Studies on the Origin of Milk Fat

2. THE SECRETION OF DIETARY LONG-CHAIN FATTY ACIDS IN MILK FAT BY RUMINANTS

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Early work, carried out chiefly by means of the arteriovenous technique, indicated that sufficient of the triglyceride fraction of blood is absorbed by the udder to account for all the fat of ruminant milk, and that no other fraction of blood fat is likely to be an important precursor (see reviews by Folley, 1940, 1949). However, it cannot be concluded that it is the fat absorbed from the blood which appears in the milk, for its metabolism in the udder is not known (Graham, Jones & Kay, 1936). This question can be examined in detail only by the use of isotopically-labelled fatty acids.

The origin of the short-chain fatty acids characteristic of the fat of ruminant milk has been explained in various ways, of which that proposed by Hilditch (1947) (see also Achaya & Hilditch, 1950) seemed at the time to be best supported by experimental evidence, though this is, admittedly, mostly indirect. According to this theory the short-chain acids are formed in the mammary gland by oxidative degradation of preformed oleoglycerides, beginning at the end of the carbon chain furthest from the carboxyl group. One molecule of oleic acid would thus give rise to one molecule of short-chain acid.

More recently, attention has been focused on the synthesis of fatty acids from smaller molecules, of which the most important is acetic acid, or a reactive derivative of acetic acid. That this occurs in the mammary gland has been shown by respiratory and isotope experiments on mammary slices *in vitro* (see Folley, 1954), and by isotope experiments on the intact goat (Popják, French & Folley, 1951) and on the isolated, perfused cow's udder (Cowie *et al.* 1951). It was found in these experiments that [^{14}C]acetate injected into the blood resulted in considerable radioactivity in all the acids of milk fat up to and including palmitic acid. Stearic and oleic acids, on the other hand, had a much lower specific activity, and it was thought that they might arise mainly from blood lipids.

This work with labelled acetate does not, however, give any indication of the quantitative

importance of acetate as a precursor of milk fatty acids, and the possibility is by no means excluded that appreciable proportions of the lower acids arise by degradation of long-chain acids, as envisaged by Hilditch. In the experiments to be described, tritium-labelled stearic acid has been administered orally, either as free acid or as triglyceride, to lactating goats and a cow, and the rate of appearance and the distribution of radioactivity in the milk have been investigated. By this means the uptake of circulating long-chain acids by the mammary gland could be detected, Hilditch's theory could be tested, and an estimate could be made of the contribution of dietary stearic acid to milk fat.

Some of the results have already been communicated in preliminary form (Duncombe & Glascock, 1954; Glascock & Reinius, 1954).

EXPERIMENTAL

Analytical methods

Milk fat was determined by the routine Gerber method.

Isolation of milk fat for isotopic analysis. About 100 ml. of each milk sample was centrifuged and the aqueous phase kept for isolation of other milk constituents. The cream was treated with boiling ethanol and ammonia and the lipids were extracted with a mixture of equal volumes of light petroleum (b.p. 40–60°) and ether (McClymont, 1951). The solution of lipids was dried over anhydrous Na_2SO_4 , the solvent evaporated under reduced pressure, and the molten fat subjected to a vacuum of a few mm. Hg in a desiccator.

Milk water was obtained from the aqueous phase by freeze-drying about 0.1 ml. in a vacuum apparatus and collecting the sublimed water.

Lactose was prepared from the aqueous phase according to the method of Popják, Hunter & French (1953), and was washed with a solution in light petroleum (b.p. 40–60°) of tristearin and stearic acid in order to remove any traces of radioactive lipids, and then crystallized from methanol. After drying at 110° the lactose was characterized by formation of the osazone (m.p. 188–189°).

Casein was precipitated from the aqueous phase (diluted 1:4 with water) by adjusting the pH to 4.6 with acetic acid. The precipitate was filtered off, washed quickly with 0.02M acetic acid, and dissolved by the addition of the minimum quantity of 0.1N-NaOH. The solution was filtered and the casein again precipitated, dissolved, reprecipitated and

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finally dried. It was then continuously extracted with ether to remove possible traces of radioactive lipids.

Fractionation of milk fat. The following fractions were obtained by the use of procedures similar to those described by Popják & Beeckmans (1950): glyceride fatty acids (non-volatile total, saturated and unsaturated; steam-volatile water-soluble and -insoluble) and phospholipid fatty acids (same fractions). Steam-volatile fractions were redistilled after addition of inactive carrier stearic acid, in order to avoid possible contamination by traces of radioactive long-chain acids.

Glycerol was isolated as the tribenzoate from the aqueous phase remaining after saponification of the glyceride fraction and extraction of the free fatty acids (Popják, Glascock & Folley, 1952), and was thoroughly washed with a carrier solution of stearic acid.

Blood total solids and blood water were obtained by freeze-drying small volumes.

Determination of fat content of diet (Experiment IV). Samples of each component of the diet were taken at intervals during the experiment and pooled. These were dried to constant weight at 100°, ground and extracted with ether in a Soxhlet apparatus. The ethereal extract was decolorized with activated charcoal, and the total pigment-free lipids were dried to constant weight after removal of the ether.

Radioactivity measurements

Solid samples were subjected to combustion in a stream of oxygen (Glascock, 1952). The tritium content of the combustion water, and of blood- and milk-water samples, was determined by conversion into butane and measurement of the radioactivity in a gas counter (Glascock, 1951). All specific activities quoted are in counts/min./mg. of water as observed with our equipment.

Radioactive materials

[³H]Stearic acid was prepared from oleic or elaidic acid by hydrogenation with tritium gas (Glascock, 1954; Glascock & Reinius, 1956). Labelled triglycerides were prepared by transesterification of tristearin or olive-oil triglycerides with the labelled acid, possible traces of free fatty acid and phospholipid being removed by passage of an acetone solution of triglyceride through a magnesium oxide column (Bergström, Borgström & Rottenberg, 1952).

Animal experiments

Experiment I. The animal was a cross-bred British Saanen goat weighing 73.5 kg., pregnant, and giving about 1.5 kg. of milk daily. The labelled material consisted of 11.7 mg. of [³H]stearic acid (prepared from oleic acid) containing a total activity of 4.05×10^9 counts/min. It was dissolved in 1 ml. of arachis oil and emulsified in 50 ml. of 5% (w/v) glucose solution (hot) together with 0.5 g. of glyceryl monostearate. The goat was injected with oxytocin and milked out, and the cooled emulsion was immediately administered quantitatively by mouth, followed by 50 ml. of similar but non-radioactive emulsion. Water, hay and stock cereal diet were provided *ad lib.*, and the goat was kept in an isolation pen and milked once daily for 15 days. Bedding and droppings were burnt daily. In this and subsequent experiments milk samples were treated in the manner already described.

Experiment II. In the preceding experiment the labelled stearic acid was synthesized from oleic acid. This is very difficult to obtain in a state of chemical purity, and impurities might give rise, on saturation with tritium-hydrogen, to labelled acids of chain length other than C₁₈. In the present experiment the starting material was elaidic acid, the crystalline *trans*-isomer of oleic acid, which can be obtained chemically pure. The labelled material administered orally to the goat was an emulsion containing 7.7 mg. of [³H]stearic acid, prepared from elaidic acid, and having a total activity of 3.94×10^9 counts/min.

This goat was a pedigree British Saanen weighing 61.1 kg. and giving 2.3 kg. of milk daily. After administration of the emulsion the goat was milked out at 2-hourly intervals for the first 10 hr., and twice daily afterwards for 14 days. Every 2 hr. during the first day 5 ml. blood samples were taken from the jugular vein and afterwards once daily.

Experiment III. This experiment was designed so that some estimate might be made of the quantitative contribution of dietary fat to milk fat. The labelled stearic acid was fed as tristearin, and the dietary fat was arranged to consist mostly of triglyceride by using a diet deficient in fat and augmented with tristearin and milk.

The animal was a pedigree British Saanen goat weighing 46.8 kg., and giving about 3 kg. of milk daily. The daily diet contained sugar-beet pulp and potato flakes (about 2 kg.); hay (about 450 g.); casein (about 100 g.); milk (about 2 kg.), and tristearin (about 75 g. as a 10% emulsion mixed with the milk). The exact amount of each component consumed was recorded daily and its fat content determined, so that the daily intake of fat could be calculated. During the 7-day experimental period the total consumption of fat was 1197 g., derived as follows: sugar-beet pulp and potato flakes, 22%; hay, 2%; milk, 32%; tristearin, 44%. Since milk fat consists largely of triglycerides (Bloor, 1943), it can be seen that at least 75% of the dietary fat must have been in this form. The goat was gradually accustomed to this diet during the 3 weeks preceding the experimental period.

The labelled material consisted of 2.72 g. of tristearin containing combined [³H]stearic acid prepared from elaidic acid. The total activity was 1.24×10^9 counts/min. This preparation was administered orally in an emulsion, and the goat was milked twice daily. Total faeces were collected daily and dried, and the tritium content was measured.

Experiment IV. A cow was used in this experiment, and here, also, the labelled fatty acid was fed as triglyceride. However, in order to overcome the objection that tristearin is not a common dietary constituent and might abnormally influence utilization of fat, a tracer dose of labelled olive-oil triglyceride was administered.

The cow was a pedigree Shorthorn in her fourth lactation, weighing 567 kg. and giving about 10 kg. of milk daily. The diet consisted of hay, concentrates and silage, each from the same stock throughout the experiment. The daily consumption of each of these was measured. Fat was determined in each component of the diet as already described and the daily intake of fat calculated.

The labelled material consisted of 4 g. of olive-oil triglycerides containing combined [³H]stearic acid prepared from elaidic acid. The total activity present was 2.25×10^{10} counts/min. This oil was administered by mouth in an

emulsion immediately after milking, and the cow was afterwards milked at 4-hourly intervals for the first 40 hr. Normal twice-daily milkings were then resumed.

RESULTS

Experiment I. The specific activity of the milk fat is shown plotted semi-logarithmically against time in Fig. 1 (a), the time for each point on the curve being the mid-point of the time during which the sample was secreted. It will be observed that milk having the highest specific activity was secreted during the first 24 hr. The specific activity then declined rapidly. The half-life was not constant, however, indicating that a number of processes with different time-constants are involved in the transport of the radioactivity from gut to milk.

in the milk. It is of interest to note that both triglyceride and phospholipid long-chain acids of milk have about the same specific activity; they are evidently derived ultimately from the same pool.

An unexpected feature of the results was the comparatively high specific activity found in the unsaturated glyceride acids. It is well known that saturation and desaturation of fatty acids occur in the body. If, however, the stearic acid is labelled with tritium only at the $C_{(9)}$ and $C_{(10)}$ atoms, as expected from the method of synthesis, then biological desaturation would be expected to lead to the loss of approximately half the radioactivity, assuming that no isotopic fractionation had occurred. Further investigation of this problem, using samples obtained in Expt. II, has, in fact, shown

Fig. 1. Specific activity of milk and blood constituents of goats (a, b and c) and a cow (d) after ingestion of $[^3H]$ stearic acid administered as free acid (a and b) or as triglyceride (c and d). $\circ$, Milk fat; $\bullet$, blood solids; $\times$, blood water; $\triangle$, milk water.

The cumulative secretion of activity in the milk fat, as a percentage of the dose administered, is shown in Fig. 2 (a). More than 17% of the dose was secreted in the first 24 hr., the total secretion being substantially complete at about 26% of the dose after 8 days.

Fractionation of the fat from the first day's milk sample (Table 1, column 2) shows that most of the activity resided in the long-chain fatty acids, though there was also measurable activity in the short-chain acids, and in glyceride glycerol, cholesterol and lactose. It thus seems probable that dietary long-chain fatty acids can be transported intact to the mammary gland and secreted

that the stearic acid was not labelled as expected (Glascock & Reinus, 1954, 1956). In the oleic acid biologically derived from it only about 3% of the total tritium present resided on the $C_{(9)}$ atom and 12% on the $C_{(10)}$ atom. Furthermore, the activity in atoms $C_{(10)}$ to $C_{(18)}$ was 1.68 times that in atoms $C_{(1)} to $C_{(9)}$. The finding that the unsaturated acids in the present experiment have quite a high specific activity is thus consistent with the occurrence of biological desaturation of long-chain acids in the intact goat.$

Experiment II. One of the objects of this experiment was to investigate more closely the early part of the milk-fat specific activity/time curve

Fig. 2. Cumulative secretion of radioactivity in milk fat of goats (*a*, *b* and *c*) and a cow (*d*) after ingestion of [^3H]stearic acid administered as free acid (*a* and *b*) or as triglyceride (*c* and *d*).

Table 1. *Specific activities (counts/min./mg. of water) of milk constituents from goats and a cow after the oral administration of [^3H]stearic acid as free acid or as triglyceride*

For experimental details see text.

Constituents	Expt. I	Expt. II		Expt. IV
		Sample 6	Sample 7	
Total lipids	11 460	12 880	10 610	14 200
Glyceride fatty acids:				
Non-volatile total	11 835	—	—	—
Non-volatile saturated	14 000	12 770	10 900	14 280
Non-volatile unsaturated	11 030	10 800	10 200	19 540
Steam-volatile water-soluble	80	34	68	24
Steam-volatile water-insoluble	206	196	450	198
Glyceride glycerol	346	144	316	—
Phospholipid fatty acids:				
Non-volatile total	13 290	—	—	—
Non-volatile saturated	—	—	—	13 000
Non-volatile unsaturated	—	—	—	19 000
Steam-volatile water-soluble	201	—	—	—
Steam-volatile water-insoluble	411	—	—	—
Cholesterol	69	—	51	—
Lactose	150	39	31	38
Casein	—	111	171	—

after the ingestion of a tracer dose of labelled free stearic acid. As will be seen from Fig. 1 (b), activity appears quite rapidly in the milk fat, an appreciable level being found in the sample taken 4 hr. after dosing; the peak activity is reached after 23 hr. Thereafter, the pattern is very similar to that found in the previous experiment.

The cumulative secretion of activity (Fig. 2b) has a similar form to that of the previous experiment. The total secretion is considerably greater here, however, reaching 47% after 8 days (cf. 26% in Expt. I) and 49% after 14 days. Appreciable activity was still being secreted after 14 days.

The pattern of distribution of activity in milk-fat fractions and other milk constituents (Table 1, columns 3 and 4) also confirms the previous results.

The peak activity of blood solids occurs at about the same time as that of milk fat (Fig. 1b). During the early part of the experiment at least it seems probable that nearly all the radioactivity associated with blood solids is due to long-chain fatty acids absorbed from the gut, and these results indicate that circulating lipids are taken up by the mammary gland and secreted in the milk very rapidly.

It will be seen from Fig. 1 (b) that the blood-water and milk-water curves do not coincide. This is unexpected because labelled water is known to equilibrate very rapidly with body water. Although this finding cannot at present be explained, it is not thought that it is due to analytical errors.

From these results it is also possible to calculate the percentage of administered activity circulating in blood solids or as body water at any time. Taking the blood volume of the goat as 6.2% of its body weight and the total solids content of the blood as 20.5 g./100 ml. (Dukes, 1947), then the solids of the blood amount to about 7.7×10^5 mg. Combustion of dry blood solids showed that 1 mg. gave 0.72 mg. of water. The highest measured specific activity of the blood solids was 156 counts/min./mg. of combustion water. The total activity circulating in blood solids at that time was therefore 8.65×10^7 counts/min., corresponding to 2.2% of the activity administered. Similarly, by taking body water to be 70% of the body weight, and assuming that blood water is characteristic of all body water, it is calculated that when the blood water is at its peak specific activity, about 41% of the total activity administered is present in the body in the chemical form of water. Because of the low specific activity of the body water, at least 41% of the ingested activity is therefore lost as far as fatty acid metabolism is concerned. Of the 59% remaining, more than 48% is secreted in the milk fat, from which it is evident that the lactating mammary gland takes a large share of the labelled stearic acid available to the whole body.

Experiment III. In this experiment the labelled material was administered as triglyceride instead of free acid. The rate of appearance of activity in the milk fat is hardly changed, however, as may be seen by comparing Fig. 1 (c) with Fig. 1 (a) and (b), and the peak activity is again reached in less than 24 hr. after dosing. The total secretion of activity in milk fat is, however, considerably less than after ingestion of labelled stearic acid, the plateau level reaching only about 13% of the ingested activity (Fig. 2c).

The controlled fat intake of the goat in this experiment enables an estimate to be made of the contribution of dietary glycerides to milk fat. During the first 7 days of the experiment 1197 g. of fat was ingested, and as an approximation it may be assumed that this amount of glyceride fat became labelled with the total radioactivity given in the dose. About 12.5% of this activity was secreted in the milk during the first 7 days; thus 12.5% (149 g.) of the dietary glyceride appeared during that time in the milk-fat of which the total output was 623 g. Thus $149/623$, or 24% of the milk fat, was derived from dietary glycerides. This is an upper limit for the contribution, the assumption being made that the dietary fat consisted entirely of glycerides. In fact, at least 75% of the dietary fat is known to have been glycerides (see Experimental), so that the lower limit to the contribution becomes about 18%. There are, of course, considerable uncertainties in such a calculation. In particular, the assumption is implicit that all long-chain fatty acids behave similarly with respect to absorption from the gut, transport and utilization by the mammary gland. If stearic acid does not behave quantitatively in the same way as other esterified long-chain acids then the calculation will not be valid.

It is of interest to note that this calculation does not depend on the assumption that all the labelled glyceride was absorbed from the gut. As a matter of interest measurements of total faecal dry matter and its specific activity were made and these showed that 33.9% of the total activity ingested was excreted, so that only 66.1% was absorbed. The figure of 12.5% of the total ingested activity appearing in milk fat in 7 days (Fig. 2c) thus corresponds to 18.9% of the absorbed activity.

Experiment IV. The rate of appearance of activity in the milk fat of a cow after oral administration of labelled triglyceride (Fig. 1d) is in general similar to the curves previously found for goats given free stearic acid or triglyceride (cf. Fig. 1a-c), and the maximum activity occurs at about the same time. Measurable activity was still present in the milk fat secreted after 37 days.

The cumulative secretion of activity was the highest found in any experiment, reaching 59%

after 10 days (Fig. 2*d*). From the specific activity of blood water 2 days after dosing it was calculated (as in Expt. II) that about 40% of the ingested activity was at that time in the chemical form of water. In this particular animal, therefore, nearly all the ingested activity could be accounted for either as milk fat or as water. Absorption must therefore have been virtually complete. This result is in contrast with that of Expt. III, which was carried out on the goat, in which 33.9% of the ingested activity was excreted. This is possibly because the diet in that experiment contained a considerable amount of tristearin, which is an unphysiological ingredient.

In the first 10 days the fat intake of the cow was 2070 g. (ether-extractable fat, decolorized with charcoal), and the production of milk fat was 4525 g. Using the figure of 59% of the dose appearing in the milk fat, a calculation similar to that employed in Expt. III shows that some 27.5% of the milk fat was derived from dietary fat, compared with 18–24% found for the goat. This is almost certainly an upper limit for the contribution, since it is assumed in the calculation that all the dietary fat becomes effectively labelled with the ingested, labelled triglyceride. If, as is possible, some of the dietary fat consists of waxes or other lipids not utilized by the mammary gland in the same way as glycerides, then the true contribution of dietary fat to milk fat would be less than that calculated.

Fractionation of the milk-fat sample of highest specific activity (Table 1, column 5) gave results generally in good agreement with those obtained from goats given labelled unesterified stearic acid, but differing in that the unsaturated long-chain acids of both triglycerides and phospholipids here have a considerably higher specific activity than the corresponding saturated acids. Whether this difference from the previous results is due to the chemical form of the ingested acid cannot at present be determined.

DISCUSSION

In the experiments reported here the labelled materials were administered by mouth. A considerable degree of randomization and transformation of lipids takes place during digestion and absorption in laboratory animals, on which most work has been carried out. The administered radioactivity thus becomes distributed in the various fractions of the blood lipids. This leads to considerable difficulties in the interpretation of milk-fat radioactivities when a specific precursor is being sought, even if detailed fractionation of blood lipids is attempted. Nevertheless, it is clear from these results that some part of the milk fatty acids

is derived from circulating lipids without extensive breakdown and resynthesis. The long-chain fatty acids of milk had a very much higher specific activity than the short-chain acids, and other fractions of the milk, including glyceride glycerol, cholesterol, lactose and casein, were found to have a comparatively low specific activity. Obviously, therefore, much of the milk long-chain acids must have arisen other than by synthesis from small molecules, and it seems certain that this is by direct transport of dietary fatty acids. The results suggest, however, that the contribution of dietary fat to milk fat may not be more than about 25%. The original estimate of 70% (Duncombe & Glascock, 1954) was based on a dietary fat content taken as the total ether-extractable matter, and is evidently considerably too high.

There are virtually no other figures available to enable a complete balance sheet of milk-fat production to be attempted. Results from non-isotopic techniques, such as measurement of arteriovenous differences, are difficult to interpret even qualitatively since the use to which absorbed fat is put by the mammary gland is unknown. In future developments of this work it is hoped to investigate the importance of the various fractions of blood lipids as precursors of milk fat. The quantitative importance of acetate and other short-chain acids absorbed from the rumen must also be studied before there is a possibility of obtaining a complete picture.

The question whether the short-chain fatty acids of milk arise by degradation of long-chain acids can now be answered with a fair degree of certainty. In all these experiments the short-chain acids have had a much smaller specific activity than the long-chain acids, and it is clear that not very much chain-shortening of the higher acids could have occurred. It should be noted that this conclusion is valid only if chain-shortening would give lower fatty acids still containing tritium. On Hilditch's theory, it is the oleic acid in glyceride combination that undergoes chain-shortening, starting at the methyl end of the molecule. As mentioned above, the oleic acid obtained biosynthetically in the present experiments was found to be labelled with tritium on atoms other than C_9 and C_{10} (Glascock & Reinius, 1956), so that acids down to C_{10} formed from it would almost certainly have a high specific activity, while shorter acids might also be appreciably active. The finding of only small specific activities in both water-soluble and -insoluble steam-volatile fatty acids suggests that these fractions did not arise primarily by degradation of oleic (or stearic) acid, and is consistent with the synthesis which is known to occur from circulating short-chain acids. This synthesis, contrary to the theory of Hilditch, may thus be the quantitatively

predominant process in the production of the characteristic short-chain acids of ruminant-milk fat.

Hilditch (1952) has more recently suggested that his analytical observations could be explained by assuming that oleic acid residues in glyceride combination might, instead of being degraded to short-chain acids, be replaced by short-chain acids derived from some other source. This would indeed help to reconcile the known facts with his observations, although other questions are raised, such as the fate of the oleic acid residues displaced from the glycerides. These might perhaps be oxidized, or re-incorporated into glycerides if more glycerol is made available by synthesis. Such possibilities might well be studied by isotopic techniques.

SUMMARY

1. Tritium-labelled stearic acid was administered orally, either as free acid or as triglyceride, to lactating goats and to a cow, and the appearance of radioactivity in milk fat and its distribution in milk constituents were studied.

2. Measurable activity was found in the milk fat 4 hr. after administration of labelled fat, the maximum specific activity occurring within 24 hr. Activity was still detectable in milk fat secreted up to 37 days after administration.

3. Up to 59% of the ingested activity ultimately appeared in the milk fat.

4. High specific activities were found in the long-chain acids, both saturated and unsaturated, of milk glycerides and phospholipids. Much lower specific activities were found in the steam-volatile water-soluble and -insoluble fatty acids of milk fat, and in milk glyceride glycerol, lactose, cholesterol, casein and in body water.

5. The results suggest that the contribution of dietary fat to milk fat was not more than about 25%, and that the characteristic short-chain acids of the milk fat of ruminants do not arise mainly by degradation of long-chain acids.

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