Metabolism of ethylmalonate to mesaconate in the rat

Evidence for *trans*-dehydrogenation of methylsuccinate

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Rats excrete increased ethylmalonate and methylsuccinate when given ethylmalonate in the diet; when given methylsuccinate they excrete methylsuccinate and mesaconate. Tenfold more labelled mesaconate was produced from *threo*-methyl[2,3-²H₂]succinate precursor than from the *erythro* isomer. Our findings suggest *trans*-dehydrogenation of methylsuccinate and are the first direct evidence linking the metabolism of ethylmalonate, methylsuccinate and mesaconate.

Mamer *et al.* (1976) described a pathway comprising an *R*-series of alloisoleucine derivatives terminating in ethylmalonate; this pathway is an analogue of the *S*-series terminating in methylmalonate. In the present paper we describe the metabolic sequence by which ethylmalonate is subsequently converted into mesaconate through methylsuccinate.

Ethylmalonyl-CoA (2-carboxybutyryl-CoA) is a substrate for methylmalonyl-CoA mutase; the product of the reaction is methylsuccinyl-CoA (3-carboxybutyryl-CoA) (Rétey *et al.*, 1978). Human patients with isovalericacidemia excrete methylsuccinate in elevated amounts, presumably by ω -oxidation of the excess free isovalerate (Nakamura *et al.*, 1976). Patients with propionicacidemia also excrete an excess of methylsuccinate, formed, along with glyoxylate, by the action of isocitrate lyase on methylcitrate (Ando *et al.*, 1972). Methylsuccinate is a substrate *in vitro* for succinate dehydrogenase; the product is mesaconate (Gawron *et al.*, 1962).

The conversion of succinate into fumarate by succinate dehydrogenase involves *trans*-elimination of two hydrogen atoms (Gawron *et al.*, 1963; Tchen & van Milligan, 1960). Conversion of methylsuccinate into mesaconate has not been defined *in vivo*. Accordingly we gave rats *threo*- and *erythro*-methyl[2,3-²H]succinates to show that oxidation of methylsuccinate involves *trans*-elimination. We also fed ethylmalonate and methylsuccinate in large

doses to rats to demonstrate that mesaconate is derived from ethylmalonate.

Experimental

Animals and materials

Animals and supplies were obtained as follows: Sprague–Dawley rats from Canadian Breeding Farms, Montreal, Quebec, Canada; deuterium gas (99.5 atom% ²H), deuterium oxide (99.7 atom% ²H) and deuteromethanol (CH₃O²H; 99 atom% ²H) from Merck, Sharp and Dohme, Montreal, Quebec, Canada; mesaconic acid, citraconic acid, methylsuccinic acid and ethylmalonic acid from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; rhodium (5%) on powdered aluminium from Matheson, Bell and Coleman, Norwood, OH, U.S.A.; solvents of appropriate purities from various local supply houses; TRISIL/BSA and stationary phases OV-101 and OV-17 from Pierce Chemical Co., Rockford, IL, U.S.A.; Chromosorb W HP (mesh size 100–120) from Chromatographic Specialties, Brockville, Ontario, Canada.

Synthesis of *threo*-methyl[2,3-²H]succinic acid

To replace the acidic carboxy hydrogen atoms with deuterium, mesaconic acid was recrystallized from hot deuterium oxide. Deuteromethanol (1 ml) was used to dissolve 100 mg of the resulting [²H₂]mesaconic acid. Approx. 1 mg of rhodium catalyst and 2 ml of deuteromethanol were placed in a small bomb and pressurized with deuterium gas (550 kPa) at room temperature. After 15 min the pressure was released, and the [²H₂]mesaconic acid

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in deuteromethanol was added. The bomb was evacuated of air by a water aspirator, and then pressurized again to 550 kPa with deuterium gas. This pressurization procedure was then repeated. The bomb was held in an ultrasonic water bath for 15 min and then shaken for 2 h at room temperature. The mixture was filtered, and the filtrate evaporated under N_2 . The product was recrystallized from hot deuterium oxide, and analysed as the trimethylsilyl derivative. We obtained pure (100%) methylsuccinic acid with the following isotopic composition: 2H_1 , 12%; 2H_2 , 71.2%; 2H_3 , 6.5%; 2H_4 , 10.3%.

Synthesis of erythro-methyl[2,3- 3H_2]succinic acid

Citraconic acid was treated in the same manner as described for mesaconic acid for the *threo* synthesis. Pure (100%) methylsuccinic acid was obtained with the following isotopic distribution: 2H_1 , 13.7%; 2H_2 , 76.3%; 2H_3 , 4.5%; 2H_4 , 5.5%.

Animal studies

Loading of rats with ethylmalonate and methylsuccinate. Eight male rats, 300–800 g, were subjected to light ether anaesthesia. Four animals received ethylmalonic acid and four others methylsuccinic acid (250 mg per kg body wt.). Five control rats were given water (1 ml) by intraperitoneal injection. The animals were housed separately in suspended metabolic cages, and their urine was collected for 24 h into acidified saline. Urine samples were stored at $-20^\circ C$. A sample (2 ml) of urine from each rat was extracted as described by Gibbs *et al.* (1972). Organic acids were converted into their trimethylsilyl derivatives in 80 μ l of TRISIL/BSA at $60^\circ C$ for 15 min and measured with the use of decanoic acid as the internal standard.

Analysis of the organic acids was performed on an LKB 9000 coupled gas chromatograph–mass spectrometer. The operating conditions were: separator and injector block, $280^\circ C$; ion source, $290^\circ C$; ionizing voltage, 70 eV; current, 60 μA . The chromatography was performed on a glass column (2 m $\times$ 6 mm) packed with 6% OV-101 on Chromosorb W HP and temperature-programmed from $80^\circ C$ at $5^\circ C/min$.

Feeding of threo- or erythro-methyl[2,3- 2H_2]succinate to rats. Two solutions (A and B) were prepared containing 20 mg of *threo*- and *erythro*-methyl[2,3- 2H_2]succinic acid respectively in 1-litre volumes of distilled water (0.15 mM). Six rats, divided into two groups of three, were housed individually in metabolic cages. Group 1 received solution A as drinking water; group 2 received solution B. Urine was collected during five consecutive 24 h periods, and treated as described above.

The operating conditions for the LKB 9000 instrument were the same as above. Chromatography was performed on a glass column (2 m $\times$ 6 mm) packed with 3% OV-17, operated isothermally at $141^\circ C$. The $M^{++}-CH_3^+$ ion (m/z 259) for mesaconic acid was monitored along with the ion at m/z 260 for [2H_1]mesaconic acid. Deuterium incorporation was measured after correction for the natural isotopic abundance of the ion at m/z 259. Total mesaconic acid was calculated with the use of decanoic acid as the internal standard. The results were expressed as mg of mesaconic acid per g of urinary creatinine.

Results

Ethylmalonate loading increased methylsuccinate excretion 3-fold. Methylsuccinate loading did not influence ethylmalonate excretion, but increased mesaconate excretion 300-fold. These findings are presented in Table 1, and indicate that ethylmalonate was converted into mesaconate *in vivo*.

The rats drank approx. 40 ml of water per day, equivalent to about 1 mg of *threo*- or *erythro*-methyl[2,3- 2H_2]succinic acid. This intake of substrate increased excretion of mesaconate slightly (3–4 times normal), a significant fraction of which contained a deuterium label. Rats given the *threo* isomer excreted about 0.5 mg of [2H_1]mesaconic acid/g of creatinine, whereas those given the *erythro* isomer excreted about one-tenth this amount. Total excretion of mesaconate was similar in both groups, indicating that they ingested comparable amounts of the labelled acids. These results are summarized in Fig. 1.

Table 1. Urinary excretion of organic acids by rats given ethylmalonate or methylsuccinate

Neutralized aqueous solutions of ethylmalonic acid or methylsuccinic acid were given to male rats (250 mg per kg body wt.). Age- and sex-matched controls received water bolus only. Urine was collected for 24 h after each load. Results are presented as means $\pm$ S.D. For further details see the text.

Substance given (number of rats)	Urinary excretion (mg of acid/24 h per kg body wt.)		
	Ethylmalonate	Methylsuccinate	Mesaconate
Ethylmalonate ($n = 4$)	56.4 ± 35.1	0.54 ± 0.22	0.06 ± 0.05
Methylsuccinate ($n = 4$)	1.25 ± 0.6	128.6 ± 34.9	18.5 ± 9.2
Water (control) ($n = 5$)	0.49 ± 0.23	0.17 ± 0.16	0.06 ± 0.03

Fig. 1. Excretion of mesaconate by rats exposed for 6 days *ad libitum* to drinking water containing ^2H -labelled methylsuccinate (0.15 mM)

Columns represent total mesaconate excreted in five successive urine collections (left to right: 0–24, 24–48, 48–72, 72–96 and 96–144 h); shaded portion of column represents the singly labelled fraction of mesaconate in that collection. Rats 1, 2 and 3 were given *threo*-methyl[2,3- $^2\text{H}_2$]succinate; rats 4, 5 and 6 received the *erythro* isomer. Excretion of labelled mesaconate is significantly different ($P < 0.005$; Student's *t* test) after exposure to *threo* and *erythro* isomers (means $\pm$ s.d. 0.507 ± 0.44 and 0.066 ± 0.04 mg/g of creatinine respectively); total mesaconate excretion is not significantly different.

Discussion

An increase in urinary excretion of methylsuccinate after feeding ethylmalonate to the rat gives evidence of a third mechanism for the formation of methylsuccinate in mammals in addition to ω -oxidation of isovalerate and degradation of methylcitrate. Recovery of mesaconate containing deuterium from urine of rats given labelled methylsuccinate demonstrates that the conversion *in vivo* involves dehydrogenation, probably by means of succinate dehydrogenase (Gawron *et al.*, 1962).

The positions of the deuterium atoms in labelled methylsuccinate after the rhodium-catalysed *cis*-addition of deuterium to mesaconate and citraconate to produce *threo*- and *erythro*-methyl[2,3- $^2\text{H}_2$]succinate respectively are illustrated in Scheme 1. Enzymic dehydrogenation *in vivo* can involve either *cis*- or *trans*-removal of hydrogen atoms. *cis*-Elimination with *threo*-methyl[2,3- $^2\text{H}_2$]succinate would yield unlabelled mesaconate, whereas *trans*-elimination would yield labelled [$^2\text{H}_1$]mesaconate. *cis*-Elimination with *erythro*-methyl[2,3- $^2\text{H}_2$]succinate would yield labelled [$^2\text{H}_1$]mesaconate, whereas *trans*-elimination would yield unlabelled mesaconate. We found that a significant fraction of mesaconate formed *in vivo* from *threo*-methyl[2,3- $^2\text{H}_2$]-

Scheme 1. Chemical synthesis of *threo*- and *erythro*-methyl[2,3- $^2\text{H}_2$]succinates by catalytic reduction with deuterium of mesaconic acid (A) and citraconic acid (B) respectively, and the possible routes of their subsequent enzymic oxidation *in vivo* to mesaconic acid

Unlabelled mesaconic acid would be produced by loss of $^2\text{H}_2$ through *cis*-elimination with the *threo* isomer or through *trans*-elimination with the *erythro* substrate. Conversely, singly labelled mesaconate would be produced from the *erythro* and *threo* acids respectively by the *cis*- and *trans*-elimination of $^1\text{H}^2\text{H}$.

succinate was labelled with one deuterium atom per molecule, whereas that formed from the *erythro* isomer was not. A *trans*-elimination mechanism is indicated.

trans-Elimination of hydrogen atoms from succinic acid occurs *in vitro* (Tchen & van Milligan, 1960). The ratio of [2,3- $^2\text{H}_2$]fumarate to total fumarate after incubation with *meso*-[2,3- $^2\text{H}_2$]succinate is about half the labelled/total succinate ratio; this finding is compatible with an equal probability of *trans*-elimination of either $^2\text{H}_2$ or $^1\text{H}_2$. Enzymic dehydrogenation of the racemic substrates [2*R*,3*R*]- and [2*S*,3*S*]-succinate unexpectedly produced a small amount of [2,3- $^2\text{H}_2$]fumarate (9% as much as in the *meso* case when compared with the $^2\text{H}_2$ enrichments in precursor succinate).

We found that *erythro*-methyl[2,3- $^2\text{H}_2$]succinate analogously provoked a modest excretion of [$^2\text{H}_1$]-mesaconate, about 10% relative to excretion after the *threo* isomer load. Our synthesis of *threo*- and *erythro*-methylsuccinates yielded products containing three deuterium atoms per molecule (6.5% and 4.5% of total product respectively). A *trans* removal of two deuterium atoms in the latter case would therefore account for nearly half of the excreted [$^2\text{H}_1$]mesaconate; the remainder could result from non-specific routes of enzymic dehydrogenation of methylsuccinate or catalytic reduction of citraconate.

Gawron *et al.* (1962) showed that *R*-methylsuccinate is a substrate of succinate dehydrogenase *in vitro*, that *S*-methylsuccinate acts as a competitive inhibitor and that the enzyme has 190-fold lower affinity for *R*-methylsuccinate relative to succinate.

Increased urinary methylsuccinate is a phenotypic condition associated with isovalericacidaemia, glutaricaciduria type II and Jamaican vomiting sickness (Goodman & Markey, 1981), resulting from decreased activity of isovaleryl-CoA dehydrogenase. The excretion of ethylmalonate is also increased in the latter two cases because of decreased butyryl-CoA dehydrogenase activity. Increased excretion of methylsuccinate and mesaconate is a potential consequence of ethylmalonate accumulation. In the interval since labelled mesaconate excretion was first reported as a result of the loading of rats with 2-methyl-[3,3,4,4,4- $^2\text{H}_5$]butyrate, a precursor of ethylmalonate (Mamer *et al.*, 1976), elevated excretion of mesaconate has been reported in a patient with acute isovalericacidaemia (Truscott *et al.*, 1981). This patient was also found to excrete increased amounts of methylsuccinate; the latter acting as a substrate for succinate dehydrogenase would be the precursor of mesaconate.

To summarize, we show that ethylmalonate is converted in the intact rat into methylsuccinate, probably by the action of methylmalonyl-CoA

mutase on the CoA ester. In a contiguous ethylmalonate/methylsuccinate/mesaconate pathway, hydrolysis of methylsuccinyl-CoA may be effected by succinyl-CoA synthase. We further provide the first direct evidence of a *trans*-dehydrogenation for the conversion *in vivo* of methylsuccinate into mesaconate, the homologues of succinate and fumarate respectively in the tricarboxylic acid cycle.

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