Incorporation of ^{14}C -Labelled Fatty Acids into the Mammary-Gland Lipids of the Lactating Rabbit

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(Received 9 November 1964)

1. [^{14}C]Stearic acid, [^{14}C]stearic acid, [^{14}C]palmitic acid and [^{14}C]decanoic acid were fed to lactating rabbits, and the fatty acids of the mammary gland were separated and partially degraded. 2. Most of the ^{14}C recovered was located in the acids fed. Stearic acid was least efficiently absorbed; decanoic acid was the most extensively metabolized. 3. Resynthesis after degradation to C_2 units led to uniform alternate labelling in the C_2 – C_{10} acids, whereas C_{12} – C_{18} acids had excess of ^{14}C at the carboxyl end. 4. Acids formed by β -oxidation down to C_{12} , but not below, were also present in the mammary-gland lipids. Desaturation of the administered acids was a very minor reaction.

Despite considerable progress, many features of fat metabolism in animals remain uncertain. In particular, knowledge of the individual pathways of degradation and synthesis is more advanced than our understanding of their relative importance. One way of investigating this more general problem is to study the fate of labelled fatty acids in intact animals. Since the labelled acids are then subjected to the full interplay of degradative and synthetic processes, with correspondingly complex results, such experiments are not always easy to interpret. In the experiments reported below we used the readily available [^{14}C]stearic acid together with three ^{14}C -labelled acids, namely stearic acid, palmitic acid and decanoic acid. When ^{14}C -labelled acids are used in experiments of this kind the label enters lipids in two ways, namely (1) incorporation of the intact acid with labelling still in C_{11} , and (2) β -oxidation, with immediate generation of [^{14}C]acetyl-CoA, some of which may be used synthetically. By using the ^{14}C -labelled acids we hoped additionally to obtain evidence of the extent of β -oxidation and of the possible importance of β -oxidation products of intermediate chain length in synthetic processes.

MATERIALS AND METHODS

^{14}C -labelled acids. [^{14}C]Stearic acid (20 mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. The ^{14}C -labelled acids were synthesized by the method of Bu'Lock, Smith & Bedford (1962) and had specific activities between 0.2 and 1.0 mc/m-mole; the [^{14}C]palmitic acid contained 1–2% of [^{14}C]octanoic acid, and the [^{14}C]stearic acid a similar amount of [^{14}C]-

decanoic acid, these contaminants having similar specific activities.

Administration of ^{14}C -labelled acids. The [^{14}C]stearic acid (2.6 mg., 0.2 mc) was converted into the sodium salt and added in aq. 20% (v/v) ethanol (1 ml.) to 4 ml. of rabbit serum; this was injected intravenously into a well-fed lactating rabbit, which was allowed to feed freely, and after 5 hr. the animal was killed (Expt. A). The ^{14}C -labelled acids (Expts. B, C and D) (stearic acid, 120 mg., 0.15 mc; palmitic acid, 300 mg., 1.0 mc; decanoic acid, 240 mg., 0.24 mc) were dissolved in corn oil (5 ml.) and fed by intubation to lactating rabbits that had been starved overnight; they were then allowed to feed freely, and were killed after 24 hr.

Lipid extraction and preliminary fractionation. The entire mammary gland was minced as finely as possible with scissors, and the liver sliced and briefly homogenized. The extraction procedure and separation into acetone-soluble 'triglyceride' and acetone-insoluble 'phospholipid' fractions were as described by Popják & Beeckmans (1950).

Samples of adipose tissue, and the phospholipid and triglyceride fractions from liver and mammary gland, were saponified and separated into non-saponifiable, steam-volatile acid and non-volatile acid fractions (Popják & Beeckmans, 1950).

The fatty acids from liver samples and the non-volatile acids from mammary-gland triglycerides were separated into 'saturated' and 'unsaturated' concentrates by three crystallizations from acetone (15–20 ml./g.) at -30° for 24 hr. periods.

Separation of steam-volatile acids (C_2 – C_{10} acids). The mixture of C_2 – C_{10} acids, predominantly octanoic acid and decanoic acid, was first fractionated by countercurrent distribution in a methanol–water–heptane mixture (73:27:50, by vol.) (120 tubes, 190 transfers), giving (i) mixed C_2 – C_8 acids, (ii) octanoic acid, (iii) decanoic acid and (iv) a fraction that was combined with the unsaturated acid fraction from low-temperature crystallization. Acids from C_2 to C_{12} were

finally purified by chromatography in aq. acetone–butanone on Amberlite CG-50 as described by Seki (1958).

Fractionation of the non-volatile acids. The 'unsaturated' fraction from low-temperature crystallization was partly resolved by countercurrent distribution in heptane–methanol–formamide–acetic acid (3:1:1:1, by vol.) (120 tubes, 400 transfers, with recycling), giving concentrates of oleic acid, linoleic acid, palmitoleic acid, myristic acid and lauric acid, which were further fractionated by conversion of the unsaturated acids into hydroxy acids (Lapworth & Mottram, 1925) followed by chromatography as described by Savary & Desnuelle (1953).

Reversed-phase chromatography of fatty acids. Palmitic acid and stearic acid in the 'saturated' fraction were separated by chromatography on silicone-treated Celite columns by the method of Howard & Martin (1950) as improved by Silk & Hahn (1954) and Crombie, Comber & Boatman (1955). The separated fatty acids were in each case mixed with an excess of the unlabelled homologue and rechromatographed to constant radioactivity, and traces of paraffin etc. were removed on a small column of silica gel.

Oxidation of dihydroxy acids. The dihydroxy acids were oxidized with the permanganate–periodate reagent as described by Lemieux & Rudloff (1955). The monocarboxylic acids were recovered by steam-distillation and purified by the method of Seki (1958); the non-volatile residue was azelaic acid, which was repeatedly crystallized from benzene–hexane and from water.

Decarboxylation. Purified acids were decarboxylated by the Schmidt reaction as described by Phares (1951); the C_1 – C_{13} amines formed were removed by steam-distillation, and purified by chromatography as the phthalimide derivatives on alumina. Pentadecylamine and heptadecylamine were conveniently recovered as the sulphates by dilution of the reaction mixture with water and then recrystallized from ethanol.

Kuhn–Roth oxidation. Oxidation with chromic acid–sulphuric acid mixture was carried out as described by Ahrens (1952). The liberated CO_2 was collected as $BaCO_3$ in approx. 40 mg. lots over a 5 hr. period. The acetic acid was purified by the method of Seki (1958) and converted into *p*-bromophenacyl acetate, which was recrystallized.

Assay of ^{14}C . All assays were carried out in an end-window counter of approx. 5% efficiency, counting at least 10^4 counts from infinitely thick (30 mg./cm.²) samples of 0.3–2.0 cm.². Solid acids and $BaCO_3$ were counted directly, C_2 – C_{10} acids as *p*-bromophenacyl esters, C_1 – C_{13} amines as phthalimides and C_{15} – C_{17} amines as sulphates, and equiva-

lent self-absorptions were assumed. All samples had been purified to constant radioactivity, with special measures (see above) when adjacent homologous compounds had widely disparate radioactivities (e.g. stearic acid and palmitic acid in Expt. A).

RESULTS

Four separate experiments were performed, referred to as Expt. A ($[1-^{14}C]C_{18}$ acid), Expt. B ($[9-^{14}C]C_{18}$ acid), Expt. C ($[9-^{14}C]C_{16}$ acid) and Expt. D ($[9-^{14}C]C_{10}$ acid). Table 1 shows the recovery and general distribution of ^{14}C lipids in each experiment; because of differences between the animals, and the different mode of administration in Expt. A, quantitative comparisons between the four experiments are not appropriate. Table 1 shows that stearic acid is the least efficiently absorbed, whereas the low retention of ^{14}C from decanoic acid coupled with low recovery from the faeces suggests that this acid is the most completely dissimilated.

The triglyceride and phospholipid fractions from the mammary-gland and liver extracts were all separately hydrolysed and divided into 'steam-volatile' acids (up to C_{12} , mainly C_8 and C_{10} acids; negligible in liver lipids), 'unsaturated' acids (mainly oleic acid and linoleic acid) and 'saturated' acids (mainly C_{16} and C_{18} acids). The values for the liver acids were not significantly different from those for corresponding mammary-gland acids and the former are not presented here; for similar reasons, the values in Table 2 for the mammary-gland acids were obtained by adding together the values for triglyceride acids and those for the small phospholipid fractions. Table 2 shows that the recovered ^{14}C is mainly present in the fractions in which the administered ^{14}C -labelled acids would themselves be found. The conversion into unsaturated acids is generally very low, though for $[9-^{14}C]$ decanoate (Expt. D) it is noticeably greater.

Table 3 shows the distribution of ^{14}C found in the mammary-gland acids. The values for $C_{(1)}$ are

Table 1. Recovery of ^{14}C in lipids after the administration of ^{14}C -labelled acids

Experimental details are given in the text.

Expt.	Acid administered	Percentage recovery of ^{14}C					
		In mammary gland		In liver		In adipose tissue (per g.)	In faeces
		Glycerides	Phospholipids	Glycerides	Phospholipids		
A	$[1-^{14}C]C_{18}$ acid	17.5	10.0	4	12	0.009	—
B	$[9-^{14}C]C_{18}$ acid	3.9	0.1	2	6	0.007	25
C	$[9-^{14}C]C_{16}$ acid	25	0.9	2	2	0.002	11
D	$[9-^{14}C]C_{10}$ acid	3.5	0.03	0.8		0.001	4

Table 2. *Preliminary fraction of lipids from mammary glands*

Experimental details are given in the text. The $\mu\text{C/g.}$ values were recalculated to a common administered dosage of 1.0 mc. The recoveries are given as percentages of the total ^{14}C -labelled fatty acids.

Acid administered.....	Expt.....A [1- ^{14}C]C ₁₈ acid			B [9- ^{14}C]C ₁₈ acid			C [9- ^{14}C]C ₁₆ acid			D [9- ^{14}C]C ₁₀ acid		
	(Re-)			(Re-)			(Re-)			(Re-)		
	(g.)	($\mu\text{C/g.}$)	covery)	(g.)	($\mu\text{C/g.}$)	covery)	(g.)	($\mu\text{C/g.}$)	covery)	(g.)	($\mu\text{C/g.}$)	covery)
Total lipids	17.0	16.2	—	11.0	3.6	—	30.0	8.1	—	18.5	2.0	—
Fatty acids	14.6	19.4	100	10.3	3.9	100	28.8	8.4	100	17.0	2.1	100
Steam-volatile fatty acids	4.1	1.2	2	1.5	1.3	5	6.0	0.8	2	3.0	10.8	86
Unsaturated fatty acids	6.6	0.7	2	5.7	0.2	3	14.1	0.6	3	8.9	0.5	12
Saturated fatty acids	3.8	70.8	96	3.1	11.0	92	8.3	28.0	95	4.9	0.1	1

Table 3. *Distribution of ^{14}C in mammary-gland triglyceride fatty acids as shown by Schmidt degradation*

Experimental details are given in the text. The ^{14}C contents are given as $\mu\text{C/mole}$ per active (alternate) atom, recalculated to a common administered dosage of 1.0 mc. Values in *italics* indicate selective incorporation of ^{14}C at carboxyl end; values in **bold** type indicate incorporation of labelled chain from substrate.

C _n acid degraded	Expt.....A [1- ^{14}C]C ₁₈ acid		B [9- ^{14}C]C ₁₈ acid		C [9- ^{14}C]C ₁₆ acid		D [9- ^{14}C]C ₁₀ acid	
	C ₍₁₎ C _{(2)-C_n}		C ₍₁₎ C _{(2)-C_n}		C ₍₁₎ C _{(2)-C_n}		C ₍₁₎ C _{(2)-C_n}	
C ₂	—	—	14	0	41	0	184	0
C ₄	19	19	32	32	140	151	630	650
C ₆	43	42	38	38	62	65	540	550
C ₈	49	49	47	48	40*	25	230	230
C ₁₀	34	34	57*	36	20	20	150	960
C ₁₂	162	33			15	32		
C ₁₄	338	63			4	97		
C ₁₆	1080	32			1	1950		
C ₁₈	51500	8			14	95		
Δ^9 -C ₁₆	—	—			0.2	18		
Δ^9 -C ₁₈	130	2			1	0.9		

* From 1- ^{14}C -labelled acids present in the [9- ^{14}C]palmitate and [9- ^{14}C]stearate.

obtained from the barium carbonate produced by Schmidt degradations and those for C₍₂₎-C_(n) from the corresponding amine phthalimides or sulphates. The latter values have been divided by $(n/2) - 1$, the number of carbon atoms in the amine that were odd-numbered in the original fatty acid C_n, to show the average value for these atoms (which are the ones labelled by way of resynthesis from [1- ^{14}C]-acetyl-CoA). The combined activities of the Schmidt-degradation products agreed to $\pm 2\%$ with the activities of the original fatty acids. We obtained, in Expt. A, corresponding values for the mammary-gland phospholipid acids and for the palmitate and stearate of the liver lipids; these showed no novel features.

In all four experiments, as shown in Table 3, there was some ^{14}C incorporation into the steam-volatile acids (C₂-C₁₀ acids), which are characteristic of the mammary triglycerides. On degradation,

these acids always showed a 'uniform' distribution of labelling (through the odd-numbered carbon atoms); e.g., in Expt. A, C₍₁₎ of octanoic acid has exactly one-fourth the activity of the whole molecule. Three exceptions to this rule occurred (**bold** type in Table 3), all due to the intact incorporation of specifically-labelled acids; in Expts. B and C these were [1- ^{14}C]decanoic acid and [1- ^{14}C]octanoic acid present as impurities in the 9- ^{14}C -labelled acids fed, whereas in Expt. D [9- ^{14}C]decanoate had been administered. The uniform labelling of the remaining acids (C₄-C₈ acids) in Expt. D was particularly striking.

In contrast, the C₁₂-C₁₆ acids in Expt. A show a different pattern of labelling, with an increasing excess in the carboxyl group, C₍₁₎. More detail of this pattern was revealed by Kuhn-Roth oxidation of the amines from Schmidt degradations of the C₁₀-C₁₄ acids (Table 4); in these oxidations the

Table 4. *Degradation of C₁₀–C₁₄ acids from Expt. A by Kuhn–Roth oxidation of the amines from Schmidt degradations*

C _n acid	Experimental details are given in the text. μC/mole per active (alternate) atom			
	C ₍₁₎	C ₍₂₎ –C _(n) amine	Successive samples of Kuhn–Roth CO ₂	Kuhn–Roth CH ₃ ·CO ₂ H
C ₁₀	34	34	34, 34	—
C ₁₂	162	33	35, 33, 34	—
C ₁₄	338	63	70, 51, 40, 13	12

evolved carbon dioxide was not collected singly but in a series of samples as the oxidation proceeded. The results confirm the uniform labelling of decanoic acid, and show that in dodecanoic acid the excess of labelling is confined to C₍₁₎, whereas in tetradecanoic acid there is a large excess in C₍₁₎ but also marked non-uniformity elsewhere in the chain, probably an excess at C₍₃₎. The stearic acid in Expt. A shows the very high activity at C₍₁₎ due to intact incorporation of [1-¹⁴C]stearate; oleic acid contained relatively little ¹⁴C, nearly all of which was at C₍₁₎.

A third pattern is shown by the C₁₂–C₁₈ acids in Expt. C (Table 3), all of which show an excess of labelling in the chain (the apparent magnitude of this excess is decreased in Table 3 by averaging over the odd-numbered carbon atoms). With palmitic acid this excess is due to intact incorporation of the [9-¹⁴C]palmitate administered, but it is also clear that dodecanoic acid, tetradecanoic acid and stearic acid must contain specifically labelled chains derived from [9-¹⁴C]palmitate. By β-oxidation the latter would afford [7-¹⁴C]tetradecanoyl and [5-¹⁴C]-dodecanoyl derivatives, thus explaining the results, but on the other hand there is no sign of any similar incorporation by way of a [3-¹⁴C]decanoyl derivative. The stearic acid in this experiment also shows an unexpectedly higher activity in C₍₁₎, which is presumably analogous to the excess of labelling at C₍₁₎ in Expt. A. As in Expt. A, there was very little incorporation of ¹⁴C into unsaturated acids. Both palmitoleic acid and oleic acid show a small degree of specific incorporation of at least the C₍₉₎–C₍₁₈₎ part of the [9-¹⁴C]palmitate. This was confirmed by oxidation studies: whereas palmitoleic acid gave azelaic acid from C₍₁₎–C₍₉₎, containing 97% of the original ¹⁴C, in oleic acid 84% of the ¹⁴C was found in nonylic acid (from C₍₁₀₎–C₍₁₈₎), with a further 13% in C₍₁₎ (Table 3).

In no experiment did we observe any ¹⁴C incorporation into linoleic acid.

DISCUSSION

In considering these results it must be emphasized that we have examined only that part of the ¹⁴C,

administered to the whole animal, that is transferred, by a complex series of processes, into the mammary-gland lipids. This sample is not large (Table 1), nor is it necessarily typical, and, as shown below, it may be made up of materials drawn off by more than one route.

Incorporation of unchanged acids. Tables 2 and 3 show that labelled palmitic acid and stearic acid were largely incorporated as such, and conversion into other acids was of very minor importance. Lossow & Chaikoff (1958) reported similar observations with lactating rats, which are in contrast with the situation in ruminants (Glasecock, 1958). Even with decanoic acid, intact incorporation accounted for nearly half of the activity recovered in the mammary-gland lipids. Intact incorporation of the small amounts of octanoic acid and decanoic acid that were administered as incidental contaminants in Expts. B and C could also be detected in the mammary-gland lipids.

Resynthesis via [1-¹⁴C]acetyl-CoA. The main pathway through which administered acids are completely oxidized is β-oxidation by way of acetyl-CoA, which in all our experiments would carry ¹⁴C in C₍₁₎. Clearly a small part of this is re-used for synthesis, and the manner of its incorporation as revealed by Tables 3 and 4 is of some interest. The incorporation of [¹⁴C]acetyl derivatives into fatty acids has been studied by many workers, and in general two types of incorporation can be distinguished. The first leads to what we have here called uniform labelling, i.e. substantially equal labelling of successive C₂ units in the fatty acid chain, and arises when intermediates in the synthetic process are not in equilibrium with pools of unlabelled acids. The second leads to an uneven distribution of labelling, with the highest activity in the C₂ units nearest the carboxyl group, and it arises when there is equilibration with unlabelled substances of intermediate chain length. Thus, in a C₁₈ acid formed in such a system, labelling of the C₍₁₎–C₍₂₎ unit is diluted only at the C₁₈ stage and is therefore higher than labelling in the C₍₃₎–C₍₄₎ unit, which has also undergone dilution at the C₁₄ stage. Both types of result have been observed in a variety of bio-

logical systems, and have been discussed in relation to different mechanisms of synthesis. Thus Wakil (1961) considers the uniform labelling to be characteristic of non-mitochondrial biotin-dependent pathways involving malonyl-CoA, and selective labelling near the carboxyl group to result from mitochondrial biotin-independent pathways, which use acetyl-CoA for chain-extension and operate mainly in the C₁₂-C₂₂ region.

In our results, both types of pattern are distinctly seen in those acids that owe their labelling to re-incorporation of [1-¹⁴C]acetyl units formed oxidatively. Uniform labelling is characteristic of the steam-volatile acids up to C₁₀, which are peculiar to the mammary gland itself, whereas selective labelling towards the carboxyl end is found in the C₁₂-C₁₆ acids in Expt. A and in stearic acid and oleic acid in Expt. C. Thus the synthetic systems accessible to ¹⁴C in our experiments differ for these two groups of acids, which are similarly distinguished in relation to β -oxidation (see below). The break at C₁₀-C₁₂ is consistent with the specificity of the mitochondrial systems described by Wakil (1961); at least one mechanism for the synthesis of oleic acid also requires a mechanistic break at this stage (Goldfine & Bloch, 1961).

Products of β -oxidation. If intermediates in β -oxidation are released into the lipids isolated, then acids with specific ¹⁴C-labelling outside the carboxyl group should be detectable down to C₁₀ in Expt. C and down to C₄ in Expt. D. In fact, this excess was found only in the C₁₂ and C₁₄ acids in Expt. C, and was absent in the C₁₀-C₄ range in either experiment. It therefore appears that in β -oxidation, as well as in synthesis, intermediates of C₁₂ and over are represented in the mammary-gland lipids but those of C₁₀ and under are not.

Unsaturated acids. In the lactating rabbit neither

orally administered nor intravenous palmitate or stearate are important precursors of the mammary-gland oleic acid, and the major route to this important lipid component is not easily accessible to ¹⁴C in our experiments. In Expt. D, where the highest ¹⁴C incorporation by synthesis occurred, incorporation into oleic acid was also highest. The observed values for palmitoleate and oleate in Expts. A and C could indicate some slight degree of direct dehydrogenation of the corresponding saturated acids but could equally well result from other pathways.

We are grateful to the Department for Scientific and Industrial Research for a maintenance grant and to the Medical Research Council for a grant for purchase of ¹⁴C-labelled acids.

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