ENZYMIC CARBOXYLATION OF CROTONYL-CoA AND THE METABOLISM OF GLUTARIC ACID

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Isotope studies have shown that glutarate is metabolized in the rat via acetate (Rothstein and Miller, 1952, Hobbs and Koeppe, 1958). The demonstration that animal tissues can synthesize glutaryl-CoA from glutarate by activation and transfer reactions (Menon and Stern, 1959) suggested that acyl-CoA derivatives are the actual intermediates in glutarate metabolism. Potential enzymic pathways of glutaryl-CoA metabolism, illustrated in the following scheme, have been explored by the use of $C^{14}O_2$ and of synthetic acyl-CoA compounds postulated as intermediates. It has been found that glutaconyl-CoA can be formed enzymically by carboxylation of crotonyl-CoA (Reaction 1).

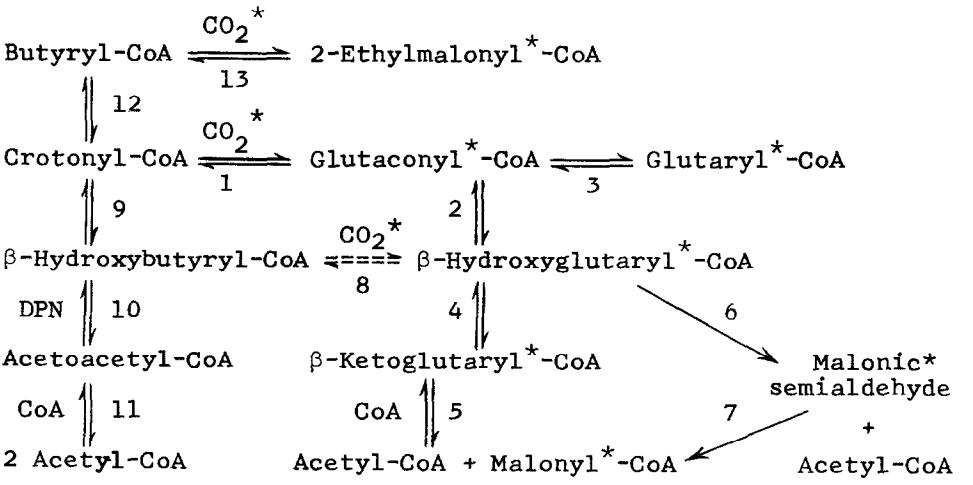

TABLE I

Fixation of $C^{14}O_2$ by Crotonyl-CoA

System	$C^{14}O_2$ fixed	System	$C^{14}O_2$ fixed
	mμmole		mμmole
Complete	13.7	No GSH	8.7
No ATP	3.6	Complete	19.8
No crotonyl-CoA	0.4	" + avidin	0.7
No Mn^{++}	1.0	" + p-CMB	21.8
Mg^{++} for Mn^{++}	11.1	Acetyl-CoA	51.2

The reaction mixture contained (in μmoles): Tris buffer pH 7.0 (100), $MnCl_2$ (1.0), ATP (5), glutathione (5), crotonyl-CoA (1.0), $KHC^{14}O_3$ (10), 97,000 counts per minute per μmole, and 9.9 mg. enzyme protein (35-65% satd. ammonium sulfate fraction of rat liver extract). Acetyl-CoA (1.0) was substituted for crotonyl-CoA, and $MgCl_2$ (5) for $MnCl_2$ as indicated. Avidin (0.25 unit) and p-chloromercuribenzoate (10^{-3} M) were preincubated with enzyme for 15 minutes before addition of components of complete system. Volume 1.0 ml. Incubation, 40 min. at 30°.

As shown in Table I, enzyme fractions from rat liver fix $C^{14}O_2$ when incubated with crotonyl-CoA, ATP and Mn^{++}. This $C^{14}O_2$ fixation is largely or completely dependent upon thioester, ATP and Mn^{++}. Mg^{++} in higher concentration replaces Mn^{++}. Like some other carboxylation reactions (Wakil et al., 1958, Lynen et al., 1959), the carboxylation of crotonyl-CoA is completely inhibited by low concentrations of avidin implicating biotin as a coenzyme of this reaction also. The enzyme fraction also contained acetyl-CoA carboxylase (Wakil, 1958, Formica and Brady, 1959).

Nature of the Carboxylation Reaction. It became necessary to determine whether carboxylation of crotonyl-CoA occurred at the α-carbon as with propionyl-CoA (Flavin and Ochoa, 1957) and the analogous butyryl-CoA (Stern et

al., 1959, Hegre et al., 1959) or at the γ-carbon as with the analogous β-methylcrotonyl-CoA (Lynen et al., 1959). The product, then, would be respectively 3-C^{14}-ethylidene-malonyl-CoA or 5-C^{14}-glutaconyl-CoA. In preliminary experiments, the products of crotonyl-CoA carboxylation were reacted with hydroxylamine and the resulting hydroxamic acids extracted and chromatographed on paper in isoamyl alcohol-formic acid solvent. Three major radioactive peaks were found on analysis in the strip counter indicating the carboxylation products were also CoA esters. In a large scale experiment the products of $C^{14}O_2$ fixation by crotonyl-CoA in rat liver were subjected to alkaline hydrolysis and the acids liberated extracted with ethanol and dried. Carrier glutaconic, β-hydroxyglutaric and malonic acids were added in equimolar amounts, and these acids then were chromatographed on a celite column. All three acids were eluted as separate C^{14} containing fractions with coincidence of C^{14} content and acid titration values (Table II).

TABLE II

Chromatography of Carboxylation Products

Fraction	Radio-activity*	Fraction	Radio-activity*
A. ?	514	D. Glutaconic	1100
B. ?	784	E. Malonic	1448
C. Ethylmalonic	494	F. β-OHglutaric	5595

In addition, three unknown radioactive fractions were observed. Fraction C was identified as 2-ethylmalonic acid on re-chromatography. Thus crotonyl-CoA was converted by

*12,400 c.p.m. (0.18 μmole $C^{14}O_2$ fixed) placed on column.

carboxylation and other reactions to glutaconyl-CoA, β-hydroxyglutaryl-CoA, malonyl-CoA and 2-ethylmalonyl-CoA.

Although crotonase, which catalyzes Reaction 9, is present in the liver fraction, crotonyl-CoA (not β-hydroxybutyryl-CoA as in Reaction 8) is regarded as the primary substrate carboxylated because treatment of the enzyme fraction with 10^{-3} M p-chloromercuribenzoate, which inhibits crotonase at least 95 per cent (the inhibited crotonase not being reactivated by glutathione as is the inhibited β-methylcrotonyl-CoA carboxylase of pigeon liver (del Campillo et al., 1959)) did not affect $C^{14}O_2$ fixation (Table I). Moreover, the product of β-methylcrotonyl-CoA carboxylation has been shown to be β-methylglutaconyl-CoA (Lynen et al., 1959). By analogy, β-hydroxyglutaryl-CoA would arise by hydration of glutaconyl-CoA (Reaction 2). Indeed we find that synthetic glutaconyl-CoA (crude) is hydrated by the liver fraction as determined by direct optical measurement. Presumably this is catalyzed by endogenous β-methylglutaconase (Hilz et al., 1958).

Potential pathways of formation of radioactive malonyl-CoA are shown in the scheme. Using synthetic compounds, no evidence could be obtained by optical and chemical methods that the rat liver fraction catalyzed the interconversion of β-hydroxyglutaryl-CoA and β-ketoglutaryl-CoA (Reaction 4) in presence of pyridine nucleotides, or the cleavage of either β-hydroxyglutaryl-CoA (Reaction 6) or β-ketoglutaryl-CoA (Reaction 5). It is more likely that labelled malonyl-CoA arose by carboxylation of acetyl-CoA formed from β-hydroxybutyryl-CoA (Reactions 10 and 11). However, it should be noted that if the rate of Reactions 4, 5 and 6 is as slow as the rate of crotonyl-CoA carboxylation, these

reactions would escape detection by the methods employed. They cannot, therefore, be completely excluded at present.

The labelled 2-ethylmalonyl-CoA could have arisen from crotonyl-CoA via the known Reactions 12 and 13; or, conceivably, by α-carboxylation of crotonyl-CoA to 2-ethylidenemalonyl-CoA followed by reduction of the latter.

The formation of glutaconyl-CoA and β-hydroxyglutaryl-CoA suggests the probable occurrence of the oxidation of glutaryl-CoA to glutaconyl-CoA (Reaction 3). Indeed the rat liver fraction catalyzes the reduction of 2,6-dichlorphenolindophenol, of cytochrome C, and of triphenyltetrazolium by glutaryl-CoA, but not by glutarate, in the absence of any deacylation of the former. The product of glutaryl-CoA oxidation, presumably glutaconyl-CoA, remains to be identified (Tustanoff and Stern, unpublished experiments). Evidence has recently been presented by Rothstein and Greenberg (1960) that glutarate-1,5-C^{14} is converted to labelled β-hydroxyglutarate by rat liver homogenates. This conversion can be explained by Reactions 3 and 2. Since carboxylases catalyze reversible reactions, the sequence of Reactions 3, 1, 9, 10 and 11 can account enzymically for the conversion of glutaryl-CoA to acetyl-CoA.

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