LXXXIV. THE FATTY ACIDS OF OX BLOOD.

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WHILST numerous data concerning the nature of the fatty acids present in depot fat and liver are available [Banks and Hilditch, 1931; Klenk and Schoenebeck, 1932; Irving and Smith, 1935], there are few observations on the nature of the fatty acids in blood. This problem is clearly of importance, since knowledge as to the transport of fatty acids from the tissues and depots *via* the blood can only be obtained when the chemical nature and relative proportions of the various fatty acids present in the blood of animals in the post-absorptive state are known. As a contribution to this problem the present work was undertaken.

EXPERIMENTAL.

The lack of knowledge on this subject is due to the great difficulty of preparing fatty acids from blood in sufficient quantity to make adequate analysis possible. Whilst it might be anticipated that such a preparation would be readily achieved by digestion of the blood with alkali, followed by acidification and extraction with a fat solvent, such as ether, great difficulties are encountered. In the first place the amount of total fatty acid present in different forms of combination in blood is relatively small (about 0.2 g. per 100 ml.), while a further difficulty arises because of the high protein concentration in blood. Repeated experiments showed that simple digestion with alkali, followed by acidification, resulted in the precipitation of such a considerable quantity of protein degradation products that extraction with any fat solvent was most ineffective and it happened quite often that the proportion of fatty acid extracted in this way was an insignificant part of the whole. Endeavours were made to overcome these difficulties by hydrolysis of the blood with 10% NaOH in the presence of alcohol. Some fifty varieties of treatment with different proportions of 10% NaOH and alcohol and variations in time of heating were investigated, but in no case was the result satisfactory. Many experiments of similar type were carried out in which the strength of alkali was varied from 15 to 30%, with heating for different time periods in the presence of varying proportions of alcohol. The method finally adopted, which was the best compromise possible in view of the large volume of blood required, was as follows. To each 100 ml. of blood were added 5 g. NaOH and the mixture was refluxed for 4 hours on a sand-bath; after cooling overnight, 50 ml. of alcohol were added and the mixture was refluxed for 2 hours more; it was then cooled, acidified with conc. HCl and the fatty acids and unsaponifiable matter extracted with ether. After washing the ethereal extract with water and evaporating to dryness, the residue was saponified with *N* sodium ethoxide and the fatty acids and unsaponifiable matter prepared in the usual manner and purified by treatment with light petroleum. As a control of this and the other methods investigated, further samples were treated by the method of Channon and Collinson [1929] and the yields compared. It appeared that the method yielded about 70% of the total fatty acids present in blood. A

large scale preparation was then carried out in which 51.7 l. of blood were treated in volumes of 3-4 l. at a time to give 78 g. of fatty acids (151 mg./100 ml.). The corresponding figures quoted from a large number of previous samples of ox blood by Channon and Collinson averaged 180 mg./100 ml. By combining the fatty acids obtained from these various trial experiments with the main bulk, the total fatty acids available weighed 96 g., i.v. 92.6; those obtained by the latter authors had i.v. 97.5.

Separation of saturated and unsaturated acids. 95.4 g. of the mixed acids were fractionated by the Twitchell process as modified by Hilditch and Priestman [1931]. Considerable difficulty was experienced in obtaining a more saturated fraction which had a reasonably low i.v., but ultimately the acids were divided into two portions: (1) 25.6 % of the mixture, i.v. 9.6, and (2) 72.4 %, i.v. 121.6, mol. wt. 302, the loss being 2.0 %. In order to determine the amount of saturated acid still remaining in the liquid fraction, Bertram's oxidation process was applied, when 3.777 g. of that fraction yielded 0.274 g. acids, (i.v. 4.6) or 7.3 %. Assuming that oleic acid was the cause of the i.v. of 4.6 and 9.6 quoted above, the true amounts of saturated and of unsaturated acids in the original mixture were calculated to be 27.9 and 70.1 % respectively, excluding the loss of 2.0 %.

Analysis of the saturated fraction. 20.7 g. acids (i.v. 9.6) were esterified and the methyl esters distilled into five fractions and a residue. The acids from each fraction were separated from traces of unsaponifiable matter and their mol. wt. and i.v. determined. The composition of each was then calculated, the results being shown in Table I.

Table I. *Fractionation of the saturated esters.*

Fraction	B.P. at 3 mm. up to ° C.	Wt. of ester g.	i.v. of acids	Mol. wt. of acids	Composition %			
					Palmitic	Stearic	Ligno- ceric*	Oleic
1	160	1.48	3.2	257	6.7	—	—	0.2
2	168	5.49	3.4	263	19.3	5.3	—	1.0
3	173	3.03	4.4	272	6.0	7.4	—	0.7
4	181	3.63	6.0	275	5.6	10.2	—	1.1
5	185	3.00	7.5	283	0.5	12.3	—	1.2
Residue	—	4.81	15.2	321	—	8.6	10.0	3.8
Total	—	21.44	—	—	38.1	43.8	10.0	8.0

* For purposes of calculation the acids higher than stearic acid have been grouped together as lignoceric acid.

Table II. *Fractionation of the unsaturated esters.*

Frac- tion	B.P. at 3 mm. up to ° C.	Wt. of esters g.	Twitchell separation (description)	i.v. of acids	Mol. wt. of acids	Saturated			Unsaturated		
						C ₁₆	C ₁₈	C ₂₄	C ₁₈	C ₂₀	C ₂₂
1 a	155	1.09	Solid	53.6	277	0.3	0.2	—	0.8	—	—
b			Liquid	106.7	291	—	—	—	2.7	1.3	—
2 a	160	2.07	Solid	35.5	283	—	1.2	—	0.8	—	—
b			Liquid	110.7	293	—	—	—	4.6	3.5	—
3 a	170	2.19	Solid	32.2	290	—	0.9	0.1	0.5	—	—
b			Liquid	106.7	291	—	—	—	6.2	2.9	—
4	175	3.26	„	115.8	293	—	—	—	9.1	6.8	—
5	176	3.22	„	118.5	294	—	—	—	8.4	7.3	—
6	176	2.90	„	130.9	309	—	—	—	—	14.1	—
Residue	—	4.87	„	152.2	325	—	—	—	—	9.3	14.4
Total	—	*19.60	—	—	—	0.3	2.3	0.1	33.1	45.2	14.4

* 20.56 g. were taken initially. Loss = 4.7 % of the total unsaturated fractions or 3.4 % of the original mixed acids.

Analysis of the unsaturated acids by bromination. The results obtained by brominating the unsaturated acids of blood were very similar to those previously found and fully discussed in an analysis of pig liver fatty acids [Irving and Smith, 1935]. 22.3 g. (I.V. 121.6) yielded 3.37 g. ether-insoluble bromide, M.P. 228° (indef.), Br 67.9%. $C_{20}H_{32}O_2Br_8$ requires Br 67.8%. The minimum amount of arachidonic acid present was therefore 4.9% of the unsaturated acids.

From the ether-soluble portion, 1.98 g. crude tetrabromostearic acid were isolated, M.P. 106°, Br 56.1%. After prolonged fractional crystallisation, the purest sample weighed 0.35 g., M.P. 110–111°. $C_{18}H_{32}O_2Br_4$ requires M.P. 114°, Br 53.3%. Obviously this derivative was still contaminated with more complex bromides from which it could not readily be separated.

By the usual method of extracting the ether-insoluble bromides with benzene, no hexabromostearic acid was found, suggesting that linolenic acid was entirely absent or present in traces only.

Oxidation of the unsaturated acids. 12.78 g. of the liquid fraction (I.V. 121.6) were oxidised and twice reoxidised by the process of Lapworth and Mottram [1925, 1], as carried out by Channon *et al.* [1934], a method which is recognised to give the greatest yield of dihydroxy-derivatives so far obtainable from such a mixture. The dihydroxystearic acid so prepared weighed 1.51 g. Recrystallisation yielded 1.02 g., M.P. 131°, mol. wt. 314, and 0.23 g., M.P. 123°. The usual 9:10-dihydroxy-derivative requires M.P. 132°, mol. wt. 316.

1.01 g. dihydroxystearic acid were now further oxidised by the method of Lapworth and Mottram [1925, 2]. The resulting dibasic acid (0.341 g.) melted after recrystallisation at 141°, mol. wt. 177. $C_8H_{14}O_4$ requires M.P. 141°, mol. wt. 174 and the theoretical weight obtained should have been 0.556 g. It can be said therefore that the bulk of the octadecenoic acid in ox blood is of the usual $\Delta^{9,10}$ type.

The tetrahydroxystearic acid arising from the original oxidation of the 12.78 g. amounted to 0.36 g. and was the usual mixture of isomerides melting indefinitely at 155°.

The amounts of di- and tetra-hydroxy-derivatives obtained correspond to 10.5 and 2.3% of oleic and linoleic acids in the unsaturated fraction, but it is well known that with such a mixture these values are always very considerably below the theoretical figures. As shown in Table III, attempts were made to

Table III. *The oxidation of the acids from each liquid fraction from the distillation of the unsaturated esters.*

Fraction	Wt. taken for oxidation g.	Dihydroxystearic acid			Tetrahydroxystearic acid		
		Weight g.	M.P. ° C.	Oleic acid in total unsaturated acids %	Weight g.	M.P. ° C.	Linoleic acid in total unsaturated acids %
1 b	0.433	0.035	126	0.3	0.004	—	0.04
2 b	1.064	0.077	125	0.5	0.098	160–163	0.6
3 b	1.330	0.225	126	1.4	0.129	160–163	0.7
4	2.029	0.351	127	2.5	0.109	160	0.7
5	2.074	0.388	127	2.6	0.213	164–165	1.3
6	2.014	0.144	126	0.9	0.034	163	0.2
Residue	3.467	None	—	—	None	—	—
Total	—	—	—	8.2	—	—	3.5

increase these yields in order to obtain more accurate values by oxidising the different fractions which resulted on distillation of the liquid esters. However, no appreciable difference was observed [*cf.* Irving and Smith, 1935].

Analysis of the unsaturated acids by fractional distillation followed by oxidation of each fraction. 21.48 g. acids (i.v. 121.6, mol. wt. 302) were esterified and the methyl esters distilled. The fractions were saponified, traces of unsaponifiable material removed and the acids prepared. The total loss in the whole process amounted to 4.7 % of the unsaturated fraction and, since much decomposition had been observed to take place in the residual fraction during the distillation with the formation of petroleum-insoluble products, it may be assumed that the loss was mainly in that residual fraction. Since the acids of the first three fractions were partly solid at room temperature, they were subjected to the Twitchell separation.

From the mol. wt. and i.v. recorded in Table II, it was possible to calculate the mean mol. wt. of each liquid fraction, had it been fully saturated. Then from these calculated figures the proportions of C_{18} , C_{20} and C_{22} acids were estimated. From the data available, only the mean degree of unsaturation can be known for each of these types of acid.

A portion of each fraction was now oxidised by Lapworth and Mottram's method, the object being to obtain as true a value as possible for the amounts of oleic and linoleic acids present in the total mixture. The results are set out in Table III.

The figures obtained in this way for the contents of oleic and linoleic acids in the unsaturated fraction were therefore 8.2 and 3.5 %, a result which is discussed later.

Hydrogenation of the liquid fraction. As a liquid saturated acid has been isolated by Turner [1931] from cat's kidney, it was desirable to investigate the possibility of the existence of such substances in blood. Such acids, if present, would be found in the liquid fraction from the Twitchell process. 4.41 g. mixed acids from a separate preparation were therefore separated by the lead soap process and 1.3 g. of the liquid fraction hydrogenated. The hydrogenated acids, which were recovered quantitatively, formed a hard solid crystalline mass, m.p. 58–60°. The absence of more than traces of liquid saturated acids was therefore established.

DISCUSSION.

The value of the general analysis just described depends in the first place on the truth of the assumption that the particular preparation of the fatty acids employed was truly representative of the whole. In the present case the amount of fatty acids extracted from the blood was approximately 70 % of the figure obtainable on a small scale by the method of Shimidzu [1910], and 85 % of that obtained by the method of Channon and Collinson [1929]. As there is no apparent reason why in the method used here any particular acid should be isolated in preference to another, it seems probable that the mixture prepared was truly representative.

It must also be observed that samples of fatty acids prepared from different small batches of ox blood vary quite appreciably both in mean i.v. and in mean mol. wt., but the results recorded in this communication may claim to be typical of the average, since the analysis was made on the mixed acids from over 50 l. of blood.

The fractional distillation method of making the general analysis has already been discussed by Hilditch [1934] and by Irving and Smith [1935]. In regard to the results in Table I, where the content of palmitic and stearic acids is calculated as a percentage of the saturated fraction and in Table II, where the C_{18} and C_{20} unsaturated acids are presented as percentages of the unsaturated

fraction, it is realised that the figures quoted for these particular acids may only approach the true values to within ± 5 units %. This possible error is considerably reduced, particularly in the case of the saturated acids, when the composition of the whole mixture is finally calculated as recorded in Table IV.

Table IV. *A comparison of the fatty acid content of the liver, blood and depots.*

		Ox depot*	Ox blood fat	Ox liver fat†		Pig liver‡ fat
		(Average of 4 analyses)		Phosphatide	Glyceride	
Saturated acids	Lower acids	6	—§	Trace	Trace	1
	C ₁₆	27	10	12.5	25	14
	C ₁₈	22	13	27	20	19
	Higher acids	Trace	3	Trace	Trace	2
	Total	55	26	39.5	45	36
Unsaturated acids	C ₁₆	—	—§	5	9	1.5
	C ₁₈	45	26	27	37	33
	C ₂₀	—	33	18	8	20
	C ₂₂	—	10	10.5	1	7.5
	Total	45	69	60.5	55	62

* Banks and Hilditch [1931].

† Klenk and Schoenebeck [1932].

‡ Irving and Smith [1935].

§ There was not sufficient material available to make possible the detection of small amounts of lower acids.

In the case of the higher saturated acids the total amount present was of the order of 3 %. It made no significant difference to this figure whether the actual acid was regarded as arachidic or lignoceric. In actual fact there are probably traces of several higher saturated acids in ox blood, but lignoceric acid was chosen for calculation purposes, as it is known to be present in sphingomyelin in blood.

Hydrogenation experiments proved the absence of anything more than minute traces of liquid saturated acids, while the absence of ordinary linolenic acid was indicated by bromination. These findings are in agreement with the corresponding results obtained in the case of pig liver fat [Irving and Smith, 1935].

Attempts were made to estimate the amounts of oleic and linoleic acids present in the liquid fraction, first by oxidising a sample of the total mixture and then by oxidation of each individual fraction obtained on distillation. The figures of 10.5 and 2.3 % resulting in the first case for the contents of oleic and linoleic acids were confirmed, within the limits of the processes involved, by those of 8.2 and 3.5 % by the second method. Obviously nothing was gained by the latter and much more laborious process. A very similar finding resulted in the case of the pig liver acids where the corresponding pairs of figures were 21.4 and 4.5 % and 22.0 and 4.3 % respectively. Again nothing was gained by the more elaborate method. It must also be noticed that in the two cases under discussion the amount of C₁₈ unsaturated acids found to be present by fractional distillation was very considerably more than that suggested by the oxidation results. The poor yields of hydroxy-derivative always obtained from such mixtures remains unexplained.

The composition for the total mixture of blood fatty acids is shown in Table IV where it is compared with the figures found for ox depot fat by Banks and Hilditch [1931] and with those obtained for ox liver fat by Klenk and

Schoenebeck [1932]. The pig liver values are also quoted for comparison. From the i.v. recorded in Table II, together with the oxidation results already quoted, it appears probable that the 26 % of C_{18} acids present in the total mixture consisted approximately of 20 % oleic and 6 % linoleic acid. From the i.v. and bromination results the C_{20} and C_{22} unsaturated acids contained anything from one to four or five double bonds.

One of the most interesting points arising from this table is the very high content of C_{20} and C_{22} unsaturated acids occurring both in blood and in liver. Apart from the possibility of minute traces, these substances are conspicuously absent both from the depot fat and from various foodstuffs, such as grass [Smith and Chibnall, 1932]. It is thus possible that these acids are synthesised in the tissues, or alternatively that their presence is explained by the fact that they are contained in the foodstuffs in traces too small for detection and that these small amounts are selectively retained in the tissues. From the relatively large amounts shown in Table IV it might be tentatively supposed that they are the result of synthesis, although further confirmation is desirable before a definite conclusion is drawn.

Another fact of interest arising from the figures in Table IV, together with the i.v. and bromination experiments already recorded here and in other papers cited, is the absence of linolenic and the relatively small proportions of linoleic acid present in the blood, liver and depots, whereas grass and other vegetable foodstuffs are known to contain very appreciable quantities of these substances. This finding confirms the view that the more unsaturated acids of the diet are those which are most readily and quickly oxidised by the body and may well be connected with the fact that these particular acids are "essential" in the diet [Burr and Burr, 1929; 1930; Burr *et al.*, 1932].

As there are no measurable amounts of higher unsaturated acids in the depots and yet the liver and blood contain them to such an extent, it might be suggested that the liver has perhaps synthesised these higher constituents and that the blood is transporting them to tissues other than the depots. Results in partial confirmation of such a theory have arisen from work being carried out in these laboratories on the total fatty acids prepared by hydrolysis of the carcass of the rat from which the liver and the alimentary tract have been removed. Some 10 % of these total acids have been shown to consist of C_{20} and C_{22} unsaturated compounds, although in the depots alone they are almost entirely lacking [Banks *et al.*, 1933].

SUMMARY.

A method of preparing the fatty acids from blood on a large scale was investigated and the acids from over 50 l. of ox blood were obtained in yields approximating to 70 % of the estimated content. These were analysed and the final results are set out in Table IV. The presence of the higher unsaturated acids in liver and blood is discussed.

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