

# Metabolism of stearate-1-C<sup>14</sup> in the isolated cow's udder\*

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## SUMMARY

One-half of a lactating cow's udder was perfused with heparinized and oxygenated cow's blood at 38° for 150 minutes; 30 minutes after the onset of the perfusion, approximately 200  $\mu$ c of stearate-1-C<sup>14</sup> (albumin-bound) was added to the perfusing blood, together with inactive acetate and glucose. Almost all the blood's stearate-1-C<sup>14</sup> was absorbed by the gland, and much of the added C<sup>14</sup> was recovered from the glycerides of the udder tissue. Stearic acid and oleic acid of the glycerides showed significant specific activities. Negligible activities were detected in the shorter chain fatty acids of the glycerides, in citric acid, and in CO<sub>2</sub>, indicating that stearic acid was broken down to only a very small extent.

It has been known for several years that part of the milk fat of the ruminant is derived as a result of lipogenesis in the mammary gland itself (short-chain fatty acids) and that the other part originates from plasma lipid fatty acids (C<sub>18</sub> fatty acids) (1).

Glascock *et al.* (2) administered tracer doses of tritium-labeled stearate, either free or combined, by mouth to lactating cows and goats and noted an efficient transfer to the milk. The total recovery amounted, in one experiment, to 60%. Radioactivity was detected in milk fat within 4 hours after the administration, and the specific activity reached a maximum after 23 hours. In the glyceride fatty acids the highest specific activity was observed in the nonvolatile fractions. The specific activity of the stearic acid was estimated to be approximately three times that of oleic acid, indicating a very considerable conversion of one acid to the other. The lower fatty acids of the glycerides showed only a very low activity, indicating that stearic and oleic acids are broken down to a very slight extent. This agrees with the view that these lower acids are not derived from the degradation of the long-chain acids. Glascock *et al.* (3) did not succeed in identifying the plasma lipid fraction which was most important for the transport of the labeled stearic acid from the digestive tract to the udder.

As Fredrickson and Gordon (4) state in a recent review, free or unesterified long-chain fatty acids

(FFA) are present in the plasma of man and the common laboratory mammals in concentrations in the range of 0.2 to 2.0 mEq/liter under normal physiological conditions. These acids are primarily bound to albumin, and the rate of transfer of FFA *in vivo* is considerable, as the average fatty acid molecule remains in the plasma for only a few minutes at a time. Garton *et al.*<sup>1</sup> have found that the FFA of cow plasma consists mostly of stearic and oleic acids, together with palmitic and palmitoleic acids.

In the experiments reported here, we studied the metabolism of albumin-bound stearic acid-1-C<sup>14</sup> by the perfused cow's udder. A preliminary report of this work has been published (5).

## METHODS

*Perfusion of Half Udder.* The perfusion experiment was carried out on one lactating half udder from a cow yielding 15 liters of milk daily. The half udder was perfused for 150 minutes by the method of Peeters and Massart (6). The experimental details of the perfusion technique and C<sup>14</sup>O<sub>2</sub> collection have been described previously (7). Immediately before slaughter the cow was milked out as completely as possible after an intravenous injection of oxytocin. The udder was removed as soon as the cow was shot, and the gland was completely bisected along the median septum and one-half

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<sup>1</sup> G. A. Garton, A. K. Lough, and W. R. H. Duncan. Private communication.

connected to the perfusion apparatus. Heparinized, fresh cow's blood (8.75 liters) was used for the perfusion of this half udder. For the preparation of stearic acid bound to blood plasma proteins we followed essentially the method described by McCalla *et al.* (8). Stearic acid-1-C<sup>14</sup> (0.5 mc = 6.56 mg) was dissolved in 1 N NaOH on a steam bath. The solution was then added to 50 ml of saline. The day before the experiment, fresh heparinized cow's blood was centrifuged and the plasma collected. The dissolved stearate was added to 200 ml of plasma, shaken, and left overnight in the refrigerator. The following day the plasma was centrifuged to remove the precipitate. The plasma thus prepared contained approximately 200  $\mu$ c of stearic acid-1-C<sup>14</sup> bound to plasma proteins. This solution was added to the blood 30 minutes after the beginning of the perfusion. After the addition of the labeled substrate, a solution containing 10 g of CH<sub>3</sub>COONa · 3 H<sub>2</sub>O and 2 g of glucose dissolved in 300 ml of water was added to the perfusion blood by means of a constant-drip device at the rate of 2.5 ml per minute. Then, 115 minutes after the addition of the isotope, 10 I.U. of oxytocin was injected intra-arterially and 78 ml of milk was collected. After perfusion, the udder was removed from the apparatus and the skin, teats, and adipose tissue were removed by dissection. The udder tissue was weighed (4.74 kg), cut into pieces, and frozen at -15° for 1 hour. Blood samples were taken before and after the perfusion experiment.

**Analysis of the Blood Samples.** The packed cell volume (hematocrit) was measured. The total lipids were extracted from the plasma with chloroform-methanol. The total fatty acids were determined following hydrolysis of the total lipids with 6 N HCl in methanol (9). Free and total cholesterol were estimated in the plasma before and after perfusion according to the method of Schoenheimer and Sperry (10).

**Isolation of Casein, Lactose, and Citric Acid from Milk.** Casein was precipitated from the skim milk by acidification to pH 4.5 (7). The whey was lyophilized and lactose isolated from the dried powder as described by Reiss and Barry (11). The solutions from the lactose crystallization were evaporated to dryness, dissolved in 20 ml of water, and passed through a Dowex 1 (HCOO<sup>-</sup> form) column (2 × 20 cm). The citric acid eluted from this column was rechromatographed on silicic acid as described previously (7).

**Isolation of Udder-Tissue Fat and Milk Fat Fractions.** Total lipid was isolated from udder tissue and milk as described previously (7). Total milk fat was not fractionated further. Lower FFA were removed from udder-tissue fat by washing the ether extract several times, first with 1% Na<sub>2</sub>CO<sub>3</sub>, then with water. The

technique of chromatography on silicic acid, originally developed by Fillerup and Mead (12) and described in detail by Garton and Duncan (13) for the separation of the plasma lipids of lactating cows, was applied to the separation of udder-tissue fat. Columns of 5 cm diameter, containing a mixture of silicic acid and Hyflo Super-Cel 200/70 (g/g) and having a capacity of separating about 6 g of total fat, were used. In the eluate, free cholesterol and esterified cholesterol were estimated according to Kenny (14), phospholipids by the phosphorus determination method of Allen (15), and glycerides by the hydroxamic acid method of Morgan and Kingsbury (16). Separation of the cholesterol esters, glycerides, free cholesterol, and phospholipids was obtained as shown in Figure 1. Evidence was obtained that on these columns the higher FFA were spread mainly over the glyceride and free cholesterol fractions. The material in the cholesterol ester peak, the glyceride peak, and the free cholesterol peak

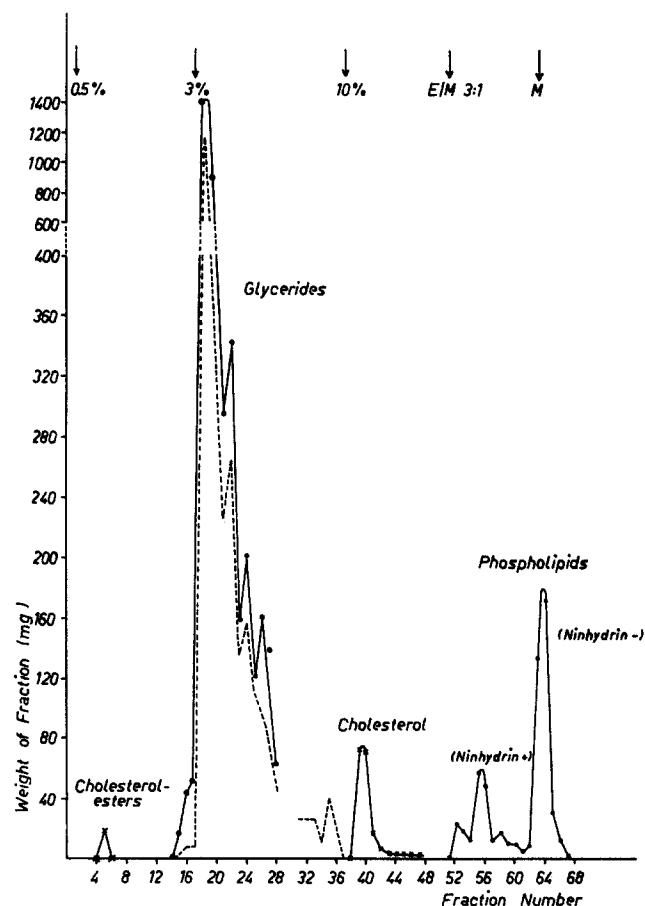


FIG. 1. Chromatography on silicic acid of udder-tissue lipids. Solvents used were 0.5%, 3%, and 10% ethyl ether-petroleum ether, ether-methanol 3:1 (v/v), and methanol. The solid lines of the glyceride peak indicate weight determinations; the dotted lines indicate hydroxamic acid determinations.

was taken up into wet ether. A large amount of non-labeled stearic acid was added to each peak and the fraction passed through two successive Dowex 1 (OH<sup>-</sup> form) columns. These columns had been previously saturated with wet ether (17). Ten milliliters of Dowex 1 (OH<sup>-</sup> form) was used per mM of added fatty acid. The neutral fat fractions were eluted with wet ether. Elution with wet ether was stopped when no further material came off the columns. The FFA were then eluted with a mixture of ether-95% ethanol-sulfuric acid 200:8:17 (v/v). Preliminary experiments on Dowex 1 (OH<sup>-</sup> form) columns with a mixture of inactive tripalmitin and highly labeled stearic acid showed that under these conditions no contamination of the neutral fat fraction with FFA occurred. However, some hydrolysis, occurring during the passage of the neutral fat fraction through the Dowex columns, could not be avoided. Finally the free cholesterol fraction was treated with digitonin; the digitonide was decomposed in benzene, and the free cholesterol isolated.

*Chromatographic Separation of Fatty Acids.* The main fraction of glycerides was saponified, acidified, and the even-numbered fatty acids were isolated by the reversed phase chromatography technique of Howard and Martin (18), as described previously (7). Odd-numbered fatty acids are not detected by this technique as they occur only in trace amounts in udder and milk lipids.

*Radioactivity Measurements.* The radioactivity measurements were made with a windowless flow counter. After wet combustion, lactose and casein were counted as BaCO<sub>3</sub>; citric acid and the sodium salts of the individual fatty acids were counted as previously described (7). The magnitude of the counting rates obtained and the thickness of the material were of the same order as those described by Verbeke *et al.* (7).

Cholesterol esters, cholesterol, glycerides, phospholipids, whole blood, and blood plasma samples were taken at the end of the perfusion experiment. They were plated on plastic disks and counted at infinite thickness until 10,000 counts.

RESULTS

*Perfusion.* In the experiments we carried out with udders from slaughterhouse cows, the O<sub>2</sub> uptake of the isolated glands in the course of the first 2 hours of perfusion compares rather favorably with the activity of the gland *in vivo*. However, for reasons which at present are but partially clear, the physiological activity of the excised glands declines rather quickly afterward. We therefore limit our perfusion experi-

ments to 2 hours. The milk production in the experiment reported here was somewhat lower than that of most of our earlier studies.

*Blood Samples.* In view of the changes in plasma volume which result from the addition of water to the blood by way of drip infusion, the concentrations of lipid constituents in the plasma following perfusion were corrected according to the hematocrit to make them comparable with values on the plasma before perfusion. Determination of the total fatty acid content of the plasma before and after perfusion gave values of 1,946 mg/liter and 1,862 mg/liter, respectively. The figures for total cholesterol were 1,376 mg/liter before, and 1,456 mg/liter after perfusion, and for free cholesterol, 274 mg/liter before, and 290 mg/liter after perfusion. The concentrations of these lipid constituents in the plasma at the end of perfusion are not considered significantly different from the preperfusion control. At the end of the perfusion no C<sup>14</sup> was detected in the red cells, and only 2 μc was present in the total plasma, indicating that virtually all the added stearate had been removed.

*Carbon Dioxide.* In total, 0.35 μc of C<sup>14</sup>O<sub>2</sub> was collected. This amount is very small compared with previous perfusion experiments performed in the presence of C<sup>14</sup>-labeled lower fatty acids, and indicates the small extent of catabolism of the added substrate.

*Milk Constituents.* No detectable incorporation of C<sup>14</sup> was observed in casein or lactose. Milk citric acid showed a small but definite C<sup>14</sup> incorporation (0.05 mμc/mg carbon). In total, 0.002 μc was recovered in this fraction.

Milk fat showed a specific activity of 0.007 mμc/mg. In total, 0.06 μc was recovered in this fraction.

*Udder-Tissue Fat.* Table 1 gives the specific activities of the different fat fractions, before and after pas-

TABLE 1. SPECIFIC ACTIVITIES OF LIPID FRACTIONS OF UDDER TISSUE BEFORE AND AFTER PASSAGE THROUGH TWO SUCCESSIVE DOWEX 1 (OH<sup>-</sup>) COLUMNS

Fat Fraction	Weight	Specific Activity*		
		Before Passage	After Passage Through	
			1st Dowex Column	2d Dowex Column
	<i>per cent</i>			
Cholesterol esters	0.45	†	†	357 ± 36
Glycerides	83.5	1220 ± 122	834 ± 83	827 ± 80
Free cholesterol	3.25	612 ± 61	202 ± 20	210 ± 21
Phospholipids 1‡	4.5	347 ± 35		
Phospholipids 2‡	8.0	653 ± 65		

\* Cpm infinitely thick. Means of triplicates ± standard error.

† Not determined.

‡ The phospholipid fractions were not passed through Dowex columns.



sage through two successive Dowex 1 (OH<sup>-</sup> form) columns. A sharp drop in activity was observed after passage of the glyceride fraction and the free cholesterol fraction through a first Dowex column, due to removal of the FFA. Passage of either of the neutral fat fractions through a second Dowex column did not alter the specific activity, indicating a complete removal of the FFA after passage through the first Dowex column. Although a sharp drop in activity of the cholesterol peak was observed after passage through the Dowex columns, some activity still remained, which we ascribed to the presence of traces of diglycerides. No attempt was made to distinguish the partial glycerides. After precipitation of the cholesterol as digitonide, followed by decomposition, no activity could be detected in this fraction. Because of the presence of unknown amounts of deterioration products, derived from the resin after elution with the acid-ethanol mixture, no accurate value for the specific activity of the FFA fraction was obtained.

Table 2 gives the specific activities of the even-numbered glyceride fatty acids from udder tissue. The highest specific activity was observed in stearic acid followed by oleic acid, the specific activity of stearic acid being approximately 2.3 times that of oleic acid. All the other acids showed very low activities. The activities of the individual fatty acids are expressed as a percentage of total fatty acid activity. More than 50% of the total fatty acid activity is observed in the oleic acid fraction. From the specific activity of the crude fat, and from the total lipid present in the udder tissue, it was calculated that 166  $\mu$ c was recovered in the total fat of the udder

tissue. In an analogous manner we found that approximately 87  $\mu$ c was incorporated into the glycerides, 7  $\mu$ c into the phospholipids, and 0.2  $\mu$ c into the cholesterol. These figures indicate that approximately 72  $\mu$ c was probably present in the FFA.

#### DISCUSSION

It is clear that albumin-bound stearate is removed from the circulating blood by the udder. Two hours after the start of perfusion, virtually all the labeled material had disappeared from the blood. Unfortunately, no greater number of blood samples was collected in the course of the experiment, though it is highly probable that the low concentration of C<sup>14</sup> was reached at a much earlier moment. After intravenous injection of palmitic acid-1-C<sup>14</sup>, bound to serum albumin into rats, Bragdon and Gordon (19) observed a very rapid disappearance of 99% of the labeled FFA from the circulating blood. The long persistence of the remaining 1% indicated that an exchange occurred between plasma FFA and the fatty acids of a tissue pool. As our results seem to indicate that an important activity was present in the FFA fraction isolated from udder tissue, a similar exchange as observed by Bragdon and Gordon (19) in the rat might have occurred.

A large percentage of the labeled substrate, absorbed from the blood, was metabolized and incorporated into the glycerides and phospholipids of the udder fat. On the basis of the respective specific activities and concentrations of these fractions in the udder fat, it is clear that most of the esterified stearate-1-C<sup>14</sup> was present in the glycerides. Although no detailed analytical work was carried out on the fat of the secreted milk, it is likely that the milk fat activity distribution resembled that of udder fat. It would be desirable to investigate this question in another perfusion experiment carried out for a longer period under physiological conditions so as to obtain a higher specific activity in the secreted milk fat than we obtained in this experiment. In previous experiments carried out in the presence of C<sup>14</sup>-labeled substances, it was always observed that C<sup>14</sup> was distributed in an analogous manner between the fatty acids isolated from milk and from udder tissue. The specific activities in the milk fat fractions, however, are always much lower than those of the udder fat because of the limited duration of the perfusion experiments. It is to be noted, however, that the fatty acid pattern (i.e., the ratio stearic to oleic acid) of the milk fat can be significantly different from that of the udder fat.

Stearic acid was dehydrogenated to a considerable extent, giving rise to oleic acid. This dehydrogenation

TABLE 2. INCORPORATION OF C<sup>14</sup> IN EVEN-NUMBERED FATTY ACIDS OF UDDER TISSUE GLYCERIDES

Fatty Acid	Specific Activity	Distribution in Glycerides as Molar Per Cent	Percentage of C <sup>14</sup> *
	<i>mμc/mM</i>		
Butyric } Caproic } Caprylic }	2.38 ± 0.36	17.72 ± 0.18	0.47 ± 0.09
Capric	4.32 ± 0.60	1.62 ± 0.03	0.08 ± 0.01
Lauric	3.74 ± 0.58	2.17 ± 0.04	0.09 ± 0.02
Myristic	3.19 ± 0.50	10.2 ± 0.1	0.37 ± 0.07
Palmitic	3.84 ± 0.19	25.21 ± 0.25	1.08 ± 0.09
Stearic	365 ± 13	11.1 ± 0.1	45.4 ± 3.4
Palmitoleic	3.26 ± 0.58	2.62 ± 0.05	0.09 ± 0.02
Oleic	159.2 ± 1.1	29.4 ± 0.3	52.4 ± 2.4

All values expressed as means ± standard errors.

\* C<sup>14</sup> recovered expressed as percentage of total fatty acid activities.

must be a very active process as it occurred to over half of the stearic acid incorporated into the udder glycerides during the relatively short time of perfusion. Using stearic acid with the label on the 1 position, it is clear that the label would not be altered in the dehydrogenation. The unequivocal position of the label, the short experimental period, and the use of an isolated organ system characterize the dehydrogenation process much more clearly than that observed in the studies of Glascock *et al.* (2), where the tritium label was not uniformly positioned and the labeled stearate was administered by mouth. It was pointed out by these authors that a considerable degree of randomization and transformation of lipids takes place during digestion and absorption if the labeled material is administered by mouth, and this may lead to difficulties in the interpretation of milk fat radioactivities. Very little breakdown of the C<sub>18</sub> acids to small molecules (acetyl-CoA) took place in the perfusion experiment, as is indicated by the very low specific activities detected in citric acid, the lower fatty acids, and CO<sub>2</sub>, and by the absence of activity in the casein fraction. The specific activities of these last fractions were very much below those observed previously in the presence of C<sup>14</sup>-acetate, -butyrate, -hexanoate and -isovalerate (20, 21). It is to be noted that Glascock *et al.* (2), in agreement with our observations, found a much higher specific activity in the long-chain fatty acids of milk glycerides than in the short-chain acids. The specific activities detected in palmitic acid and palmitoleic acid of the perfusion experiment deserve some further consideration. The direct conversion of stearic to palmitic acid would probably not be detected with the carboxy-labeled stearic acid used in these studies. It therefore appears probable that C<sup>14</sup> was introduced into palmitic acid by way of acetyl-CoA, and it seems reasonable to assume that palmitic acid was dehydrogenated, giving rise to palmitoleic acid. Therefore it would be of interest to study the metabolism of C<sup>14</sup>-labeled palmitic acid in another perfusion experiment.

Glascock (1) did not observe an especially high activity in the FFA fraction of the plasma of the cow in his experiments. He found that a highly active fraction, amounting to about 1% of the total plasma lipids, was precipitated with the phospholipids using acetone. According to Glascock (1), this fraction, which was not identified definitively, behaved chromatographically like neutral fat.

Several workers have observed that lipid was lost from the blood during its passage through the udder (22, 23, 24). Voris *et al.* (25) found that it was mainly the triglyceride fraction which was absorbed by the udder. Lough *et al.* (26) found that esterified higher

fatty acids were taken up by the perfused cow's udder from the plasma, and always noted a loss of triglycerides. In some of their experiments the plasma content of both esterified and free cholesterol decreased, whereas in other experiments it increased during perfusion. In the stearate-1-C<sup>14</sup> experiment, however, only a small decrease of the total fatty acid content of the plasma was observed during perfusion. The difference in total fatty acids before and after perfusion amounted to only 84 mg/liter of plasma, and this value was well within the limits of experimental error. In this experiment no determination of plasma triglycerides was carried out. Glascock (1) infused a tritium-labeled triglyceride emulsion in a lactating cow. The emulsion was "unphysiological," and caused acute symptoms because of agglutination of fat particles. However, 20% of the activity injected was removed in the milk fat, showing that circulating triglycerides can be used as precursors for the synthesis of milk fat. Riis *et al.* (27) infused cow plasma containing P<sup>32</sup> and C<sup>14</sup>-labeled lipids and proteins into a lactating cow. Little radioactivity was found in the expired CO<sub>2</sub>, and none was found in the plasma volatile acids. They found only a trace of activity in casein and lactose, but significant activity was found in the butter fat.

The results reported in this paper show that circulating albumin-bound stearic acid can be used as a precursor for the synthesis of milk fat. As noted by Fredrickson and Gordon (4), FFA serve in men and the common laboratory animals during fasting as a transport form of fatty acids, being added to the blood mainly by peripheral adipose tissue and removed as blood perfuses the viscera. FFA may be considered as a major source of energy in the fasting state. The contribution of incoming chyle FFA to total plasma turnover would be very small. FFA-C<sup>14</sup> appeared in the expired air as C<sup>14</sup>O<sub>2</sub>, and was incorporated into triglycerides and phospholipids of tissues.

It may well be that plasma FFA are of significance in the formation of milk fat glycerides, though physiological factors involved in the living cow remain to be determined. The hypothesis could be formulated that the FFA fraction would be of great importance quantitatively in the process of milk fat synthesis of the ruminant in the fasting state, but less important in normal circumstances.

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