CXXXIV. BLOOD-FAT.

II. THE ACETONE-ETHER-SOLUBLE FRACTION.

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IN a previous paper [Channon and Collinson, 1929] a method of preparing blood-fat has been described and the general nature of a number of samples has been discussed. In the large-scale preparation, 40 l. of ox blood at fasting level yielded 158.9 g. of fat, which contained 1.57 % P, 2.39 % N, 17.04 % sterol precipitated by digitonin, 6.43 % sterol present in ester form, and 8.24 % unsaponifiable matter other than sterol; it yielded 32.46 % of its weight as fatty acids which had molecular weight 294 and iodine value 100.

136 g. of the fat were dissolved in 400 cc. ether and the warm solution was poured into 1.8 l. acetone with vigorous shaking, after which the mixture was maintained at 0° for 7 days. The precipitated phosphatide fraction was removed by filtration, ground in a mortar with acetone and the mixture filtered. This operation was repeated three times, and the acetone washings were united to the ether-acetone mother liquor. The acetone-insoluble fraction was then shaken with ether, when a portion of it was found to be insoluble. After standing, the ether solution was siphoned off, and the residue again treated with ether three times, and the ether washings combined. In this way, the 136 g. of fat were resolved into three fractions: (a) soluble in acetone-ether, 47 g.; (b) precipitated from ether solution by excess of acetone and redissolved in ether, 69.6 g.; (c) precipitated from ether solution by excess of acetone, but found to be no longer soluble in ether, 10 g.

The present communication discusses the nature of fraction (a), the acetone-ether-soluble fraction. It was an orange mass which contained 0.87 % N and 0.05 % P. About 87 % of the nitrogen present in the original material was precipitated in the phosphatide fractions (b) and (c), while the phosphorus content of the fraction corresponds to about 1.3 % of lecithin. The material was stored in the dark in a flask completely filled with ether for 12 months before opportunity to investigate it arose.

EXPERIMENTAL.

A small amount of precipitate which had formed on standing was removed by filtration¹. The remaining material was found by saponification to contain 70.13 % of unsaponifiable matter, 91.81 % of which was cholesterol, and 27.28 % of fatty acids. There were thus present in the material (45.53 g.), 29.31 g. of free and combined cholesterol, 2.63 g. of unsaponifiable matter other than sterol² and 12.43 g. of fatty acids.

The material contains free cholesterol and cholesterol esters together with glycerides, and possibly traces of free fatty acids and soaps.

The object of the subsequent work was to attempt to gain some idea as to what fatty acids were in combination with cholesterol and to what extent the fraction was composed of glycerides. On account of the similar solubilities of cholesterol and its esters in the usual fat solvents, the separation by fractional crystallisation of small amounts of cholesteryl esters in any state of purity from a mixture containing a high percentage of free cholesterol is a matter requiring large amounts of material, and even then the complete identification of the esters is difficult unless they be obtained in quantity. Hence the nature of the constituents of the ester mixtures obtained by Hürthle [1895] and Bloor [1924] remains uncertain. In view of these difficulties, therefore, it was considered advisable to attempt the separation of the material into two fractions, (*a*) cholesterol and cholesteryl esters, and (*b*) glycerides, instead of attempting to isolate the individual cholesteryl esters present. If such a separation, based on the fact that cholesteryl esters have a lower solubility in fat solvents than glycerides, were possible, the identification of the fatty acids produced by the hydrolysis of fraction (*a*) would indicate what cholesteryl esters were present. The material was dissolved in 400 cc. acetone and on cooling yielded 20.25 g. of crystalline solid (fraction 1). Further crystallisation was carried out and four fractions, two of which were oils, were obtained, together with 9.06 g. of sticky solid obtained from the mother liquor (fraction 3). The amount of free cholesterol in fraction 1 was determined, and the remainder was saponified and the weight of unsaponifiable matter and fatty acids obtained. The results of these estimations showed that the amount of fatty acid was greater than that required by the esterified cholesterol present, and demonstrated that the fractional crystallisation was unsuccessful. Accordingly, the four intermediate fractions were united to form fraction 2.

¹ After repeated boiling with charcoal and crystallisation from acetone, a mixture of needles and an amorphous substance was obtained. This mixture, 0.2 g., contained 35.08 % N.

² The analytical figures determined on the original blood-fat show that there should be present 31.9 g. of total cholesterol and 11.20 g. of unsaponifiable material other than cholesterol. There thus appears to have been a loss of 2.59 g. of cholesterol and 8.57 g. of unsaponifiable material other than cholesterol. The former loss is attributable to the manipulations involved. The apparent loss of about 80 % of the unsaponifiable material other than cholesterol may be due either to its being precipitated together with the phosphatides or alternatively to its production by the materials used in the alkaline saponification. The latter view is almost certainly correct.

Quantities of about 1 g. of fractions 2 and 3 were set aside for the purpose of estimating glycerol. After determination of the amount of free cholesterol, the remainder of each fraction was then resolved after saponification with sodium ethoxide into its constituent unsaponifiable matter and fatty acids. These results together with those obtained from the whole of fraction 1 are recorded in Table I, together with the iodine values of the fatty acids.

Table I.

Fraction	Weight (g.)	Unsap. matter (%)	Free sterol (%)	Fatty acids (%)	Iodine value of fatty acids
1	20.24	78.3	56.9	20.11	110.1
2	9.18	69.2	37.3	27.39	137.9
3	10.17	49.5	22.2	38.80	122.6

In Table II are recorded the weights of the components corresponding to the percentages recorded in Table I. The percentage of total cholesterol present in each unsaponifiable fraction was not estimated, because, even if the unsaponifiable fractions had been wholly cholesterol, there would still be an excess of fatty acid in each fraction over that required by the cholesteryl ester present. It has been mentioned already that the unsaponifiable fraction determined on an aliquot part of the whole material prior to resolution into three fractions contained 91.81 % of cholesterol; and accordingly this figure has been used for the calculations of the total sterol in each unsaponifiable fraction. As the mean molecular weight of the fatty acids in these three fractions was 285.2, the weight of fatty acid necessary for combination with the amount of cholesteryl ester present has been calculated as stearic acid (m.w. 284). The amount of fatty acid in excess of that required by the cholesteryl ester is recorded in the last column. The weights of the fractions are the original weights prior to removal of the aliquot parts used for other determinations.

Table II.

Fraction	g.	Unsap. matter (g.)	Total sterol (g.)	Free sterol (g.)	Sterol ester (g.)	Fatty acid equivalent of sterol ester (g.)	Fatty acids found (g.)	Fatty acids present in other forms (glyceride)
1	20.24	15.85	14.55	11.52	3.03	2.23	4.07	1.84
2	9.18	6.35	5.83	3.43	2.40	1.77	2.51	0.74
3	10.17	5.03	4.62	2.26	2.36	1.74	3.95	2.21
Total	39.59	27.23	25.00	17.21	7.79	5.74	10.53	4.79

From these results it is clear that the 39.59 g. of material contains no more than 4.79 g. of fatty acid in excess of that required for the cholesteryl esters present. If allowance be made for the 4.53 g. withdrawn from the main bulk before fractional crystallisation, this amount of fatty acid becomes 5.34 g. Further, if the phosphorus content of the original 45.53 g. (0.05 % P) be due to the presence of lecithin, the latter would give rise to 0.4 g. of fatty acid on hydrolysis. Hence the amount of fatty acid obtained by hydrolysis of the ether-acetone fraction which is not in combination with cholesterol is 4.94 g.

from 34.2 l. of blood, or about 15 mg./100 cc. blood. Although, as already pointed out [Channon and Collinson, 1929], the method used for extracting the blood has resulted in not more than 80 % of the fat being extracted, it seems probable that the figure of 15 mg./100 cc. is a maximum, for it is to be anticipated that what little glyceride is present in blood is readily extracted, whereas the phosphatide and cholesteryl esters will be removed completely only with difficulty.

THE FATTY ACIDS.

The remainder of the fatty acids, 9.58 g., were separated into a solid fraction, 2.04 g., and a liquid fraction 6.06 g., by the lead salt and ether process. These two fractions had iodine values of 30.1 and 150.8 respectively.

The solid fraction. After boiling the acetone solution of the saturated acids with charcoal, two fractions (1) 0.23 g., (2) 0.86 g. were obtained. These analysed as follows.

Fraction (1) 0.1366 g. substance required for neutralisation 5.0 cc. 0.1 N KOH.

4.920 mg. substance: 13.65 mg. CO₂ and 5.68 mg. H₂O.

Fraction (2) 0.1127 g. substance required for neutralisation 4.22 cc. 0.1 N KOH.

4.400 mg. substance: 12.08 mg. CO₂ and 4.96 mg. H₂O.

	C	H	M.W.	M.P.
Calculated for C ₁₆ H ₃₂ O ₂ :	75.01	12.50	256	63–64°.
C ₁₈ H ₃₆ O ₂ :	76.07	12.68	284	70–71°.
Found, fraction (1)	75.65	12.82	273	57.5–58°.
(2)	74.85	12.52	267	54–55°.

The amount of material available was obviously too small for the definite identification of the acids present, but the saturated acids appear to be a mixture of stearic and palmitic acids.

The liquid fraction. 5.8 g. were brominated in anhydrous ether at 0°. After standing overnight, the precipitate 0.6 g. (fraction 1) was filtered off, and washed with ether. The excess bromine was removed from the mother liquor by shaking with sodium thiosulphate solution. The ether solution after drying with anhydrous sodium sulphate was concentrated to 25 cc. and cooled to 0° when a further precipitate (0.2 g.) was removed. Excess of light petroleum was added and the ether removed by evaporation until the solution was turbid at room temperature. The solution was cooled to 0° overnight and 1.3 g. of precipitate (fraction 2) were removed. After a further 5 days at 0° a second precipitate 0.4 g. (fraction 3) was obtained. The mother liquor from fraction 3 was completely soluble in light petroleum. The fractions analysed as follows.

Fraction (1) 4.73 mg. substance: 4.58 mg. CO₂ and 1.74 mg. H₂O.

0.1067 g. substance required (Stepanoff) 8.9 cc. 0.1 N thiocyanate.

Fraction (2) 4.582 mg. substance: 5.710 mg. CO₂ and 2.09 mg. H₂O.

0.0753 g. substance required 5.23 cc. 0.1 N thiocyanate.

Fraction (3) 4.841 mg. substance: 6.420 mg. CO₂ and 2.58 mg. H₂O.

0.0971 g. substance required 6.49 cc. 0.1 N thiocyanate.

Mother liquor 0.1488 g. substance required 8.0 cc. 0.1 *N* thiocyanate.

	C	H	Br	M.P.
Calculated for $C_{20}H_{32}O_2Br_8$:	25.42	3.39	67.79	(decomp.) 240°.
$C_{18}H_{30}O_2Br_6$:	28.49	3.99	63.26	181°.
$C_{18}H_{32}O_2Br_4$:	36.00	5.33	53.28	114°.
$C_{18}H_{34}O_2Br_2$:	48.88	7.69	36.2.	
Found, fraction (1)	26.4	4.09	66.71	(decomp.) 238°.
(2)	33.98	5.07	55.60	105–107°.
(3)	36.16	5.92	53.48	114°
Mother liquor			43.0	

The analytical figures for fraction 1 approximate to those required by arachidonic acid octabromide. Fraction 2 appears to consist of a mixture of tetrabromostearic acid and of the bromides of linolenic acid or arachidonic acid. Fraction 3 is tetrabromostearic acid. The mother liquor yielded a syrup containing 43.0 % bromine intermediate between the values required by the tetrabromide (53.28 %) and the dibromide (36.2 %). The finding of arachidonic acid in the acetone-soluble fraction is of interest since it was shown to be present in liver-lecithin by Levene and Simms [1922]. Since the small amount of phosphatide present in the fraction would give rise to 0.2 g. of unsaturated acids, it is just possible that the amount found could have been derived from the phosphatide present. This, however, seems improbable, since the phosphatides isolated from varying sources give rise on hydrolysis to mixtures of unsaturated acids.

DISCUSSION.

The source of the residual fatty acids. During the subsequent discussion, the fatty acids which are present in the blood in forms other than as cholesteryl esters or as phosphatide will be referred to as the residual fatty acids, for the sake of ease of description. Information as to whether these residual fatty acids are present in the blood in the post-absorptive state as neutral fat, free fatty acid or possibly as soap is almost entirely lacking. It is of interest to note, however, that Letsche [1907] concluded that if glycerides were present, they constituted an insignificant portion of the fat. Since it was not possible to separate the glycerides from the cholesterol and cholesteryl esters present, estimations of the glycerol content were carried out on quantities of about 1 g. of fractions 2 and 3 by the method of Zeisel and Fanto [1903]. The results showed that these fractions contained glycerol corresponding respectively to 4.69 % and 9.59 % of neutral fat calculated as tristearin, or 0.43 and 0.97 g. These quantities account for 56 % of the residual fatty acids in fraction 2 and 42 % in the case of fraction 3. A determination was not carried out on fraction 1 as material was not available, but the results on fractions 2 and 3 suggest that about 50 % of the residual fatty acids are coupled to glycerol as neutral fat, *i.e.* the blood contains 7–8 mg. of neutral fat per 100 cc. Whether the remainder is present as free fatty acids or soaps,

we have no information, but the method of Zeisel and Fanto applied to materials containing so small a percentage of glyceride will tend to give low values. Jarisch [1923] concluded, from a study of the behaviour of soaps in solutions of buffers at varying p_H values, that the reaction of the blood is such as to preclude the presence of soaps.

The amount of the residual fatty acids in blood. In view of the dearth of information regarding the analysis of blood-fat on a large scale, it is of interest to compare our findings with those obtained by micro-methods. For the determination of the various fatty compounds in blood, two methods, differing both in the technique employed for the extraction of the blood and the subsequent treatment of the extracts obtained, have been worked out by Bloor [1915] and by Bang [1918, 1]. Bloor's method entails nephelometric determinations of total fatty acids and ether-soluble phosphorus on an alcohol-ether extract of blood, together with a colorimetric determination of cholesterol. The amount of the residual fatty acids may then be calculated by subtracting the sum of those present as phosphatide (obtained from the ether-soluble phosphorus figure) and of those present as cholesteryl esters from the total fatty acids. Bang's method depends on the extraction of air-dried blood with light petroleum followed by alcohol. The former solvent is reported by Bang [1918, 1] and by Blix [1925, 1, 2] to remove free cholesterol and neutral fat only, and the latter, cholesteryl ester and phosphatide. The amount of the components present in the two extracts is estimated after suitable treatment by oxidation under prescribed conditions with potassium dichromate. Hence Bang's method entails a direct estimation of the glyceride content, while Bloor's figures are obtained indirectly. Some of the results recorded in the literature are presented in Table III.

Table III. *Amount and distribution of fatty acids of blood expressed as mg./100 cc.*

Animal	Mean of	Method	Total	As lecithin	As cholesteryl ester	Residual (glyceride)	Observer
Dog	10	Bloor [1915]	552	238	34*	280	Bloor [1915]
	7	"	594	243	44*	307	Bloor [1916, 1]
	6	"	713	208	40	465	Knudson [1917]
	12	Bang [1918, 1]	276	181	66	29	Bang [1918, 2]
Man	14	Bloor [1915]	360	210	52†	98	Bloor [1916, 2]
Woman	9	"	360	203	57†	100	"
Man	7	Bang [1918, 1]	225	131	70	24	Bang [1918, 2]
	15	"	—	—	—	20	Blix [1925, 2]
Woman	21	"	—	—	—	30	"

The figures recorded for the fatty acids present as phosphatide and cholesteryl ester have been calculated by the writers from those in the papers referred to (fatty acids = $0.70 \times$ lecithin, fatty acids as cholesteryl ester = ester cholesterol $\times \frac{333}{400}$).

* Total cholesterol only was determined. This figure has been calculated from the results of Knudson [1917] who found 26.4 % of the total sterol in dog's blood to be in ester form.

† Calculated similarly from the results of Bloor and Knudson [1917] who found that 34 % of the total cholesterol present in human blood was in ester form.

The results recorded in Table III show that the figures yielded by Bloor's method are consistently greater than those obtained by that of Bang. The

differences in the figures appear to be much in excess of what might be expected to be due to normal variation in the amount of blood-fat at fasting level, for each is a mean value derived from determinations on the blood of six or more animals. The main difference appears in the residual fatty acids, for those obtained by Bloor's method are from 6 to 15 times as great as those recorded by Bang for dog's blood, and from 3 to 5 times as great in the case of human blood. Since from the study of blood-fat in bulk we have arrived at a figure of 15 mg. per 100 cc. for the residual fatty acids of ox blood, a figure which is of the same order as those reported by Bang and Blix for blood from a large number of dogs, men and women (20–30 mg./100 cc.), our results, as do those of Letsche [1907], may suggest that Bang's method yields results for the residual fatty acids more reliable than that of Bloor. It may perhaps be unsafe to compare the results obtained from the blood of a herbivorous animal with those from that of carnivorous animals. Horiuchi [1920], however, using Bloor's method on the blood of rabbits, obtained results which suggested that 40–50 % of the fatty acids were present as residual fatty acids, figures of the same order as are recorded in Table III for the blood of dog and man by this method. The possibility exists, however, that figures for the residual fatty acids, even though they be obtained from blood in the post-absorptive stage, may vary considerably among themselves on account of the state of previous nutrition of the animal. The finding of relatively small amounts of fatty acid not in combination with cholesterol or present as phosphatide bears out the results previously recorded on twelve samples of blood [Channon and Collinson, 1929].

The degree of unsaturation of the fatty acids of the phosphatide and acetone-soluble fraction. During the investigations of the nature of the fatty acids of the phosphatide fraction, it became apparent that the phosphatide fatty acids were definitely more saturated than those of the acetone-soluble fraction. Further samples of blood-fat were prepared and were approximately separated by the addition of excess of acetone to their solution in ether into phosphatide and acetone-soluble fractions: without further purification the fractions were saponified and, after removal of the unsaponifiable matter, the fatty acids were obtained and their iodine values were determined. For comparison with the figures so obtained, there are recorded in Table IV the iodine values of the corresponding fractions of the liver, heart and kidney of rats, which had received for some weeks a synthetic diet in which butter was the source of fat. These figures on rat tissues are recorded instead of figures on the organs of the ox, because they are representative average values, since the two groups (a) and (b) contained 25 and 17 animals respectively.

Bloor [1927; 1928] obtained for the iodine values of the fatty acids of the phosphatide fractions of the liver, kidney and heart of the ox 113, 110 and 120, and for those from the acetone-soluble fraction 87, 82 and 75 respectively. It is clear from the data recorded in Table IV, firstly, that the phosphatide fatty acids of blood are considerably less unsaturated than those of the tissues, and

Table IV. *Iodine values of the fatty acids of the phosphatide and acetone-soluble fractions of the fat of ox blood at fasting level.*

Ox	Blood	Phosphatide fatty acids		Fatty acids of acetone-soluble fraction	
		71		123	
		77		137	
		86		143	
		90		121	
		(a)	(b)	(a)	(b)
Rat	Liver	153	149	76	80
	Kidney	129	113	64	66
	Heart	133	128	83	75

secondly, that the fatty acids in the acetone-soluble fraction are considerably more unsaturated than those of the phosphatide fraction. These figures are of the same order as those of Bloor [1924] who found that the fatty acids of the phosphatide fraction of ox plasma had an average iodine value of 71 determined on 16 samples, whereas the average iodine values of the fatty acids of the successive fractions obtained by crystallisation of the acetone-soluble fraction from alcohol were 139, 147, 111, 119 and 94.

Before discussing the possible physiological significance of the degree of unsaturation of the fatty acids of the phosphatide and acetone-soluble fractions, the evidence as to whether the unsaturated acids in the acetone-soluble fraction are present as glyceride or as cholesteryl ester will be considered. Meanwhile, it may be noted here that, as in blood, the fatty acids of yeast-phosphatides are more saturated than those of the acetone-soluble fraction [Daubney and Smedley-MacLean, 1927], and the same finding holds for the corresponding acids from cabbage leaf cytoplasm [Chibnall and Channon, 1929].

The compounds in which the unsaturated acids are present. Earlier in this paper, it was shown that about 55 % of the fatty acids of the acetone-soluble fraction are coupled to cholesterol as cholesteryl esters, and that the remaining 45 % are present probably chiefly as glyceride. The amounts of the fatty acid obtained from the fractions 1, 2 and 3 were 82, 42 and 127 % in excess of that required by the cholesteryl ester present, and the iodine values of the mixed fatty acids from the three fractions were 110, 138 and 123 respectively. It was shown also that the combined fatty acids of the three fractions contained arachidonic, linoleic and oleic acids. Since the fatty acids obtained by hydrolysis of each fraction contain so large an amount of residual acid, no reliable deduction can be drawn from consideration of their iodine values as to the degree of unsaturation of that fraction of the acids which was coupled to cholesterol. Thus, if those acids not present as cholesteryl esters were arachidonic and linoleic acids, the acids coupled to cholesterol would have a relatively low iodine value. Hence analysis of our own results leaves us unprepared to express a definite view on this point. On the other hand, Bloor [1924], from a study of the proportions of unsaponifiable matter and fatty acid produced by hydrolysis of the first two fractions obtained by crystallisation from alcohol of the acetone-soluble fraction of 16 samples of plasma-fat,

concludes that these fractions consist of cholesteryl esters and deduces from the iodine values of the fatty acids that the unsaturated acids of plasma are mainly present as esters of cholesterol. Since Bloor's calculated figures for the fatty acids are obtained by regarding the unsaponifiable matter as entirely cholesterol, the agreement with the amounts of fatty acid found is more apparent than real, for it is stated later that the unsaponifiable matter consisted of from 70 to 90 % of sterol only. Hence, disregarding the fact that any free cholesterol in the fractions will lower still further the theoretical amount of fatty acid required, analysis of Bloor's figures shows that if the sterol content of the unsaponifiable fractions be taken as 80 % on the average, the fatty acids obtained by hydrolysis were in excess of the theoretical amount in 11 cases in fraction 1 from 21 to 54 % and in fraction 2 from 23 to 40 %. Hence for the reasons mentioned in the case of our own results, no conclusion can be safely drawn as to what proportion of the unsaturated acids is present as cholesteryl esters.

The transport of unsaturated acids from the liver to the tissues. On the evidence of Leathes and Meyer-Wedell [1909] and Hartley [1909], Leathes and Raper [1925] consider that one function of the liver is to desaturate fatty acids in preparation for their utilisation by the tissues. Hence, evidence regarding the unsaturation of the fatty acids in the various fat compounds in blood should afford some evidence as to the form in which these desaturated fatty acids are carried to the tissues from the liver.

Since the fatty acids of the acetone-soluble fraction of blood-fat are considerably more unsaturated than those of the phosphatide, it might seem reasonable to suggest that the unsaturated acids are carried to the tissues in this fraction.

On the other hand, the fact that the phosphatide fatty acids of blood are more saturated cannot be taken as evidence that the phosphatides are not playing a part in fat transport. If the researches of Levene and his colleagues on the phosphatides of tissues, which have shown that the fatty acids of these compounds are made up of equimolecular proportions of saturated and unsaturated acids, apply to the blood-phosphatides, then the iodine value of the unsaturated half of the mixed fatty acids, which have an iodine value of 80, will be 160. If these unsaturated acids contain oleic acid, the iodine value is sufficiently high for there to be present in the phosphatide molecule acids considerably more unsaturated than oleic. Hence the relatively low iodine value of the blood-phosphatide acids does not preclude the phosphatides from being considered as carriers of unsaturated acids from the liver to the tissues.

SUMMARY.

1. The acetone-soluble fraction of the fat from 34 l. of ox blood at fasting level has been investigated.
2. The fatty acids present in the fraction are arachidonic, linoleic, oleic, stearic and palmitic acids.
3. Of the fatty acids produced by hydrolysis of the fraction, 55 % are present as cholesteryl ester. Of the remainder, about one-half is present as neutral fat.
4. The amount of fatty acid in the fraction which is not in combination with cholesterol corresponds to no more than 15 mg./100 cc. blood. The bearing of this finding on certain micro-methods of blood-fat determination is discussed.
5. The acids obtained by hydrolysis of the fraction are considerably more unsaturated than those of the phosphatide fraction; but whether the unsaturation is mainly due to the fatty acids present as glyceride or as cholesteryl ester has not been determined.
6. The bearing of the results on the question of the transport of unsaturated acids from the liver to the tissues is discussed.

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