Mammalian Metabolism of Glutaric Acid*

ABRAHAM BESHAT,‡ CARL E. POLAN,§ AND L. M. HENDERSON

From the Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55101

SUMMARY

Rat liver mitochondria metabolize glutarate at a very slow rate as compared with glutaryl coenzyme A. The stimulatory effect of citric acid cycle intermediates, NAD, and coenzyme A on glutarate metabolism was interpreted as a manifestation of their involvement in the activation of glutarate by a thiol transferase with succinyl-CoA as the coenzyme A donor.

Glutaryl-CoA dehydrogenase, which catalyzes the stoichiometric conversion of glutaryl-CoA to 1 mole each of carbon dioxide and crotonyl-CoA or its intermediate metabolite, was purified approximately 44- and 100-fold from bovine liver and kidney mitochondria, respectively. The enzyme was shown to require an artificial electron acceptor and to be further stimulated by exogenous FAD. Of the various electron acceptors tested, methylene blue and phenazine methosulfate were most effective. The enzymes from both sources have similar properties. Catalysis was optimum at pH 7.5 in potassium phosphate buffer and at pH 8.0 in Tris-HCl buffer. The enzyme activity was inhibited by p-chloromercuribenzoate. The $K_m$ for glutaryl-CoA was 3.3 $\mu$M.

Glutaconyl-CoA, the suspected primary intermediate of glutaryl-CoA metabolism, was synthesized, and its effect on the oxidation of glutaryl-CoA and its role as an alternative substrate for the partially purified enzyme were studied. Kinetic results indicated that glutaconyl-CoA competitively inhibited the release of $^{14}$CO$_2$ from glutaryl-CoA-$^{1,5}$H$_4$C possibly because of the metabolism of glutaconyl-CoA by the enzyme. Thus far, attempts to isolate enzymatically formed glutaconyl-CoA and to separate the dehydrogenation and decarboxylation activities of the enzyme have been unsuccessful.

EXPERIMENTAL PROCEDURE

Methods

Glutarate has been implicated as an intermediate in the degradative pathways of tryptophan (1-3), lysine (4, 5), and 5-hydroxylysine (6) in the intact rat by use of the metabolite overloading technique. Meghal el al. (7) showed that glutaryl-CoA was the de facto intermediate of lysine catabolism by the rat. Glutarate is apparently derived by hydrolysis of the metabolically formed glutaryl-CoA, which in turn is formed by oxidative decarboxylation of $\alpha$-ketoadipate by $\alpha$-ketoglutarate dehydrogenase (3, 8). Utilization of glutarate in the mammalian system (9, 10) and in Pseudomonas fluorescens (11) requires the prior activation to glutaryl-CoA.

On the basis of glutamate-labeling experiments using glutarate-$^{3,14}$C, Hobbs and Koeppel (12) concluded that no direct conversion of glutarate to glutamate occurred in the intact rat, but acetate-$^{1,14}$C was the immediate precursor of $^{14}$C-glutamate. A subsequent report by Bagchi et al. (13) presented evidence that acetocetate is an intermediate in this conversion. Likewise, Nishizuka, Kuno, and Hayashi (11) observed the conversion of 1 mole of glutarylpantetheine to 2 moles of acetyl pantetheine by extracts prepared from P. fluorescens. Further work by Numa et al. with extracts of this organism, showed that $\beta$-hydroxybutyryl-CoA was an intermediate in glutaryl-CoA oxidation (14).

Although the metabolism of glutarate in P. fluorescens is now reasonably well understood (14, 15), our understanding of the mammalian system is not so complete. The present report deals with the metabolism of glutarate by mammalian systems in vitro, with special emphasis on the enzyme system which catalyzes dehydrogenation and subsequent decarboxylation of glutaryl-CoA. The partial purification of this enzyme from bovine liver and kidney is described. Evidence will be presented for the involvement of glutaconyl-CoA as the primary product of glutaryl-CoA dehydrogenation as catalyzed by the partially purified enzyme from bovine kidney.

Experimental Procedure

Preparation of Thioesters—Glutaric anhydride was prepared by heating the free acid under reflux for 30 min with redistilled acetic anhydride and glutaric anhydride by a modification of the method described by Bland and Thorpe (16). The coenzyme A esters of glutaric, glutarconic, crotonic, and succinic acids were made from the corresponding anhydrides by the method of Simon and Shemin (17). $\beta$-Hydroxybutyryl-CoA was prepared from $\beta$-butyrolactone by essentially the same procedure.
Pantetheine was reduced with sodium amalgam to yield pantetheine, which was employed for the preparation of glutaryl pantetheine-1,5-14C by means of the same procedure as for the corresponding coenzyme A derivatives. The completion of the esterification reaction was determined by the disappearance of free sulfhydryl groups with the nitroprusside reagent (18) or 5,5'-dithio-bis-(2-nitrobenzoic acid) (19). The increase in absorbance at 293 m\(\mu\) was also utilized to monitor the formation of thioester bonds (20). In some cases, preparations of acyl-CoA derivatives were purified by descending paper chromatography on Whatman No. 3 filter paper with ethanol and 0.1 M potassium acetate buffer, pH 4.5 (1:1) as the solvent system (21). The concentrations of thioesters were determined by a modification of the hydroxamate method of Lipmann and Tuttle (20), with commercial glutaric anhydride as a standard.

**Enzyme Assays**—Since glutaryl-CoA dehydrogenase catalyzes the oxidative deamination of glutaryl-CoA, the assay involved the rate of carbon dioxide production or the reduction of a suitable artificial electron acceptor. A unit of enzyme activity is defined as that amount which catalyzes the production of 1 pmoles of carbon dioxide or the reduction of 1 pmoles of the appropriate dye in 1 min at 37°C.

 Routinely, the enzyme activity was assayed by trapping 14CO2 evolved from glutaric acid 1,5 14C or its thioester. The standard incubation mixture, which consisted of 0.1 pmoles of FAD, 1.0 pmoles of methylene blue, 50 pmoles of potassium phosphate buffer, pH 7.5, and 0.1 pmoles of glutaryl-CoA-1,5-14C in a total volume of 1.0 ml, was placed in a test tube (25 X 100 mm) fitted with a rubber serum cap. A culture tube (10 x 75 mm) containing a KOH-impregnated filter paper was placed inside the reaction vessel to trap the 14CO2. The reaction was initiated by the addition of the substrate and was terminated by injecting 0.5 ml of 25% trichloroacetic acid. Shaking of the tubes in the water bath at 37°C was continued for at least 30 min after the termination of the reaction to allow maximum trapping of the 14CO2. The strips of filter paper were placed in a counting fluid to scintillation fluid, and radioactivity was estimated with a liquid scintillation spectrometer. The scintillation fluid was prepared by dissolving 4.0 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis-[2-(5-phenylxazoaryl)]benzene in a liter of a solution composed of 40% absolute ethanol and 60% toluene.

In assays which involved the reduction of an artificial electron acceptor, 0.07 pmoles of 2,6-dichlorophenolindophenol and 0.1 pmoles of phenazine methosulfate were substituted for methylene blue in the standard 14CO2 incubation mixture already described. Since free sulfhydryl groups readily reduce 2,6-dichlorophenolindophenol, the glutaryl-CoA preparation employed for this assay was treated with a water-insoluble organomercurial copolymer of ethylene and maleic acid to remove the free thiol compounds. The resin was a gift of Dr. I. Lieber (22). The rate of decrease in absorbance at 660 mp\(\mu\), due to the reduction of 2,6-dichlorophenolindophenol, was measured at room temperature. In 110 cases was the 2,6-dichlorophenolindophenol limiting.

Crotone was assayed by decrease in absorbance at 263 mp\(\mu\) as reported by Stern (23), or by the indirect method of Wakil and Mahler (24), with a commercial preparation of 1,β-hydroxyacyl-CoA dehydrogenase. β-Hydroxybutyryl-CoA dehydrogenase was assayed by an adaptation of the method described by Wakil et al. (25).

**Miscellaneous Methods**

Large scale reactions were conducted for the purpose of identifying the products of glutaryl-CoA oxidation. The reaction mixture contained the following compounds at the concentrations shown: 0.1 mM FAD, 0.4 to 1.0 mM methylene blue, 12.5 to 50 mM Tris or phosphate buffer, pH 7.5, purified substrate 1.0 mM, and NAD, when used, 7.5 mM with a total volume of 4 ml. The reaction was terminated by the addition of 0.2 ml of 6 N KOH. To the alkaline incubation mixture 100 pmoles each of glutaric and glutaric acid 14C and 200 pmoles each of crotonic, α,β-hydroxybutyric, and acetoacetic acids were added as carriers. The mixture was then shaken in a 37°C water bath for 1 hour to effect the hydrolysis of the acyl-CoA derivatives.

After acidifying the sample the volume was reduced in a vacuum to approximately 5.0 ml. The organic acids were separated on a silica gel column (2 x 30 cm) essentially as described by Marvin and Rands (26). The solvent schedule employed was 100 ml of 5% (v/v), 200 ml of 10%, 100 ml of 15%, and 100 ml of 20%; 1-butanol in chloroform saturated with 0.5 N H2SO4. Fractions (5 ml) were collected, 0.5-ml aliquots were removed for radioactivity measurements, and the remainder of each fraction was titrated to a phenol red end point to locate the elution position of the carrier acids added.

High voltage paper electrophoresis was done using Whatman No. 1 filter paper, according to the method of Ryle et al. (27). Samples were applied as a band of appropriate width about 15 cm from one end of the filter paper, 54 cm in length. The longer end of the filter paper was placed toward the anode and electrophoresis was carried out at a potential of 2500 volts for 1 hour with pyridine-acetic acid-water (5:2:493), pH 5.3.

Protein concentrations were estimated by the method of Lowry et al. (28) with crystalline bovine plasma albumin as a standard. In some cases the spectrophotometric methods of Warburg and Christian (29) was also employed.

**Enzyme Preparation**

Mitochondria were prepared from rat liver and from bovine liver and kidney by the method described by Schneider (30). The homogenizing medium used for the isolation of mitochondria was 0.25 M sucrose, which was made 2 mM with respect to tetra-sodium ethylenediaminetetraacetate, 1 mM with respect to cysteine, and then adjusted to pH 7.5. The mitochondrial suspension was washed with 1.5 volumes of the sucrose solution and finally suspended in 0.02 M potassium phosphate buffer, pH 7.5, which was made 1 mM with respect to cysteine. The final volume in milliliters was equal to the weight of the tissue in grams. The mitochondrial suspension was kept frozen until used.

The freeze-thaw-treated mitochondrial suspension was subjected to sonic disruption for a period of 5 to 15 min. The disrupted mitochondria were centrifuged at 78,000 X g for 1 hour. The resulting supernatant solution, which contained most of the glutaryl-CoA dehydrogenase activity, was used for further purification.

**RESULTS AND DISCUSSION**

**Preliminary Studies**—The present investigations as well as earlier studies by Bagchi et al. (13) and Rothstein and Greenberg (31) have shown that the mitochondrion is the major site for the metabolism of glutarate in mammalian tissues. The location of the glutarate-oxidizing system is hardly surprising, since many
of the enzymic steps involved, as will be seen later, are similar if not identical with those in the fatty acid oxidation system.

Rat liver mitochondria lost their capacity to metabolize glutaric acid when they were stored in the frozen state. Addition of coenzyme A and ATP did not restore the activity. Since these preparations readily metabolized the coenzyme A ester of glutarate, it was concluded that freezing labilized the system in mitochondria responsible for the conversion of glutarate to glutaryl-CoA. Coenzyme A, but not ATP, enhanced \( ^{14} \text{C} \) evolution from glutarate-1,5-\( ^{14} \text{C} \) by fresh rat liver mitochondria.

Likewise, citric acid cycle intermediates enhanced the ability of fresh mitochondria to oxidize glutarate. Citrate and \( \alpha \)-ketoglutarate increased the activity 4- and 20-fold respectively (Table I). Succinate and fumarate, on the other hand, enhanced the activity only slightly. The nonparticipation of ATP and the effect of coenzyme A, citrate, and \( \alpha \)-ketoglutarate strongly suggest that a thiol transferase-type reaction was involved in the activation of glutarate. Similar effects by citric acid cycle intermediates on the metabolism of \( \beta \)-hydroxy-\( \beta \)-methylglutarate led Burch, Rudney, and Irias (32) to propose succinyl-CoA as the CoA-donating compound in rat kidney. In the present studies, succinyl-CoA was shown to be stimulatory to the metabolism of glutarate by rat liver mitochondria. The effect of citrate and \( \alpha \)-ketoglutarate could be the result of their generating succinyl-CoA. The activation of glutarate by rat liver mitochondria under the conditions employed here does not therefore appear to proceed by the ATP-dependent reaction reported by Menon, Friedman, and Stern (33) in pigeon and rat liver supernatant fluids and by Ichihara and Miyake (34) in pseudomonad extracts.

Tustanoff and Stern (9, 35), in their studies of the ATP-dependent carboxylation of crotonyl-CoA, reported the isolation of glutaroyl-CoA as one of the products. They envisioned the reversal of the ATP-dependent carboxylation as one of the steps involved in glutaryl-CoA metabolism by rat liver. However, no dependence on adenine nucleotide could be demonstrated for the oxidative decarboxylation of glutaryl-CoA in the present studies. Furthermore, avidin was without effect on the oxidation of glutaryl-CoA by rat liver mitochondrial extracts. These results are similar to those of Numa et al. (15), who reported that the decarboxylation of glutaroyl-CoA in \( P. \) fluorescens did not proceed via the reversal of the ATP-dependent carboxylation of crotonyl-CoA.

**Glutaryl-CoA Dehydrogenase from Bovine Liver Mitochondria**—The first attempts to free glutaryl-CoA dehydrogenase of other mitochondrial enzymes were made with liver. Bovine liver mitochondria were prepared and solubilized as described above. Purification of the enzyme present in the supernatant solution obtained after centrifugation at 78,000 \( \times g \) for 1 hour involved exposure to pH 5.0 for 10 min and ammonium sulfate fractionation. The enzyme precipitated at 38 to 60% saturation. These two steps increased the specific activity 25-fold without loss. Further purification was attempted with DEAE-cellulose column chromatography, but lability of the enzyme prevented success.

The enzyme exhibited maximum activity at pH 7.5 in potassium phosphate buffer and at pH 8.0 in Tris-HCl buffer. The partially purified enzyme was more active in the presence of FAD, but flavin mononucleotide, NAD, NADP, and cytochrome c had very little effect on the enzyme activity. The omission of methylene blue from the reaction mixture resulted in complete loss of activity as measured by \( ^{14} \text{CO}_2 \) release.

Preliminary investigations indicated that higher enzymic activity was obtained when cysteine or other sulphur compounds were added to the homogenizing medium in the isolation of mitochondria. As a result, all of the buffers used for fractionation and dialysis were routinely made 1 mM with respect to cysteine. The requirement for sulphur compounds was further investigated by the use of \( p \)-chloromercuribenzoate. CMB (1 \( \mu \) mole) in the standard incubation mixture inhibited enzyme activity 90% when added simultaneously with the substrate. Incubation of the enzyme with the same amount of CMB for 10 min, prior to the initiation of the reaction with substrate, resulted in 90% inhibition.

Earlier studies with rat liver mitochondria and extracts thereof, had suggested a requirement for Mg\( ^{++} \) when glutarate or glutaryl-CoA was the substrate. Partially purified enzyme preparations from bovine liver did not display such a requirement. Ethylenediaminetetraacetate had no significant effect on the enzyme activity in the presence or absence of Mg\( ^{++} \). None of the common metal ions tested displayed any stimulatory effect on activity. On the contrary, Co\( ^{++} \), Cu\( ^{++} \), and Zn\( ^{++} \) inhibited \( ^{14} \text{CO}_2 \) release from glutaryl-CoA-1,5-\( ^{14} \text{C} \) by at least 50%.

**Glutaryl-CoA Dehydrogenase from Bovine Kidney Mitochondria**—Preliminary studies indicated that the specific activity of the enzyme in bovine kidney mitochondria was 5 to 10 times as high as that in bovine liver mitochondria. Consequently, the kidney mitochondria were used as the source in an extensive effort to purify this enzyme. The purification steps were identical with those used for the liver enzyme with the exception of the acid step. The pH 5.0 treatment which was found effective in the purification of the liver enzyme was omitted because it was of no value for the kidney enzyme.

The fraction, which precipitated between 35 and 55% saturated ammonium sulfate, and which had a specific activity 15 to 20-fold greater than the mitochondria (Table II), was dialyzed.

1 The abbreviation used is: CMB, \( p \)-chloromercuribenzoate.

### Table I

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration</th>
<th>Radioactivity released as ( ^{14} \text{CO}_2 ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10(^{-4})</td>
<td>0.2</td>
</tr>
<tr>
<td>Succinate</td>
<td>10(^{-4})</td>
<td>0.4</td>
</tr>
<tr>
<td>Fumarate</td>
<td>10(^{-4})</td>
<td>0.1</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate</td>
<td>10(^{-4})</td>
<td>4.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>10(^{-4})</td>
<td>8.8</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate</td>
<td>10(^{-3})</td>
<td>2.3</td>
</tr>
<tr>
<td>( \alpha )-Ketoglutarate + boiled homogenate supernatant</td>
<td>10(^{-4})</td>
<td>5.7</td>
</tr>
<tr>
<td>NAD</td>
<td>10(^{-3})</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Glutaryl-CoA dehydrogenase activity was recovered in the void volume of a Sephadex G-200 or Bio-Gel A-300 column, which suggests that the enzyme has a molecular weight in excess of 200,000. Gel filtration was, however, ineffective in rendering the enzyme free of the other enzymes of the glutarate pathway.

A substantial degree of purification was achieved when the active fractions from a hydroxylapatite column were subjected to sucrose density gradient centrifugation with the procedure of Martin and Ames (38). Centrifugation at 130,000 \( \times g \) for 14 hours in a model L Spinco preparative ultracentrifuge partially separated the dehydrogenase from crotonase and \( \beta \)-hydroxybutyryl-CoA dehydrogenase. Although this procedure partially separated the contaminating enzymes of concern it did not lend itself to large scale preparative work.

Glutaryl-CoA dehydrogenase from bovine kidney mitochondria displayed characteristics similar to its liver counterpart. Purification attempts were hampered by the unstable nature of the activity. Effluents from a DEAE-cellulose column lost as much as 50\% of their activity upon standing at 4\(^{\circ}\) for 2 days. The extent of the loss of activity was dependent upon the relative purity and protein concentration of the preparation. Crude preparations were more stable than the partially purified enzyme, and concentrated fractions were usually more stable than dilute fractions. The loss of the dehydrogenase activity appeared to be irreversible since adding mercaptans or FAD was not effective in restoring the lost activity. No useful method was found to control this gradual loss of activity, which takes place during fractionation and dialysis at 4\(^{\circ}\). Storing the enzyme preparation as a suspension in a 55\% ammonium sulfate solution at -20\(^{\circ}\) minimized the loss of activity.

The partially purified dehydrogenase displayed a maximum rate of catalysis between pH 7.5 and 8.0 with a variety of buffers, including those described by Good et al. (39). The rate of catalysis decreased sharply when the pH was either increased or decreased. Thus, at pH 6.5 or 9.5, the activity was approximately 50\% of that obtained at pH 8.0.

The data in Table III, obtained with the fraction which precipitated between 35 and 55\% saturated ammonium sulfate, show that the kidney glutaryl-CoA dehydrogenase displayed requirements for an artificial electron acceptor and was stimulated further by exogenous FAD. Potassium ferricyanide was only 25\% as effective as methylene blue in this system. Molecular oxygen and 2,3,5-triphenyltetrazolium chloride were not suitable terminal electron acceptors. Phenazine methosulfate at a
concentration of 0.1 mm was as good as methylene blue at 1.0 mm. Glutarylpantheine was oxidized about 8% as fast as the natural substrate.

The function of FAD in this partially purified system is not clear. Attempts to remove the presumably bound FAD were not successful and added FAD did not enhance the activity of the dehydrogenase more than 2- to 3-fold (Table III).

The kidney enzyme activity was stimulated by incubating the protein with sulfhydryl compounds prior to assay. All of the free thiol compounds tested, including dithiothreitol (40), appeared to be equally effective in augmenting the activity of glutaryl-CoA dehydrogenase; p-chloromercuribenzoate at a concentration of 10 μM inhibited activity by as much as 67%. From the Lineweaver-Burk plot, the apparent \( K_m \) for glutaryl-CoA was shown to be 3.3 μM whether reaction rates were measured by the \( ^{14} \text{CO}_2 \) release or by the assay with phenazine methosulfate and 2,6-dichlorophenolindophenol as the artificial electron acceptors.

Product Identification—Numa et al. (15) reported the conversion of 1 mole of glutaryl-CoA to 1 mole of carbon dioxide and 1 mole of 4-carbon acyl-CoA derivatives (crotonyl-CoA and \( \beta \)-hydroxybutyryl-CoA) by glutaryl-CoA dehydrogenase from \( P. \) fluorescens. In the present studies, a similar stoichiometric relationship was obtained with the mammalian enzymes. The data in Table IV indicate that the average recovery of isotopic \( \text{CO}_2 \) was 38% from glutaryl-CoA-1,5-\( ^{14} \text{C} \). The maximum yield is 50% as only 1 of the radioactive carbon atoms (carbon 5) of the substrate is converted to carbon dioxide. Since the \( ^{14} \text{CO}_2 \)-trapping method employed was only 70 to 80% efficient, as determined by using radioactive sodium carbonate, it was concluded that 50% of the radioactivity in the substrate was being converted to carbon dioxide. Similarly, it could be argued from Experiments 5 and 6 in Table IV that equimolar amounts of carbon dioxide and 4-carbon acyl-CoA derivatives were formed by the action of the enzyme on glutaryl-CoA-1,5-\( ^{14} \text{C} \).

Glutaryll-CoA and \( \beta \)-hydroxybutyryl-CoA were identified by silicic acid chromatography as the major products formed by the action of bovine kidney and liver mitochondria on glutaryl-CoA. When the crude homogenates, mitochondria, or disrupted mitochondria were the enzyme source, the ratio of concentrations of \( \beta \)-hydroxybutyryl-CoA to crotonyl-CoA as measured by the radioactivity in the combined fractions from the silicic gel column was less than 2 (Table V). This value is comparable to that (1.4) observed by Wakil and Mahler (24) for crotonase partially purified from an acetone powder of beef liver mitochondria, but somewhat lower than the value of 3.45 reported by Stern and del Campillo (41) for the crystalline enzyme. With the use of a sonic supernatant fraction and subsequent fractions from either kidney or liver, ratios ranging from 11 to 13 were observed. This apparent substantial deviation in the position of the equilibrium of the crotonase reaction from the values previously reported has not been explained. Similarly high ratios were observed by Numa et al. (15) when an excess of crotonase was added to the

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Methyline blue</th>
<th>FAD</th>
<th>(^{14} \text{CO}_2 ) release</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,5-( ^{14} \text{C} )-glutaryl-CoA</td>
<td>4.0</td>
<td>0.1</td>
<td>15,087</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>1,5-( ^{14} \text{C} )-glutaryl-CoA</td>
<td>4.0</td>
<td>0.1</td>
<td>5,469</td>
<td>36</td>
</tr>
<tr>
<td>3</td>
<td>1,5-( ^{14} \text{C} )-glutaryl-CoA</td>
<td>4.0</td>
<td>0.1</td>
<td>92</td>
<td>0.6</td>
</tr>
<tr>
<td>4</td>
<td>1,5-( ^{14} \text{C} )-glutaryl-CoA</td>
<td>4.0</td>
<td>0.1</td>
<td>44</td>
<td>0.3</td>
</tr>
<tr>
<td>5</td>
<td>1,5-( ^{14} \text{C} )-glutarylpantheine</td>
<td>4.0</td>
<td>0.1</td>
<td>1,193</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>1,5-( ^{14} \text{C} )-glutarylpantheine</td>
<td>4.0</td>
<td>0.1</td>
<td>224</td>
<td>18.7</td>
</tr>
<tr>
<td>7</td>
<td>1,5-( ^{14} \text{C} )-glutarylpantheine</td>
<td>4.0</td>
<td>0.1</td>
<td>53</td>
<td>4.4</td>
</tr>
<tr>
<td>8</td>
<td>1,5-( ^{14} \text{C} )-glutarylpantheine</td>
<td>4.0</td>
<td>0.1</td>
<td>32</td>
<td>2.7</td>
</tr>
</tbody>
</table>

**Table V**

**Radioactivity distribution in enzymic products of glutaryl-CoA-1,5-\( ^{14} \text{C} \)**

The conditions for large scale incubation for silicic acid chromatography are described in the text. The reaction mixture was made alkaline after the indicated period of incubation in order to effect hydrolysis of the coenzyme A esters. The free acids were then separated by silicic acid chromatography.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Bovine kidney enzyme source</th>
<th>Radioactivity</th>
<th>Ratio of ( \beta )-hydroxybutyrate to crotonate</th>
<th>Period of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>600 ( \times ) g homogenate</td>
<td>9,700</td>
<td>16,260</td>
<td>11,200</td>
</tr>
<tr>
<td>2</td>
<td>Mitochondria</td>
<td>1,230</td>
<td>18,220</td>
<td>24,450</td>
</tr>
<tr>
<td>3</td>
<td>Disrupted mitochondria</td>
<td>4,640</td>
<td>8,540</td>
<td>18,490</td>
</tr>
<tr>
<td>4</td>
<td>Sonic supernatant</td>
<td>2,580</td>
<td>29,680</td>
<td>3,340</td>
</tr>
<tr>
<td>5</td>
<td>0 to 55% ( \text{NH}_4 )\text{SO}_4 fraction</td>
<td>5,150</td>
<td>68,350</td>
<td>6,050</td>
</tr>
<tr>
<td>6</td>
<td>DEAE-cellulose fraction</td>
<td>5,200</td>
<td>56,650</td>
<td>850</td>
</tr>
<tr>
<td>7</td>
<td>DEAE-cellulose + NAD</td>
<td>4,400</td>
<td>47,800</td>
<td>9,800</td>
</tr>
</tbody>
</table>

1 mole of 4-carbon acyl-CoA derivatives (crotonyl-CoA and \( \beta \)-hydroxybutyryl-CoA) by glutaryl-CoA dehydrogenase from \( P. \) fluorescens. In the present studies, a similar stoichiometric relationship was obtained with the mammalian enzymes. The data in Table IV indicate that the average recovery of isotopic \( ^{14} \text{CO}_2 \) was 38% from glutaryl-CoA-1,5-\( ^{14} \text{C} \). The maximum yield is 50% as only 1 of the radioactive carbon atoms (carbon 5) of the substrate is converted to carbon dioxide. Since the \( ^{14} \text{CO}_2 \)-trapping method employed was only 70 to 80% efficient, as determined by using radioactive sodium carbonate, it was concluded that 50% of the radioactivity in the substrate was being converted to carbon dioxide. Similarly, it could be argued from Experiments 5 and 6 in Table IV that equimolar amounts of carbon dioxide and 4-carbon acyl-CoA derivatives were formed by the action of the enzyme on glutaryl-CoA-1,5-\( ^{14} \text{C} \).

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incubation mixture containing glutaryl-CoA and the purified dehydrogenase from *P. fluorescens*.

The presence of a decarboxylase which acts preferentially on β-hydroxybutyryl-CoA could account for the high ratios observed. That this is not the explanation was indicated by the results of experiments with fractions which gave high ratios. When the incubation mixture was chromatographed directly without alkali treatment to remove the coenzyme A, no free β-hydroxybutyrate-¹⁴C appeared in the effluent from the silica gel column.

There was no significant radioactivity in the acetate-acetoacetate fractions when a DEAE-cellulose preparation was incubated with glutaryl-CoA-¹⁴C and the hydrolyzed products were separated by silicic acid chromatography. The inclusion of NAD at a concentration of 10 mM in the incubation mixture gave rise to radioactivity in the acetate-acetoacetate region with a concomitant decrease in radioactivity in the β-hydroxybutyrate region. It appeared, therefore, that the DEAE-cellulose enzyme was not only contaminated with crotonase, but also with β-hydroxybutyryl-CoA dehydrogenase and possibly β-ketothiolase. It should be recalled that all of these contaminants of the dehydrogenase are enzymes of the well established fatty acid degradative system.

After successive futile attempts to free the dehydrogenase from the other enzymes on the pathway of glutaryl-CoA metabolism, we have recently obtained a minor protein fraction from a hydroxylapatite column which was free of these contaminants. The protein was further purified by sucrose density gradient centrifugation and incubated with glutaryl-CoA-¹⁴C. Crotonate was the only product observed in the effluent from a silica gel column (Fig. 2). The β-hydroxybutyrate was unlabeled, confirming the observation that crotonate had been separated on the hydroxylapatite column. The recovery of ¹⁴C in crotonate was 69.5% of that released as ¹⁴CO₂.

Attempts to isolate the presumed primary dehydrogenation product of glutaryl-CoA have been unsuccessful. Glutarate and glutaconate do not separate on a silica gel column. Resolution of the radioactivity in the glutarate-glutaconate region of the effluent by high voltage paper electrophoresis showed that there was no incorporation of radioactivity into glutaconate. The failure to observe ¹⁴C in the carrier glutaconate after isolation

Fig. 2. Silicic acid chromatography of the enzymic product of glutaryl-CoA by means of an enzyme fraction obtained by hydroxylapatite column chromatography and sucrose density gradient centrifugation. The procedure for the incubation and for enzyme preparation are described in the text. Before chromatography 100 amoles of glutaric acid and 200 amoles each of crotonic and dl-β-hydroxybutyric acid (dl-β-oh-butyrate) were added to the incubation mixture.

Attempts to isolate the presumed primary dehydrogenation product of glutaryl-CoA have been unsuccessful. Glutarate and glutaconate do not separate on a silica gel column. Resolution of the radioactivity in the glutarate-glutaconate region of the effluent by high voltage paper electrophoresis showed that there was no incorporation of radioactivity into glutaconate. The failure to observe ¹⁴C in the carrier glutaconate after isolation

**Table VI**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Glutaryl-CoA</th>
<th>Glutaconyl CoA</th>
<th>CO₂ produced</th>
<th>¹⁴CO₂ released</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.79</td>
<td>41,500</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.0</td>
<td>1.44</td>
<td>43,700</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>3.26</td>
<td>37,000</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.1</td>
<td>0.87</td>
<td>3,090</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>1.91</td>
<td>2,720</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>3.24</td>
<td>2,780</td>
<td></td>
</tr>
</tbody>
</table>

The double side-arm Warburg flask contained 0.3 µmole of FAD, 12 µmole of methylene blue, 150 µmole of potassium phosphate buffer, pH 7.5, and 35 to 55% (NH₄)₂SO₄ fraction as enzyme source in the main compartment. One side-arm contained the substrates, and the other 0.2 ml of 5 N H₂SO₄. The reaction was initiated by tipping in the substrate and it was allowed to proceed for 30 min. The acid was then tipped in and shaking continued for 1 hour to allow the complete release of the CO₂. The CO₂ was then trapped in KOH for ¹⁴C determination.
does not, of course, rule out enzyme-bound glutaryl-CoA as the primary product, which is then decarboxylated to give crotonyl-CoA. The nonaccumulation of glutaryl-CoA can be explained on the basis that the decarboxylation reaction is much faster than the dehydrogenation. Thus far, it has not been possible to resolve glutaryl-CoA dehydrogenase into two activities, one for dehydrogenation and the other for decarboxylation. Numa et al. (1b) implicated glutaryl-CoA as a primary intermediate in a pseudomonad by their observation that glutaryl-CoA, generated in situ by the action of crotonase on &-hydroxylglutaryl-CoA, was converted to CO2 and 4-carbon acyl derivatives by a partially purified glutaryl-CoA dehydrogenase from this organism. The approach employed in the present studies was to synthesize the suspected intermediate and study its metabolism by the mammalian enzyme.

Preliminary studies of the effect of glutaryl-CoA on glutaryl-CoA-1,5-14C metabolism showed that the amount of 14CO2 released in 5 min was inhibited 28%, 50%, and 73% by including 0.1, 0.2, and 0.4 pmole, respectively, of glutaryl-CoA in the standard assay system, which contained 0.1 pmole of glutaryl-CoA-1,5-14C. Metabolism showed that the amount of 14CO2 released in 5 min was inhibited 28%, 50%, and 73% by including 0.1, 0.2, and 0.4 pmole, respectively, of glutaryl-CoA in the standard assay system, which contained 0.1 pmole of glutaryl-CoA-1,5-14C.

Further experiments were done in which manometry was used to determine the quantity of CO2 released during the exposure of the partially purified enzyme to both substrates for 30 min (Table VI). These conditions do not permit kinetic interpretation, but the results clearly indicated that glutaryl-CoA was metabolized by the enzyme preparation, and that in the longer experimental period the glutaryl-CoA was largely metabolized in spite of the presumed reduction in rate caused by glutaryl-CoA. These results do not permit a choice among the explanations presented above (Fig. 3) but they are consistent with the point of view that enzyme-bound glutaryl-CoA is an intermediate in glutaryl-CoA oxidation in mammalian systems, as appears to be the case in bacteria (15).

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REFERENCES

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