Metabolism of Methylmalonic Acid in Rats

IS METHYLMALONYL-COENZYME A RACEMASE DEFICIENCY SYMPTOMATIC IN MAN?

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ABSTRACT Vitamin B_{12}-deficient and normal rats were loaded with methylmalonic (MMA) and ethylmalonic acids labeled with ^{13}C in the carboxyl groups and with ^{2}H in the alkyl groups. Significant fractions of the administered acids were excreted in both the B_{12}-deficient and the normal animal, having undergone exchange of both their ^{13}C-labeled carboxyl groups with endogenous ^{12}C. The exchange of the α-^{1}H of MMA in $^{2}H_2O$ at 25°C and pH 7.5 was found by ^{1}H-nuclear magnetic resonance to have a half-life of 28.3 min.

These results show that a fraction of in vivo metabolism through the propionate-to-succinate pathway occurs via a shunt involving free MMA. The enzymes of this pathway are thought to utilize only coenzyme A (CoA) esters. To allow for the exchange of the second CoA-bound carboxyl group, we propose the deacylation of the once exchanged acid with spontaneous racemization (relative to the ^{13}C-carboxyl group), followed by reacylation, thus exposing the labeled carboxyl to decarboxylation. The significance of this mechanism involving free MMA is that racemization of methylmalonyl (MM)-CoA may also occur without the intervention of MM-CoA racemase. A deficiency of this enzyme need not result in symptomatic methylmalonic aciduria.

INTRODUCTION

Methylmalonic acid (MMA)[1] is a metabolite derived in mammals from a wide variety of substrates, chiefly valine and isoleucine. There has been considerable controversy concerning the metabolism of valine. After incubating racemic [4,4-$^{14}C_2$]valine with rat liver homogenate, Kinnory et al. (1) recovered labeled propionic, isobutyric, and 3-hydroxyisobutyric acids. To explain the presence of labeled propionate, they suggested that methylmalonyl semialdehyde (MMSA) was first decarboxylated to propionaldehyde, which, in turn, was oxidized to propionic acid. Robinson and co-workers (2) found that 3-hydroxyisobutyryl-coenzyme A (CoA), the precursor of MMSA, must be deacylated to the free acid before oxidation to MMSA; they proposed further that direct oxidation of MMSA to methylmalonyl-CoA (MM-CoA) followed by decarboxylation would also yield propionyl-CoA (P-CoA), implying that reversal of the P-CoA carboxylase reaction would be the final step in valine metabolism. Lane and Halenz (3) demonstrated the reversible nature of P-CoA carboxylase with MM-CoA, and also showed that ethylmalonyl-CoA and butyryl-CoA (B-CoA) are similarly interconvertible. In the MMA system, Rétey and Lynen (4) showed that the MM-CoA produced by the action of P-CoA carboxylase has the *S* configuration. Tanaka and colleagues (5) have more recently demonstrated that P-CoA is an obligate intermediate between MMSA and *S*-MM-CoA. Scheme 1 summarizes the conversion of propionate to succinate as it is now generally accepted for mammals.

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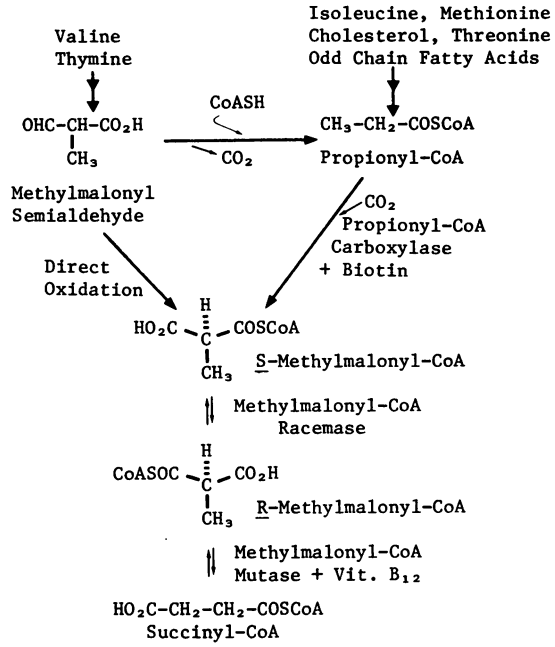
[1] *Abbreviations used in this paper:* B-CoA, butyryl CoA; CoA, coenzyme A; CoASH, free CoA; EMA, ethylmalonic acid; [$^{2}H_5$]EMA, 2-[Et-$^{2}H_5$]ethylmalonic acid; [$^{2}H_5$]-[$^{13}C_2$]EMA, 2-[Et-$^{2}H_5$]-1,3-[$^{13}C_2$]ethylmalonic acid; GCMS, gas chromatography-mass spectrometry; MMA, methylmalonic acid; [$^{2}H_3$]MMA, 2-[Me-$^{2}H_3$]methylmalonic acid; [$^{2}H_3$]-[$^{13}C_2$]MMA, 2-[Me-$^{2}H_3$]-1,3-[$^{13}C_2$]methylmalonic acid; MMAuria, methylmalonic aciduria; MM-CoA, methylmalonyl CoA; MMSA, methylmalonyl semialdehyde; m/z, mass/charge ratio; NMR, nuclear magnetic resonance; P-CoA, propionyl CoA; TMS, trimethylsilyl.



SCHEME I

The importance of reversible decarboxylation of *S*-MM-CoA by P-CoA carboxylase and the reversible hydrolysis-reesterification of *R*- and *S*-MM-CoA in the metabolism of MMA in the intact mammal has been largely overlooked until now.

To determine the extent to which *S*-MM-CoA is decarboxylated, we synthesized ethylmalonic acid (EMA) and MMA having ^{13}C-labeled carboxyl groups and fully deuterium-labeled alkyl side-chains (2-[Et-$^{2}H_5$]-1,3-[$^{13}C_2$]ethylmalonic and 2-[Me-$^{2}H_3$]-1,3-[$^{13}C_2$]methylmalonic acids, abbreviated as [$^{2}H_5$]-[$^{13}C_2$]EMA and [$^{2}H_3$]-[$^{13}C_2$]MMA, respectively, in this work), and we administered them to normal and vitamin B_{12}-deficient rats. We anticipated that a portion of the administered substrates would be converted to the CoA derivative and become eligible for a replacement of ^{13}C label by endogenous ^{12}C mediated by P-CoA carboxylase. We analyzed the resulting urine by gas chromatography-mass spectrometry (GCMS) for evidence of depletion of ^{13}C label in the administered acids.

Deuterium labeling permits clear discrimination in the mass spectrometer between the trimethylsilyl (TMS) derivatives of endogenous (i.e., unlabeled) metabolites and those of administered (exogenous) analogues. Thus, [$^{2}H_3$]-[$^{13}C_2$]MMA produces molecular and fragment ions that are 5 atomic mass units (amu) heavier than those produced by the endogenous acid. There are several advantages that accrue from this technique. The deuterium isotope effect inhibits the mutase step and retards metabolic outflow of labeled MMA substrate to the Krebs cycle via succinate and allows greater opportunity for ^{13}C-carboxyl exchange. More important, however, exchange of both ^{13}C-labeled carboxyl groups can be readily observed. Without ^{2}H-labeling, MMA so produced is indistinguishable from the endogenous species.

EMA is a homologue of MMA and undergoes similar metabolic reactions. EMA is a poor substrate for MM-CoA mutase (6); hence, conversion of [$^{2}H_5$]-[$^{13}C_2$]EMA to methylsuccinate should be additionally retarded. Furthermore, incorporation of five ^{2}H atoms into the ethyl moiety provides a separation of fragment ions derived from the endogenous and exogenous EMA that is 2 amu greater than that in the MMA study.

We demonstrate that exogenous MMA and EMA excreted by the loaded rats have undergone partial decarboxylative exchange of ^{13}C label in the carboxyl groups for endogenous ^{12}C, presumably by the action of P-CoA carboxylase on the corresponding CoA esters. More significantly, we also demonstrate that the ^{13}C label in the CoA ester function is similarly subject to depletion. This last finding indicates that a portion of the flow through the propionyl- to succinyl-CoA pathway passes through a free MMA intermediate. The currently accepted pathway includes only the CoA-esterified derivatives of these acids.

The significance of these findings lie in an interpretation of the Mendelian phenotype known as methylmalonic aciduria (MMAuria). Over 100 cases of MMAuria have been reported. Of these, only one might be attributable to an error of MM CoA racemase; that patient has since been shown to have mutase deficiency (7, 8). Our results suggest that putative racemase deficiency need not be associated with symptomatic MMAuria, because a free MMA shunt may allow a nonracemase-mediated conversion of *S*-MM-CoA to *R*-MM-CoA.

METHODS

Materials

3-wk-old male Sprague-Dawley rats were purchased from Canadian Breeding Farms, Montreal, Quebec. Standard rat chow was obtained from Ralston Purina Canada, Inc., Montreal; the B_{12}-deficient diet, vitamin B_{12}, and pectin came from ICN Pharmaceuticals, Inc., Cleveland, OH. Stable isotope-labeled compounds were purchased from Merck, Sharp and Dohme, Montreal. The isotopic purities were as follows: deuterium oxide (99.7 atom % ^{2}H), deuterium chloride (35% in $^{2}H_2O$, 99 atom % ^{2}H), sodium deuteroxide (40% in $^{2}H_2O$, 98 atom % ^{2}H), [$^{2}H_5$]ethyl iodide (99 atom % ^{2}H), [$^{2}H_3$]methyl iodide (99.5 atom % ^{2}H), and diethyl malonate-1,3-[$^{13}C_2$] (90 atom % ^{13}C). Solvents and inorganic materials of appropriate purities were obtained from various suppliers in Montreal. Derivatizing reagents, TRI-SIL/BSA and *N*-trimethylsilylimidazole, the stationary liquid phases, OV-101 and OV-17, and Chromosorb W HP, mesh size 100/120, were purchased from Chromatographic Specialties, Brockville, On-

tario. Dexsil 300 was obtained from Analabs, Inc., North Haven, CT. Column packings for gas chromatography were prepared as required by slurrying in methylene chloride, followed by rotary evaporation.

Procedures

Vitamin-B_{12} deficiency protocol. A newly weaned male rat was housed in a hanging metabolic cage to prevent coprophagy and fed a powdered B_{12}-deficient diet in which pectin was thoroughly mixed (15 g pectin/100 g diet). Periodic urine collections were analyzed for MMA and EMA, with 2-[Me-$^2H_3$]methylmalonic ([$^2H_3$]MMA) and 2-[Et-$^2H_5$]ethylmalonic acids ([$^2H_5$]EMA) as internal standards, by a method similar to that reported recently for MMA by Trefz et al. (9). The rat was considered B_{12} deficient when the MMA excretion exceeded 500 mg/g urinary creatinine. Control rats used in these loading studies were fed a regular diet.

Synthesis of substrates labeled with stable isotopes

[$^2H_3$]-[$^{13}C_2$]MMA. This acid was synthesized from diethyl malonate-1,3-[$^{13}C_2$] (500 mg, 3.1 mmol), [$^2H_3$]methyl iodide (500 mg, 3.7 mmol), sodium (230 mg, 10 mmol), and absolute ethanol (20 ml) in the manner of a standard malonate ester synthesis (10). The product was 285 mg (2.36 mmol) of labeled MMA (76%) and was shown to have the following isotopic distribution: [$^2H_3$]-[$^{13}C_2$]MMA, 80.71%; [$^2H_3$]-[$^{13}C_1$]MMA, 15.96%; [$^2H_3$]-[$^{13}C_0$]MMA, 1.93%; [$^2H_0$]-[$^{13}C_0$]MMA (i.e., unlabeled), 1.40% by monitoring the $M^{+\cdot}-CH_3^{\cdot}$ ions of the *bis*-TMS derivative (mass-to-change ratio [m/z] 252, 251, 250, and 247, respectively).

[$^2H_5$]-[$^{13}C_2$]EMA. A standard malonate ester synthesis was used (10), starting with diethyl malonate-1,3-[$^{13}C_2$] (500 mg, 3.1 mmol), [$^2H_5$]ethyl iodide (600 mg, 3.7 mmol), sodium (230 mg, 10 mmol), and absolute ethanol (20 ml). Purity of the final product (320 mg, 2.3 mmol or 74% yield) was confirmed by GCMS. By monitoring the $M^{+\cdot}-CH_3^{\cdot}$ ions at m/z 268, 267, 266, and 261 for the *bis*-TMS derivative, the product was shown to have the following isotopic distribution: [$^2H_5$]-[$^{13}C_2$]EMA, 78.90%; [$^2H_5$]-[$^{13}C_1$]EMA, 17.91%; [$^2H_5$]-[$^{13}C_0$]EMA, 3.04%; [$^2H_0$]-[$^{13}C_0$]EMA (i.e., unlabeled), 0.15%.

[$^2H]_5$EMA and [$^2H_3$]MMA. The first acid was synthesized by the malonate ester method previously reported (11). [$^2H_3$]Methyl iodide was used for synthesis of the latter acid in the place of labeled ethyl iodide.

Synthesis of sodium hydrogen methylmalonate. MMA (1.2 g, 10 mmol) was dissolved in 1 N aqueous sodium hydroxide (10 ml), rotary evaporated to dryness under reduced pressure, and the residue taken up and recrystallized from hot ethyl acetate. The product, a dense white powder, was heated for 3 h at 50°C in vacuo to remove entrapped ethylacetate (final yield 0.90 g, 6.4 mmol, 64%).

Analytical and instrumental conditions

Organic acids were isolated by ether extraction (12) and converted to their TMS derivatives for GCMS analysis on an LKB 9000 GCMS (LKB Instruments, Inc., Gaithersburg, MD) or a Hewlett-Packard 5984A GCMS (Hewlett-Packard Co., Palo Alto, CA). The operating conditions for the LKB 9000 were: separator and injector block, 280°C; ion source, 290°C, ionizing voltage, 70 eV, and current, 60 μA; and for the Hewlett-Packard 5984A: glass jet separator, 300°C; injector block, 200°C; ion source, 200°C, ionizing energy 70 eV. Gas chromatographic conditions were chosen that were appropriate for each analysis and are described below.

In vivo studies in the rat

[$^2H_3$]-[$^{13}C_2$]MMA administration. Under light ether anaesthesia, four normal rats received [$^2H_3$]-[$^{13}C_2$]MMA loads (~0.5 mmol/kg) by intraperitoneal injection of an aqueous solution neutralized with sodium hydroxide. They were housed individually in hanging cages with access to water only. Urines were collected into acidified salt solutions over intervals of 0–5, 5–24, 24–30, and 30–48 h. Aliquots (2 ml) of urine from each collection containing 20 μg of capric acid as internal standard were extracted as described (12). The extracts were derivatized with 0.1 ml of TRI-SIL/BSA and were analyzed on the Hewlett-Packard 5984A with a 2-m column containing a 1:1 column-packing mixture of 4% Dexsil 300 and 6% OV-17 on Chromosorb W HP operated isothermally at 110°C. This particular composition of column packing was required for the separation of MMA from 2-ethyl-3-hydroxybutyric acid, which is not separated by more conventional packings. MMA has a retention time of 4.4 min under these conditions. The $M^{+\cdot}-CH_3^{\cdot}$ ions for MMA were monitored at m/z 252, 251, 250, and 247, corresponding to the deuterium-labeled $^{13}C_2$, $^{13}C_1$, and $^{13}C_0$, and endogenous MMA species, respectively. All peak areas were corrected for isotopic impurities and natural isotopic abundances. Total MMA was measured relative to capric acid on the LKB 9000 in a similar mixed column.

[$^2H_5$]-[$^{13}C_2$]EMA administration. The B_{12}-deficient rat (220 g) and a normal littermate (500 g) each received 0.29 mmol [$^2H_5$]-[$^{13}C_2$]EMA/kg body wt by gastric tube. A second normal littermate (500 g) similarly received a larger load (1.15 mmol/kg). A third normal (500 g) received 1.15 mmol/kg of the same acid solution but by intraperitoneal injection. The acids were dissolved in minimum amounts of aqueous sodium hydroxide and administered to each rat under light ether anaesthesia. Urine samples were collected and extracted as described above. The extracts were derivatized in 100 μl of TRI-SIL/BSA and were analyzed on a 2-m, 6% OV-101 column operated isothermally at 110°C in the Hewlett-Packard 5984A. The $M^{+\cdot}-CH_3^{\cdot}$ ions were monitored at m/z 268, 267, 266, and 261, representing the respective deuterium-labeled $^{13}C_2$, $^{13}C_1$, $^{13}C_0$, and endogenous EMA species. The retention time of EMA was 5.2 min. All peak areas were corrected for isotopic impurities and natural isotopic abundances. Total EMA was measured on the LKB 9000 by total ion current peak height comparison with capric acid. The results were expressed as nanomoles of EMA excreted per kilogram of body weight per hour.

Exchange of the methylmalonyl-α-^1H for ^2H in deuterium oxide at physiological pH. A buffer solution pH 8.0 was made from 0.68 g KH_2PO_4, 0.20 g NaOH, and deuterium oxide (final vol, 2.0 ml). To this was added 0.28 g sodium hydrogen methylmalonate with rapid dissolution by shaking. The time of this final mixing was noted and an aliquot was pipetted into a standard nuclear magnetic resonance (NMR) tube. ^1H-NMR spectra were obtained on a Varian T-60A spectrometer over the next 70 min (Varian Associates, Inc., Palo Alto, CA). In all, 17 scans were taken with integration of the portion of the spectrum that included the resonances for the three methyl protons and the α-proton. The sample was mixed and was maintained at 25°C throughout the experiment. The pH of the mixture did not change over the 70 min (pH 7.5).

RESULTS

Mass spectra of labeled synthesized acids

Mass spectra of TMS derivatives of the labeled acids synthesized for this study are shown in Fig. 1.

Fate of labeled acids in vivo

$[^2H_3]$-$[^{13}C_2]$MMA. The urines collected following the administration of $[^2H_3]$-$[^{13}C_2]$MMA to rats were analyzed for isotope content in MMA. Table I summarizes the excretion data for unlabeled MMA, $[^2H_3]$-$[^{13}C_2]$-, $[^2H_3]$-$[^{13}C_1]$-, and $[^2H_3]$-$[^{13}C_0]$MMA, which represent, respectively, endogenous MMA, the unchanged administered substrate, and the administered substrate that has undergone exchange of one and both ^{13}C-labeled carboxyl groups. It may be seen that the unchanged administered loads were largely excreted within 24 h by all four rats. Significant amounts of MMA derived from the load (identified by the residual $^2H_3$ label) that had been depleted of one or both ^{13}C labels in the carboxyl groups were coexcreted. For rats 1, 2, and 4, the rate of excretion of $[^2H_3]$-$[^{13}C_0]$MMA did not slow as quickly as the excretion rate for the unchanged acid in going from the first to the second collection periods. This apparent delay in excretion for the former acid may relate to labeled MMA that is accumulated by mitochondria. In rat 4, endogenous MMA in the first two urine samples after the load was a small negative quantity after being corrected for unlabeled MMA in the labeled acid load. This finding may reflect preferential metabolism of unlabeled acid over labeled acid. A large primary kinetic isotope effect is known for this enzyme/substrate combination (13).

$[^2H_5]$-$[^{13}C_2]$EMA. Unlabeled EMA and $[^2H_5]$-$[^{13}C_2]$-, $[^2H_5]$-$[^{13}C_1]$-, and $[^2H_5]$-$[^{13}C_0]$EMA monitored in the urine collections represent, respectively, the endogenous acid, the administered substrate, and the substrate having one and both carboxyl groups replaced with endogenous $^{12}CO_2$. Table II summarizes these findings. Exchange of two labels is clearly evident and is represented by the $[^2H_5]$-$[^{13}C_0]$EMA excreted. In all rats, the administered substrate was largely excreted in the first 24 h; excretion of $[^2H_5]$-$[^{13}C_1]$EMA followed the same pattern. The rate of $[^2H_5]$-$[^{13}C_0]$EMA excretion appeared somewhat delayed relative to that for the other two labeled acids.

In other experiments, MMA and EMA isolated from rat urine after large or small doses of $[^2H_3]$MMA and $[^2H_5]$EMA retained all of their initial deuterium labeling. Further confirmation that the label lost was ^{13}C and not ^2H was obtained by periodically monitoring the McLafferty rearrangement ion for $[^2H_5]$-$[^{13}C_2]$EMA at m/z 251 which has been shown not to contain the $[^2H_5]$ethyl group (14). Although of lower relative in-

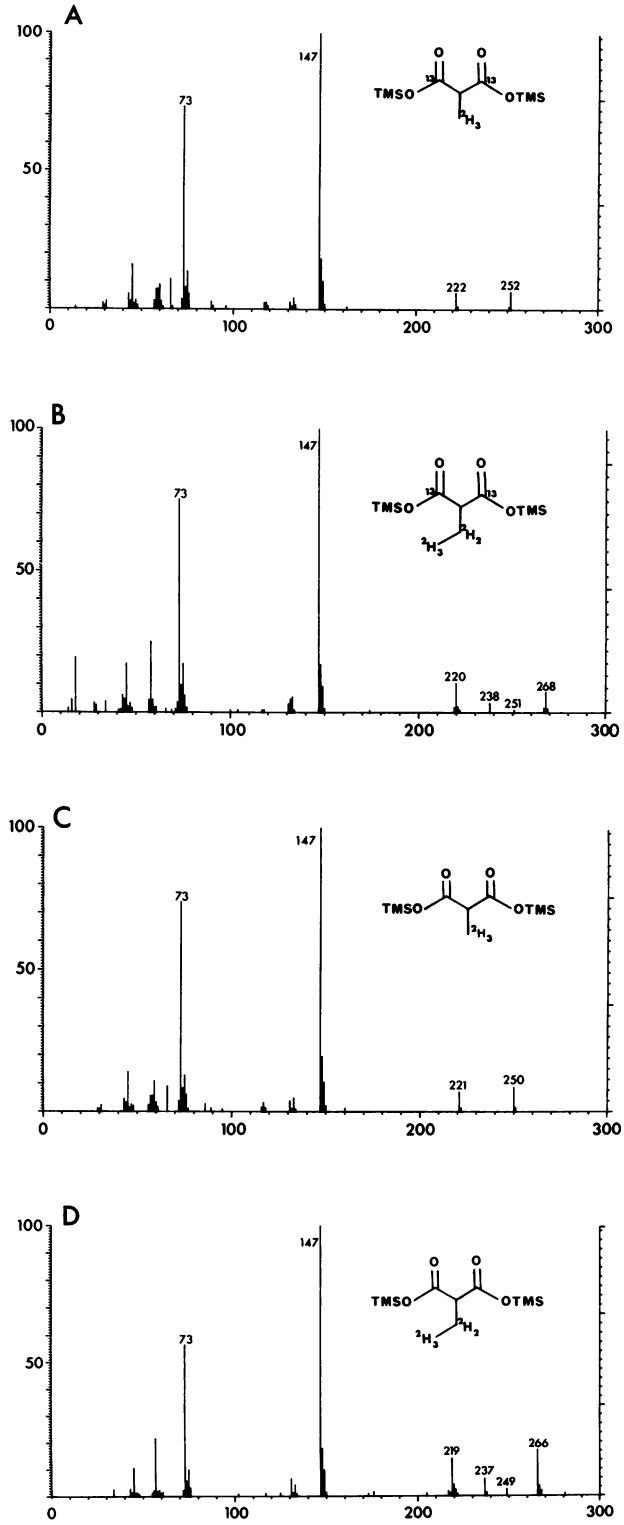


FIGURE 1 The 70-eV electron impact spectra of the trimethylsilyl derivatives of the following acids: (*A*) $[^2H_3]$-$[^{13}C_2]$MMA, (*B*) $[^2H_5]$-$[^{13}C_2]$EMA, (*C*) $[^2H_3]$MMA, (*D*) $[^2H_5]$EMA, TMSO: trimethylsilyl ester.

TABLE I

Methylmalonic Acid Excretion after Intraperitoneal Injection of [$^2H_3$]-[$^{13}C_2$]MMA to Normal Rats

Rat, weight, load	Time	Urinary MMA excretion rate*			
		Endogenous	[$^2H_3$]-[$^{13}C_2$]-	[$^2H_3$]-[$^{13}C_1$]-	[$^2H_3$]-[$^{13}C_0$]-
	h	*nmol/kg/h*			
Rat 1, 470 g, 0.52 mmol/kg	0–5	158	52,900	951	199
	5–24	17.3	134	2.96	2.11
	24–30	0.14	0.35	0.05	0.04
	30–48	2.16	0.54	0.03	0.04
Total excreted‡		1,159	267,000	4,810	1,040
Rat 2, 450 g, 0.54 mmol/kg	0–5	324	36,000	204	123
	5–24	29.4	1,620	21.5	25.5
	24–30	0.72	0.63	0.04	0.04
	30–48	0.68	0.54	0.02	0.04
Total excreted‡		2,190	211,000	1,490	1,100
Rat 3, 480 g, 0.51 mmol/kg	0–5	—§	—§	—§	—§
	5–24	39.9	14,500	127	57.0
	24–30	0.30	0.34	0.05	0.05
	30–48	2.07	0.24	0.02	0.03
Total excreted‡		797	276,000	2,410	1,080
Rat 4, 550 g, 0.52 mmol/kg	0–5	neg‖	26,300	20.9	72.1
	5–24	neg‖	1,660	20.4	12.6
	24–30	1.69	15.2	0.25	0.05
	30–48	0.51	1.37	0.04	0.03
Total excreted‡		—	163,000	495	601

* Determined as amount excreted per kilogram of body weight during collection period, divided by period length.

‡ During whole collection period, 0–48 h (nmol/kg).

§ Rat did not urinate during this period.

‖ After correction for isotopic purity, a small negative value was residual.

tensity, this ion also demonstrated the exchange of two ^{13}C-labeled carboxyl groups.

The B_{12}-deficient rat was given labeled EMA (by the gastric route) to determine whether an acquired reduction of MM-CoA mutase activity would further retard metabolic outflow of labeled substrate to the Krebs cycle and permit additional enhancement of EMA carboxyl exchange. B_{12} depletion was confirmed by measurement of serum B_{12} level (undetectable, <20 pg/ml) and in the urinary MMA level (>500 mg/g creatinine). Relative to the control rat that was offered the same load by the same route, the B_{12}-deficient rat had elevated excretion rates for all three labeled EMA species. Although two control rats offered larger loads had comparable total excretion of acids depleted in ^{13}C, the rat given the load by intraperitoneal injection had a higher initial rate of excretion of the administered acid than the rat given the load by gastric tube.

Determination by NMR of the rate of exchange of the α-hydrogen of MMA for ^2H in ^2H_2O at pH 7.5 and 25°C. Over the period of 70 min, the doublet for the three methyl protons was transformed gradually into a singlet without significant change in the overall integrated intensity. On the other hand, the integrated quartet signal due to the α-proton experienced gradual decay. Fig. 2 illustrates the first-order decay of the α-proton signal (normalized to the invariant methyl proton signal). The half-life for exchange was 28.3 min.

DISCUSSION

Our observation of the replacement of ^{13}C by ^{12}C in one of the carboxyl groups of MMA (Table I), when

TABLE II

Ethylmalonic Acid Excretion after Administration of $[^2H_5]$-$[^{13}C_2]$EMA to a Vitamin B_{12}-deficient and Normal Rats

Experiment*	Time	Urinary EMA excretion rate‡			
		Endogenous	$[^2H_5]$-$[^{13}C_2]$-	$[^2H_5]$-$[^{13}C_1]$-	$[^2H_5]$-$[^{13}C_0]$-
	h	*nmol/kg/h*			
B_{12} deficient,	0–5	516	30,300	250	46
220 g, 0.29	5–24	358	1,270	20	36
mmol/kg,	24–30	107	10	3.3	9.9
gastric	30–48	286	75	9.9	0.4
Total excreted§		15,200	177,000	1,820	991
Normal, 500 g,	0–5	439	6,320	78	29
0.29 mmol/kg,	5–24	258	849	7.2	10
gastric	24–30	76	19	2.9	2.9
	30–48	333	76	0	2.9
Total excreted§		13,500	49,200	546	410
Normal, 500 g,	0–5	94	17,800	200	47
1.15 mmol/kg,	5–24	176	23,300	310	96
gastric	24–30	171	101	15	77
	30–48	85	230	3	0.1
Total excreted§		6,400	537,000	7,140	2,090
Normal, 500 g,	0–5	227	72,300	435‖	292
1.15 mmol/kg,	5–24	76	17,300	145	103
i.p.	24–30	68	117	4.3	1.4
	30–48	44	59	1.4	0.4
Total excreted§		3,780	692,000	4,890	3,420

* Nature of rat, weight of rat, amount of acid loaded per kilogram of rat, and route of administration (gastric tube or intraperitoneal injection).

‡ Determined as amount excreted per kilogram of body weight during collection period divided by period length.

§ During whole collection period, 0–48 h (nmol/kg).

‖ Estimation because of interference of broad defocused skirt of intense m/z 268 produced by administered acid.

MMA was originally labeled with ^{13}C in both carboxyl groups, supports the view now generally held that *S*-MM-CoA achieves P-CoA carboxylase-mediated equilibrium of the free carboxyl group with endogenous CO_2. We further show that the carboxyl group esterified to CoA is also subject to decarboxylative exchange with endogenous CO_2. The known pathway of MMA metabolism however fails either to predict or allow for this last exchange (Scheme 1). We also present evidence that EMA will undergo analogous exchange during its own metabolism (Table II).

P-CoA carboxylase and acetyl-CoA carboxylase are reversible enzymes (15, 16), and both can carboxylate P-CoA and B-CoA (17, 18). Waite and Wakil (18), however, suggest that the rate of carboxylation of P-CoA and B-CoA by acetyl-CoA carboxylase is too slow to have physiological significance. It is most probable then that P-CoA carboxylase is the principal enzyme involved in the decarboxylative loss of the ^{13}C label and the recarboxylation of these alkylmalonic acids. Before this reaction, it is presumed that the administered acids are esterified to CoA.

Our studies show that one or both MM-CoA enantiomers must undergo deacylation (i.e., enzymic hydrolysis to free coenzyme A [CoASH] and the free acid) and subsequent reesterification at the other carboxyl group. The ^{13}C label, found originally in the esterified carboxyl group of *S*-MM-CoA before hydrolysis, must reappear by some mechanism in the free carboxyl group of *S*-MM-CoA formed by reesterification of the free acid. This is necessary because, for P-CoA carboxylase to remove this last label, the substrate must be *S* (that is, *S* in the conventional sense, ignoring ^{13}C priority over ^{12}C). A mechanism is proposed in Fig. 3. Central to this proposal are two assumptions: first, that free MMA can be easily esterified (and hence deesterified)

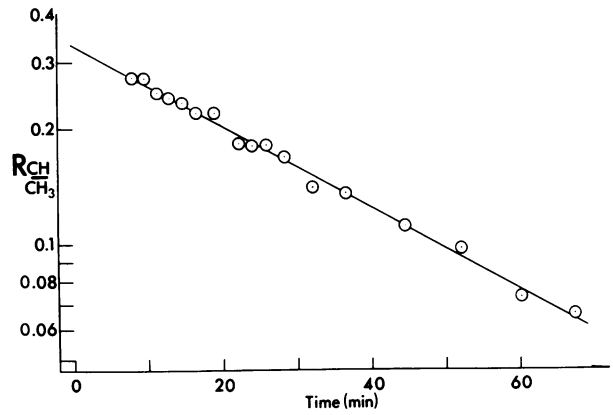


FIGURE 2 Semilogarithmic plot of the ratio of the integrated signals for the α-proton and the methyl protons of methylmalonic acid in buffered ^2H_2O (apparent pH 7.5) at 25°C vs. time. The α-proton presented as a quartet (δ = 3.20, J = 7.0 Hz) that decreased in intensity with time, and the methyl protons presented as a doublet initially (δ = 1.28, J = 7.0 Hz) that was converted to a singlet (δ = 1.28) with time. The decay in α-proton signal strength was kinetically first order ($\ln R = -0.02446T - 1.1060$; $r = -0.9973$) with a half-life of 28.3 min and an intercept at $T = 0$ calculated as $R = 0.331$. The expected ratio is 0.333 for one α-proton to three methyl protons.

with CoA; second, that reaction C (Fig. 3), the interconversion of the free *R* and *S*-1-[^{13}C]MMA, is rapid compared with the overall rate of MMA metabolism.

The first assumption is supported by our observation of metabolic loss of label from the administered malonic acids: some of the load must have been esterified and then metabolized as the CoA derivative. Further support is provided by the observation of the massive excretion of free MMA seen in methylmalonic acidemia. If MMA remained irreversibly bound to CoA, in this disorder, one would further expect to see a urinary glycine conjugate of MMA produced by the hepatic enzyme, glycine-*N*-acylase from the accumulating *R*-MM-CoA. Glycine conjugation appears to be an important detoxification mechanism in several inborn errors of metabolism, such as isovaleric acidemia (19), but not in methylmalonic acidemia.

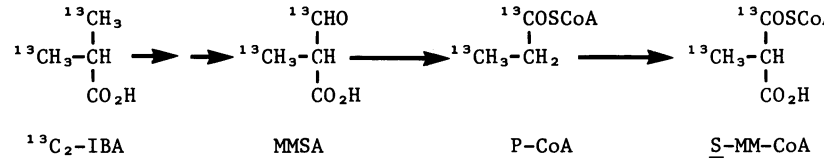
The differences reported between the ratios of liver CoA intermediates of propionate metabolism and the corresponding urinary free acids (20) can be interpreted to demonstrate the relative ease of deacylation of MM-CoA. In B_{12}-deficient rats that had elevated P-CoA and MM-CoA in liver, the ratio of P-CoA to MM-CoA was 1.8. In control rats, this ratio was ~1.0. However, the urinary free acids in B_{12}-deficient rats poorly reflect this ratio; excretion of MMA was much higher relative to propionic acid. The corresponding urinary pattern is seen in humans with methylmalonic acidemia. It is, therefore, likely that deacylation of MM-CoA is more efficient than that of P-CoA and serves as a means for elimination of MMA while sparing CoA. Deacylation to free MMA has been demonstrated in homogenates of liver, kidney, and brain in both control and B_{12}-deficient rats (21). The process, however, is not understood. Frenkel et al. (20) suggest two mechanisms to account for the elevated tissue P-CoA in B_{12}-deprived rats. The first is the reverse action of P-CoA carboxylase on the *S*-MM-CoA, particularly when it is in large concentrations. The second is the relative ease of deacylation of MM-CoA compared with P-CoA. It is probable that both mechanisms are effective in vivo.

Our NMR analysis of the rate of exchange of the α-hydrogen of MMA with ^2H addresses our second assumption that the rate of interconversion of the free acids *R*- and *S*-1-[^{13}C]MMA is metabolically significant. Exchange at physiological pH and 25°C in ^2H_2O, via the generally accepted enolic intermediate, had the expected first order (in MMA) kinetics and a half-life of 28.3 min. Since an enolic intermediate requires loss of optical activity, the actual rate of inversion of the $^{13}C_1$-labeled free acid about the α-carbon at physiological pH and 37°C cannot be slower than the observed rate of α-hydrogen equilibration with solvent water at similar pH but at 25°C. Since the serum half-life of MMA has been estimated at 3 h (22), it would appear that there is ample opportunity for the spontaneous racemization of free MMA bearing one remaining carboxyl label. Reesterification could therefore occur on either of the labeled or unlabeled carboxyl groups with roughly equal probability, thereby exposing the remaining label in the *S*-MM-CoA enantiomer to decarboxylative loss by P-CoA carboxylase (as proposed in Fig. 3).

The loadings of labeled EMA, summarized in Table II, produced overall yields of once-exchanged EMA ([$^2H_5$]-[$^{13}C_1$]) that are generally greater than the yield of twice-exchanged EMA ([$^2H_5$]-[$^{13}C_0$]). It should be noted that the excretion of the twice-exchanged acid was delayed somewhat after the smaller loads and continued into the second period (5–24 h), where it exceeded the excretion of the once-exchanged acid. In the loading study with labeled MMA (Table I), the yields of the twice-exchanged and once-exchanged acids ([$^2H_3$]-[$^{13}C_0$] and [$^2H_3$]-[$^{13}C_1$], respectively) are comparable. The fact that the carboxyl label remaining in the P-CoA intermediate is also lost suggests that hydrolysis and free MMA racemization (Fig. 3, reactions B and C, respectively) followed by reesterification to Coa may occur more rapidly than the excretion of free MMA. One possible explanation for this result is that nascent MMA and CoASH are maintained in close association with the acylase enzyme, so that escape of MMA into free solution is slow compared with the effective rate of carboxyl exchange of the reesterified

S-MM-CoA by P-CoA carboxylase. When both labeled carboxyl groups have been lost, further equilibration with endogenous $^{12}CO_2$ apparently continues; but ultimately some free MMA will be excreted, retaining its distinguishing [$^2H_3$]methyl label which is refractory to exchange. Since MMA is excreted very efficiently (23), probably little of its load and, by analogy, little of EMA also succeeds in crossing into the mitochondria before excretion.

Whereas we believe our observation of exchange of the carboxyl group bound to CoA in MM-CoA is without precedent, other workers have published related studies with labeled substrates that support this proposal. Baretz and Tanaka (24) examined urinary metabolites from rats after administration of various stable isotope-labeled isobutyrates. After feeding control and B_{12}/folate-deficient rats [3,3-$^{13}C_2$]isobutyric acid, they analyzed urinary MMA for ^{13}C content. The conventional pathway established by them suggests that this acid should be metabolized through MMSA and P-CoA to S-MM-CoA. P-CoA should be obtained with both ^{13}C labels intact as shown in Scheme 2.

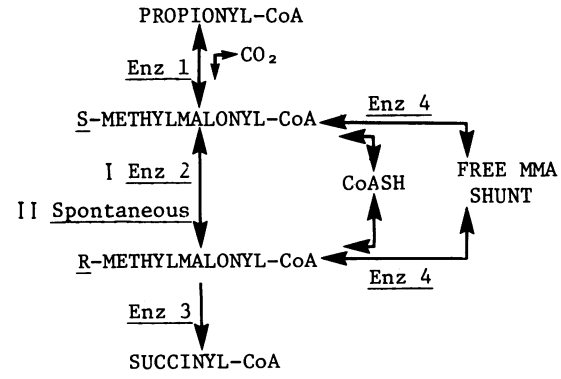


SCHEME 2

Carboxylation of P-CoA should therefore yield MM-CoA with the same ^{13}C content as the starting acid (80% $^{13}C_2$, 18% $^{13}C_1$, and 2% $^{13}C_0$). The MMA from a normal rat after the load was reported to be 35% $^{13}C_2$, 12% $^{13}C_1$, and 53% $^{13}C_0$. The ratio $^{13}C_1/(^{13}C_2 + ^{13}C_1)$ can be used to normalize the $^{13}C_1$ label content in the administered isobutyrate and the MMA produced; this ratio should be the same for both acids, if there is no exchange of the labeled ester carbonyl in S-MM-CoA. This ratio is calculated for the isobutyrate load to be 18/(80 + 18), or 0.184. The ratio obtained for the MMA excreted by their normal rat (12/[35 + 12] = 0.255) is 39% larger than for the starting acid. The concentration of $^{13}C_1$-labeled MMA is therefore 39% larger than can be accounted for by the $^{13}C_1$ content of the isobutyrate. This same calculation applied to their B_{12}/folate-deficient rat (25/[63 + 25] = 0.284) shows that the concentration of $^{13}C_1$-MMA is 54% larger than can be expected from the label content of the isobutyrate. These relative increases in $^{13}C_1$ content must be at the expense of the label in the CoA-esterified carboxyl group. This can occur if there has been deacylation of MM-CoA to free MMA followed by reacylation of the other carboxyl group in the same manner proposed here. It is significant that their B_{12}/folate-deficient rat exhibited greater loss of ^{13}C than did the control. The deficient rat has a reduced rate of outflow from the MM-CoA pool, which would permit greater opportunity for multiple CoA transesterifications and P-CoA carboxylase-mediated decarboxylations.

Baretz et al. (25) investigated the metabolism of labeled 2-methylbutyric and butyric acids via the *R*-pathway (11) in rats to examine the relationship between ethylmalonyl semialdehyde and EMA. After [1-^{13}C]butyrate administration, excreted EMA had less label per mole than coexcreted *N*-butyrylglycine. Metabolic loss of the ^{13}C carboxyl label in [1-^{13}C]butyryl-CoA was discounted; the discrepancy could represent EMA produced from other unrelated sources, or from labeled precursor through exchange of the labeled carboxyl in B-CoA with endogenous $^{12}CO_2$ by a mechanism homologous to that depicted in Fig. 3.

We propose that reversible deacylation-reacylation of MM-CoA may function as a free MMA shunt operating in parallel with MM-CoA racemase (Scheme 3). In other words, hydrolysis of S-MM-CoA followed by reesterification of the free acid would produce a partly racemic mixture and provide *R*-MM-CoA for the mutase step. Others have published work describing nonracemase-mediated racemization of S-MM-CoA.



Enzymes
1 Propionyl-CoA Carboxylase
2 Methylmalonyl-CoA Racemase
3 Methylmalonyl-CoA Mutase
4 Acylase/Deacylase

SCHEME 3

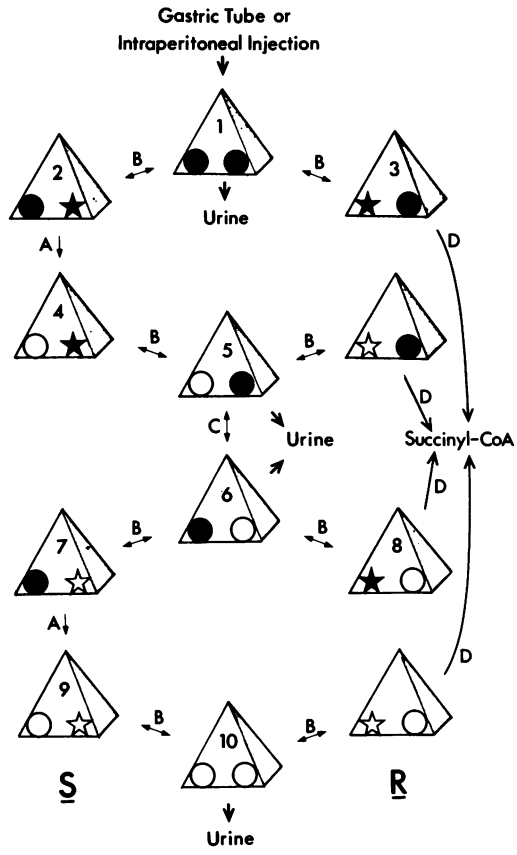


FIGURE 3 Proposed mechanism to account for the observed exchange of both ^{13}C-labeled carboxy groups in MMA. The four points of a tetrahedral model demonstrate the stereochemistry of the various reactions affecting the four functional groups attached to the 2-carbon of MMA. Adopting the convention that the upper corner is always the hydrogen atom and the rear corner is always the methyl group, the symbols represent the following: ●, $^{13}COOH$; ○, $^{12}COOH$; ★, $^{13}COSCoA$; ☆, $^{12}COSCoA$.

Reaction *A*: S-MM-CoA $\underset{^{13}CO_2}{\rightleftharpoons}$ P-CoA $\underset{^{12}CO_2}{\rightleftharpoons}$ S-MM-CoA.

Reaction *B*: Nonspecific CoASH acylase.

Reaction *C*: Spontaneous enolization of free MMA.

Reaction *D*: Mutase conversion of *R*-MM-CoA to succinyl-CoA.

The administered, labeled free acid is represented by tetrahedron *1*. It is esterified to CoA by a nonspecific acylase to give a racemic mixture of *S*- and *R*-MM-CoA, tetrahedra *2* and *3*, respectively. The left column maintains an *S* stereochemistry throughout, and the right column the *R*. Reaction sequence *A* accounts for the exchange of one ^{13}C atom, giving *4*. Deacylation (reaction *B*) yields the free acid *5*, which is optically active by virtue of having one ^{13}C-labeled carboxyl group. This acid can spontaneously enolize in solution (reaction *C*) to give the racemic mixture *5* and *6*, which can either be excreted or reesterified. The *R* free acid, *6*, on esterification to CoA yields a mixture of *7* and *8*. Ester *7* exchanges the second $^{13}COOH$ by reaction *A* to yield *9*, which by reaction *B* yields the free acid *10*. Acid *10* can leave the cell and be found in the urine, as can *5* and *6*. At all times *R*-MM-CoA can be converted by the mutase reaction *D* to succinyl-CoA and lost to the Krebs cycle. As can be seen, this mechanism provides a nonracemase-mediated path for racemization of MM-CoA. Not represented here for the sake of clarity is spontaneous racemization which can slowly interconvert *R* and *S*-MM-CoA (e.g., *2* ↔ *3* and *4* ↔ *8*).

Mazumder et al. (26) demonstrated that *S*-MM-CoA produced by crystalline P-CoA decarboxylase from P-CoA could be slowly racemized; aliquots of the preparation of increasing age were found to give larger yields of succinyl-CoA on treatment with purified mutase. They also studied enzyme dependence for conversion of a mixture of 1- and 4-[^{14}C]succinyl-CoA to P-CoA and $^{14}CO_2$ in the presence of mutase, racemase, and P-CoA carboxylase (26). Even in the absence of racemase, some $^{14}CO_2$ was liberated, possibly through spontaneous racemization of *S*-MM-CoA. The authors concluded further that there was no intermolecular exchange of CoA between ^{14}C-labeled MMA and unlabeled racemic MM-CoA in their in vitro system, and thus dismissed this as a mechanism for racemizing *S*-MM-CoA. However, an acylase that is not present in the purified in vitro preparation might achieve a nonracemase-mediated racemization in vivo.

Overath et al. (27) showed that 89% of the succinyl-CoA formed from racemic MM-CoA in the presence of hepatic racemase and mutase incorporated ^{3}H-labeling from $^{3}H_2O$ added to the incubation mixture, and 1% was labeled when the racemase was absent. This slow incorporation of ^{3}H into succinyl-CoA in the absence of racemase also suggests that MM-CoA can spontaneously racemize, presumably through an enolic intermediate. They further showed that the model compound, *S*-methylmalonyl-*N*-succinyl, cysteamine, spontaneously exchanges the α-proton for deuterium at pH 7.5 in $^{2}H_2O$ at 2% of the rate obtained when racemase was present. Hegre et al. (28) demonstrated that *S*-[1-^{14}C]MM-CoA incubated with mutase produced succinyl-CoA labeled in the 1- and 4-carbons in the ratio 86:14, respectively, while *S*[3-^{14}C]MM-CoA yielded the ratio 29:71. Scrambling of the label in succinyl-CoA through the action of succinyl-CoA deacylase was suggested. These results are also consistent with a deacylase-mediated inter- or intramolecular transfer of CoA to the other carboxyl group that may operate in vivo as a racemizing mechanism for MM-CoA. Racemase-independent racemization could occur by two mechanisms: option I, deacylase-mediated hydrolysis to the free acid and CoASH, followed by random reesterification with CoASH to either carboxyl group; option II, enolization of MM-CoA and consequent loss of chirality.

Accordingly, we propose that the propionate pathway for conversion to succinate is accommodated by both spontaneous racemization and the appearance of free

MMA as shown in Scheme 3. Formation of free acid may not be obligatory and would depend upon factors such as the ratio of the effective racemization rate to the rate of the mutase-catalyzed conversion of *R*-MM-CoA, as well as the rate of input of propionate substrate. The hydrolytic mechanism will spare CoA and facilitate excretion of large amounts of free MMA in the mutase-deficient phenotype and EMA in the case of multiple dehydrogenase defects. This mechanism is important in this last disorder, because EM-CoA is not the preferred substrate for a competent mutase enzyme (6).

Finally, we must ask whether both these mechanisms are sufficient to offset a hypothetical racemase deficiency in vivo.

Only one case of racemase defect has been reported (7). Subsequent studies of fibroblast enzymes from this patient confirmed mutase apoenzyme deficiency instead of racemase deficiency (8). More than 100 patients have been reported with isolated methylmalonic acidemia; none has had a racemase defect. There are various interpretations for the preponderance of mutase-deficient MMAuria: (*a*) racemase deficiency is a lethal defect in the fetus; (*b*) it is a benign disorder that escapes detection because of the free MMA shunt; (*c*) mutations do not occur at a racemase locus; (*d*) there is no racemase locus. The first and third proposals await evidence; the fourth seems unlikely because of evidence for a racemase protein (26); the second is favored by our findings reported here.

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