CCXLI. STUDIES IN THE SECRETION OF MILK FAT

II. THE EFFECT OF INANITION ON THE YIELD AND COMPOSITION OF MILK FAT

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IN the course of research work on the secretion of milk fat, the effect of inanition on the lipoids of both the blood and the milk of actively lactating cows has been studied in detail. The previous paper records the experiments on the blood lipoids, while the present communication describes the changes brought about in the fat of the milk during the fast. At the same time certain modifications are described for the analysis of butter fat by the fractional distillation process.

EXPERIMENTAL

The yield of fat was estimated daily by noting the yield of milk and determining its fat content by the Gerber process. The results are recorded in Fig. 1.

Fig. 1. The effect of inanition on the total daily yields of milk fat. The extremely low yield from Cow no. 2 on the 3rd and 4th days was due mainly to the incidence of milk fever.

For the determination of iodine and Reichert Meissl values, small samples of the milk fat were prepared daily by centrifuging some of the milk, chilling the resulting product in the refrigerator and carefully removing the cake of fat so formed from the top of the aqueous phase. The fat was then further purified by suitable treatment with light petroleum. The larger samples essential for fractional distillation were prepared by separating the milk, churning the

cream and purifying the fat finally by filtration. To ensure that the sample obtained was truly representative of the whole, tests were always made to ensure that the aqueous phase never contained more than the merest trace of fat. The figures for the iodine and Reichert Meissl values are shown diagrammatically in Fig. 2, where very regular changes can be observed for the fat secreted by each of the three cows during the fast.

Fig. 2. The effect of inanition on the iodine values and Reichert Meissl values of the milk fat.

Analysis by fractional distillation. Owing to the complex nature of butter fat the analysis of its component fatty acids has always presented some difficulty. Two main procedures have been used. Hilditch & Jones [1929] and Hilditch & Thompson [1936] first saponify the fat and then distil the acidified products for some hours in a current of steam. This is followed by the fractional distillation of the steam-volatile compounds, while the non-volatile residue is separated by the Twitchell process into solid and liquid acids. These are then converted into methyl esters and fractionated. Owing to other sources of error which seem to be unavoidable in any process of this type which can at present be devised, this procedure probably gives results which are as accurate as possible, but the present authors believe that there are slight disadvantages in the actual technique. One of these lies in the fact that during steam distillation, the non-volatile fatty acids have to be heated to about 100° in the presence of steam for several hours, a process which ought to be avoided when unsaturated acids are

present; another is that, unless very large quantities of the original butter fat are available, the amount of volatile compounds resulting from the steam distillation must be exceedingly small for their accurate fractionation. In the modified method now suggested distillation in steam is dispensed with, and the lower acids can be estimated more readily on smaller amounts of initial material. Bosworth & Brown [1933] also avoided the use of steam distillation in making a detailed investigation of the lower components of butter fat and were thus able to establish the presence of decenoic and tetradecenoic acids, but they did not adapt the method to the complete analysis of the total fatty acids. To do this the Twitchell separation of liquid and solid acids must be introduced at some stage in the process. The scheme which was therefore adopted in the present work may be summarized briefly by saying that the fat was converted directly into methyl esters, the lower components were fractionally distilled from the whole bulk, the higher members being separated into liquid and solid acids, methylated and also fractionally distilled. In order to explain the method more fully reference will be made in detail to the analysis of a typical butter fat, that secreted by Cow no. 1 prior to starvation.

The analysis of butter fatty acids by the modified method. The butter fat (about 287 g., Reichert Meissl value 26, i.v. 36.6) was first refluxed in 2 l. methyl alcohol containing 5 % H_2SO_4 for 24 hr. to convert the glycerides into methyl esters. Most of the alcohol was then boiled off and kept, while the esters were exhaustively extracted with ether and the united ether extracts washed three times with a little water. The ether solution was desiccated over sodium sulphate, filtered and the solvent almost completely removed in the water bath at ordinary pressure. The flask containing the esters was then connected to an electrically heated and packed fractionating column similar to that described by Longenecker [1937] and the last traces of solvent were carefully removed and collected under a very slight vacuum. Saponification values were then obtained for the methyl alcohol and ether was distilled from the esters at the two different stages, while the united aqueous portions were saponified with alkali, acidified with H_2SO_4 , steam-distilled and the distillate was titrated. As all the methyl esters higher than the butyrate are insoluble in water it is reasonable to assume that all the acidity present in the various solvents was due to butyric acid. The amount of this compound present in the original fat could thus be calculated. A blank determination was carried out with a similar quantity of lard for which the Reichert Meissl value was negligible. The amounts of "butyric acid" estimated to be present for both the milk fat and the lard were 0.09 and 0.03 % in the aqueous portion, 2.73 and 0.03 % in the methyl alcohol and 0.68 and 0.25 % in the ether, making totals of 3.5 and 0.31 % of the butter and lard respectively. It is evident from these results that the estimated figure for butyric acid by this method tends to be somewhat high.

In the fractional distillation which followed, details of which appear in Table I, the first two fractions passed over at a pressure of 15 mm. and were kept separate. A large primary fraction containing all the esters distilling below $158-160^\circ$ at 2 mm. was then collected, for it had been shown by preliminary trials that practically all the lower compounds up to and including most of the myristate pass over below that point, while the distillation of oleate is still relatively slight. This large primary fraction was then distilled into some 20 smaller fractions for which the iodine values and molecular weights were determined, and as these data suggested that the last fraction and residue consisted mainly of palmitate and oleate, these two final fractions were added again to the higher compounds still remaining undistilled. All these higher esters were

Table I. *The fractionation of the methyl esters prepared from 287 g. of the normal butter fat secreted by Cow no. 1 prior to inanition*

Frac- tion	B.P. at 2 mm. up to	% of total esters	Mol. wt.	i.v.	Frac- tion	B.P. at 2 mm. up to	% of total esters	Mol. wt.	i.v.
Lower esters									
1	(Methyl butyrate)	3.88	—	—	12	118	0.76	216.4	9.1
2	45*	0.35	132.1	0.6	13	124	1.05	232.4	11.6
3	45*	0.32	131.8	0.6	14	127	1.12	233.4	10.8
4	56	0.22	158.5	2.6	15	127	1.61	238.3	10.7
5	56	0.42	157.4	2.7	16	128	2.38	239.8	10.4
6	56	0.07	159.2	—	17	131	1.66	242.1	9.1
7	70–80	0.56	177.8	12.8	18	141	1.31	248.4	9.0
8	82	0.67	184.8	16.5	19	145	1.56	260.9	8.9
9	84	0.61	183.1	16.4	20	148	3.44	266.2	9.5
10	102	0.51	195.5	12.9	21	149	1.76	269.5	11.3
11	106	0.94	209.5	7.9	Total		25.20		
Solid esters									
22	140	0.45	245.0	0.7	29	162	3.16	281.0	8.2
23	149	0.67	262.3	0.4	30	158	3.25	283.2	9.0
24	153	1.61	268.4	1.3	31	161	2.73	284.0	9.8
25	153	2.38	274.4	4.0	32	165	2.92	289.2	11.2
26	159	2.93	274.5	4.1	33	165	1.93	290.2	13.7
27	157	3.47	277.8	5.0	34	170	2.69	297.3	13.9
28	161	3.20	278.0	5.6	35	Residue	3.05	307.7	17.9
					Total		34.44		
Liquid esters									
36	132	0.30	237.2	17.1	44	163	3.27	287.8	77.6
37	132	0.46	244.2	17.4	45	164	2.76	288.7	79.9
38	145	0.37	254.9	27.4	46	165	2.78	290.5	82.1
39	153	0.60	268.9	46.3	47	166	4.72	295.1	85.9
40	155	1.20	281.5	67.9	48	168	4.91	295.3	87.4
41	155	2.40	282.2	72.2	49	168	4.26	295.5	88.7
42	161	2.18	284.7	72.4	50	170	4.47	294.8	90.3
43	163	2.40	287.1	76.6	51	Residue	3.28	303.1	93.1
					Total		40.36		

* At 15 mm. pressure.

then saponified and separated into liquid and solid acids and the liquid portion was freed from any small amounts of unsaponifiable matter which might be present. They were then esterified again and fractionally distilled through the electrically heated column. The iodine values, molecular weights and other data for the various fractions are recorded in Table I, the molecular weights being obtained either from the saponification values of the esters or by titration of the acids prepared from them. Corresponding data were then obtained for samples of starvation fat secreted by Cows 1 and 2, but in order to illustrate the marked differences between ordinary fat and that secreted during inanition, only the figures for the first of these need be recorded as shown in Table II.

Before the composition of the various fractions could be calculated, according to the method adopted and discussed by Irving & Smith [1935], it was first necessary to oxidize several fractions to ensure that the assumptions made as to their actual nature were justified. There is, however, no necessity to describe these oxidations in detail. In order to show the type of fractionation obtained without the use of steam distillation, the intermediate results obtained for the composition of the three main fractions, the lower, solid and liquid esters of both

Table II. *The fractionation of the methyl esters prepared from 285 g. of the butter fat secreted by Cow no. 1 on the 11th and 12th days of inanition*

Frac- tion	B.P. at 2 mm. up to	% of total esters	Mol. wt.	i.v.	Frac- tion	B.P. at 2 mm. up to	% of total esters	Mol. wt.	i.v.
Lower esters									
1	(Methyl butyrate)	1.27	—	—	6	140	0.47	242.8	24.9
2	30-40	0.05	164.7	—	7	145	0.45	258.8	22.2
3	70-90	0.13	178.0	14.4	8	150	0.74	267.0	21.3
4	90-120	0.15	193.9	15.0	9	153	1.25	270.6	21.1
5	120-130	0.20	222.3	20.8	10	157	1.11	275.4	29.0
Total							5.82		
Solid esters									
11	147	0.26	250.9	2.5	18	154	2.86	279.8	4.4
12	150	0.74	262.4	0.8	19	160	1.81	282.0	6.3
13	152	1.79	269.0	1.0	20	165	3.32	294.2	9.9
14	152	3.50	271.7	2.0	21	167	4.06	298.0	11.1
15	153	3.15	275.6	3.0	22	168	3.55	300.4	11.8
16	153	4.25	275.2	2.1	23	Residue	3.08	303.7	14.9
17	154	3.86	277.3	3.5	Total		36.23		
Liquid esters									
24	140	0.33	243.6	40.9	33	170	5.17	294.0	86.1
25	150	0.46	260.0	49.2	34	175	1.30	293.0	87.5
26	160	0.96	274.6	61.6	35	180	2.47	294.3	86.9
27	164	1.49	288.3	79.3	36	180	7.16	296.2	87.4
28	166	1.59	291.6	83.0	37	180	8.12	296.8	89.2
29	170	2.08	291.1	83.2	38	182	7.63	297.8	90.1
30	170	1.46	294.2	84.0	39	182	4.95	299.0	91.5
31	170	2.47	294.7	85.3	40	Residue	6.20	306.0	91.9
32	170	4.11	294.4	85.9	Total		57.95		

Table III. *Cow no. 1, the composition of the lower, liquid and solid fractions before and during inanition*

Fraction	Methyl esters of the normal fat secreted before inanition				Methyl esters of the fat secreted on the 11th and 12th days of inanition			
	Lower	Liquid	Solid	Total	Lower	Liquid	Solid	Total
Saturated:								
C ₄	3.88	—	—	3.88	1.27	—	—	1.27
C ₆	0.64	—	—	0.64	—	—	—	—
C ₈	0.98	—	—	0.98	0.11	—	—	0.11
C ₁₀	1.85	—	—	1.85	0.18	—	—	0.18
C ₁₂	2.51	0.05	—	2.56	0.14	—	—	0.14
C ₁₄	8.07	3.08	0.74	11.89	1.00	1.25	0.53	2.78
C ₁₆	5.32	0.31	17.83	23.46	1.96	0.23	17.84	20.03
C ₁₈	—	0.15	11.38	11.53	—	0.04	14.27	14.31
C ₂₀	—	—	1.08	1.08	—	—	0.89	0.89
Unsaturated:								
C ₁₀	0.24	—	—	0.24	—	—	—	—
C ₁₂	0.24	—	—	0.24	—	—	—	—
C ₁₄	0.76	0.18	—	0.94	0.19	0.25	—	0.44
C ₁₆	0.71	2.44	—	3.15	0.28	1.17	—	1.45
Oleic	—	32.16	3.41	35.57	0.69	49.23	2.70	52.62
Linoleic	—	1.17	—	1.17	—	2.47	—	2.47
C ₂₀	—	0.82	—	0.82	—	3.31	—	3.31
Total	25.20	40.36	34.44	100.00	5.82	57.95	36.23	100.00

Table IV. *The component fatty acids of the different butter fats expressed in weight and molar percentages*

Acid	Wt. per 100 g.			Molecules per 100 molecules		
	Cow no. 1		Cow no. 2 During inanition†	Cow no. 1		Cow no. 2 During inanition†
	Before inanition	During inanition*		Before inanition	During inanition*	
Saturated:						
C ₄	3.5	1.2	2.7	9.7	3.5	7.9
C ₆	0.6	—	0.1	1.2	—	0.1
C ₈	1.0	0.1	0.1	1.6	0.2	0.2
C ₁₀	1.8	0.2	1.0	2.5	0.3	1.5
C ₁₂	2.5	0.1	0.6	3.0	0.2	0.7
C ₁₄	11.9	2.8	3.8	12.5	3.2	4.3
C ₁₆	23.5	20.0	22.1	22.1	20.9	22.1
C ₁₈	11.6	14.3	9.9	9.8	13.5	8.9
C ₂₀	1.1	0.9	0.9	0.8	0.8	0.8
Total	57.5	39.6	41.2	63.2	42.6	46.5
Unsaturated:						
C ₁₀	0.2	—	0.2‡	0.3	—	0.2
C ₁₂	0.2	—	0.2‡	0.3	—	0.3
C ₁₄	0.9	0.4‡	0.4‡	1.0	0.5	0.5
C ₁₆	3.2	1.4	2.0	3.0	1.5	2.0
Oleic	35.9	52.8	51.7	30.5	50.1	46.9
Linoleic	1.2	2.5	0.8	1.0	2.4	0.7
C ₂₀	0.8	3.3	3.5	0.6	2.9	2.9
Total	42.4	60.4	58.8	36.7	57.4	53.5
Total of all the acids up to C ₁₄	22.6	4.8	9.1	32.1	7.9	15.7

* A sample obtained by mixing the fat secreted on the 11th and 12th days of inanition.

† A pooled sample from the fat secreted on the last 6 days of inanition.

‡ Where the amount of lower acids present is very low and oleic acid predominates as in the fats of inanition, it is probable that the figures marked ‡ are somewhat higher than the true values.

normal and "fasting" fat are arranged in Table III, while the final figures for all three fats are recorded in Table IV.

Linoleic acid. As it was at first thought that inanition might cause marked changes in the amount of linoleic acid present in the different fats, its presence was sought by oxidation methods in various fractions, but in no case could tetrahydroxystearic acid be isolated. This was doubtless due to the fact suggested by the final results of the fractionation that the linoleic acid content of the milk fat from these particular cows was as low as 1–2% and that the amount of fat available was limited. But as the presence of octadecadienoic acid in normal butter fat has been shown beyond dispute by Green & Hilditch [1935], there can be little doubt that it also existed in smaller amounts in the present case.

Arachidonic acid. The presence of small amounts of arachidonic or some such highly unsaturated acid in the milk fat both before and during inanition was shown by brominating both the last fraction and the residue in the distillation of the liquid esters. The amount of bromo-derivative (Br 65.5%, m.p. 228° with decomposition), obtained in both cases was less than 0.1% of the whole.

DISCUSSION

The yields of fat. For all three animals the period of inanition was characterized by a very considerable decrease in the daily secretion of milk fat. Thus it can readily be seen from Fig. 1 that for the first cow, in which inanition was not

complicated by other factors, the yield fell quickly from 568 g. before the fast to 353 g. on the 3rd day, and then more slowly to a mean of 227 g., or 40 % of the original during the last 3 days of the fast. On re-alimentation a further slight drop was noticeable, but 5 days later the daily yield began to increase and at the end of about 6 weeks it had returned to some 60 % of the pre-fast value. With Cow no. 2, in which milk fever symptoms developed on the 3rd day, the secretion of fat and milk almost ceased while this complication lasted, but by the 5th day, the fat production had risen to 12 %, and before the end of the fast to 20 % of the original value. On re-alimentation the yield followed a course almost parallel with that of Cow no. 1 during the same period.

The composition of the fats. Reference to Fig. 2 shows clearly that inanition caused a marked progressive change in the nature of the fats secreted by each of the three cows as judged by their iodine and Reichert Meissl values, and that these changes were similar for all three animals. It will be observed that the initial iodine values of 36.6 and 37.1 for Cows 1 and 2 rose quickly to 46.1 and 47.3 on the 1st day of the fast, and then more slowly to 52.5 and 54.9 by the 12th day, the figures for Cow no. 3 following a similar course till the 7th day when this particular animal was withdrawn from the experiment. Then again the Reichert Meissl values for Cows 1 and 2 fell rapidly in the first 2 days from 26.0 and 33.3 to 14.5 and 22.9 and then still further to 9.8 and 13.8 by the end of the fast. On re-alimentation a slight tendency to return to normal was observed in both values after the first few days and this tendency continued somewhat spasmodically for the next few weeks until figures similar to the pre-fast values were attained. These results suggest that inanition causes a considerable increase in the content of unsaturated acids and a marked fall in that of lower fatty acids normally present as glycerides in butter fat. This was confirmed by more detailed analyses based on fractional distillation methods, for reference to the totals recorded in Tables I and II shows that while the proportion of higher solid esters remained almost unaffected as a result of inanition, the lower esters were reduced from a pre-fast value of 25.2 to 5.8 % during inanition, and that the corresponding figures for the liquid esters showed an almost equal increase from 40.4 to 58 %. Again it will be observed from the weight percentages in Table IV that the sum of all the acids up to and including C_{14} amounted to 22.6 % before the fast and to no more than 4.8 % during inanition, a decrease of 17.8 units, while the oleic acid content rose by 16.9 units from 35.9 to 52.8 %. Calculated also on a molar basis, as shown in Table IV, the fall of 24.2 molecules % in the lower acids is mainly accounted for by a rise of almost 20 molecules % in the oleic acid. It is therefore evident that the chief effect of inanition is the replacement of about 80 % of the lower constituents of the fat mainly by oleic acid, and that the whole of this decrease is distributed over all the lower members from C_4 to C_{14} , although the first and last of these appear to suffer least. Thus the butyric acid content only fell from 3.5 to 1.2 % a trifle more than a third of the original, the myristic by a somewhat similar amount and the others to less than an eighth of their pre-fast values. Of the other saturated acids, the amount of palmitic fell slightly from 23.5 to 20.0 %, while the stearic increased from 11.6 to 14.3 %. That the latter rise had probably some significance is shown best by reference to the molar percentages, where it will be observed that stearic acid increased from 9.8 to 13.5 %, almost a 40 % increase, whereas the fall in palmitic acid only amounted to some 5 % of the pre-fast value. In fact the constancy of the palmitic acid in all three sets of results is worthy of particular notice. The saturated acids represented as C_{20} also remained virtually unchanged. In the unsaturated acids higher than C_{14} and other than oleic, the chief change appeared to be a decrease

in palmitoleic acid from 3.2 to 1.4% and a most marked rise from 0.8 to 3.3% in higher unsaturated components represented as " C_{20} ".

The results for the fat secreted by Cow no. 2 during inanition are also recorded in Table IV, where it will be seen that with the probable exception of stearic and linoleic acids, the general effect of the fast was the same for both animals.

The relationship between the lipoids of the blood and milk. In discussing the relationship between the blood lipoids and the milk fat it must be remembered that at present there is no means of analysing the triglyceride fatty acids of the blood by themselves but only when mixed with those originally present as cholesteryl esters. There is therefore every possibility that the triglyceride acids of the blood may change and yet the change may be impossible to detect, and as these acids probably form one of the most important sources of milk fat, the disadvantage inherent in this fact for research work of the present type cannot be overestimated. At the same time, bearing this limitation in mind, it is well worth observing the almost complete absence of change already noted in the blood fatty acids and particularly in those of the non-phosphatide fraction of the plasma during inanition as compared with the very striking alterations brought about in the fat of the milk. In fact, the latter suggest that the normal equilibrium existing between the amount of fat actually formed in the gland and that arising directly from the blood was very considerably disturbed. For example, the enhanced concentration of higher unsaturated compounds of the C_{20} type during the fast might well be taken as indicating that there was an increase in the unsaturated acids arising directly from the blood in which the proportion of these acids has been shown by Parry & Smith [1936] to be exceedingly high, and that the proportion of fat synthesis in the gland was considerably decreased.

The greatest change, however, was in the amount of lower fatty acids present and these are worthy of special consideration. With regard to their source in normal butter fat there are probably three main possibilities. In the first place, they may pre-exist in the blood and arise from it directly. Secondly, they may be formed in the mammary gland itself by breakdown of oleo-glycerides as suggested by Hilditch & Thompson [1936] and by Hilditch & Paul [1936]. Thirdly, they may arise, again in the gland itself, as by-products in the synthesis of the fat from some form of carbohydrate material. The theory that they may arise directly from the blood gains support from the suggestion that lower fatty acids may be formed as by-products of rumination in the cow and that these may then be transported by the blood to the mammary gland. In human milk the content of these lower constituents is exceedingly small. From the present work this theory might also appear to receive confirmation from the fact that when rumination ceased as a result of the enforced fast, the lower fatty acid content of the milk also fell. In this connexion two points should, however, be noted. In the first place it would be natural for these lower compounds, if they exist in blood, to be present in the non-phosphatide fraction of the plasma. But the results recorded in Table II of the previous paper (p. 1861) suggest that if any of these lower acids were present as esters in this fraction, their amount must have been very small, and that they certainly did not appear to decrease during inanition. It is always possible, however, that during the analysis of the blood lipoids free water-soluble acids were lost, for such an occurrence would be exceedingly difficult either to detect or avoid. In the second place, if the reduction in these lower constituents of milk fat were due in the present instance to a deficiency of the same acids in the blood, it would be natural to expect that their decrease in the milk fat during inanition would be counterbalanced by increases fairly evenly distributed over the other constituents, whereas it has already been

shown that almost the whole loss is made good by oleic acid alone. Indeed this last observation might well be taken as evidence in support of Hilditch's suggestion that the lower acids arise by the oxidation of oleo-glycerides in the gland itself, for if this were so, the catabolism of these glycerides might not be so essential during inanition when the general activity of the gland was considerably reduced, with the consequence that the content of the lower constituents would fall and that of oleic acid rise. Recent work, however, by Graham *et al.* [1938], based partly on respiratory quotient measurements, has definitely suggested that fat metabolism in the mammary gland probably takes the form of synthesis rather than of breakdown. The explanation of the present results may therefore be that the lower compounds are formed as by-products in the synthesis of oleic acid, but that during inanition, when the gland is producing much less than its usual amount of fat, the smaller amount of synthesis taking place is able to proceed to its final stages, so that the lower acids are almost completely converted into oleic. This suggestion gains support from the well-known fact that diets rich in carbohydrates and poor in fat cause an increase in the lower fatty acids of the milk fat, while those poor in carbohydrates and rich in fat have the reverse effect, indicating that with diets on which fat synthesis would probably be most necessary the lower fatty acids increase. At present it is impossible to decide as to which of these various theories is correct but it is hoped that future work now contemplated will throw more light on this particular aspect of the subject. At present the fact of chief importance emerging from the results just described is the inverse relationship existing between the oleic acid on the one hand and the lower acids on the other.

SUMMARY

1. Although in the previous paper no appreciable change was observed during inanition in the nature of the blood lipoids of three lactating cows, very considerable changes have been found in the milk fat, and these have been studied in detail.

2. The chief change was a decrease of about 80 % in the original content of lower acids up to and including C_{14} , a deficiency which was almost entirely made good by an increase in the content of oleic acid.

3. The fact that oleic acid tended so completely to replace the lower constituents has been discussed in connexion with different theories previously put forward to explain the occurrence of these lower homologues in milk.

4. A description is given of a modified procedure for the analysis of butter fat by the fractional distillation method from which prolonged distillation of the saponified fat in steam is omitted.

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