The Role of *n*-Valeric Acid in the Synthesis of the Higher Saturated Straight-Chain Acids Containing an Odd Number of Carbon Atoms in Bovine Milk Fat

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The synthesis in the udder of the *n*-saturated acids by the successive addition of acetate units has been confirmed by the work of numerous investigators. This subject has been reviewed by Popják (1952), Folley (1952), Glascock (1954), and more recently by Shorland & Hansen (1957).

The discovery of the saturated straight-chain acids containing an odd number of carbon atoms ('*n*-odd-numbered acids') in the body and milk fats of ruminants (Hansen, Shorland & Cooke, 1957; Shorland, Gerson & Hansen, 1955*a, b*) raises the question of their origin. Some light has been thrown on the mechanism of synthesis of these acids by the work of James, Peeters & Laurysse (1956), who found that labelled propionate perfused through a cow's udder gave rise to the higher '*n*-odd-numbered acids' by chain elongation with acetate. The small amounts of propionate found in the blood by Annison (1954) could therefore be utilized for the formation *in vivo* of higher '*n*-odd-numbered acids' present in the milk fats. It could be assumed that valerate is formed by the addition of acetate to propionate as an intermediary in the synthesis of the higher '*n*-odd-numbered acids'. This is difficult to reconcile with the results of Popják, French, Hunter & Martin (1951), who detected no [¹⁴C]valeric acid in goat-milk fat after the injection of labelled acetate into the jugular vein, suggesting that the valeric acid present is not formed endogenously but is derived from the rumen. On the other hand, such an origin seems improbable in view of the failure of Annison (1954) to detect valeric acid in sheep's blood.

To explore further the role of valeric acid in fatty acid synthesis we have determined the ¹⁴C activity of the constituent fatty acids in butterfat from a cow which had been given an intrajugular injection of sodium [carboxy-¹⁴C]valerate (M. Kleiber, personal communication, 1956).

EXPERIMENTAL

The dairy cow used in this experiment weighed 449 kg., and produced approximately 10 kg. of milk daily. After

milking, sodium [carboxy-¹⁴C]valerate (5.0 mc) was injected into the jugular vein. The milk yields and activities of the milk fats are shown in Table 1.

Isolation of fatty acids. The butterfat (96 g.) was saponified by the method of Henry & Kon (1942) and the soaps converted into acids with 40% (v/v) H₂SO₄ (10% excess), and steam-distilled. The steam-volatile fatty acids were neutralized with KOH, evaporated to dryness and analysed by gas-liquid chromatography (James & Martin, 1952). The non-volatile fatty acids were extracted with diethyl ether, washed with water until neutral to Congo red and dried *in vacuo* on a water bath. The acids were then separated into 'solids' and 'liquids' by crystallization three times from 15 vol. of acetone at -38°. The 'solids' and 'liquids' respectively were converted into methyl esters and fractionated in a spiral column (column E; Shorland, 1952), and further resolved into their components by gas-liquid chromatography, the purified constituents being collected as described by James *et al.* (1956). The fatty acid composition was calculated as outlined by Hilditch (1956) after allowance for the (+)-*anteiso* acids. The proportions of the (+)-*anteiso* acids were determined from the optical rotation of the methyl ester fractions (Table 2), by using as the standard the values given by Milburn & Truter (1954) for the pure substances.

Isolation of glycerol. Glycerol was estimated by the procedure described by Abraham & Hassid (1957). The fat (approx. 4.0 g.) was saponified (Henry & Kon, 1942) and the soaps were acidified with 7 ml. of 25% (v/v) sulphuric acid. The volatile acids were removed by steam distillation for 2 hr. The residue was extracted with diethyl ether and the aqueous layer neutralized with aq. 0.5N-KOH. The aqueous solution was then evaporated on a water bath and the solid residue dissolved in 20 ml. of boiling methanol. On cooling, the precipitate was filtered off and the filtrate evaporated to remove the solvent. The residue was taken up in 2-5 ml. of methanol and again filtered to remove a slight precipitate. Repetition of this last stage resulted in the removal of the remaining salt, and the filtrate was then freed from the solvent on a water bath, the last traces being removed *in vacuo*.

Determination of specific activities. The samples were adsorbed on carbon-free asbestos and packed into a platinum combustion boat. The combustion was carried out in a semi-micro furnace, with a stream of CO₂-free oxygen. The CO₂ formed was passed over heated CuO, dried over Anhydron (British Drug Houses Ltd.) and P₂O₅, and collected in traps cooled with liquid oxygen, the surplus

oxygen being passed out of the system through a soda-lime tube and an H_2SO_4 bubbler.

The counting technique used was based on that of Eidinoff (1950). The gas counters were of a conventional type, constructed of Pyrex glass with thin sheet copper sprung against the glass envelope and an anode made of tungsten wire 0.004 in. in diameter.

Table 2 shows the reproducibility of the method when tested with radioactive methyl palmitate from the butterfat and with inactive acetic acid.

The specific activities ($\mu\text{C/g. atom of carbon}$) of the samples were calculated assuming no end- or wall-effects. However, all determinations are comparable, since they have always been referred to the same counter. The milk-

fat sample, which was burned and counted repeatedly in different counters, over a period of approximately one year, gave specific activities constant to within $\pm 1\%$.

RESULTS AND DISCUSSION

The fatty acid composition of the milk fat (Table 3) falls within the range normally found (e.g. Hilditch, 1956) but, in addition, some of the minor constituents not usually quantitatively determined have been included. Popják, French, Hunter & Martin (1951) showed that propionic acid and valeric acid were present in the milk fat of goats, but in the bovine milk fat used in the present investigation these acids were not detected, as in a New Zealand milk-fat sample examined earlier (Hawke, 1957).

The specific activities of *n*-pentadecanoic and *n*-heptadecanoic acids were similar to those of palmitic acid and stearic acid (Table 3). It therefore appears that most of the [*carboxy*- ^{14}C]valerate is not utilized directly in the biosynthesis of the '*n*-odd-numbered acids' by addition of C_2 , but is degraded to labelled acetate and unlabelled propionate. The labelled acetate would then be used

Table 1. *Milk yield and milk-fat radioactivity after the injection of [carboxy- ^{14}C]valerate*

Milk sample	Time after injection (hr.)	Milk yield (kg.)	Activity of milk fat ($\mu\text{C/g. atom of C}$)
1	3.3	1.36	2.95
2*	10.0	2.45	6.00
3	22.2	4.99	1.62

* Saponification equiv., 240.7; iodine val. (Wijs), 35.6.

Table 2. *Recovery of carbon dioxide and radioactivity after the combustion and gas counting of labelled methyl palmitate and inactive acetate*

Substance	Wt. (mg.)	Vol. of CO_2 at 23° (ml.)		Activity ($\mu\text{C/g. of C}$)
		Found	Calc.	
Methyl palmitate (labelled)	12.8	17.3	18.4	0.410
Methyl palmitate (labelled)	5.1	6.5	7.3	0.411
Methyl palmitate (labelled)	59.2	74.9	85.2	0.409
Methyl palmitate (labelled)	7.0	8.6	10.1	0.395
Acetic acid (inactive)	245 $\mu\text{equiv.}$	12.2	12.4	—

Mean $\pm$ s.d.,
0.406 $\pm$ 0.007

Table 3. *Fatty acids of butterfat and their labelling 10 hr. after injection of [carboxy- ^{14}C]valerate*

Fatty acid	Composition (% by wt.)	$\mu\text{C/100 g. of total fatty acids}$	$\mu\text{C/g. atom of C}$
C_2 saturated	0.12	0.02	4.32
C_4 saturated	3.11	2.25	15.96
C_6 saturated	2.08	1.48	13.80
C_8 saturated	1.03	0.41	7.20
C_{10} saturated	3.13	0.11	0.60
C_{10} unsaturated	0.08	—	—
C_{12} saturated	6.03	4.34	12.00
C_{12} unsaturated	0.39	0.23	9.84
C_{14} saturated	7.69	4.54	9.60
C_{14} unsaturated	1.04	0.38	5.88
C_{16} * saturated	0.80	0.21	4.20
C_{16} anteiso saturated	0.36	0.03	1.32
C_{16} saturated	26.91	8.88	5.28
C_{16} unsaturated	4.94	0.52	1.68
C_{17} * saturated	0.80	0.23	4.56
C_{17} anteiso saturated	0.41	0.04	1.68
C_{18} saturated	8.38	2.29	4.32
C_{18} unsaturated	32.70	2.00	0.96
Total	100.00	27.96	—
Glycerol	—	—	15.0

* The amounts of *n*- C_{15} and *n*- C_{17} saturated acids have not been determined in the present work. The figure recorded for the *n*- C_{15} saturated acid is based on an earlier analysis of New Zealand butterfat by Shorland, Gerson & Hansen (1955*b*). From results previously obtained on butterfat (Shorland & Hansen, 1957) it is estimated that the proportion of *n*- C_{17} acids is of the same order as that of *n*- C_{15} acids.

equally for the synthesis of 'odd'- and 'even'-numbered fatty acids. In the light of the investigations of James *et al.* (1956) the unlabelled propionate liberated would participate in the formation of '*n*-odd-numbered acids'. The degradation of valeric acid suggests an explanation for its absence from the blood (Annison, 1954), despite its occurrence as a normal component in the rumen.

Table 3 shows the distribution of ^{14}C among the fatty acids. On the basis of their specific activities the fatty acids may be divided into two groups. The acids of C_4 – C_{10} form one group with maximum specific activity at C_4 , which is followed by a steep decline to C_{10} . The second group comprises the acids of C_{12} and above with a maximum specific activity at C_{12} , followed by a decline with increasing molecular weight. This pattern of distribution of specific activities differs from that found by Popják, French, Hunter & Martin (1951) in the goat-milk fat, where the specific activities rose to a maximum at C_{10} during the 12 hr. period after injection of labelled acetate. In the 12–48 hr. period after injection the specific activities increased from C_4 to C_{16} . This led Popják, French, Hunter & Martin (1951) to the conclusion that all the acids up to C_{16} were synthesized chiefly in the udder. Shaw, Powell & Knodt (1942) had already shown that blood fat, which consists mainly of non-volatile fatty acids (Lough & Garton, 1957), contributes to cow-milk fat. In the present experiment the distribution of specific activities (Table 3) is consistent with the origin of the milk fat from both sources. The specific activities appear to indicate, however, that the fatty acids C_4 – C_{10} , rather than C_4 – C_{16} , are predominantly synthesized in the udder, while C_{12} and above are synthesized at other sites, such as the liver, from separate pools containing [^{14}C]acetate. The decline in specific activities (Table 3) following the maxima at C_4 and C_{12} could be explained by the replacement of labelled by unlabelled acetate during the course of synthesis (Popják, French & Folley, 1951; McClymont, 1949). The dilution of [^{14}C]acetate with inactive acetate is supported by the low specific activity of the acetate isolated by us.

The C_{15} and C_{17} (+)-*anteiso* acids, as well as the C_{12} , C_{14} , C_{16} and C_{18} unsaturated acids, show a lower specific activity than do the saturated straight-chain acids of similar chain length. The formation of these acids from acetate therefore appears to take place at a lower rate than does that of the saturated straight-chain acids.

The specific activity of glycerol ($15.0 \mu\text{C/g. atom}$ of carbon) was identical with that reported by M. Kleiber (personal communication, 1956). This indicates that, like propionate (Kleiber, Black, Brown & Tolbert, 1953), a large part of the intact valerate is utilized for carbohydrate synthesis.

SUMMARY

1. The distribution of ^{14}C among fatty acids in the milk fat of a lactating cow, which had been injected with [*carboxy*- ^{14}C]valerate, showed that the saturated straight-chain acids of similar molecular weight, containing odd and even numbers of carbon atoms, had closely similar specific activities. Valeric acid is not, therefore, a direct precursor in the formation of saturated straight-chain acids of milk fat containing an odd number of carbon atoms. The present results are consistent with the degradation *in vivo* to acetate and propionate.

2. The present results suggest that the volatile fatty acids up to and including C_{10} are synthesized predominantly in the udder, and that those from C_{12} to C_{18} are synthesized elsewhere.

3. The high specific activity of glycerol carbon shows valerate to be glycogenic rather than lipogenic.

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