β-HYDROXYBUTYRYL-CoA, AN INTERMEDIATE IN GLUTARATE CATABOLISM

Shosaku Numa, Yuzuru Ishimura, Yasutomi Nishizuka
and Osamu Hayaishi

Department of Medical Chemistry
Kyoto University Faculty of Medicine
Kyoto, Japan

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In an earlier communication from our laboratory (Nishizuka et al., 1960) it was shown that one mole of glutarylpantetheine or glutaryl-CoA is converted to 2 moles of the corresponding acetyl-compound and one mole of CO_2 by an enzyme preparation from Pseudomonas fluorescens (ATCC 11250); the free carboxyl carbon (C-5) of glutarylpantetheine is converted to CO_2, and both C-3,4 and C-1,2 of glutarylpantetheine become C-1,2 of acetylpantetheine, respectively. Further attempts to elucidate intermediates in glutarate catabolism in the same organism have revealed that β-hydroxybutyrylpantetheine, instead of acetylpantetheine, accumulates when glutarylpantetheine is incubated with either an acetone powder extract which has been subjected to repeated freezing and thawing, an extract from an aged acetone powder which has been stored at -10°C for several months, or an ammonium sulfate fraction thereof. This is the subject of the present communication.

Cells were grown and cell-free extracts prepared from acetone powders of the cells as previously described (Nishizuka et al., 1960). The reaction mixture (1.2 ml.) containing (in μmoles)

glutarylpantetheine-1,5-C^{14} (1.3; 52,200 c.p.m.), FAD* (0.01; Takeda), potassium phosphate buffer pH 6.5 (100) and 0.3 ml. of a crude cell-free extract prepared from the aged acetone powder (5 mg. protein) was incubated in a Warburg vessel at 37°C for 60 min. The reaction was stopped by the addition of 0.2 ml. of 2 N KOH, and then carrier acids (20 to 40 μmoles each of glutaric, acetic, crotonic, D,L-β-hydroxybutyric and β-hydroxyglutaric acids) were added. The mixture was subjected to alkaline hydrolysis at 37°C for 30 min., and then acidified with 0.2 ml. of 5 N H_2SO_4. The carbon dioxide evolved was trapped in alkali. The acidified mixture was chromatographed on a silica gel column (45 cm. X 1.2 $cm.^2$) (method of Bulen et al., 1952, with minor modifications).

The major radioactive product appeared in the β-hydroxybutyric acid fraction with a coincidence of C^{14}-content and acid titration values (Table I). The C^{14}-β-hydroxybutyric acid was further identified by paper chromatography in xylene-phenol-formic acid (5:5:2) (R_F: 0.76) and in ethanol-ammonia-water (20:1:4) (R_F: 0.58). The same radioactive product was also obtained when glutarylpantetheine-1,5-C^{14} was replaced by glutarate-1,5-C^{14} or -3-C^{14}, CoA, ATP and Mg^{++}.**

In other experiments neutralized hydroxylaminehydrochloride at a final concentration of 0.5 M was added to the reaction mixture (glutarylpantetheine-1,5-C^{14} as substrate) at the end of the incubation. The resulting hydroxamic acids, after extraction

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* Preliminary experiments showed that FAD is almost obligatorily required for CO_2 formation from glutarylpantetheine, when extracts treated with both charcoal and Dowex 1 (Cl^- form) were employed. Neither FMN nor pyridine nucleotides could replace FAD.

** The reaction mixture (1.0 ml.) contained (in μmoles): glutarate-1,5-C^{14} (0.2; 118,000 c.p.m.) or glutarate-3-C^{14} (0.4; 120,400 c.p.m.), CoA (0.1), ATP (5), $MgCl_2$ (10), reduced glutathione (10), FAD (0.01), potassium phosphate buffer pH 6.5 (100) and 0.5 ml. of the extract (7 mg. protein).

TABLE I

Chromatography of Reaction Products

Fraction	Eluted with	Radioactivity (c.p.m.)
Crotonic	4% n-Butanol in chloroform	976
Acetic	"	903
Glutaric	10% n-Butanol in chloroform	20,474
β-Hydroxybutyric	"	8,597
β-Hydroxyglutaric	20% n-Butanol in chloroform	0
Unknown	50% n-Butanol in chloroform	1,672

Experimental conditions; see the text. $C^{14}O_2$ evolved: 11,640 c.p.m.

with ethanol or acetone, were chromatographed on paper with 3 different solvent systems, xylene-phenol-formic acid (5:5:2), n-amyl alcohol-formic acid-water (3:1:3) and n-butanol-isobutyric acid-water (2:2:1). There was a peak of radioactivity on the chromatograms at the position corresponding to β-hydroxybutyrohydroxamic acid. The R_F values of this hydroxamic acid in the 3 solvent systems were 0.74, 0.34 and 0.48, respectively.

A preliminary degradation study of the C^{14}-β-hydroxybutyric acid was carried out by the dichromate oxidation method of Peters and van Slyke (Peters et al., 1932). When the C^{14}-β-hydroxybutyric acid obtained from glutarate-1,5-C^{14} was treated with dichromate, 65%* of the radioactivity was recovered in CO_2 and 9%

* The conversion of β-hydroxybutyric acid to acetone and CO_2 is no more than 75% under the conditions used (Peters et al., 1932).

in the mercury-acetone complex. On the other hand, the C^{14}-β-hydroxybutyric acid obtained from glutarate-3-C^{14} yielded, under the identical conditions, 40%** of its radioactivity in the mercury-acetone complex and 14% in CO_2. This labeling pattern indicates that the carbon chain of glutarate remains intact during its conversion to the β-hydroxybutyryl-derivative.

Rothstein et al. postulated β-hydroxyglutarate as an intermediate in glutarate catabolism in the rat liver system (Rothstein et al., 1960a, b). We carried out a trapping and dilution experiment with 8 μmoles of non-radioactive β-hydroxyglutarylpantetheine added to the reaction mixture described above (glutarylpantetheine-1,5-C^{14} as substrate), but the formation of $C^{14}O_2$ was not diminished. Moreover, neither β-hydroxyglutaric acid nor its monohydroxamic acid isolated from this reaction mixture by paper chromatography was radioactive, despite the fact that a considerable amount of these compounds could be detected on the chromatogram. Bagchi et al., using β-hydroxyglutarate as well as β-ketoglutarate as trapping agents, also failed to show these acids to be dissociable intermediates in glutarate catabolism in the rat liver system (Bagchi et al., 1961). They found, however, that acetoacetate was an intermediate in their system. In a similar trapping and dilution experiment with 10 μmoles of non-radioactive malonylpantetheine we noted no decrease in $C^{14}O_2$ formation. Furthermore, no radioactivity was found in malonomonohydroxamic acid isolated by paper chromatography. These results speak against glutarate breakdown via the β-hydroxyglutaryl- and β-ketoglutaryl-derivatives, but this degradative pathway can not be completely excluded, since equilibration between intermediates and the trapping agents might not have occurred.

** Radioactivity was determined with thin samples and no correction was made for self-absorption.

Tustanoff et al. demonstrated with rat liver fractions the oxidation of glutaryl-CoA to glutaconyl-CoA (Tustanoff et al., 1961) and the carboxylation of crotonyl-CoA to glutaconyl-CoA (Tustanoff et al., 1960). In trapping experiments with 8 μmoles of non-radioactive crotonylpantetheine we found that the major radioactive product was again β-hydroxybutyrate or its hydroxamic acid. However, the failure in these experiments to demonstrate an appreciable amount of radioactivity in crotonate or in its hydroxamic acid probably is due to the high activity of crotonase present in the crude extract employed. After the incubation no detectable amount of the crotonylpantetheine added was recovered, as revealed by staining the hydroxamic acids on the chromatogram; instead, a large amount of β-hydroxybutyrohydroxamic acid was seen.

The results presented, together with those of our earlier communication (Nishizuka et al., 1960) as well as of others (Tustanoff et al., 1960, 1961; Bagchi et al., 1961), suggest that a plausible pathway of glutarate catabolism proceeds via β-hydroxybutyryl-CoA, which may be formed through the decarboxylation of glutaconyl-CoA.

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