CXLIV. THE FATTY ACIDS IN THE LIVER OF THE SHEEP.

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WHILE it is well known that the fatty acids that can be isolated from the liver and other organs of animals differ from those that are found as glycerides in the adipose tissue, where reserve fat is held in store, the precise nature of the unsaturated acids in these organs has been defined only in the case of certain acids from the liver of the pig. The difference is particularly marked in the iodine value, which is usually about 135 in the case of the liver fatty acids as against 65 or less for the acids of the reserve fat. Hartley [1909] isolated an oleic acid from pig's liver in which he proved that the double bond was between C_{12} and C_{13} and a linoleic acid with double bonds between C_9 and C_{10} and between C_{12} and C_{13} . No evidence of the presence in pig's liver of the ordinary $\Delta^{9,10}$ -oleic acid was obtained, though this appeared to be the only oleic acid present in the adipose tissue fat of the pig. He interpreted these results as pointing to the formation in the liver of the new oleic acid from stearic acid, and of the linoleic acid, which also was not found in the adipose tissue fat, from the oleic acid found there, by the introduction of a new double linkage between C_{12} and C_{13} in each case. The occurrence of these acids in the liver but not in the fat stored in adipose tissue was thus regarded as further evidence for the view that stored fat of low iodine value when called up for use is taken up more particularly in the liver, where the fatty acids are desaturated and assume the properties characteristic of the fatty acids found in this and other organs.

But part of the evidence for this view consists in the fact that the liver is found frequently to contain large amounts of fat, and it is only when it does not yield more than about 3 % of fatty acids on treatment with concentrated potassium hydroxide that the iodine value of these acids is as high as 130 or 135. The larger the yield of fatty acids the lower their iodine value, and, as was shown by Imrie [1914], the actual iodine value observed is in the human liver such as would correspond with an admixture of fatty acids from the adipose tissue stores having the iodine value 65 with the ordinary 3 % of fatty acids found in the liver and having the iodine value 135; when there is 6 % of total acids the iodine value is $(135 + 65)/2$ or 100, when 9 % $(135 + 130)/3$ or 88 and so on up to 20 % or more. Had the pigs' livers that Hartley [1909] worked with contained larger amounts of fatty acids than about 3 % and included some that had been freshly imported from the adipose tissue it is to be expected that he would have found not only the $\Delta^{12,13}$ -oleic acid but also the $\Delta^{9,10}$ -acid.

The yield of fatty acids obtained by treating with potassium hydroxide the livers with which he worked is not recorded, but the fact that he found only that oleic acid which is not found in the adipose tissue seems to imply that the yield was not more than about 3 %.

In a reinvestigation of the nature of the oleic acid in pigs' livers containing about 4.5 % of fat certain data were obtained which pointed to the presence of both oleic acids in the organs used. But in sheep's liver no evidence was to be obtained that any oleic acid was present other than the $\Delta^9,^{10}$ -acid which is commonly found in adipose tissue fat. On the other hand, the same linoleic acid was obtained from sheep's liver as Hartley obtained from pig's liver. The occurrence of this linoleic acid in the liver but not in adipose tissue fat of the sheep lends itself to the same interpretation as that given by Hartley [1909], that it is formed from the $\Delta^9,^{10}$ -oleic acid by the introduction of a new double bond between C_{12} and C_{13} . But there is no evidence of the desaturation of stearic acid by this reaction at this point in the sheep's liver and if stearic acid is desaturated in this animal's liver it must be by conversion into the ordinary $\Delta^9,^{10}$ -oleic acid.

EXPERIMENTAL.

The livers were minced, the pulp was weighed, mixed with $\frac{1}{3}$ of its weight of 50 % potassium hydroxide solution and heated on a water-bath. When most of the material had gone into solution an equal volume of alcohol was added, and heating was continued to complete saponification of the compounds of fatty acids. The fluid, diluted with alcohol to 50 %, was shaken out with light petroleum (B.P. about 60°) three times to remove unsaponifiable soluble material and then after acidification shaken with the same solvent to remove the fatty acids. Sheeps' livers on various occasions treated in this way together with preparations from other species gave results such as those shown in Table I.

Table I.

	Wt. of liver g.	Fatty acids		Iodine value
		g.	% of liver	
Sheep's liver	960	36.5	3.9	123
"	2451	85.5	3.5	124
"	1500	44.7	3.0	116
"	Sample	—	3.3	135
"	"	—	5.7	—
Pig's liver	"	79.5	4.3	133
Ox liver	—	—	4.5	113
Cat's liver	—	—	2.6	125
Rabbit's liver	—	—	3.3	123

In dealing with large quantities the operations take longer and the iodine value tends to be lowered. Small samples can be carried through more rapidly and then a smaller proportion of the more unsaturated acids undergoes changes that render these insoluble in light petroleum. The molecular weight of the acids tends to be high, 300 or more, but the more thorough the extraction of the alkaline soap solution for removal of unsaponifiable substances and also

sometimes of some solid material that tends to separate out at the junction between the two fluid layers, the more nearly does the molecular weight of the fatty acids approximate to that of the common higher fatty acids (about 280). The fatty acids in this process also become less deeply coloured. If the acids finally obtained are kept for any length of time in the air they do not redissolve completely in petroleum; a product either of oxidation or possibly of polymerisation forms which is not soluble in this solvent. Two specimens of sheep-liver acids extracted on a larger scale were supplied by the kindness of the British Drug Houses, each amounting to about a kilogram. One of these had the iodine value 112 and the apparent m.w. 302, the other iodine value 92.5 and m.w. 334. For the purpose of defining the nature of the acids they were oxidised by the following methods.

(1) OXIDATIONS OF MIXED FATTY ACID ETHYL ESTERS
BY ARMSTRONG'S METHOD.

The oxidation of unsaturated fatty acids by means of powdered potassium permanganate in boiling acetone, as shown by Armstrong and Hilditch [1925], causes scission of the molecule at the site of the double bond. In this way, for example, the commonly occurring $\Delta^9,^{10}$ -oleic acid gives rise to pelargonic and azelaic acids:

The common linoleic acid, with its double bonds in the $\Delta^9,^{10}$ - and $\Delta^{12,^{13}}$ -positions, similarly breaks down to caproic and azelaic acids, together with malonic acid which loses carbon dioxide and is isolated mainly as acetic acid.

It was decided to apply this method to the mixed fatty acids from the mammalian livers to discover what acids were mainly produced. It was first necessary to make absolutely certain that in this method no oxidation of saturated fatty acids took place. 5 g. of palmitic acid were accordingly dissolved in 50 cc. of acetone and 5 g. of powdered potassium permanganate were added. After boiling for several hours the permanganate was still quite pink, showing that little, if any, oxidation had occurred. The palmitic acid was recovered quantitatively and unchanged. Armstrong and Hilditch [1925] state that for a successful oxidation it is necessary to employ a large excess of permanganate and to continue the oxidation for several hours. They also state that it is advisable to employ the fatty acids in the form of their esters, since protection of the terminal carboxyl group minimises the risk of uncontrolled oxidation. All these observations were confirmed in a series of preliminary oxidations of oleic acid and of the acids from poppy-seed oil.

A. Sheep-liver acids. Iodine value = 92.5. Equivalent wt. = 334. 40 g. of the ethyl esters (iodine value 86) were dissolved in 400 cc. of acetone and to the boiling solution 150 g. of powdered potassium permanganate were

added in small amounts during 2 hours, and the whole was boiled for a further 9 hours. The acetone was then distilled off, 1 litre of water was added to the residue and a stream of sulphur dioxide was passed in until the solution was free from manganese dioxide. The volatile acids were removed by steam distillation and extracted from the distillate with ether. The extract was dried over calcium chloride and evaporated. The yield of volatile acids was 7.18 g. These were distilled from a small flask and gave the following fractions:

Fraction	Temp. range	Wt. of fraction g.	Equivalent wt.	% Ba in Ba salt
1	Up to 190°	1.06	—	—
2	190–215°	0.88	131.4	—
3	215–245°	2.22	146.3	34.7
4	245–260°	1.02	200.0	31.5
Residue—almost non-acidic.				
Theoretical values	Acid	B.P.	Equivalent	% Ba
	Caproic	203°	116	37.37
	Pelargonic	253°	158	30.44

There were also recovered from the residue after steam distillation 17.0 g. of neutral products with iodine value 16.2, and 10 g. of acidic products, not volatile in steam, which after two recrystallisations from hot water gave an acid of equivalent 100.2 and m.p. 95°. Azelaic acid has an equivalent of 94 and m.p. 106°.

B. Ox-liver acids. Iodine value = 103. The oxidation was carried out in the same way as described for sheep-liver acids. 36.5 g. of esters (iodine value 94.5) gave 6.23 g. of volatile acids. On distillation as before:

Fraction	Temp. range	Wt. of fraction g.	Equivalent wt.
1	Up to 110°	0.06	—
2	110–200°	0.71	—
3	200–210°	0.53	131
4	210–245°	1.48	145
5	245–260°	0.67	188

The residue was almost non-acidic.

There is therefore a very close correspondence between the results obtained for sheep- and ox-liver fatty acids. The fatty acids appear to consist almost entirely of a mixture of caproic and pelargonic acids, the latter preponderating in amount. Such evidence as these figures afford of the presence of caproic acid as well as pelargonic acid furnishes no proof of the presence of $\Delta^{12,13}$ -oleic acid, since caproic acid will be formed, as shown later, from the oxidation of the linoleic acid.

(2) OXIDATIONS USING HYDROGEN PEROXIDE.

On several occasions oxidations were performed on unsaturated fatty acids using Hilditch's [1926] method, in which the fatty acids are dissolved in glacial acetic acid, concentrated hydrogen peroxide is added and the mixture is heated at 70° for several hours. The yields of dihydroxy-acids obtained in this manner were much better than those from the permanganate oxidations to be described below. Oxidations were carried out on the fatty acids from sheep's liver, pig's liver and cat's kidneys, and also on technical linoleic acid

(iodine value 180), and in each case a dihydroxy-acid was obtained (M.P. 92°). Instead of sativic acid, however, a dark brown, viscous oil was invariably obtained. Bauer and Kutscher [1925] had reported that the use of hydrogen peroxide or perbenzoic acid on linolenic acid gave rise to oils of low iodine value and high molecular weight instead of to a crystalline hexahydroxystearic acid. They suggest that linolenic acid is polymerised when these reagents are employed and it seemed worth while to see whether linoleic acid itself would give rise to sativic acid when oxidised with hydrogen peroxide; 10 g. of linoleic acid, prepared from the tetrabromide by the method described by Rollett [1909], were dissolved in 100 cc. of glacial acetic acid to which was next added 50–60 cc. of 90/100 vols. hydrogen peroxide. The solution was heated for 12 hours at 70° with occasional agitation, and at the end of this time all the upper oily layer had disappeared. The liquid was then poured into 300 cc. of water and steam-distilled until free from acetic acid. Excess of potassium hydroxide was added and the solution was boiled for 6 hours under reflux to hydrolyse any esters which might have formed. Acidification with sulphuric acid caused separation of a light oil and of a very small amount of a white crystalline solid. The oil was readily soluble in ether but the white solid remained undissolved. The ethereal extract, after removal of the ether, gave 5.0 g. of a thick orange-coloured oil, insoluble in petroleum, with an iodine value 7.86. The white solid weighed 0.12 g. (crude) and when recrystallised from hot water melted at 144°. Another 10 g. of linoleic acid were oxidised as before except that the temperature was maintained at 37° for three days. This yielded 4.4 g. of an orange oil of iodine value 5.0, and a small quantity of white solid which had M.P. 145°. One must conclude that hydrogen peroxide employed in this way is not suitable for the preparation of sativic acid.

Hydroxy-acids from the bromo-acids. An attempt was made to prepare the hydroxylated acids from the original unsaturated fatty acids by way of the bromine addition compounds. Specimens of dibromo- and tetrabromo-stearic acids were prepared by bromination of oleic acid and linoleic acid respectively in ice-cold ether. These bromo-compounds were then boiled with silver acetate in glacial acetic acid for 3–4 hours. The resulting products were further boiled with alcoholic potassium hydroxide for 2 hours. Small amounts of dihydroxy- and tetrahydroxy-stearic acids were obtained in this way but the yields were no better than those from the oxidation methods.

(3) OXIDATION OF THE FATTY ACIDS WITH ALKALINE PERMANGANATE.

(a) *Oxidation process.* The method used for the oxidation of the unsaturated acids to the corresponding hydroxylated acids was the same in principle as that devised by Hazura [1888] and described by Hartley [1907]. But the modifications introduced make a new description necessary since, with their aid, large quantities of material can be dealt with easily and rapidly. 50 g. of the mixed fatty acids were heated on a water-bath with 50 cc. of alcohol until solution was complete, and, while still warm, 50 cc. of potassium hydroxide

solution (sp. gr. 1.145) were added. The resulting soap solution, which contained a good excess of alkali, was poured into 2 litres of water in the reaction vessel. This consisted of a stout enamel vessel surrounded by a good freezing-mixture of ice and salt, and fitted with a mechanical stirrer. When the contents of the vessel were at 0°, 2 litres of 1.5 % potassium permanganate solution were added in a fine stream from a tap-funnel, the temperature of the reaction mixture being maintained throughout at 0°, if necessary by the addition of crushed ice. When all the permanganate had been added the solution was stirred for a few minutes longer, and then made strongly acid by addition of concentrated hydrochloric acid. It was found that acidification, before passing in a stream of sulphur dioxide to remove the manganese dioxide, caused the precipitated fatty acids to rise to the surface more quickly and in a more compact form. The decolorisation with sulphur dioxide was best carried out in a large aspirator bottle, since this allowed the lower aqueous layer to be drawn off without disturbing the fatty acids. By removing most of the water in this way the subsequent filtration of the fatty acids was greatly accelerated. Filtration through a Büchner funnel is not recommended since the fatty acids pack into a compact mass and prevent the passage of the water through them. The fatty acids were left to drain overnight on the filters and were then spread on porous plates to dry. The drying can be hastened by changing the plates once or twice and by allowing them to rest on a bed of filter-paper. It was found advantageous to carry out seven or eight oxidations in one day and to collect all the products for extraction.

(b) *Separation of the hydroxy-acids.* Soxhlet extraction of the dried acids was tried several times but it has many disadvantages. There is produced in the oxidations a considerable amount of a viscous oil, insoluble in petroleum, which collects in the syphon tubes and prevents the proper functioning of the apparatus. Also, unless the fatty acids are perfectly dry, channelling by the solvent takes place, and the extraction is incomplete. It was found far quicker and simpler to pour the dry melted fatty acids quickly into a litre of warm light petroleum (B.P. 60°–80°) in a flask, and then to boil the mixture under reflux for a few minutes. While still warm it was filtered through a fluted filter and the residue was again treated with more petroleum; this process was continued until no more fatty acids were extracted. If during the first extraction with petroleum an appreciable amount of water settled to the bottom of the flask, this was removed, before filtration, by pouring the whole into a separating funnel and drawing off the bottom aqueous layer. When the unoxidised saturated fatty acids had been extracted in this way with petroleum, the residue, after drying *in vacuo*, was boiled up under reflux with about 1 litre of ether. The ethereal extract was poured while warm on to a filter, and the filtrate on cooling gave a fine white deposit of dihydroxystearic acid. This process was repeated two or three times until the ethereal extract failed to deposit any more hydroxy-acid. The ethereal filtrates were then concentrated to about 100 cc., when, on cooling, a further crop of dihydroxy-acid was

obtained. On concentrating the ethereal extracts to dryness, the residue was usually a thick dark-brown oil from which nothing of value could be obtained. The ether-insoluble residue was then boiled out with 2 litres of water; the hot filtered extract deposited sativic acid in fine white crystals. These were filtered off and dried in an air-oven at 100°. The process was repeated as long as any sativic acid crystallised out on cooling the filtrate. If any residue, insoluble in boiling water, remained, it was found usually to consist of a little dihydroxy-acid which had escaped extraction by the ether. The petroleum-soluble acids from the cold permanganate oxidation usually amounted to about 60 % of the weight of the mixed acids taken. The oxidation of the unsaturated acids is never complete since the petroleum-soluble acids have an iodine value of 25–45 according to the conditions of the experiment. If the original mixed liver acids are subjected to a separation by the lead soap-ether process the unsaturated acids obtained form about 50 % of the whole and their iodine value is in the neighbourhood of 170. This shows that acids more unsaturated than linoleic acid must be present, and this can be confirmed by the preparation of polybromides which are insoluble in almost all organic solvents.

FURTHER OXIDATION OF THE HYDROXYLATED ACIDS.

The dihydroxy- and tetrahydroxy-stearic acids were next oxidised separately by the method of Armstrong and Hilditch [1925] as previously described.

A. Dihydroxystearic acid. This acid after recrystallisation from ethyl acetate or 70 % aqueous alcohol was a white crystalline solid m.p. 131–2°.

0.2226 g. required for neutralisation 7.05 cc. *N*/10 NaOH.

Equivalent wt. 315.9.

Calculated for $C_{18}H_{34}O_2(OH)_2$ 316.

20 g. of this substance after oxidation yielded 6.9 g. of acid volatile in steam and soluble in ether. This, after drying over calcium chloride, was distilled from a small flask heated in an oil-bath. Owing to the presence of a small amount of water the temperature of the distillate rose slowly to 100°; when all the water had distilled over, the temperature rose immediately to 235° at which point distillation again commenced and continued steadily up to 249°. The whole of the volatile acid distilled between these temperatures. The following fractions were collected:

No.	Temperature	Wt. g.	Equivalent wt.
1	235°	1.25	168.5
2	235–245°	1.73	167.4
3	245–249°	1.79	161.0
Residue 1.26 g.			
Equivalent wt. data			
Fraction (1)	0.1700 g. required for neutralisation	10.08 cc. <i>N</i> /10 NaOH	168.5
" (2)	0.1850 " "	11.05 cc. <i>N</i> /10 NaOH	167.4
" (3)	0.1963 " "	12.20 cc. <i>N</i> /10 NaOH	161.0
Equivalent wt.			
Theoretical for pelargonic acid $C_9H_{18}O_2$		158	253°
Theoretical for caproic acid $C_6H_{12}O_2$		116	203°

The acid amides were prepared by the method of Aschan [1898] from fractions (1) and (2) combined and from fraction (3). They melted at 93° and 94° respectively. Pelargonamide melts at 93–94°.

0.1744 g. amide from fractions (1) and (2) gave 11.2 cc. *N*/10 NH_3 (Kjeldahl).

$\text{N} = 9.0\%$.

0.4480 g. amide from fraction (3) gave 29.40 cc. *N*/10 NH_3 (Kjeldahl).

$\text{N} = 9.2\%$.

Calculated for pelargonamide $\text{C}_9\text{H}_{19}\text{ON} = 8.9\% \text{ N}$.

„ caproamide $\text{C}_6\text{H}_{13}\text{ON} = 12.17\% \text{ N}$.

All three fractions therefore consisted of pelargonic acid and there was no evidence whatever of the presence of caproic acid.

After removal of the volatile acids from the oxidation products by steam distillation, the solid residue, insoluble in water, consisted of unchanged dihydroxy-acid mixed with the non-volatile dibasic acid formed simultaneously with the volatile pelargonic acid in the oxidation. This residue was boiled with about 500 cc. of petroleum, in which dihydroxystearic acid is insoluble but which dissolves azelaic acid to a small extent when boiling. The extract was filtered hot and, on cooling, a small amount of a white crystalline solid separated out. This was recrystallised from hot water and had m.p. 104°.

0.1556 g. required 16.30 cc. *N*/10 NaOH for neutralisation.

Equivalent wt. = 95.4.

Azelaic acid $\text{C}_7\text{H}_{14}(\text{COOH})_2$ has equivalent wt. 94 and m.p. 106°.

Hence the dihydroxystearic acid from sheep-liver oleic acid yields, on oxidation with permanganate, a mixture of pelargonic and azelaic acids and must itself have arisen from $\Delta^9,^{10}$ -oleic acid.

B. Tetrahydroxystearic or sativic acid. Hartley [1909] found that the tetrahydroxystearic acid obtained by oxidation of pig-liver fatty acids was not a single substance but that it could be resolved into several fractions having different melting-points and solubilities in water. The same behaviour was observed with the sativic acid from sheep-liver. On boiling out the crude acids with successive volumes of water, it was found that the first fractions always gave an acid which melted at about 155°, and that the successive fractions had increasingly higher melting-points until a limit of 171–173° was reached. The earlier fractions also were more soluble in water than those with the higher melting-point. Unfortunately the quantities of the lower melting form were insufficient (about 3 g.) for a further oxidation, and it is therefore impossible to state whether the two or more sativic acids which are present are derived from geometrical isomerides of the same linoleic acid, or whether they correspond to linoleic acids in which the double linkages are situated in different positions in the molecule. 6 g. of the sativic acid (m.p. 171° uncorr.) were oxidised in a similar manner to the dihydroxystearic acid with powdered potassium permanganate in acetone. The volatile acids were extracted from

the steam distillate with ether, and after drying over calcium chloride the ether was removed and the acid amide prepared. It melted at 99°.

0.2420 g. of amide gave 22.15 cc. $N/10$ NH_3 (Kjeldahl).

N = 12.8 %.

Calculated for caproamide 12.17 %.

The dibasic acid is the same as that obtained from the dihydroxystearic acid, namely azelaic acid. The production of caproic acid and azelaic acid in the oxidation suggests that the double bonds in the original molecules of linoleic acid occupy the $\Delta^9,^{10}$ - and $\Delta^{12,^{13}}$ -positions. This is the most commonly-occurring form of linoleic acid.

Octabromoarachidic acid. A polybromide was prepared from the mixed sheep-liver acids by bromination in ice-cold glacial acetic acid. The white insoluble bromide was filtered through a Jena glass crucible and thoroughly washed with cold ether.

0.1570 g. of bromide gave 0.2462 g. AgBr.

Br = 66.73 %.

The bromide was then extracted thoroughly with boiling benzene, followed by boiling ethyl acetate.

0.1913 g. of purified bromide gave 0.3596 g. AgBr.

Br = 67.06 %.

$\text{C}_{20}\text{H}_{32}\text{O}_2\text{Br}_8$ requires 67.77 % Br.

The compound was therefore octabromoarachidic acid. The low solubility of this substance in all organic solvents makes its absolute purification a task of great difficulty. The formation of this bromide is evidence of the presence of arachidonic acid $\text{C}_{20}\text{H}_{32}\text{O}_2$ in the mixed acids from the sheep-liver.

SUMMARY.

An investigation has been carried out on the fatty acids obtained from the liver of the sheep.

When the ethyl esters prepared from these fatty acids are oxidised by the method of Armstrong and Hilditch [1925] a mixture of pelargonic and caproic acids is obtained. Oxidation of the mixed fatty acids with hydrogen peroxide according to the method described by Hilditch [1926] gives rise to dihydroxystearic acid but not to tetrahydroxystearic acid. Dilute alkaline permanganate, however, produces a mixture of both these acids.

The oleic acid present in sheep-liver fat has been identified as the $\Delta^9,^{10}$ -modification. Linoleic acid and arachidonic acid are also present, but there is no evidence of a linolenic acid.

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