306	0.312	1.8
	—	0.317	0.317	—	1.8

In this table the fat has been calculated as tripalmitin.

We have attempted to modify the method so as to permit of using much smaller quantities of blood. Even with careful technique, however, the errors in measuring with the ordinary pipette are sufficient to vitiate the results. By substituting for pipettes measuring instruments based on the principle of the Rehberg burette, it would, no doubt, be possible to obtain moderately accurate results with quantities of blood less than 0.5 cc. The method, even as we have used it, calls for very careful work, and can hardly be used as a routine method by the busy technician; it is primarily a method for research. However, the use of a modified Rehberg burette for the measurement of the alkali used in hydrolysis and the acid subsequently added renders the method less delicate and more suitable for routine use.

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