Catabolism of 2-Methyloctanoic Acid and 3 β -Hydroxycholest-5-en-26-oic Acid

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(Received 12 April 1966)

1. 2-Methyl[1-¹⁴C]octanoic acid was synthesized from 2-bromo-octane and ¹⁴CO₂. 2. 2-Methyl[1-¹⁴C]octanoic acid was readily oxidized to propionic acid and carbon dioxide by mitochondrial preparations from liver, less readily oxidized by adrenal and kidney (mitochondria), and only poorly oxidized by heart, spleen and brown fat (mitochondria). 3. 3 β -Hydroxy[26-¹⁴C]cholest-5-en-26-oic acid was rapidly oxidized by mammalian-liver mitochondria to propionic acid and carbon dioxide. Caiman-liver and toad-liver mitochondria also oxidized this steroid acid. 4. The oxidation of propionic acid, octanoic acid and palmitic acid by mitochondrial preparations from these various tissues was also studied. 5. Added carnitine did not stimulate 2-methyloctanoic acid oxidation and feebly stimulated 3 β -hydroxycholest-5-en-26-oic acid oxidation. 6. The significance of these results is discussed in relation to sterol catabolism in mammals and non-mammalian species.

Branched-chain fatty acids are formed as intermediates in the catabolism of branched aliphatic amino acids (Atchley, 1948; Kinnory, Takeda & Greenberg, 1955); for example, α -methylbutyrate is an intermediate in the enzymic degradation of isoleucine to propionyl-CoA (Robinson, Bachhawat & Coon, 1956). There is evidence that at least one C₂₇ α -methyl carboxylic acid (3 α ,7 α ,12 α -trihydroxycoprostanic acid) is formed as an intermediate in sterol catabolism by mammalian-liver mitochondria (Suld, Staple & Gurin, 1962; Danielsson, 1960). This acid is either excreted into the bile after conjugation with taurine or glycine (Carey & Haslewood, 1963; Staple & Rabinowitz, 1962), or further oxidized to cholanolic acids (Masui & Staple, 1965; Briggs, Whitehouse & Staple, 1961) with cleavage of the terminal C₃ unit as propionyl-CoA (Suld *et al.* 1962). The terminal isopropyl group of cholesterol itself is oxidized and cleaved by rat-liver mitochondrial preparations, yielding propionyl-CoA (Mitropoulos & Myant, 1965).

In this paper we present evidence that rat-liver mitochondria can rapidly degrade both acyclic and steroidal α -methyl carboxylic acids, e.g. 2-methyl-

octanoic acid and 3 β -hydroxycholest-5-en-26-oic acid, to propionic acid or propionyl-CoA, whereas mitochondrial preparations from other extrahepatic mammalian tissues can only oxidize 2-methyloctanoic acid. 3 β -Hydroxycholest-5-en-26-oic acid is formed by liver preparations as an intermediate in the oxidation of 26-hydroxycholesterol *in vitro* (Dean & Whitehouse, 1966a, and unpublished work), which in turn may be formed from cholesterol by isolated rat-liver and mouse-liver mitochondria (Danielsson, 1961; Mitropoulos & Myant, 1966) and has been found in at least one extrahepatic tissue (Steel, Brooks & Harland, 1966).

EXPERIMENTAL

Chemicals. 3 β -Hydroxy[26-¹⁴C]cholest-5-en-26-oic acid was prepared from K¹⁴CN and 25-bromo-3 β -hydroxy-27-norcholest-5-ene (Dean & Whitehouse, 1966a). [2-¹⁴C]-Propionic acid, [1,4-¹⁴C]succinic acid and Ba¹⁴CO₃ were purchased from The Radiochemical Centre, Amersham, Bucks.

Preparation of 2-methyl[1-¹⁴C]octanoic acid. 2-Bromo-octane was prepared by the method of Shiner & Young (1930) from octan-2-ol and PBr₃ in 80% yield, with b.p. 83–84° at 18 mm., n_D^{20} 1.4500.

2-Bromo-octane (300 mg.) in dry ether (5 ml.) was treated with magnesium turnings. The reaction was allowed to subside and the mixture (protected from atmospheric moisture with CaCl₂) was heated under reflux on a water bath for $\frac{1}{2}$ hr. The Grignard reagent so formed was used immediately.

¹⁴CO₂ was prepared *in vacuo* by the action of conc.

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H₂SO₄ (2 ml.) on Ba¹⁴CO₃ (142 mg.) in a vessel attached to a vacuum line designed for the subsequent purification of the ¹⁴CO₂ as described by Weinhouse, Medes & Floyd (1944). Purified ¹⁴CO₂ was condensed into the reaction vessel, by cooling with liquid N₂, which contained the frozen solution of the Grignard reagent described above. The reaction vessel was isolated from the main manifold and allowed to warm up to -80° for 1 hr. and -40° for 1 hr.; it was finally kept, sealed, at room temperature and under N₂ at 15 lb./in.² overnight. Dilute HCl (2 ml.) was added to the reaction mixture and after shaking was separated off; further extraction with the same dilute HCl (1 × 2 ml.) was followed by extraction with 2N-NaOH (3 × 2 ml.). The alkaline extracts were combined and neutralized to litmus and stored at -15°. The yield from Ba¹⁴CO₃ was 30%.

Biochemical methods. Ox-heart sarcosomes were isolated by the method of Sanadi & Fluharty (1963). Subcellular fractions from other tissues were prepared and incubated exactly (except that the supernatant fraction was replaced by 10% sucrose) as described by Dean & Whitehouse (1966a), usually for $\frac{1}{2}$ hr. (but 1 hr. in experiments with caiman-liver or toad-liver preparations). ¹⁴CO₂ was trapped in 2.5N-NaOH and assayed as described by Dean & Whitehouse (1966a). [¹⁴C]Propionic acid was determined after adding 2 mg. of unlabelled sodium propionate to each incubation flask, acidified with 0.2 ml. of 12N-H₂SO₄ after incubation. The mixture was centrifuged to precipitate denatured protein. The supernatant and pellet were separately extracted with ether (4 × 1 ml.) and the extracts combined and evaporated at 35° to 0.5 ml. Propionic acid in the extracts was separated from other constituents either by paper chromatography of the ammonium salts in butanol-aq. 3N-NH₃-ethanol (8:10:3, by vol.) (cf. Brown, 1950) or by gas-liquid chromatography with a column packed with diethylene glycol adipate + pentaerythritol + phosphoric acid on Chromosorb W (80-100 mesh). The effluent gas was bubbled into Ditol D (see Dean & Whitehouse, 1966a) and the Ditol changed periodically and assayed for radioactivity by liquid-scintillation

counting. The retention time for propionic acid was between 1.5 and 2 min. and that for 2-methyloctanoic acid was about 35 min.

RESULTS

2-Methyloctanoic acid catabolism. 2-Methyl-[1-¹⁴C]octanoic acid was rapidly oxidized to ¹⁴CO₂ by fortified mouse-liver, rat-liver and guinea-pig-liver mitochondrial preparations, more than 80% of added ¹⁴C being liberated as ¹⁴CO₂ within 1 hr. on incubation at 37°.

CoA and ATP were essential for ¹⁴CO₂ production but the ATP requirement could be met by either GTP or ITP. In mouse-liver mitochondria, the pH optimum for ¹⁴CO₂ formation (in tris hydrochloride buffers) was 7.7. Mitochondrial fractions isolated from homogenates of caiman and toad livers, suitably fortified with cofactors, oxidized 2-methyloctanoic acid much less rapidly than mammalian-liver mitochondria when compared by the yields of ¹⁴CO₂ (per mg. of protein N in the preparation) after 1 hr. incubation at 37°.

Homogenates and mitochondrial fractions of mammalian heart muscle, adrenal cortex, kidney, spleen and brown fat oxidized both 2-methyloctanoic acid and propionic acid to carbon dioxide (Table 1).

3 β -Hydroxycholest-5-en-26-oic acid catabolism. Only mammalian-liver and toad-liver mitochondrial preparations oxidized this steroid acid *in vitro* at a rate comparable with that of 2-methyloctanoic acid. Caiman-liver 'mitochondria' consistently showed some capacity to oxidize this steroid acid, which is of especial interest as this species normally

Table 1. *Oxidation of some carboxylic acids by mitochondrial fractions of different tissues*

Results are given as averages of the percentage yields of ¹⁴CO₂ from 30 min. incubations at 37° in tris hydrochloride buffer, pH 7.5, from added substrate (0.01 μ C), computed as $100 \times \frac{^{14}\text{CO}_2}{[^{14}\text{C}]\text{substrate}}$.

Tissue source	Percentage oxidation of				
	2-Methyl-octanoic acid	Propionic acid	Octanoic acid	Palmitic acid	Hydroxy-cholestenoic acid
Mouse liver*	80	15†	8	15	70
Ox heart	8	14	20	50	0.01
Ox adrenal	20	9	4	2	1
Rabbit kidney	33	12	6	5	1
Guinea-pig brown fat	1	7	2	5	0.1
Rat spleen‡	4	2	1	—	—
Toad liver	14	3	—	—	10
Caiman liver	10	22	—	—	2

* Rat-liver and guinea-pig-liver mitochondria show the same relative order of oxidation but the overall conversion was always lower for the same weight of tissue.

† See the text.

‡ Homogenate, not isolated mitochondria.

excretes C₂₇ bile acids (Haslewood, 1964). Mitochondrial fractions isolated from heart, kidney, adrenal cortex and brown fat were virtually incapable of oxidizing this steroid acid (not more than 5% of the efficiency of 2-methyloctanoic acid oxidation).

With mouse-liver mitochondria, the pH optimum for ¹⁴CO₂ formation (in tris hydrochloride buffers) was 7.7.

Propionic acid formation. [¹⁴C]Propionic acid was isolated after coincubating either 2-methyl-[1-¹⁴C]octanoic acid or 3β-hydroxy[26-¹⁴C]cholest-5-en-26-oic acid with non-radioactive propionic acid (20 μmoles/incubation mixture≡8 mm) and

liver mitochondria. The results of paper chromatography of an incubation-mixture extract are shown in Fig. 1. Identical results were obtained with incubations of 3β-hydroxycholest-5-en-26-oic acid and non-radioactive propionic acid except that the *R_F* of the steroid acid was even higher than 2-methyloctanoic acid. These results were confirmed by gas-liquid chromatography. In these coincubation experiments, the yield of ¹⁴CO₂ from these acids was much decreased (Table 2) and was always much less than the yield of ¹⁴CO₂ from unbranched fatty acids (octanoic acid, palmitic acid) under the same conditions. The presence of propionic acid decreased the oxidation of unbranched fatty acids by 50% or less, indicating some competition for CoA or dilution of radioactive intermediates in the tricarboxylic acid cycle etc.

Propionic acid oxidation. The oxidation of added [1-¹⁴C]propionic acid by liver mitochondria was rather variable, the yield of ¹⁴CO₂ in 1 hr. varying from 5% to 60%, but in all experiments with liver mitochondria propionic acid was less readily oxidized than 2-methyloctanoic acid (with the sole exception of caiman-liver mitochondrial preparations). Table 1 shows that several extrahepatic tissues oxidized propionic acid to carbon dioxide.

Effect of carnitine. When DL-carnitine hydrochloride (1.0 mm) was added to incubations containing ox-heart or rat-liver mitochondria, palmitic acid oxidation was consistently stimulated 100% or more. The oxidation of 3β-hydroxycholest-5-en-26-oic acid by liver mitochondria was stimulated by not more than 30% under the same conditions. Carnitine failed to stimulate the oxidation of either octanoic acid or 2-methyloctanoic acid by both heart and liver mitochondrial preparations.

DISCUSSION

These experiments show that both 2-methyloctanoic acid and 3β-hydroxycholest-5-en-26-oic

Fig. 1. Paper chromatograms of ether-soluble acids extracted from incubations of 2-methyl[1-¹⁴C]octanoic acid (in the presence of non-radioactive propionic acid) with rat-liver mitochondria. A (solid histogram), position of non-radioactive propionic acid; B (solid histogram), position of non-radioactive 2-methyloctanoic acid. (a) incubation with mitochondria; (b) incubation with boiled mitochondria. Incubation conditions were as given in Table 2, with the propionate concentration given in Table 2. The background radioactivity was 200 counts/min.

Table 2. *Effect of coincubating non-radioactive propionic acid with ¹⁴C-labelled acids and mammalian mitochondrial preparations*

Results are given as 100 × ¹⁴CO₂ evolved in the presence of propionic acid (20 μmoles)/¹⁴CO₂ evolved when no propionic acid was present.

Tissue source	Percentage residual oxidation to CO ₂ of				
	2-Methyl-octanoic acid	Octanoic acid	Propionic acid	Palmitic acid	Hydroxy-cholest-enoic acid
Rat liver	20	50	11	75	13
Ox heart	7	75	13	—	*
Ox adrenal cortex	14	45	5	—	*
Rabbit kidney	5	50	12	—	*

* Not oxidized.

acid are degraded by β -oxidation, forming propionic acid (as the CoA derivative), which is then further oxidized to carbon dioxide. The relative ability of different tissues to form propionic acid from 2-methyloctanoic acid parallels observations by Griffin & Green (1948) and Cheldelin & Beinert (1952), who showed that odd-numbered fatty acids were readily oxidized by kidney and liver but only poorly by heart.

2-Methyloctanoic acid was used in this study as an analogue of the (fatty acid) side chain (C-20 to C-27) of a sterol acid (cholest-5-en-26-oic acid). Our findings indicate that the presence of the steroid nucleus protects the terminal isopropyl group of the C₈ side chain from those extrahepatic tissues that can cleave the propionyl group from 2-methyloctanoic acid (e.g. kidney and adrenal cortex). Evidently, only liver mitochondria can activate and β -oxidize a C₂₇ sterol acid and this must be one of the factors effectively restricting cholesterol degradation (by loss of the terminal isopropyl group) to liver tissue. Another determinant would be the tissue distribution of the 26-hydroxylase, which probably initiates this mode of cholesterol catabolism and provides the precursor of cholestan-26-oic acids.

The distribution of this side-chain-cleaving activity is not apparently 'all-or-none' in the livers of different species. We could find such activity, i.e. ability to oxidize both 2-methyloctanoic acid and 3 β -hydroxycholest-5-en-26-oic acid, in the livers of two non-mammalian species, the caiman and toad. Caimans and alligators secrete C₂₇ bile acids (Haslewood, 1964; Dean & Whitehouse, 1966b). The toad species used (*Bufo vulgaris*) secretes C₂₇ sterols, and only traces of C₂₄ acids have been detected in its bile (G. A. D. Haslewood, personal communication). However, we have some evidence (from thin-layer chromatography and mass-spectrographic studies) that C₂₄ acids may be very minor components of alligator bile.

No good evidence was obtained that the rate of oxidation of these two particular branched-chain acids by liver mitochondria depended on exogenous carnitine. Fritz (1963) found that the oxidation of short-chain fatty acids (e.g. octanoic acid) by heart mitochondrial preparations was carnitine-insensitive, in contrast with the carnitine-activated oxidation of palmitic acid (confirmed in these

experiments with liver preparations). The presence of the C₁₉ steroid nucleus does not seem to confer much carnitine-sensitivity on the oxidation of the 2-methylheptanoic acid side chain of 3 β -hydroxycholest-5-en-26-oic acid.

We are indebted to Mr J. M. Haslam for supplying sarcosome preparations, Professor G. A. D. Haslewood for a confirmatory analysis of toad bile, Mr A. Renshaw for building a gas chromatograph, and the Medical Research Council for a Scholarship (to P. D. G. D.).

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