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 re-crystallized before use). Citraconic acid was obtained from the H. M. Chemical Company, Ltd. D- Citramalic acid was kindly provided by Dr. H. A. Barker, and C4-mesycolic acid was obtained from the Volk Radio-Chemical Company. α-Hydroxyglutaric acid was prepared by catalytic hydrogenation of L-ketoglutaric acid in the presence of PtO2. Hydrogenation was carried out 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°). C4-itaconic acid was prepared as previously described (1). C4-mesaconic acid was obtained by boiling C4-itaconic acid with 10% NaOH for 7 hours (3). The solution was treated with Amberlite IRC-50 (H+ form) to remove Na+. C14-itaconic and the formed C14-mesaconic and citramalic 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-, citramalyl-, citraconyl- and α-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, Ringelmann, and Lynen (7); β-hydroxy-β-methylglutaryl-CoA cleavage enzyme according to Bachhawat, Robinson, and Coon (8), except that the acetoxacetate 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 C4O2 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 (1 = 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 mμ.

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 C4-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 removed as potassium perchlorate and the solution was adjusted to pH 11 with KOH, and 0.1 ml of 0.1 M HgCl2 was added to hydrolyze the acyl-CoA derivatives. The solution was neutralized with perchloric acid again after 1 hour and chromatographed on Dowex 1, along with 10 μg each of authentic mesaconic, itaconic, and citramalic acids. The fractions were dried on planchets and counted for radioactive carbon; the added authentic acids were titrated with 0.1 N NaOH. 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...
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nyl- or mesaconyl-CoA is probably hydrated to form citramalyl-
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ineffective. The CYO fixation as a function of time is shown
the presence of chemically prepared itaconyl-CoA, mesaconyl-
to form oxaloacetate.

It probably is an addition of CO₂ to pyruvate
fixation occurs after the formation of acetyl-CoA and pyruvate
acetate.

The extracts were found to catalyze the fixation of CO₂ in the
presence of chemically prepared itaconyl-CoA, mesaconyl-
Citraconyl-CoA was found to be
without exception, but the reaction ceases at about 5 minutes, pre-
catalyze the fixation of C⁴O₂ in the presence of itaconate. The
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It was observed that succinate-activating enzyme
acetyl-CoA is complete in the presence of DPNH oxidation, but the latter reaction soon drops to the endogenous
rate. These data again indicate that chemical thioesterification
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
acytetyl-CoA is complete in the presence of DPNH and endoge-
ous 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 cu-
vette 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 re-
quired in the itaconate-dependent DPNH disappearance reac-
tion (1). To study ATP and CoA requirements, the extract
was treated with Dowex 1 (12). To study the Mg⁺⁺ require-
ment, 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
effectors 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

RESULTS

Assay by C⁴O₂ Fixation—Adler et al. (1) have shown that
extracts of guinea pig or rat liver mitochondrial acetone powder
catalyze the fixation of C⁴O₂ 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.

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disappearance assay, do not require these three ingredients.
Incidentally, it was observed that succinate-activating enzyme

Fig. 1. The rate of O₂ fixation with synthetic itaconyl-, mesa-
conyl-, and citramalyl-CoA. Reaction mixture contained 1.2
µmoles of ATP; 1.2 µmoles of Mg⁺⁺; 5 µmoles of Tris buffer pH
8.5; 4.0 µmoles of NaHCO₃; 2.2 mg of enzyme protein; and H₂O in 0.25 ml. Incubated at 37°. The reaction was started by adding acyl-CoA. O, citramalyl-CoA; O, itaconyl-CoA; O mesaconyl-CoA. At 3 minutes, the amount of C⁴O₂ fixed corresponds to approximately 16, 50, and 76% of that theoretically possible if all the active forms of mesaconyl-, itaconyl-, and citramalyl-CoA, respectively, were cleaved and if all the pyruvate produced fixed C⁴O₂ to form oxalo-
acetate. The proportion of active forms of the CoA derivatives are estimated in the text.

Fig. 2. Assay for pyruvate formation from itaconyl-, mesa-
conyl-, and citramalyl-CoA. The reaction mixture contained 25
µmoles of phosphate buffer pH 7.4; 0.3 µmole of DPNH; 0.35 µmole
of acyl-CoA, enzyme, and water to 1 ml. Incubated at 22°; 1-cm
light path. O, endogenous; O, citramalyl-CoA; O, itaconyl-
CoA; O, mesaconyl-CoA. The arrows indicate the addition of
another 0.2 µmole of DPNH.
(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 disap-
pearance 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 frac-
tionation because of its higher activity and especially because
its endogenous DPNH oxidation was lowest. DPNH disap-
pearance 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 WM
was used as substrate. Lactic dehydrogenase was always added
to the extract in a bath at -10° and the temperature
appeared to give a linear response with time during the assay
period. One unit of enzyme was defined as that amount of
enzyme which gave ΔA 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 0.1 M phosphate buffer, pH 7.4, for 20 minutes in the
ratio of 100 mg of acetone powder per ml of buffer. After cen-
trifuging 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 ace-
tone 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 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 ace-
tone 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 frac-
tion was designated as Fraction 1. The fraction collected at
acetone concentrations of 50 to 65% (volume per volume) was
in the ratio of 100 mg of acetone powder per ml of buffer. After cen-
trifuging 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 ace-
tone 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 frac-
tion was designated as Fraction 1. The fraction collected at
acetone concentrations of 50 to 65% (volume per volume) was
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 pres-
ence 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 ac-
tivity in the other, indicating that at least two enzymes are in-
volved 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 unfrac-
tionated extracts.

Metabolic Pathway.—To test Fraction 1 and Fraction 3 for
hydrating activity, a spectrophotometric method based on the
absorption of the α,β-unsaturated carbonic acid was used.
The spectra of synthetic mesaconyl-CoA before and after hy-
drolysis showed that the maximal absorption resulting from its
coujugated 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-mesa-
conyl-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 itaco-
nyl-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 de-
tected in the region where citraconic acid was expected.

The ratio of citramalic and mesaconic acids estimated from
the areas of radioactivity in Fig. 5 is 2:1. Since the radioac-
tivity 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,
FIG. 5. Chromatogram of acids produced from $^{14}$C-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 $^{14}$C-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:

\[
\text{Itaconyl-CoA} \rightleftharpoons \text{Citramalyl-CoA} \rightleftharpoons \text{Mesaconyl-CoA}
\]

14% 57% 29%

Hils et al. (7) have shown that methylglutaconase catalyzes the interconversion of $\beta$-methylglutaconyl-CoA and $\beta$-hydroxy-$\beta$-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 DPNH disappearance assay.

Since the cleavage of itaconyl-CoA to acetyl-CoA and pyruvate (presumably via citramalyl-CoA) is analogous to the cleavage of $\beta$-hydroxy-$\beta$-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 $\beta$-hydroxy-$\beta$-methylglutaryl-CoA to liberate acetoacetate. Addition of $\beta$-hydroxy-$\beta$-methylglutaryl-CoA cleavage enzyme (8) to either fraction did not result in disappearance of DPNH in the presence of mesaconyl-CoA and lactate 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 lactate dehydrogenase. However, its $\beta$-hydroxy-$\beta$-methylglutaryl-CoA cleavage enzyme activity remained. Also, in the presence of mesaconyl-CoA, $\beta$-hydroxy-$\beta$-methylglutaryl-CoA cleavage enzyme, along with methylglutaconase, Mg$^{++}$, cysteine, and lactate 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 $\alpha$-hydroxyglutaric-CoA (presumably some of both possible isomers would be formed) was used as substrate with crude mitochondrial extract. It did not support CO$_2$ 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, $^{14}$C-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. 7. Metabolic pathway of itaconic acid](https://example.com/path/to/image)
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 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 Chlamydomonas 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 A (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.

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