The Incorporation of [1-¹⁴C]Acetate into the Methyl Ketones that Occur in Steam-Distillates of Bovine Milk Fat

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1. The ¹⁴C-labelling of the fatty acids and the methyl ketones in steam-distillates of milk fat from a lactating cow that had been injected intravenously with [1-¹⁴C]acetate was determined. 2. The labelling patterns of the C₆–C₁₆ fatty acids and the corresponding methyl ketones with one fewer carbon atoms were similar, particularly so for the C₅–C₁₀ compounds at 9 and 22 hr. after the injection of [1-¹⁴C]acetate. The isolation of ¹⁴C-labelled methyl ketones in the range C₃–C₁₅ is evidence that the β-oxo acid precursors, which are glyceride-bound in the milk fat, are synthesized in the mammary gland from acetate. The absence of heptadecan-2-one in steam-distillates and the extremely low specific radioactivity of stearic acid are further evidence for this biosynthetic pathway. 3. The specific radioactivities of the C₅–C₁₅ methyl ketones were higher (with the exception of C₉ methyl ketone in the second milking) than the specific activities of the corresponding fatty acids with one more carbon atom. This is consistent with the methyl ketone precursors being formed during the biosynthesis of fatty acids rather than being products of β-oxidation of fatty acids.

When milk fat is heated in the presence of water a homologous series of methyl ketones from C₃ to C₁₅ is produced (Wong, Patton & Forss, 1958; Patton & Tharp, 1959). Exhaustive distillation indicated that only limited quantities of the methyl ketones could be obtained (about 0.88 μmole/g. of milk fat) and that the precursors were thus present in relatively small amounts (Lawrence, 1963). Further, it has been shown that the C₅–C₁₅ series of methyl ketones originate from the even-numbered β-oxo acids with 6–16 carbon atoms (Van der Ven, Begemann & Schogt, 1963), which are present as triglyceride components in milk fat containing one β-oxo acid and two fatty acid moieties (Parks, Keeney, Katz & Schwartz, 1964).

Preliminary work (Lawrence & Hawke, 1963) indicated that these triglyceride-bound β-oxo acids arise as intermediates in the biosynthesis of fatty acids from acetate, since the homologous series of methyl ketones obtained from the milk fat of a cow that had been given an intravenous injection of sodium [1-¹⁴C]acetate possessed a labelling pattern similar to that obtained for fatty acids of similar chain length from goat milk fat (Popják, French, Hunter & Martin, 1951).

When *n*-[1-¹⁴C]valeric acid was injected intravenously into a lactating cow two maxima occurred

in the distribution of ¹⁴C-label in the fatty acids of the milk fat (Gerson, Hawke, Shorland & Melhuish, 1960). From this it was concluded that the volatile fatty acids up to C₁₀ were synthesized predominantly in the udder and that the C₁₂–C₁₈ fatty acids were synthesized predominantly elsewhere. These results contrasted with those obtained by Popják *et al.* (1951), in which the specific activities of the fatty acids showed only one maximum at any given time. In view of these results, T. Gerson & F. B. Shorland commenced a further experiment in which 5 mc of [1-¹⁴C]acetate was injected into the jugular vein of a cow and the specific activities of the fatty acids in the milk fat were determined for comparison with the above-mentioned experiments. Milk fat from this work was made available to the present authors in order to further their work on the origin of the methyl ketones obtained on distillation. In addition, T. Gerson & F. B. Shorland, pending publication of their results, have made available to us data relating to the specific activities of the fatty acids. This has enabled the labelling pattern of the methyl ketones to be compared directly with the labelling pattern of the fatty acids from the same source and to determine whether the methyl ketone precursors are being formed during synthesis or oxidation of fatty acids.

MATERIALS AND METHODS

A lactating Jersey dairy cow, after milking, was injected in the jugular vein with 5.0mc of [1-¹⁴C]acetate. The cow was milked 3, 9 and 22hr. after injection, the milk yields and activities of the milk fats being shown in Table 1. The milk fats were stored at -10° until required for analysis.

Chemicals. Sodium [1-¹⁴C]acetate and *n*-[¹⁴C]hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., activated magnesia (Seasorb 43 and Magnesia 2665) was from Fischer Scientific Co., Silver Spring, Md., U.S.A., Celite 545 was from Johns-Manville Co., New York, N.Y., U.S.A., and 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

Isolation and separation of fatty acids and measurement of their ¹⁴C activities. By using the procedure described by Gerson *et al.* (1960) the fatty acids of the milk fat were separated into steam-volatile and non-volatile acids, the latter being further resolved into 'solids' and 'liquids'.

The steam-volatile acids were separated by gas-liquid chromatography, as were the solids and liquids, as their methyl esters. The specific activities of the purified fractions were determined in a liquid-scintillation counter (T. Gerson & F. B. Shorland, personal communication).

Isolation of methyl ketones. A 20g. sample of the milk fat from each of the three milkings was steam-distilled at 100°. It was necessary to collect 10l. of steam-distillate to ensure that the maximum quantities of methyl ketones were obtained. However, only the fat from the second milking was steam-distilled exhaustively because the volume of liquid in the distilling flask increases slowly as the distillation proceeds, making complete recovery of higher methyl ketones a lengthy process. Table 2 shows that, except for the lower yields of C₁₅ methyl ketone, the yields of the methyl ketones did not seem to be affected by the shorter time of distillation.

The distillates were led directly into 2,4-DNP-hydrazine reagent (2g./l. of 2N-HCl) at 0°, and the distillate-reagent mixture was kept at room temperature in a stoppered container for 24hr. The 2,4-DNP-hydrazones were then extracted with carbonyl-free hexane, prepared according to the method of Schwartz & Parks (1961), and the extract was evaporated to dryness.

Purification of methyl ketones. The 2,4-DNP-hydrazones were subjected to a series of chromatographic procedures to remove neutral fat, fatty acids, alkanals and alkenals, which, as trace contaminants, might have contributed to the radioactivity of the 2,4-DNP-hydrazones of the methyl ketones.

Traces of neutral fat were removed from the 2,4-DNP-hydrazones by using a column of a 1:1 mixture of activated

magnesia (Seasorb 43) and Celite 545. Neutral fat was eluted in hexane (Schwartz, Haller & Keeney, 1963), and the 2,4-DNP-hydrazones were eluted with nitromethane-chloroform (1:3, v/v). The 2,4-DNP-hydrazones were then added to an alumina column (2g. packed in about 4ml. of chloroform) in a small volume of chloroform and the column was eluted with further chloroform until the effluent became colourless. Any contaminant fatty acids remained on the column.

To separate the 2,4-DNP-hydrazones of the methyl ketones from the hydrazones of other carbonyls the dry effluent from the alumina column was taken up in hexane-chloroform (17:3, v/v) and added to a column containing a 1:1 mixture of Magnesia 2665 and Celite 545, according to the method of Schwartz, Parks & Keeney (1962). The 2,4-DNP-hydrazones of the methyl ketones were eluted with the same solvent mixture.

Naturally occurring alkanals and alkenals have been reported in milk fat (Parks, Keeney & Schwartz, 1961) and were isolated in steam-distillates. In the present investigation small amounts of alka-2,4-dienals were also detected, indicating chemical deterioration during storage at -10°. The concentration of carbonyls other than methyl ketones varied between 5 and 10% of the total carbonyls obtained, but were not radioactive. Methyl ketones have not, however, been reported as products of the autoxidation of fats.

Separation of methyl ketones. The isolated 2,4-DNP-hydrazones of the methyl ketones were evaporated to dryness, taken up in a minimum amount of hexane and separated into individual compounds on Celite columns by eluting with nitromethane-hexane mixtures (Day, Bassette & Keeney, 1960). The fractions were identified and their purity was established by the paper-chromatographic methods of Klein & de Jong (1956) and Huelin (1952). The concentrations of methyl ketones were determined spectrophotometrically at 345mμ as the 2,4-DNP-hydrazones in hexane.

Determination of specific radioactivities. Isolated lipid fractions and the 2,4-DNP-hydrazones (less than 0.2mg.) were counted in a liquid-scintillation counter (assembly NE8301; Nuclear Enterprises Ltd.) in toluene solution containing 0.05% of 2,5-diphenyloxazole and 0.03% of 1,4-bis-(5-phenyloxazol-2-yl)benzene.

Quenching corrections for 2,4-DNP-hydrazones were established by using *n*-[¹⁴C]hexadecane as an internal standard. Quenching curves were prepared for the 2,4-DNP-hydrazones of the C₈-C₁₅ methyl ketones. In all cases the quenching of radioactivity was found to be proportional to the amount of 2,4-DNP-hydrazone added, up to a maximum of 0.2mg. The corrections necessary to compensate for quenching were calculated from the quenching curves and, also as a check, by adding a known amount of the standard *n*-[¹⁴C]hexadecane dissolved in toluene to the vial containing the radioactive 2,4-DNP-hydrazone.

Correction factors necessary to compensate for quenching by the steam-volatile fatty acids and methyl esters were never greater than 1.1.

RESULTS

Table 1. *Milk yield and milk-fat radioactivity after the injection of [1-¹⁴C]acetate*

Milk sample	Time after injection (hr.)	Milk yield (oz.)	Sp. activity of milk fat (μc/g. atom of C)
1	3	24	2.27
2	9	88	12.00
3	22	88	4.92

Table 2 shows the yields of methyl ketones obtained by steam-distillation of fat from each of the three milkings. No methyl ketones with carbon

Table 2. Yields of methyl ketones obtained by steam-distillation at 100° of milk fat from a cow milked 3, 9 and 22 hr. after the intravenous injection of 5 mc of [1-¹⁴C]acetate

A 6l. volume of distillate was collected from each of the 3 hr. and 22 hr. milkings, and 10l. was collected from the 9 hr. milking.

Methyl ketone	Time of milking after injection.....	Yield of methyl ketone (μmole/g. of milk fat)		
		3 hr.	9 hr.	22 hr.
C ₃		0.31	0.34	0.25
C ₅		0.07	0.10	0.11
C ₇		0.12	0.12	0.06
C ₉		0.08	0.07	0.05
C ₁₁		0.06	0.08	0.08
C ₁₃		0.09	0.09	0.09
C ₁₅		0.09	0.17	0.09
Total C ₅ -C ₁₅		0.51	0.63	0.48

number greater than C₁₅ were detected in any of the distillates.

Fig. 1 shows the relationship between the specific activities of the individual methyl ketones obtained from milk fat from the three milkings and the chain length of the methyl ketones. The very similar distribution of ¹⁴C between the methyl ketones in the second and the third milkings contrast with that of the first milking. Whereas in the first milking the relatively low specific activities of the methyl ketones decrease from 2.65 μC/g.atom of C to 0.72 μC/g.atom of C, the higher specific activities of the methyl ketones in the other two milkings increased to a maximum at C₁₁ (22.5 and 12.5 μC/g. atom of C for the second and third milkings respectively). The specific activities of tridecan-2-one and pentadecan-2-one in the third milking were considerably greater than for the ketones below C₁₁.

Although appreciable proportions of acetone were obtained in the steam-distillates of each milking (Table 2), radioactivity was detected only in the acetone from the second milking and even in this sample the acetone had a very low specific activity.

The fatty acid composition of the milk fat, as shown by relative peak areas on gas-liquid chromatograms, fell within the range normally found (e.g. Hawke, 1957). In addition, propionic acid and a C₅ acid, assumed to be valeric acid, were also detected in trace amounts but their radioactivities were not determined.

Fig. 1 also shows the distribution of ¹⁴C in the fatty acids of milk fat obtained from the three milkings. The fatty acids from the fat of the first milking (3 hr. after injection) had uniformly low specific activities, the C₆-C₁₂ acids having the highest activity (about 0.85 μC/g.atom of C). The

Fig. 1. Relationship between the specific activity and the number of carbon atoms in the fatty acids (even number of carbon atoms) and the methyl ketones (odd number of carbon atoms) isolated from the milk fat of a lactating cow injected with [1-¹⁴C]acetate 3 hr. (a), 9 hr. (b) and 22 hr. (c) before milking.

fatty acids of highest radioactivity were in the second milking (9 hr. after injection), peak activity being found at C₁₀ (12.9 μC/g.atom of C).

The pattern of labelling of the fatty acids of the third milking, 22 hr. after injection, was almost identical with that of the second milking, with peak activity again at C₁₀ acid (2.94 μC/g.atom of C). The specific activities were, however, three- to four-fold lower than in the second milking, whereas there was only a two- to three-fold decrease in specific activities of the methyl ketones from the second to the third milking. Except for acetone in all milkings and C₉ methyl ketone in the second milking, the methyl ketones had greater specific activities than the corresponding fatty acids. In the two later milkings comparisons of labelling in

fatty acids and methyl ketones show that the principal features were the close similarities in the specific activities of the two series of compounds in the C₅–C₁₀ range and the much higher specific activities of the methyl ketones in the C₁₁–C₁₆ range.

DISCUSSION

Although there are several points of difference in the labelling patterns of the fatty acids and the corresponding methyl ketones with one less carbon atom, the results in general are consistent with the view that the β -oxo acids, from which the methyl ketones are formed during steam-distillation, form part of the same metabolic pool as the fatty acids undergoing incorporation into triglycerides. Consequently, the higher methyl ketones are very weakly labelled in the first 3 hr., yet contain the major part of the activity at the end of 22 hr. The change in labelling pattern of the fatty acids with time is in accord with similar experiments with the goat by Popják *et al.* (1951), and points to the existence of a relatively large pool of fatty acids in the mammary gland and to the relatively slow rate of secretion of fat and fat precursors in the alveoli after its synthesis in the cells (Hardwick, Linzell & Mephram, 1963).

The high specific activities of the methyl ketones relative to the corresponding fatty acids may be a reflection of the formation of β -oxo acids as intermediates in fatty acid synthesis prior to a rate-limiting step or of a greater rate of incorporation of the β -oxo acids than of the fatty acids into the triglycerides. The close similarity between the labelling patterns and the specific activities of the low-molecular-weight fatty acids and the corresponding methyl ketones with one less carbon atom in the last two milkings suggest that the β -oxo acids and the corresponding fatty acids have a common precursor and are together incorporated into the triglycerides. Insufficient evidence is available to give a reliable explanation of the finding that the specific activities of the C₁₂–C₁₆ fatty acids in the 9 hr. and 22 hr. milkings are considerably lower than the activities of the corresponding methyl ketones. A possible explanation is that there is a dilution of the labelled fatty acids being synthesized from acetate in the mammary gland by fatty acids synthesized by less direct pathways, or that the differences are related to the size of the metabolic pools. The higher specific activities of the methyl ketones relative to the fatty acids is evidence that the β -oxo acids are not synthesized from fatty acids by β -oxidation. The relationship between the labelling of fatty acids or methyl ketones when radioactive acetate is used provides additional understanding of the biosynthetic origin of the fatty acid components in milk. The generally accepted view (e.g.

Folley & McNaught, 1961) is that in the biosynthesis of milk the fatty acids, up to and including palmitic acid, are synthesized from an acetate pool, whereas C₁₈ acids and above are synthesized by less direct pathways or from the blood triglycerides (Dils & Popják, 1962). It appears that, apart from acetoacetate, the β -oxo acids which are incorporated into milk triglycerides arise from the direct utilization of acetate in the mammary gland, because neither radioactive nor unlabelled C₁₇ methyl ketone or higher-molecular-weight ketones were detected in the milk fat, and stearic acid had extremely low specific activities in all milkings. The presence of small amounts of β -oxo acid in triglycerides with similar specific activity to fatty acids of the same carbon number implies that there is some dissociation of the β -oxoacyl-enzyme (Lynen, 1961) or β -oxoacyl-S-acyl-carrier protein (Alberts, Majerus, Talamo & Vagelos, 1964) in the mammary gland before the dissociation of acyl-enzyme or acyl-(acyl-carrier protein) during triglyceride synthesis. In an extension of the present study coconut oil, which like milk fat is characterized by a high proportion of saturated C₈–C₁₆ acids, was also steam-distilled, but no methyl ketones except acetone were found. This suggests that any C₈–C₁₆ β -oxo acids incorporated into the triglycerides of coconut oil are presumably reduced *in situ* and that the presence of β -oxo acids in the triglycerides of milk fat possibly results from their rapid removal from the site of synthesis during milk secretion.

Though most attention has been given to the reduction of D- β -hydroxybutyric acid of the blood stream to butyric acid (e.g. Popják *et al.* 1951; Kumar, Lakshmanan & Shaw, 1959; Annison, Leng, Lindsay & White, 1963), the results of Black, Kleiber & Brown (1961) indicate that the cow can metabolize butyrate via the classical β -oxidation pathway and the tricarboxylic acid cycle. Similarly, the present work suggests that the mammary gland can oxidize β -hydroxybutyric acid to acetoacetic acid, because in all milkings butyric acid had a higher specific activity than the corresponding methyl ketone. The presence of unlabelled acetoacetate, but labelled higher β -oxo acids, in milk triglycerides after the injection of labelled acetate could be explained by the pathway outlined in Scheme 1 and based on the mechanism for fatty acid synthesis proposed by Bressler & Wakil (1962). The quantitative importance of acetone in the mixed methyl ketones from milk fat suggests that more acetoacetate than any of the higher β -oxo acids is present in the metabolic pool.

Since both the C₅–C₁₅ methyl ketone precursors and the short-chain fatty acids in milk fat are synthesized from acetate, the amounts of methyl ketone obtained on distillation might be expected to exhibit a seasonal variation similar to that

Scheme 1. Hypothetical mechanism for the formation and incorporation of β -oxo acids into milk fat.

observed with the quantitatively important short-chain fatty acids (Hansen & Shorland, 1952; Keeney, 1956). The greater amounts of C_5 – C_{15} methyl ketones reported by Lawrence (1963) in samples of New Zealand butterfat (about $0.88 \mu\text{mole/g.}$ compared with $0.63 \mu\text{mole/g.}$ in the present study) might have been due to a seasonal effect.

The very low radioactivity of the unsaturated C_{18} fatty acids appears to exclude the possibility, suggested by Wong *et al.* (1958), that methyl ketones are products of the autoxidation of milk fat.

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