LXXVI. BLOOD-FAT[1].

I. PREPARATION AND GENERAL CHARACTERISTICS.

By HAROLD JOHN CHANNON
AND GEORGE ARTHUR COLLINSON.

From the Department of Experimental Pathology and Cancer Research, University of Leeds.

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UNTIL adequate information regarding the chemical nature of the substances in which fatty acids are present in blood-fat is available, little progress can be made with the problem of the part which phosphatides, cholesteryl esters or other compounds may play in the transport of fat to the tissues. That such information is lacking is demonstrated by the following brief reference to the literature on blood-fat.

The earlier results record figures for the total fatty acids determined in many cases by use of the modification of the Kumagawa-Suto saponification method [1908] devised by Shimidzu [1910]. Latterly, an endeavour has been made, notably by Bloor [1915] and Bloor, Pelkan and Allen [1922] to obtain more detailed information by micro-analysis, in which estimations of total fatty acids, free and combined cholesterol and ether-soluble phosphorus have been carried out; and in this way evidence has been brought to bear on the question of the distribution of the fatty acids in the different combinations in which they may occur in blood. Consideration of the many results recorded shows, however, that very wide variations in any given value exist, and in some cases the findings are directly opposed. It is true that some of these variations may be accounted for by the fact that insufficient attention has been paid to the state of absorption of the animal whose blood has been investigated, but the impression remains that many of the results are due to the employment of methods ill-suited to the problem.

Although this abundance of quantitative data exists, there is a dearth of information concerning attempts to prepare substances in any state of purity from blood-fat. From time to time, claims to have obtained individual substances from blood have been made, such as those of Hürthle [1895] to have

[1] It will be shown during the discussion of the results that glycerides are virtually absent from the blood of the fasting ox and that almost all the material consists of phosphorus-containing compounds of fatty acids, together with cholesterol and its esters. Although "fat" is thus absent, we shall refer to the ether-soluble material throughout as "blood-fat" for the sake of ease of description, for the generic word "lipide" proposed by the International Congress of Applied Chemistry and used by Bloor [1925] has not found acceptance in this country, and the variety of terms used by other workers is confusing not only to the reader but to those engaged in the same field.

prepared the oleyl, palmityl and stearyl esters of cholesterol from serum, and of Bürger and Beumer [1913], who prepared sphingomyelin and kephalin from the stromata of red cells. Bloor [1923] prepared the fatty acids of plasma and corpuscles and studied their iodine values, and the same author [1924] prepared the fat from quantities of plasma up to 2 litres. To an ether solution of this fat he added excess of acetone, hydrolysed the precipitate and studied the iodine values of the fatty acids so obtained. The acetone-ether-soluble fraction was fractionally crystallised from alcohol and each fraction was then saponified and the iodine values of its constituent fatty acids determined. As a result of these latter researches, Bloor suggests that the unsaturated cholesteryl esters present are either the linoleate or a mixture of the oleate with the esters of acids more unsaturated than oleic acid. It will be remembered also that the ether-soluble phosphorus of blood, of which so many estimations have been made, is usually recorded as lecithin, although little information other than that just mentioned is available regarding its nature, obviously on account of the difficulty of working on an adequate scale. Our object, therefore, was to attempt to prepare blood-fat in a quantity sufficient to enable us to submit it to chemical examination with a view to obtaining more definite information as to the compounds in which phosphorus and cholesterol occurred and in the hope that such a study might result in our being able to express an opinion as to the validity of the results obtained by the many micro-methods of blood-fat estimation.

Another question which we have borne in mind is whether calcium phosphatidate, which may be regarded as the parent acid of lecithin and kephalin [Channon and Chibnall, 1927], is present in blood. Kephalin preparations usually contain calcium which can be removed by shaking with mineral acid [Diaconow, 1867; Thudichum, 1884; Parnas, 1909]. Calcium phosphatidate, like kephalin, is insoluble in alcohol and its calcium may be similarly removed, so that it is possible that this substance may be responsible for the calcium which usually contaminates kephalin preparations. If calcium phosphatidate is present in blood, the rise in ether-soluble phosphorus of the blood after a fatty meal, which has been observed and investigated by Bloor [1915], might be due to that substance which is a possible intermediate in the synthesis of lecithin and kephalin from the absorbed fat. Further, the study of the nature of blood-fat in a fasting animal may throw light on the hypothesis of Leathes and Raper [1925], who consider that the fatty acids of adipose tissue may be desaturated in the liver and that after being converted into highly unsaturated liver phosphatides they may be transported to the tissues in this form.

Before attempting to submit a large amount of blood-fat to fractionation into its various constituents, it was considered wise to prepare a number of samples and to make determinations of the fat constants and of phosphorus and nitrogen on the material in order to gain some idea as to its constancy of composition or otherwise, and to obtain some guidance for the treatment of the large preparation.

Preparation of blood-fat.

Our object in the preparation has been to obtain as much fat from blood with as little alteration of its original character, either by oxidation or by enzyme action, as possible. Blood contains rather less than 0·5 g. of fat per 100 cc., some of which is held by the protein in a form which prevents it from being easily removed. The difficulties encountered in attempting to remove this fatty material from so complex a tissue as blood are such that any method must necessarily be a compromise at many points, and one of them is well illustrated by the work of Shimidzu [1910], who found that the well-known saponification method of Kumagawa and Suto [1908] for the estimation of the total fatty acids in tissues cannot be applied to blood, a finding which we confirm. Shimidzu, therefore, devised a method in which blood is run into several volumes of alcohol and, after shaking, the mixture is allowed to stand for some hours. It is then filtered, and the protein residue is extracted for 6–8 hours by exposure to the vapours of boiling alcohol by suspension in a thimble over the surface of the solvent in a flask fitted with a reflux condenser. This alcohol extract is united with the original alcohol filtrate and the mixture is saponified and the fatty acids prepared by the Kumagawa and Suto method. In spite of this rigorous treatment, some material which on hydrolysis gives rise to fatty acids still remains in the protein residue, and these fatty acids, which can only be recovered by hydrolysis of this residue, amount to not more than 5 % of the total [Mayer and Schaeffer, 1913]. The material obtained by the alcohol extraction (prior to saponification) by the Shimidzu method is usually dark red in colour and contains a large proportion of substances which do not contain fatty acids but which are soluble in fat solvents, possibly because of the presence of the blood-phosphatides. Hence such an extraction method, although of great use for estimating the total fatty acids in blood, cannot be utilised for the preparation of blood-fat on account of the high proportion of extraneous material introduced, the length of time necessary for extraction with likelihood of consequent oxidation, and the impossibility of extracting large amounts of blood-protein in an atmosphere of hot alcohol vapour.

The alternative methods for obtaining the blood in a suitable form for extraction by fat solvents are precipitation by a solvent miscible with water or direct drying with suitable precautions against oxidation. No satisfactory method of direct drying being found, we were compelled to adopt a precipitation procedure in which we used alcohol.

Little advantage is gained by adding more than three volumes of alcohol to each volume of blood, for the increase in the percentage of alcohol in the filtrate by the addition of one or more volumes beyond three is relatively insignificant. It was decided to submit the protein residue to extraction with ether and not with alcohol as in the Shimidzu method, in order to avoid obtaining in the extract much material not of fatty nature to which reference

has been made. Such ether extraction will leave a definite amount of the fat unextracted, but a compromise at this point in favour of obtaining a less contaminated product which can be more rapidly prepared appeared desirable. The alcohol filtrate from the precipitation was evaporated to dryness and the dry residue also extracted with ether, and the extracts from the two fractions were combined. The relative amounts of material extracted from the protein residue and the alcoholic filtrate depended on the time during which the mixture of blood and alcohol was allowed to stand, but usually up to 80 % of the final material was obtained from the alcohol filtrate. Consideration was also given to the question as to whether it would be worth while to draw off the oxygen of the blood by exposure in a vacuum before precipitation in order to minimise oxidation (the solubility of oxygen in alcohol is about 20 times that in water). Accordingly experiments were carried out in which blood was treated by the method outlined above before and after removal of its oxygen, and iodine values were determined on the samples of fat obtained. Typical of these may be quoted the results of one experiment in which the iodine value of the fat of the blood directly precipitated was 58·2, and that of the same blood which had been previously sprayed into an evacuated flask was 58·9. In view of the fact that we were dealing with large quantities of blood, this difference was not considered sufficiently great to render it essential to remove the oxygen before treating the blood with alcohol. The material obtained by ether extraction of the protein residues was in all cases a waxy mass, golden yellow in colour, while that from the ether extract of the residue from the alcohol filtrates was reddish brown and contained much foreign material which was found to consist of nitrogenous substances. Since the bulk of the fat was obtained in the latter fraction, no useful purpose was served by keeping the two fractions apart and they were accordingly mixed.

Method.

Ox blood was received into oxalate at the slaughter-house and run into three volumes of alcohol within 30 minutes of being drawn. As the animals usually receive no food for 24 hours before slaughter, the fat of such blood may be regarded as at fasting level. The mixture was then vigorously shaken and allowed to stand for varying times, usually 0·5 to 2 hours. In the case of the 40 l. quantity, it was necessary to precipitate large volumes of blood at one time, and these stood in alcohol for varying times up to 24 hours. After standing, the mixture was filtered on a Büchner funnel. The residue so obtained needed considerable pressing before it became reasonably solid, and even then it was found to contain large amounts of solvent, for the volume of filtrate was never greater than 1·2 times the volume of alcohol added. The residue was then extracted in a Soxhlet apparatus until the ether extract was colourless. The original alcohol filtrate was evaporated to dryness in the water-bath at low pressure: after the volume had been rapidly reduced to about one-half, the solution frothed excessively. This frothing could only be

overcome by lowering the temperature of the water-bath at this stage and reducing the water pressure to the minimum necessary to maintain a vacuum, and evaporation was extremely slow. When the volume was reduced to about 10 % of the original extract frothing ceased and the evaporation could be continued as before. The last stages of the evaporation were also slow, for the fatty material which separated at the half-way stage then formed an emulsion, and only by raising the temperature of the water-bath to 80° could the water be effectively removed. In the final stages, absolute alcohol was repeatedly added to ensure efficient removal of the remaining water. Evaporation of the filtrate obtained from 1 litre of blood took 7–10 hours. The ether extract from the protein residue was also evaporated to dryness. The two residues were then washed out with ether into a stoppered cylinder and allowed to stand overnight. The solutions were filtered into weighed vessels, the solvent was removed, and a weight obtained after drying on the water-bath *in vacuo*. The material so obtained is usually reddish brown in appearance and, when warm, transparent; when cold it has the appearance (apart from colour) of a mixture of phosphatide and cholesterol, and is a sticky translucent mass. It contains, as we shall see later, a considerable amount of materials which are not truly ether-soluble. Its solution in ether is slightly fluorescent, and is usually turbid.

Determination of constants.

Aliquot portions of the ether solutions were used for duplicate determinations as set out in Table I. Nitrogen was determined by the micro-Kjeldahl method on 20–25 mg. quantities. The low phosphorus content (1–2 %) of the material would entail the employment of quantities of the order of 0·3 g. for a gravimetric estimation as magnesium pyrophosphate and we have been compelled in the routine determination of constants, and in the subsequent fractionation of the large batch of material on which a large number of phosphorus determinations has been necessary, to use the quinol-sulphite colorimetric method after oxidation of 8–12 mg. portions with sulphuric acid and "perhydrol." Cholesterol was determined by the Windaus [1910] digitonin method. The saponification values were determined on 0·5 to 1·5 g. portions by heating with 0·25 *N* (approx.) alcoholic potassium hydroxide for 4 hours. After titration, a considerable part of the alcohol was removed, and after being made strongly alkaline with potassium hydroxide the solution was poured into water in a separating funnel and exhaustively extracted with ether. The ether extract, after thorough washing with water, was evaporated to dryness and resaponified for one hour with sodium ethylate in alcoholic solution to ensure the complete hydrolysis of cholesteryl esters. After dilution with water, the mixture was again ether-extracted. The ether extract was thoroughly washed with water, and evaporated to dryness in a weighed flask. In this way the unsaponifiable fraction was obtained. It was, in all cases, a hard crystalline mass, of deep orange colour, the colour being

due to the lipochrome pigments carotene and xanthophyll: on this material total cholesterol was determined. The soap solutions from both saponifications, together with the washing of the ether solutions, were united and acidified with hydrochloric acid, and then extracted with ether. Difficulty has been encountered at this point, because acidification always causes the precipitation of a certain amount of slimy material which is insoluble in ether, and forms an emulsion at the ether-water interface. It has been our practice to leave this material suspended in the funnel with the first ether extract, and then to add to this ether extract the four or five successive ether extracts of the aqueous acid phase. The combined extracts were washed with water, until free from mineral acid, and then filtered. The insoluble material to which reference has just been made renders this filtration tedious. As to its nature we have no information, but such emulsions are encountered in the Shimidzu method of estimating blood-fat as pointed out by Mayer and Schaeffer [1913] and seem unavoidable with this type of material. The ether solutions of the fatty acids were evaporated, and dried *in vacuo* on the water-bath. The residues were dissolved in warm light petroleum (B.P. 40–60°) and allowed to stand overnight for the so-called "resinous" materials to separate out. After further filtration and washing of the residues, the light petroleum extracts containing the fatty acids were evaporated to dryness and weighed. Determinations of the iodine values (Wijs) and the molecular weight by titration were then carried out. The results are recorded in Table I.

Table I. *Analysis of the ether extract.*

(All figures are expressed as a percentage of the ether extract.)

Sample No. ...	1	2	3	4	5	6	7	8	9	10	11	12	Mean
Vol. of blood used, cc.	400	300	40,000	1200	1000	1000	350	350	350	350	500	400	—
Yield, g./l.	4·60	5·11	3·97	—	4·73	4·19	4·72	4·90	4·56	5·47	6·01	6·65	4·99
N	2·48	2·09	2·39	2·15	2·39	2·99	2·37	2·38	1·96	2·11	2·44	2·46	2·35
P	1·51	1·57	1·57	1·65	1·62	1·63	1·66	1·64	1·56	1·57	1·37	1·52	1·57
Unsap. matter	31·55	32·08	31·81	34·85	26·44	27·16	27·83	32·29	31·45	34·45	29·16	30·14	30·76
Free cholesterol	17·74	16·04	17·04	22·46	14·40	15·68	17·41	15·80	17·3	15·1	13·50	12·88	16·27
Cholesterol as ester	8·22	6·96	6·43	7·88	8·85	7·14	8·59	7·18	10·39	14·08	13·21	15·50	9·53
Unsap. matter other than cholesterol	5·59	9·08	8·34	4·51	3·19	4·34	1·83	9·31	3·76	5·27	2·45	1·76	4·95
Fatty acids	36·0	30·0	32·5	34·8	37·7	34·9	37·9	30·7	38·3	35·5	32·7	35·3	34·7
Iodine value	—	—	100	91·6	87·8	89·3	87·5	94·4	92·9	97·3	116·2	117·7	97·5
Mol. wt.	—	—	294	298	298	304	311	294	310	294	307	305	301

Discussion.

The proportion of fat extracted.

The figures in Table I show that the mean average phosphorus content of 12 samples of fat is 1·57 % P (extreme values 1·37, 1·66 % P), which corresponds to 7·78 mg. P per 100 cc. blood. Determinations of the ether-soluble phosphorus of 15 samples of ox blood gave a mean value of

9·8 mg./100 cc., which suggests that the method used has resulted in the extraction of 80 % of the ether-soluble phosphorus present. In order to gain further information as to the proportion of material obtained, the fat was prepared from a number of samples of blood, and at the same time determinations of the sum of the amounts of unsaponifiable matter and fatty acids were made on 50 cc. portions of blood by the Shimidzu [1910] method, save that the protein residues were not hydrolysed. In the calculations of the percentage yield allowance has been made for the fact that up to a further 5 % of material may be obtained from the hydrolysis of these residues. The results are recorded in Table II.

Table II. *Fatty acids and unsaponifiable matter extracted, calculated on the yields obtained by the Shimidzu method* [1910].

(Results expressed in mg./100 cc. blood.)

Fatty acids	179·1	150·4	174·9	194·4	212·0	239·0
Unsaponifiable matter	152·4	136·3	143·5	189·0	170·4	180·4
Total	331·5	287·6	318·4	383·4	382·4	419·4
Fatty acids and unsap. matter (Shimidzu)	369·6	361·4	365·2	433·8	430·0	515·6
Percentage yield	85·2	75·3	82·8	84·0	84·5	77·2

The mean figure for the yield expressed as a percentage of that of the Shimidzu method is 81·5 %. This figure is similar to that obtained for the proportion of phosphorus extracted, and it seems justifiable therefore to consider that the fat extract is probably fairly representative of the whole.

One further point needs mention. With small quantities of blood, ether extraction of the protein residue is readily accomplished. With larger quantities, such extraction is more difficult, and although every care was taken to see that thorough extraction was obtained, it is possible that some of the variations in yield may be accounted for to some extent by this fact. This remark applies in particular to the 40 l. quantity.

We have discussed the method of preparation and the question of the proportion of fat extracted somewhat fully because it was the one finally adopted, and because that used by Bloor [1924] in the simpler problem of preparing fat from plasma seemed to us unsatisfactory.

Consideration of Bloor's results, in which 16 samples of ox blood-plasma varying in volume from 500 to 2880 cc. were used, shows that the yield of phosphatide fatty acids varies from 0·0021 to 0·0799 g./100 cc., while in 5 cases apparently no phosphatide fraction was obtained, for the yields of fatty acids are not recorded. These results may be calculated in terms of ether-soluble phosphorus (fatty acids = P × 18·26), and such calculation shows that the average yield of fatty acids from the phosphatide fractions corresponds to an extraction from the plasma of 1·06 mg. of ether-soluble phosphorus per 100 cc. (extreme values 0·11 and 4·37 mg.), whereas the average figure obtained by us in direct determination of the ether-soluble phosphorus of 12 samples of ox blood-plasma is 6·62 mg./100 cc. Hence the method used by Bloor appears to have resulted in his obtaining an average

yield of not more than 16 % of the phosphatide fatty acids present in the plasma. Bloor also points out that early in his work it was discovered that the fat obtained contained free fatty acids which were removed and weighed. In this connection, analysis of the results from the four samples for which the data are most complete shows that in these four cases the material contained from 10 to 33 % of its content of fatty acids as free fatty acid, and these free fatty acids must have resulted from decomposition of some part of the fat during the preparation. Bloor comments on the low yield of fatty acids from the phosphatide fractions, and suggests that "insoluble residues are formed at certain points in the procedure which probably contained the altered phospholipoid; also, that in carrying out the various processes some of the lipoid was probably decomposed. The fact that a considerable amount of free fatty acid was always present in the lipoidal material, which would hardly have existed in the plasma itself, bears out the latter explanation." A material such as this, containing a large proportion of decomposition products, and only a small proportion of the phosphatide present in the plasma, which phosphatide may or may not be representative of the whole in the degree of unsaturation of its acids, appears to us to be unsatisfactory, and the conclusion drawn from its analysis open to doubt.

The fat prepared from blood by our method has never contained free acid, while it has been pointed out that its phosphorus content corresponds to an extraction of about 80 % of the ether-soluble phosphorus present in the blood.

In view of Bloor's experience with plasma, we submitted 25 cc. portions of 11 samples of plasma to the method which we have used for whole blood, in order to determine what proportion of the ether-soluble phosphorus was extracted, and whether the material contained free acid. The results are recorded in Table III. In no case did the extracted fat contain free acid. It is seen that about 80 % of the ether-soluble phosphorus present in the plasma has been obtained in the fat.

Table III.

(Plasma: ether-soluble phosphorus determinations and percentage of phosphorus extracted.)

													Mean
Ether-soluble P, mg./100 cc.	6·04	7·62	9·57	7·60	6·39	8·43	6·53	5·59	4·21	5·79	6·73	4·92	6·62
Wt. of fat, g./100 cc.	0·512	0·504	0·721	0·621	0·512	0·770	—	0·452	0·326	0·489	0·555	0·433	0·536
P as % of fat	1·03	0·91	1·19	1·08	0·92	1·02	—	1·04	0·89	0·91	0·87	1·01	0·99
P in fat as mg./ 100 cc. plasma	5·27	4·56	8·62	6·73	4·71	7·88	—	4·76	2·91	4·43	4·84	4·37	5·37
Percentage of P extracted	87	60	90	89	74	93	—	85	69	75	72	89	80

Absence of glycerides.

The initial observation to be made from the figures of Table I is that the sum of the average values of the total unsaponifiable matter (30·76 %) and of the fatty acids (34·69 %) is only 65·45 % of the material. This will be

accounted for in part by the fact that lecithin on hydrolysis yields but 70·3 % of its weight of fatty acids, but the greater part of the difference is due to the presence of extraneous nitrogen in the material. Before discussing this matter further, attention may be drawn to the amount of fatty acid which percentages of phosphorus and cholesteryl ester require[1], and which are set out in Table IV. It will be seen that there is, in general, fair agreement between the percentage of fatty acids found and those calculated on the basis of the phosphorus being present as lecithin and the cholesteryl ester as stearate[2].

Table IV. *Fatty acids.*

(Figures are expressed as a percentage of the fat.)

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Phosphatide fatty acids (P × 18·26)	27·6	28·7	28·7	30·1	29·6	29·8	30·3	29·9	28·5	28·7	25·0	27·7
Fatty acids combined with cholesterol (cholesteryl ester × $\frac{284}{388}$)	6·0	5·1	4·7	5·8	6·5	5·2	6·3	5·3	7·6	10·3	9·9	11·4
Total	33·6	33·8	33·4	35·9	36·1	36·0	36·6	35·2	36·1	39·0	34·9	39·1
Found	36·0	30·0	32·5	34·8	37·7	34·9	37·9	30·7	38·3	35·5	32·7	35·3

If such calculations are justified, it is clear that there can be little glyceride present, a conclusion which the later fractionation of the fat from 40 l. of blood showed to be correct. In Table V are recorded the percentages of lecithin, cholesterol, cholesteryl ester and unsaponifiable matter other than sterol in the fat, what little glyceride there is present being neglected, the object being to gain an approximate figure for the percentage of non-fatty material present.

Table V.

(All figures are expressed as a percentage of the fat.)

Sample No.	1	2	3	4	5	6	7	8	9	10	11	12
Lecithin (P × 26)	39·22	40·78	40·78	42·85	42·07	42·34	43·11	42·59	40·52	40·78	35·82	39·48
Cholesterol	17·74	16·04	17·04	22·46	14·40	15·68	17·41	15·80	17·36	15·10	13·50	12·88
Cholesteryl stearate	13·89	11·76	10·86	13·30	14·95	12·06	14·51	12·13	17·54	23·77	22·32	25·00
Unsap. matter other than cholesterol	5·59	9·08	8·34	4·51	3·19	4·34	1·83	9·31	3·76	5·27	2·45	1·76
Total	76·44	77·66	77·02	83·12	74·61	74·42	76·86	79·83	79·18	84·92	74·09	79·12
Residue; not fat	23·56	22·34	22·98	16·88	25·39	25·58	23·14	20·17	20·82	15·08	25·91	20·88

[1] From this point onwards, calculations involving the phosphatide will be made as has been done by previous workers on the basis of stearyl-oleyl-lecithin (N = 1·74 %, P = 3·85 %, fatty acids = P × 18·26); the cholesteryl ester will be regarded as cholesteryl stearate, an assumption which will subsequently be shown to be sufficiently accurate for the purpose of calculation.

[2] As pointed out on p. 668, difficulty has been encountered in extracting the fatty acids from the acidified soap solution on account of the presence of emulsions. Another difficulty which we have failed to overcome is that on no occasion have we obtained fatty acids which appeared "clean," in spite of the fact that attempts were made to remove what appeared to be resinous materials by second and third treatments with light petroleum. The same difficulty has been encountered by Rosenthal and Trowbridge [1915] using the Shimidzu [1910] method. Hence we cannot feel confident that the figures recorded in the above table are more accurate than to within possibly 5 %. We are investigating this matter further.

Presence of excess of nitrogen.

The sum total of these constituents is never greater than 83 %, and up to 25 % of the material consists of nitrogenous substances, as is shown by the excess of nitrogen over what is required by the phosphatide present. In the following table, the nitrogen percentage in excess of what is required by the phosphorus content and a N : P ratio of unity is shown, together with the percentage of non-fatty material (from Table V); the nitrogen percentage of the latter has been calculated.

Table VI. *The excess nitrogen expressed as percentage of the fat.*

Sample No. ...	1	2	3	4	5	6	7	8	9	10	11	12
N required if P is calculated as lecithin	0·68	0·71	0·71	0·74	0·73	0·74	0·75	0·74	0·70	0·71	0·62	0·68
N found	2·48	2·09	2·39	2·15	2·39	2·99	2·37	2·38	1·96	2·11	2·44	2·46
Excess N	1·80	1·38	1·68	1·41	1·66	2·25	1·62	1·64	1·26	1·40	1·82	1·78
Residue not fat (from Table V)	23·56	22·34	22·98	16·88	25·39	25·58	23·14	20·17	20·82	15·08	25·91	20·88
N percentage in residue not fat	7·66	6·18	7·31	8·35	6·54	8·80	7·00	8·13	6·05	9·34	7·03	8·54

The fact that an alcohol extract of a tissue may yield phosphatide preparations which contain far more nitrogen than is required by the amount of phosphorus present was shown by MacLean [1912] in his investigations on horse kidney, where the alcohol extracts yielded phosphatide material having N : P ratios varying from 4 : 1 to 1·5 : 1. He found that this excess of nitrogen was due to the fact that in the presence of phosphatides certain unknown nitrogenous substances are carried into solution and act as though they possessed all the properties of the phosphatides themselves. They can be removed in one way only, namely, by precipitation of the phosphatide emulsion in water by the addition of acetone. The nature of the complex mixture of these nitrogenous materials which accompany the phosphatides is discussed at length by MacLean and Smedley-MacLean [1927] under the generic name carnithin, where it is shown that the average nitrogen content of various samples was 6 % nitrogen and that substances with a nitrogen content of 28·5 and 40 % have been isolated from the mixture. It may be wondered why no attempt was made to remove this material from the blood-fat. The reasons are that acetone precipitation of an aqueous emulsion of the material results, as would be expected, in considerable losses of material which remains in the aqueous phase. The process needs repetition, and further it was considered preferable to analyse the fat, contaminated as it was with this material, rather than to remove it and obtain a purer preparation which might not be representative of the whole. Attempts were made to obtain fat from blood which did not contain this excess nitrogen by the use of different solvents, but these were unsuccessful. That the nitrogen percentage of the non-fatty residue in our material is higher (7·58 %) than that recorded by MacLean (6 %) may be due to the fact that calculation has been made of the nitrogen

required by the phosphorus on the basis of a N : P ratio of 1 (lecithin and kephalin) whereas it will be shown subsequently that some part, at least, of the blood-phosphatide may be sphingomyelin (N : P = 2 : 1, fatty acids = P × 12), the presence of which in red cells Bürger and Beumer [1913] claim to have demonstrated. On the other hand, the high and variable contents of nitrogen in the different substances obtained from samples of carnithin show how complex a mixture it is, and hence a constant nitrogen content is not to be expected.

Fatty acids.

The average figures for the molecular weights and iodine values of the fatty acids are 301 and 97·5 respectively. Of the figures available concerning the iodine value of blood-fatty acids, mention may be made of the finding by Imrie [1915] of an iodine value of 73 in a case of lipaemia, and of the results of Boggs and Morris [1909], who found that the iodine value of the fatty acids of the blood of rabbits rendered lipaemic by bleeding varied from 105 to 134. Csonka [1918] obtained a value of 87 for the liquid fraction of the mixed fatty acids of normal human blood (48 % of the total fatty acids) which corresponds to a value of 41. Bloor [1923] found an average iodine value of 147 for the liquid fraction of the fatty acids from 16 samples of ox blood-plasma. The liquid acids constituted 74 % of the total acids which, therefore, had an iodine value of 109 as against 97·5 found by us for those of whole blood. The higher figures obtained by Bloor may be due to the fact that he removed from the acids during the lead salt separation about 15 % of material, the lead salt of which was insoluble in hot alcohol and which on decomposition yielded a residue of low iodine value. The weight of this material was not included in the calculation of the percentage of the total acids which the liquid fraction represented, and its inclusion would lower the iodine value of the mixed acids to about 100. There is little doubt that the iodine values of the blood-fatty acids are higher than those recorded in this paper, for the nature of the starting material and the lengthy operations involved in their preparation must result in some degree of oxidation occurring in spite of the precautions taken to avoid it. Further, as has been pointed out already (p. 671 note 2), we think that the fatty acids have been contaminated by some other material which would also tend to lower the iodine values. That such contamination may exist appears to be borne out by the fact that the average molecular weight of the fatty acids is 301 (stearic acid has M.W. 284). Although, as will be shown subsequently, we have obtained from blood what appears to be lignoceric acid (M.W. 368), the amount present does not seem to be sufficiently great to account for the high molecular weight of the blood-fatty acids.

We have discussed all results in terms of percentages of the fat itself, and for the more physiological considerations some of them have been converted into yields expressed as mg. per 100 cc. of blood in Table VII.

Table VII.

(All figures are expressed as mg./100 cc. blood.)

Sample No. ...	1	2	3	5	6	7	8	9	10	11	12
P	6·95	8·02	6·23	7·66	6·83	7·83	8·03	7·11	8·59	8·23	10·10
Fatty acids combined with P (P ×18·26)	126·9	146·4	113·8	139·9	124·7	143·0	146·7	129·9	156·8	150·3	184·5
Fatty acids combined with cholesterol	27·83	26·16	18·78	30·80	22·01	29·83	25·88	34·85	56·63	58·41	75·82
Total fatty acids	154·7	172·6	132·6	170·7	146·7	172·8	172·6	164·8	213·4	208·7	260·3
Total fatty acids found	165·6	153·5	129·0	178·3	146·2	178·8	150·4	174·7	194·2	196·4	235·0
Free cholesterol	81·62	81·96	67·64	68·13	65·69	82·15	77·43	78·89	82·6	81·14	85·66
Cholesterol as ester	37·82	35·56	25·53	41·86	29·91	40·54	35·18	47·37	77·00	79·39	103·0
Unsap. matter other than sterol	25·71	46·39	33·11	15·09	18·19	8·64	45·6	17·15	28·82	14·72	11·70

If allowance be made for the amount of material other than fat present in the ether extract, it may be calculated that the yield of fat varies from 3·05 to 5·34 g./l. with a mean value of 3·97 g./l. The yield of fatty acids varies from 1·29 to 2·35 g./l., giving an average figure of 1·73 g./l., while the corresponding figures for the unsaponifiable fraction are 1·138 and 2·004 g./l., and 1·520 g./l. respectively. Thus the sum of the yields of unsaponifiable matter and fatty acids is 3·267 g./l., and the unsaponifiable fraction thus constitutes 46·8 % of this material, with values varying not greatly from the mean, viz. 42·3 to 49·5. Bloor [1923] obtained for the sum of the unsaponifiable matter in fatty acids an average yield of 3·01 g./l. from plasma, and found that the unsaponifiable matter constituted 43 % of this total. The unsaponifiable matter contains on the average 84 % of sterol precipitable by digitonin.

Although the saponification values of the fats were determined, they have not been recorded because the presence of the nitrogenous material already discussed vitiates them. They were carried out in the hope that if any considerable amount of glyceride were present in any sample of fat it would make itself obvious by raising the saponification value obtained. The mean of these values was 117.

Summary.

1. A method is described by which 80 % of the fat present in blood may be extracted. The material so obtained contains considerable quantities of nitrogenous substances.

2. Fat has been prepared from 12 samples of ox blood at fasting level, and a study has been made of its composition by determination of the fat constants and of phosphorus and nitrogen.

3. The amount of fatty acid present varies little from that required if the phosphorus of the fat be calculated as lecithin, and the cholesteryl ester as cholesteryl stearate, which suggests that glycerides are not present in appreciable quantity.

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REFERENCES.

Bloor (1915). *J. Biol. Chem.* **23**, 317.
—— (1923). *J. Biol. Chem.* **56**, 711.
—— (1924). *J. Biol. Chem.* **59**, 543.
—— (1925). *Chem. Reviews*, **2**, 243.
—— Pelkan and Allen (1922). *J. Biol. Chem.* **52**, 191.
Boggs and Morris (1909). *J. Exp. Med.* **11**, 553.
Bürger and Beumer (1913). *Biochem. Z.* **56**, 446.
Channon and Chibnall (1927). *Biochem. J.* **21**, 1112.
Csonka (1918). *J. Biol. Chem.* **33**, 401.
Diaconow (1867). *Hoppe-Zeyler's Med. chem. Untersuch.* **2**, 221.
Hürthle (1895). *Z. physiol. Chem.* **21**, 331.
Imrie (1915). *J. Biol. Chem.* **20**, 87.
Kumagawa and Suto (1908). *Biochem. Z.* **8**, 212.
Leathes and Raper (1925). The fats, London.
MacLean (1912). *Biochem. J.* **6**, 333.
—— and Smedley-MacLean (1927). Lecithin and allied substances, London.
Mayer and Schaeffer (1913). *J. Physiol. Path. Gén.* **15**, 984.
Parnas (1909). *Biochem. Z.* **22**, 411.
Rosenthal and Trowbridge (1915). *J. Biol. Chem.* **20**, 711.
Shimidzu (1910). *Biochem. Z.* **28**, 237.
Thudichum (1884). Chemical constitution of the brain; quoted by MacLean and Smedley-MacLean [1927].
Windaus (1910). *Z. physiol. Chem.* **65**, 110.