The Pathway of Itaconate Metabolism by Liver Mitochondria*

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Earlier work in this laboratory (1) has shown that soluble enzymes from guinea pig or rat liver mitochondria, fortified with adenosine triphosphate, Mg++, and coenzyme A, convert itaconate to pyruvate and acetyl coenzyme A. In this metabolic process, itaconate is first activated to itaconyl-CoA by succinate-activating enzyme, and a CoA derivative is cleaved to acetyl-CoA and pyruvate. This paper reports further work on the cleavage reaction, its intermediates, and the enzymes involved.

EXPERIMENTAL PROCEDURE

Materials and Methods

The following materials were of the origin indicated. Itaconic acid was from the Eastman Kodak Company, and mesaconic acid from the Aldrich Chemical Company, Inc. (both were recrystallized before use). Citraconic acid was obtained from the H. M. Chemical Company, Ltd. DL-Citramalic acid was kindly provided by Dr. H. A. Barker, and CY4-pyruvic acid was obtained from the Volk Radio-Chemical Company. Cu-Hydroxyglutaric acid was prepared by catalytic hydrogenation of cr-ketoglutaric acid in glacial acetic acid solution and the solvent removed under reduced pressure. The residue was dissolved in moist ethyl acetate (2); crystals formed upon addition of acetone to the solution and had a melting point of 71-72° (value reported in literature, 72-73°). CY4-itaconic acid was prepared as previously described (1). CY4-mesaconic acid was obtained by boiling CY4-itaconic acid with 10% NaOH for 7 hours (3). The solution was treated with Amberlite IRC-50 (H+ form) to remove Na+. CY4-itaconic and the formed CY4-mesaconic and citraconic acids were then separated by chromatography on Dowex 1. The fractions of the mesaconic acid region (1) were combined and lyophilized. The compound had a melting point of 201-202° and showed the same melting point when mixed with authentic mesaconic acid. Itaconyl-, mesaconyl-, citraconyl-, and cr-hydroxyglutaryl-CoA were prepared by the mixed anhydride method (4). It should be emphasized that these CoA derivatives comprise structural isomers (in which CoA is bound to one or the other of the carboxyl groups) and in some cases optical isomers as well.

Chromatography on Dowex 1 was carried out according to Busch, Hurlbert, and Potter (5).

Mitochondrial acetone powder and its extract were prepared by the method of Drysdale and Lardy (6). Methylglutaconase was prepared and assayed according to Hilz, Krappe, Ringeimann, and Lynen (7); β-hydroxy-β-methylglutaryl-CoA cleavage enzyme according to Bachhawat, Robinson, and Coon (8), except that the acetoacetate formed was determined according to Walker (9). Crystalline crotonase was prepared according to Stern (10). In some experiments, enzyme kindly supplied by Dr. Stern was used. Lactic dehydrogenase was purchased from Worthington Biochemical Corporation.

Acyl-CoA-dependent CY402 fixation and DPNH disappearance experiments were carried out as described (1) except that CoA derivatives were used as substrate instead of free itaconate. Also, in the DPNH disappearance reaction, Mg++, ATP, and CoA were omitted and phosphate buffer, pH 7.4, was used in place of Tris buffer, pH 8.5, as in previous work (1).

To test the hydrating enzyme activity of various protein fractions toward mesaconyl-CoA and itaconyl-CoA, a spectrophotometric method was used. The assay cuvette (I = 1.0 cm) contained 25 μmoles of phosphate buffer, pH 7.4, 0.16 μmole of acyl-CoA, enzyme fraction, and water to 1.0 ml. The blank cuvette contained enough adenylic acid to compensate for the absorption of the adenine moiety of the acyl-CoA compounds. The absorption change was followed at 270 μm.

In the identification of the products of enzyme fractions acting on mesaconyl-CoA or itaconyl-CoA, the reaction mixture contained 25 μmoles of phosphate buffer, pH 7.4, 2 μmoles of CY4-acyl-CoA, enzyme fraction, and water to 1.0 ml. The reaction was carried out at room temperature for 2 hours and stopped by adding 0.13 ml of 30% perchloric acid. After removal of the protein by centrifugation, the perchloric acid was neutralized with perchloric acid again after 1 hour and chromatographed on Dowex 1, along with 10 mg each of authentic mesaconic, itaconic, and citramalic acids. The fractions were dried on planchets and counted for radioactive carbon; the positions of mesaconic, itaconic, and citramalic acids on Dowex 1 chromatogram have been reported (1).

The reversibility of itaconate cleavage was studied in reaction mixtures containing 75 μmoles of phosphate buffer, pH 6.5 (pH 10-12) with authentic itaconic and itaconyl-CoA.

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1 Itaconic acid is methylene succinic acid; mesaconic is methylfumaric acid; citraconic is methylmalic acid; citramalic is α-methylmalic acid.
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The rate-limiting step. The greater initial rate with synthetic period, but citramalyl-CoA does not. This suggests that itaconyl- or mesaconyl-CoA is probably hydrated to form citramalyl-CoA before cleavage and its fixation, and that the hydration is ineffective. The CYO₂ fixation as a function of time is shown in Fig. 1. Both itaconyl-CoA and mesaconyl-CoA show a lag period, but citramalyl-CoA does not. This suggests that itaconyl- or mesaconyl-CoA is probably hydrated to form citramalyl-CoA before cleavage and CYO₂ fixation, and that the hydration is the rate-limiting step. The greater initial rate with synthetic citramalyl-CoA has been observed in several experiments without exception, but the reaction ceases at about 5 minutes, presumably because of the relatively small proportion of the active isomer. Synthetic mesaconyl-CoA had the highest percentage of the active isomer as judged by the extent of reaction.

**RESULTS**

Assay by CYO₂ Fixation—Adler et al. (1) have shown that extracts of guinea pig or rat liver mitochondrial acetone powder catalyze the fixation of CYO₂ in the presence of itaconate. The fixation occurs after the formation of acetyl-CoA and pyruvate from itaconate. It probably is an addition of CO₂ to pyruvate to form oxaloacetate.

The extracts were found to catalyze the fixation of CYO₂ in the presence of chemically prepared itaconyl-CoA, mesaconyl-CoA, or citramalyl-CoA. Citraconyl-CoA was found to be ineffective. The CYO₂ fixation as a function of time is shown in Fig. 1. Both itaconyl-CoA and mesaconyl-CoA show a lag period, but citramalyl-CoA does not. This suggests that itaconyl- or mesaconyl-CoA is probably hydrated to form citramalyl-CoA before cleavage and CYO₂ fixation, and that the hydration is the rate-limiting step. The greater initial rate with synthetic citramalyl-CoA has been observed in several experiments without exception, but the reaction ceases at about 5 minutes, presumably because of the relatively small proportion of the active isomer. Synthetic mesaconyl-CoA had the highest percentage of the active isomer as judged by the extent of reaction.

Assay by Pyruvate Reduction—Extracts of rat liver mitochondrial acetone powder catalyzed the disappearance of DPNH (followed at 340 mμ) in the presence of citramalyl-CoA, itaconyl-CoA, or mesaconyl-CoA. Fig. 2 shows that, initially, both itaconyl-CoA and mesaconyl-CoA give linear responses. Addition of DPNH after the curves level off restores mesaconyl-CoA activity but does not do so with itaconyl-CoA. Citraconyl-CoA is very rapidly cleaved to pyruvate, as measured by DPNH oxidation, but the latter reaction soon drops to the endogenous rate. These data again indicate that chemical thiosterification gives more of the active isomer with mesaconate than with itaconate, and that only very little of the active isomer is formed with citramalate.

If one assumes that the cleavage of acyl-CoA to pyruvate and acetyl-CoA is complete in the presence of DPNH and endogenous lactic dehydrogenase, the metabolically active species of citramalyl-CoA and itaconyl-CoA estimated from Fig. 2 is 2% and 8%, respectively, of the thiol esters present. In another experiment in which DPNH was added continuously to the cuvette containing mesaconyl-CoA until no more disappearance of DPNH was observed, the active species of mesaconyl-CoA was found to be 38% of the total thiol ester.

ATP (or GTP or ITP), Mg⁺⁺, and CoA are absolutely required in the itaconate-dependent DPNH disappearance reaction (1). To study ATP and CoA requirements, the extract was treated with Dowex 1 (12). To study the Mg⁺⁺ requirement, this metal ion was omitted from the reaction mixture and different levels of ethylenediaminetetraacetate were added. With itaconyl-CoA or mesaconyl-CoA as substrate, neither ATP, CoA, nor Mg⁺⁺ is required. This indicates that these cofactors are required only to activate itaconate to itaconyl-CoA; further steps, including the cleavage reaction in the DPNH disappearance assay, do not require these three ingredients. Incidentally, it was observed that succinate-activating enzyme...
(P enzyme) does not activate citramalate. We cannot confirm the previously reported (1) slow activation of citramalate.

Fractionation of Mitochondrial Extract—The results described above suggested the possibility that itaconyl- and mesaconyl-CoA are hydrated to citramalyl-CoA, and that the latter is the derivative which undergoes cleavage to pyruvate and acetyl-CoA. Fractionation of the mitochondrial extract was then undertaken to ascertain the number and nature of the enzymes involved.

Extracts of liver mitochondrial acetone powder prepared from rat, guinea pig, hog, and beef, all catalyzed DPNH disappearance in the presence of mesaconyl-CoA. The average specific activities of these extracts are: rat, 20 units; guinea pig, 10 units; hog, 8 units; and beef, 16 units per mg of protein. Beef liver mitochondrial acetone powder was chosen for fractionation because of its higher activity and especially because its endogenous DPNH oxidation was lowest. DPNH disappearance was chosen as the assay method, and mesaconyl-CoA was used as substrate. Lactic dehydrogenase was always added in excess to the assay mixture. The amount of enzyme used as substrate. Lactic dehydrogenase was always added in excess to the assay mixture. The amount of enzyme was chosen to give a linear response with time during the assay period. One unit of enzyme was defined as that amount of enzyme which gave ΔΔ420 of 0.01 in 14 minutes at 20° under the assay conditions described in the legend to Fig. 2.

Beef liver mitochondrial acetone powder was extracted, at 0°, with 0.01 M phosphate buffer, pH 7.4, for 20 minutes in the ratio of 100 mg of acetone powder per ml of buffer. After centrifuging at 8000 × g for 10 minutes, the soluble portion was diluted with the same buffer to a protein concentration of about 10 mg per ml. Acetone previously chilled to -10° was slowly added to the extract in a bath at -10° and the temperature of the enzyme preparation reached -10° when the final acetone concentration was 35% (volume per volume, assuming that the volumes were additive). The mixture was centrifuged at 25,700 × g for 30 minutes at -10° and the precipitate dissolved in a small amount of 0.01 M phosphate buffer, pH 7.4; this fraction was designated as Fraction 1. The fraction collected at acetone concentrations of 50 to 65% (volume per volume) was treated in the same manner and designated as Fraction 3. The activities of these two fractions are shown in Fig. 3. Fraction 1 had practically no activity, Fraction 3 had none, and combined they catalyzed DPNH disappearance appreciably in the presence of mesaconyl-CoA and lactic dehydrogenase. Ethylene-diaminetetraacetate, up to a concentration of 0.01 M, did not affect the activity. Boiled Fraction 1 or 3 did not induce activity in the other, indicating that at least two enzymes are involved in the conversion of mesaconyl-CoA to acetyl-CoA and pyruvate. The reason for the increase in rate with time, shown in Fig. 3, is unknown. Linear rates were observed with unfractionated extracts.

Metabolic Pathway—To test Fraction 1 and Fraction 3 for hydration activity, a spectrophotometric method based on the absorption of the α,β-unsaturated carbonic thioester was used. The spectra of synthetic mesaconyl-CoA before and after hydrolysis showed that the maximal absorption resulting from its conjugated double bond was at 267 to 270 μm. Fig. 4 shows that Fraction 1 decreases the absorption of mesaconyl-CoA and increases that of itaconyl-CoA at 270 μm. The increase with the latter probably results from the formation of mesaconyl-CoA, as will be shown later. When citramalyl-CoA was used as substrate, however, no absorption change at this wave length was observed. This was probably due to the insufficient amount of the active isomer present in the citramalyl-CoA preparation. Fraction 3 did not show any activity in this assay.

To identify the products of the above reaction, both C14-mesaconyl-CoA and C14-itaconyl-CoA were treated with Fraction 1, and the reaction mixtures were hydrolyzed to liberate the free acids. Figs. 5 and 6 show the Dowex 1 chromatogram of itaconyl-CoA and mesaconyl-CoA, respectively, after treatment with Fraction 1. In the absence of Fraction 1, both C14-itaconyl-CoA and C14-mesaconyl-CoA yielded only a single peak at which itaconic or mesaconic acid was expected. Peak A in both Figs. 5 and 6 is identified as citramalic acid because it coincides with authentic citramalic acid estimated by titration. Peak B in Fig. 6 is identified as itaconic acid and Peak C in Fig. 5 as mesaconic acid by the same criterion. No radioactivity was detected in the region where citramalic acid was expected.

The ratio of citramalic and mesaconic acids estimated from the areas of radioactivity in Fig. 5 is 2:1. Since the radioactivity of Peak B originates from unreacted itaconic acid, as well as both the active and inactive isomers of itaconyl-CoA, it has no quantitative significance in estimating the equilibrium among these three acyl-CoA compounds. The ratio of citramalic acid to itaconic acid estimated by the same method is 4:1. Thus,

![Figure 3](image-url)  
**Fig. 3.** Activities of protein fractions from beef liver mitochondria. The assay mixture contained 50 μmoles of phosphate buffer pH 7.4, 0.2 μmole of DPNH, 0.64 μmole of mesaconyl-CoA, lactic dehydrogenase, enzyme fraction, and water to 2 ml. ○, Fraction 1, 0.21 mg of protein. ●, Fractions 1 plus 3, 0.17 mg. Recorded values are corrected for DPNH oxidation in absence of mesaconyl-CoA.

![Figure 4](image-url)  
**Fig. 4.** Optical density change catalyzed by Fraction 1. The assay cuvette (1 cm) contained 25 μmoles of phosphate buffer pH 7.4, 0.10 μmole of acyl-CoA, 0.05 mg of Fraction 1, and water to 1 ml. Incubated at 22°. ○, itaconyl-CoA; ●, mesaconyl-CoA.
FIG. 5. Chromatogram of acids produced from C14-citronyl-CoA. Experimental conditions are described under "Materials and Methods." The areas under the heavy line represent radioactivity. The diagonal lines designate titration of carrier acids: A = citramalic, B = itaconic, and C = mesaconic acid. The titration scale is linear but set arbitrarily to coincide with the peak of the radioactivity scale. The counting error was <5%.

FIG. 6. Chromatogram of acids produced from C14-mesaconyl-CoA. Conditions as in Fig. 5.

the equilibrium, at pH 7.4, catalyzed by Fraction 1 among these three acyl-CoA compounds may be shown as:

\[
\text{Itaconyl-CoA} \leftrightarrow \text{citramalyl-CoA} \leftrightarrow \text{mesaconyl-CoA}
\]

14% 51% 29%

Hils et al. (7) have shown that methylglutaconase catalyzes the interconversion of β-methylglutaconyl-CoA and β-hydroxy-β-methylglutaryl-CoA. When assayed for this enzyme activity, Fraction 1 was found to be active, but Fraction 3 was not. Methylglutaconase could be used in place of Fraction 1 to stimulate the activity of Fraction 3 in the assay for DPNH disappearance, and results similar to those shown in Fig. 3 were obtained. Methylglutaconase also increased the absorption at 270 m\(\mu\) of itaconyl-CoA and decreased that of mesaconyl-CoA as Fraction 1 did. Dowex 1 chromatograms of these reaction mixtures were almost identical with Figs. 5 and 6, and the equilibrium among these three acyl-CoA compounds was found to be itaconyl-CoA, 12%; citramalyl-CoA, 59%; mesaconyl-CoA, 29%; a verification of that observed when Fraction 1 was used. No radioactivity other than that of the starting materials was found when crystalline crotonase was used in place of methylglutaconase. Crotonase could not replace Fraction 1 to stimulate the activity of Fraction 3 in the DPNH disappearance assay.

Since the cleavage of itaconyl-CoA to acetyl-CoA and pyruvate (presumably via citramalyl-CoA) is analogous to the cleavage of β-hydroxy-β-methylglutaryl-CoA (8), Fractions 1 and 3 were tested for the latter cleavage enzyme, although it is reported that this enzyme requires Mg\(^{++}\) absolutely for its activity and ours does not. Both fractions cleaved β-hydroxy-β-methylglutaryl-CoA to liberate acetoacetate. Addition of β-hydroxy-β-methylglutaryl-CoA cleavage enzyme (8) to either fraction did not result in disappearance of DPNH in the presence of mesaconyl-CoA and lactic dehydrogenase. Fraction 1 was stable when stored at \(-10^\circ\) and assayed as methylglutaconase, but the activity of Fraction 3 was lost gradually at this temperature. After 2 weeks at \(-10^\circ\), it did not catalyze DPNH disappearance in the presence of mesaconyl-CoA, methylglutaconase, and lactic dehydrogenase. However, its β-hydroxy-β-methylglutaryl-CoA cleavage enzyme activity remained. Also, in the presence of mesaconyl-CoA, β-hydroxy-β-methylglutaryl-CoA cleavage enzyme, along with methylglutaconase, Mg\(^{++}\), cysteine, and lactic dehydrogenase, did not catalyze DPNH disappearance. Fraction 3 catalyzed the formation of pyruvate from enzymically prepared (from mesaconyl-CoA and methylglutaconase) citramalyl-CoA as rapidly as mesaconyl-CoA was cleaved in the presence of this fraction and an excess of methylglutaconase.

The possibility that one of the 5 carbon branched chain CoA derivatives is isomerized to form a straight chain derivative was investigated. Synthetic α-hydroxyglutaryl-CoA (presumably some of both possible isomers would be formed) was used as substrate with crude mitochondrial extract. It did not support C\(^{14}\)O\(_2\) fixation, nor did it enhance DPNH oxidation above the endogenous rate.

Reversibility of Cleavage—To study the reversibility of the cleavage of itaconyl-CoA to acetyl-CoA and pyruvate, C\(^{14}\)-pyruvate and acetyl-CoA were incubated with the extract of beef liver mitochondrial acetone powder as described under "Materials and Methods." No itaconic or mesaconic acids were detected when the hydrolyzed reaction mixture was chromatographed on Dowex 1. There was radioactivity in the citramalic region, but malic acid is also expected there, and the radioactivity was most likely in the latter. If citramalyl-CoA had been formed, radioactivity should also be found in the itaconic and mesaconic regions. Fraction 3, together with methylglutaconase, has also been used to study the reversibility, with the same negative results.

DISCUSSION

The metabolism of itaconate in liver mitochondria involves three main steps, which are summarized in Fig. 7: (a) activation

"FIG. 3. Metabolic pathway of itaconic acid"
of itaconate to itaconyl-CoA by succinate activating enzyme (P enzyme); (b) hydration of itaconyl-CoA to citramalyl-CoA by methylglutaconase; and (c) cleavage of citramalyl-CoA to acetyl-CoA and pyruvate. Since itaconate has not been reported as a common component of animal diets or animal tissue, it is not surprising that its metabolism does not involve specific enzymes in Steps 1 and 2. However, \( \beta \)-hydroxy-\( \beta \)-methylglutaryl-CoA cleavage enzyme, the only known enzyme\(^2\) which cleaves a substrate similar to citramalyl-CoA, does not catalyze Step 3. Further purification of the Step 3 cleavage enzyme and a study of its substrate specificity will be necessary before its physiological role can be ascertained.

It has been shown that mesaconate is also metabolized to the common products of itaconate metabolism by rat liver mitochondria, and that it too is activated by succinate-activating enzyme (1). Since methylglutaconase catalyzes the interconversion of itaconyl-, citramalyl-, and mesaconyl-CoA, the metabolism of mesaconate in animal tissue would proceed via the same enzymes (Fig. 7). It is interesting to contrast this pathway with that occurring in Clostridium tetanomurphum (13, 14) in which free mesaconate is converted to citramalate, and the latter is cleaved to pyruvate and acetate without involving CoA.

The reversibility of the cleavage of citramalyl-CoA to acetyl-CoA and pyruvate could not be demonstrated. Recently Losada et al. (16) have reported that Chrumutium synthesizes citramalate from pyruvate and acetyl-CoA. Further studies will be necessary to establish whether an additional energy donor is required to accomplish this synthesis.

**SUMMARY**

1. Citramalyl coenzyme \( \Lambda \) (CoA) is found to be the intermediate in the conversion of itaconyl-CoA to acetyl-CoA and pyruvate by an extract of rat liver mitochondrial acetone powder. The hydration is catalyzed by methylglutaconase.

2. Methylglutaconase catalyzes the interconversion of itaconyl-, mesaconyl-, and citramalyl-CoA. The equilibrium catalyzed by this enzyme at room temperature is estimated to be itaconyl-CoA, 13%; citramalyl-CoA, 58%; and mesaconyl-CoA, 29%.

3. Extracts of an acetone powder of beef liver mitochondria can be separated into two fractions by acetone fractionation. Only in the presence of both can mesaconyl-CoA be cleaved to acetyl-CoA and pyruvate. One fraction contains, and is replaceable by, methylglutaconase. The other fraction cleaves citramalyl-CoA to acetyl-CoA and pyruvate.

4. The reversibility of the cleavage of citramalyl-CoA was not demonstrated.

**REFERENCES**
