CC. THE DETERMINATION OF TOTAL FATTY ACIDS IN BLOOD.

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THE more important methods at present used for the estimation of fatty acid in blood are oxidimetric [Bloor, 1928] or titrimetric [Stoddart and Drury, 1929; Stewart *et al.*, 1931; Man and Gildea, 1932-33; Smith and Kik, 1933].

Bloor's method may be objected to on the ground that it depends upon the relative solubilities of the fatty acids in light petroleum. It is possible that this solvent will extract oxidisable substances other than fatty acids and cholesterol from the acidified hydrolysate, *e.g.* pigments or their hydrolytic products, bases derived from the phospholipins *etc.*, thus giving high results.

So far as the titration methods are concerned they are open to the criticism (applicable also to Bloor's method) that the extraction of lipoids from blood may be incomplete. In all cases this extraction is accomplished by adding the blood slowly to a mixture of alcohol (36 volumes) and ether (12 volumes). Man and Gildea [1932-33] claim, contrary to the generally accepted opinion, that 30 % of the lipoids may escape extraction in this way. The methods of Stoddart and Drury [1929] and of Stewart *et al.* [1931] depend upon the precipitation of the free fatty acids after hydrolysis and will therefore fail to estimate any soluble fatty acids which may be present. In considering this possible error, it must, of course, be taken into account that the acids are precipitated from sodium chloride solution, and that the presence of this salt will modify the solubility of acids actually soluble in distilled water. Man and Gildea [1932-33] have extracted the filtrate from the fatty acids by means of ether and failed to find any appreciable amount of fatty acid in the extract; since, however, they washed the ethereal extract thoroughly with water to remove mineral acid, this result is of little value. For the same reason the method of Smith and Kik [1933]—extraction of the fatty acids by means of benzene and washing the extract—cannot be accepted as estimating the hypothetical soluble fatty acids. Indeed, this method may well involve a greater loss of fatty acid than does filtration and washing of the precipitate with sodium chloride solution.

Man and Gildea have reported the further surprising result that hydrolysis with NaOH allows the recovery of only 65 % of the lecithin fatty acids, while with KOH 82 % are recovered. This is of importance, since phospholipins appear to account for most of the fatty acid present in blood [Channon and Collinson, 1929]. It is noteworthy in this connection that the exact nature of the blood phospholipins is unknown and that MacLean and MacLean [1927] state that, while lecithin is easily hydrolysed by acid or alkali, the complete hydrolysis of kephalin is much more difficult. Yasuda [1931] has found that cholesteryl esters may be hydrolysed completely by heating for 20 minutes with sodium ethoxide, and the hydrolysis of glycerides should present no difficulties.

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EXPERIMENTAL.

Extraction of lipoids from blood. The normal procedure consists in adding 2 ml. blood slowly to about 30 ml. of the alcohol-ether mixture, raising to the boiling-point, cooling and adding more of the solvent mixture to make the total volume 50 ml. It is just possible that there may be fat-protein complexes which are not disintegrated by merely bringing the blood extract up to the boiling-point of the solvent mixture. As a variant of this procedure therefore we boiled the mixture of blood and solvent under reflux condenser for periods up to 18 hours. We found, in accordance with Man and Gildea [1932-33] that the refluxed extracts appeared to contain more fatty acid than those treated in the usual way. The increase amounted to as much as 40% in some cases and was maximum with about 5 hours' refluxing. The fatty acids are finally estimated by titration with alkali so that, actually, carboxyl groups are determined, and it is possible that the apparent increase may be due, not to an increased extraction of lipid, but to oxidation of any double bonds in unsaturated acids to extra carboxyl groups. That this is actually the case is shown by experiments in which the blood was refluxed in a continuous current of purified hydrogen. Under such conditions, oxidation is impossible, and analyses show that there is no increase in carboxyl groups due either to increased extraction of lipoids or to oxidation of unsaturated acids. Experiments in which the extract, previously separated from the protein precipitate, was refluxed, showed no increase in the titration value, but it must be remembered that when whole blood is refluxed, iron is present and may act as a catalyst for the oxidation. The results of these experiments are shown in Table I. It is noteworthy that even in presence of air, refluxing causes no increase in the lipid phosphorus.

Table I.

All values are in mg. per 100 ml. of whole blood.

Figures in columns (1) are obtained by heating the extract for 30 seconds only. Those in columns (2) are the values for the refluxed extracts.

Experimental details		Total fatty acids		"Lipoid phosphorus"	
Atmosphere	Length of time of refluxing (hours)	(1)	(2)	(1)	(2)
Air	1	282	331	—	—
Air	2	285	351	11.9	11.4
Air	3	299	421	11.7	11.4
Air	4.5	370	451	—	—
Air	5	311	405	11.6	11.8
Air	12	261	392	9.6	9.8
Air	18	279	404	12.0	12.6
Hydrogen	4	273	272	10.4	9.6
Hydrogen	4	418	415	12.0	11.9
Hydrogen	5	370	363	—	—

Since an increase of 45%, which generally occurs after 5 or more hours' boiling, could be shown gravimetrically, an attempt was made to weigh the fatty acids obtained from a comparatively large volume of blood. After the washed fatty acids had been extracted with alcohol, the solution contained varying amounts of sodium chloride depending upon how thoroughly the filter-paper had been drained. Aliquot portions of the extract were evaporated in a platinum crucible, dried in the steam-oven and weighed. Incineration of the organic matter was carried out at as low a temperature as possible in order to avoid loss of chloride by volatilisation, and after cooling the weight of the chloride was found. The difference gives the weight of fatty acids

plus cholesterol in both the ordinary and in the refluxed specimens. Owing to the difficulty of evaporating alcohol-water solutions containing sodium chloride and the comparatively small differences in weight, these experiments yielded no reliable results. They may be instructive, however, if large quantities of animal blood are available.

As a further control on the completeness of the alcohol-ether extraction, we carried out a series of experiments in which samples of the same blood were extracted with different solvents including 1:1 alcohol-chloroform, pure acetone, and 1:1 alcohol-light petroleum (B.P. 40–60°). While acetone, as expected, owing to the insolubility of the phospholipins in it, gave low results, it is remarkable that both alcohol-chloroform and alcohol-petroleum gave results identical with those obtained using alcohol-ether as the solvent. Such results are shown in Table II. In no case did the alternative solvent give a significantly higher value for the fatty acid than the ordinary alcohol-ether mixture, and the identity of the results with all solvents except acetone is strong evidence that the extraction is practically complete.

Table II.

All values are expressed in mg. per 100 ml. of whole blood.

	Alcohol-ether	Alcohol-chloroform	Alcohol-light petroleum	Acetone
Blood sample No. 1:				
Total fatty acids	300	303	309	232
"Lipoid phosphorus"	10.6	10.7	11.3	7.90
Total cholesterol	132	136	138	—
Free cholesterol	83	86	—	73
Blood sample No. 2:				
Total fatty acids	298	298	286	—
"Lipoid phosphorus"	11.5	11.45	11.6	—

Hydrolysis of the extracted lipoids. Alcoholic NaOH and sodium ethoxide are in general use for the saponification of lipoids extracted from blood. Both Stoddard and Drury [1929] and Stewart *et al.* [1931] have used the former, and the following experiments were designed to test the completeness of the saponification by this reagent. Normally, an aliquot of the alcohol-ether extract of blood is mixed with 5 ml. of *N*/10 NaOH and the mixture, after being taken down to about 1 ml. on the hot-plate, is finally evaporated to dryness in the steam-oven—care being taken to avoid prolonged overheating after the soaps are dry. Hydrolysis may be extended by the addition of 5 ml. water *plus* 5 ml. alcohol when the mixture has boiled down almost to dryness, and this process may be repeated *ad lib.* Table III shows the effect of varying numbers of such hydrolyses, compared with the result normally obtained after a single hydrolysis.

Table III.

Total fatty acids are expressed in mg. per 100 ml. of whole blood.

Case	Number of hydrolyses		
	1	2	3
Mixed blood	326	336	—
D.B.	279	—	279
Mixed blood	404	—	398
H.M.	237	229	—

It is at once obvious that there is no object in prolonging the hydrolysis beyond the ordinary time, and since at the end of each hydrolysis the alkali becomes fairly concentrated, it can safely be assumed that saponification is complete. Furthermore, it appears that so long as the fatty acids

remain in an aqueous alkaline medium, there is no tendency towards oxidation of the unsaturated acids. If the soaps be left in the steam-oven for any considerable time after they are dry, there is a large increase in the number of carboxyl groups present, as demonstrated in the volume of alkali required for their neutralisation. In an endeavour to avoid the possibility of oxidation occurring at this stage, the hydrolysate was taken down to 1 ml., the flask was filled with hydrogen and evacuated, and the soaps were dried at 70–90° on a water-bath. Control experiments by the ordinary method showed that such a modification gave definitely lower results, but this is to be ascribed to a loss of material and not to reduction in the amount of any oxidation which may have occurred in the controls. During the process of drying the soaps *in vacuo*, it is impossible to avoid frothing, and when dry, the soaps are distributed round the sides of the flask in such a manner as to prevent the action of the HCl in liberating fatty acid quantitatively. However desirable such a modification would appear, it has been found impossible to overcome the defects in such a manner as to produce a reliable routine method along these lines. When the soaps are allowed to dry for periods of 24 hours, there is a variable increase in the number of carboxyl groups, but it is usually of the order of 50–75%. Such variation is to be expected, since it will depend upon the iodine value of the fatty acids.

If care be taken to avoid oxidation of the fatty acids while drying, by removing the flasks as soon as all moisture has disappeared, duplicate analyses may be regularly obtained to within 2% by Stewart, Gaddie and Dunlop's method.

Precipitation of the free fatty acids. Excess of HCl used to liberate the fatty acids from the soaps appears to have little effect upon the final titration. As a routine, we have added sufficient $N/10$ HCl to make the mixture definitely acid to litmus. Even an extra 1 ml. of $N/10$ HCl, however, is without effect on the final result. In order to facilitate the filtration of the precipitated fatty acids it is advisable to add the HCl without agitation and to shake gently only after the mixture has stood for some time. Addition of the acid to the hydrolysate before complete evaporation results generally in the precipitation of the fatty acid in so finely divided a form that filtration without loss becomes almost impossible.

The hypothetical water-soluble fatty acids. Ordinarily the precipitated fatty acids are filtered through a fat-free filter-paper and the flask and paper are washed three times (or until the washings are neutral) with 5% NaCl. The filter-paper is then returned to the flask and extracted 4 or 5 times with boiling absolute alcohol. The combined extracts are made up to 10 ml. with alcohol, and 2 ml. of this are at once titrated with $N/10$ NaOH from a Rehberg burette, using phenolphthalein or (better) thymol blue as indicator. The extraction of the precipitated fatty acids from the paper is complete, since further extraction has been shown to give no further amount of fatty acid, and since estimation of known solutions of tripalmitin gives the correct result. There is a "blank" correction in the titration which is due in part to the amount of alkali required to change the colour of the indicator and in part to the presence of traces of acid in the alcohol. Since alcohol absorbs CO_2 on exposure to air, this blank titration must be made under the same conditions as the actual estimation, *i.e.* with freshly boiled alcohol.

There still remains, however, the question of the water-soluble fatty acids which are not estimated by this method. Whilst various authors consider that they constitute about 5% of the total, it is as yet unknown if they are soluble in 5% saline, and NaCl has the general effect of reducing the solubility of fatty

acids in water. In an attempt to solve this problem and eliminate a source of error, experiments have been carried out in which the fatty acids were extracted from the acidified hydrolysate by means of organic solvents, H_2SO_4 being used in place of HCl for acidifying the mixture. Preliminary experiments showed that a dilute solution of H_2SO_4 could be repeatedly extracted by light petroleum without the latter removing any of the mineral acid. This process could also be carried out with benzene, but not with ether. Control experiments have been carried out by extracting H_2SO_4 solution, containing both phosphoric and lactic acids, with these three solvents. Phosphoric acid (from phospholipins) will, and lactic acid may, occur in the alcohol-ether hydrolysate. Extraction of 2 ml. of $N/2$ H_2SO_4 , *plus* 0.5 ml. syrupy phosphoric acid, *plus* 0.5 ml. pure lactic acid, by either light petroleum or benzene yielded a "blank" exactly equal to that on boiled alcohol alone. It is therefore unnecessary to wash the light petroleum or benzene layer after extraction, and thus loss of water-soluble extracted material can be avoided. Ether is useless, even in the absence of lactic acid, for the solubility of water in it is too great and very high "blanks" are obtained.

To test the effect of extraction in place of filtration, the following procedure was adopted. One drop of an aqueous solution of phenol red was added, and the mixture was made just acid with $N/2$ H_2SO_4 . The fatty acids were allowed to stand overnight and were then extracted with 5 ml. portions of light petroleum (B.P. 40–60°, redistilled over solid potassium hydroxide); 4 or 5 extractions were made. The petroleum layer was then evaporated to about 1 ml. on the hot-plate and the last traces of the solvent were removed by a current of purified hydrogen. The mixture of fatty acids and cholesterol was then thoroughly extracted with 3 ml. portions of boiling alcohol and the volume made up to 10 ml. 2 ml. of this solution were then titrated with $N/10$ NaOH in the usual manner. Typical results showing a comparison between this modification and Stewart, Gaddie and Dunlop's method are given in Table IV.

Table IV.

All results are expressed in mg. per 100 ml. whole blood.

Case	Total fatty acids		Percentage increase of (2) over (1)
	(1) by the filtration method	(2) by the extraction method	
F 1	216, 220	225, 222	+2.5
L 1	211, 211	221, 216	+3.6
F 2	199	202	+1.5
R	221	218	-1.4
T	495	516	+4.2
F 3	245	260	+6.1
L 2	247	262	+6.1
M 1	273	286	+4.6
L 3	244	248	+1.6
M 2	245	250	+2.0
		Average increase	+2.58

It is evident that extraction with light petroleum gives results which are slightly higher than those obtained by the filtration method. In all probability, this is due to the fact that the former technique will estimate all the soluble fatty acids with the possible exception of any hydroxy-acids which may be present. The extraction modification differs from Bloor's method only in the final stages—the difference between the two hydrolysing agents being negligible,

as stated above. If the fatty acids can be estimated by the titration method, they must necessarily be estimated by the oxidation procedure also; and furthermore, the former is undoubtedly preferable, since the occurrence of any oxidisable matter in the light petroleum extract, other than fatty acids and cholesterol, is of no account during the titration. This possibility is more than a mere suggestion, for, when the matter soluble in light petroleum is extracted with alcohol, some insoluble matter is always found in suspension. If this be organic, and there are indications that it is, it will seriously interfere with the chromate oxidation, but not with the titration.

It must be admitted, however, that the light petroleum extraction method followed by titration is not entirely satisfactory and, for some unknown reason, duplicates occasionally fail to agree. The cause of these failures has not been discovered, but there are sufficient comparisons with the filtration method in Table IV to show that agreement is not accidental. However, the difference between the extraction method and the filtration is so small that the latter may legitimately be accepted as substantially correct, and in our hands, at least, it provides by far the simpler and more reliable technique.

SUMMARY.

It has been shown that various solvent mixtures other than alcohol-ether will extract the same amount of fatty acid from blood.

The yield of fatty acids is not increased by refluxing the blood with the usual alcohol-ether mixture, but a certain amount of oxidation occurs in the process, giving an increase in the number of carboxyl groups. Refluxing does not increase the recovery of "lipoid phosphorus".

Alcoholic sodium hydroxide and sodium ethoxide are equally good hydrolysing agents, and a single hydrolysis by the former will give the maximum yield of fatty acids.

The filtration method has been compared with a method in which the fatty acids are extracted by light petroleum. It has been shown that the soluble fatty acids in blood amount to less than 3 % of the total.

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