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The Inhibition by Methylmalonic Acid of Malate Transport by the Dicarboxylate Carrier in Rat Liver Mitochondria

A POSSIBLE EXPLANATION FOR HYPOGLYCEMIA IN METHYLMALONIC ACIDURIA

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ABSTRACT We report the effects of methylmalonic acid (MMA) on the mitochondrial transport systems for malate, α-oxoglutarate, and isocitrate. MMA is shown to be a substrate for all three carrier systems, and an inhibitor of the malate-phosphate exchange carrier. The effects of MMA on the metabolism of malate, oxoglutarate, and isocitrate by rat liver mitochondria are demonstrated to be mediated by the influence of MMA on the transport step. A hypothesis regarding the metabolic impairments responsible for hypoglycemia and ketonemia in methylmalonic aciduria is formulated in relation to these findings.

INTRODUCTION

Methylmalonic aciduria has been observed in patients whose tissues are low in methylmalonyl CoA mutase (E.C. 5.4.99.2) activity (1–8), and in organisms rendered deficient in vitamin B_{12} (9–12). During these states, there is a tendency for hypoglycemia and ketoacidosis, which is exacerbated after the administration of propionate or one of its precursors (1).

Increased hepatic concentrations of methylmalonyl CoA (MMCoA)[1] could inhibit gluconeogenesis by decreasing pyruvate carboxylase activity (13) (Scheme 1). Utter and coworkers (13) have demonstrated that acetyl CoA or propionyl CoA is required for activation of the enzyme which catalyzes the conversion of pyruvate to oxaloacetate. In contrast, malonyl CoA and MMCoA competitively inhibit this activation (13). Since hepatic concentrations of MMCoA have not yet been measured, the physiological significance of the inhibition of pyruvate carboxylase by MMCoA cannot be directly assessed. The high K'_i for MMCoA (0.43 mM) in the pyruvate carboxylase reaction, and the relatively low K'_a for acetyl CoA and propionyl CoA (< 50 μM and 100 μM, respectively, at physiological pH [13]) do not favor the likelihood of a simple control of the pyruvate carboxylase reaction by these competitive intermediates.

An alternate site for regulation of gluconeogenesis is the transport of malate from mitochondria to the cytoplasmic compartment (14, 15). Butylmalonate, an inhibitor of the malate-phosphate exchange reaction across the mitochondrial membranes (16), has recently been shown to inhibit gluconeogenesis from pyruvate in perfused livers (17). It appeared possible that methylmalonate, which accumulates when MMCoA cannot be readily converted to succinyl CoA, might inhibit malate transport across mitochondrial membranes, just as do other malonate derivatives such as butylmalonate (16) and pentylmalonate (18).

In this report, we show that methylmalonic acid (MMA) does inhibit the transport of L-malate across rat liver mitochondrial membranes, and that MMA itself may be transported by all of the three carrier systems for malate previously detailed by Robinson, Williams, Halperin, and Leznoff (18). In addition, we offer a hypothesis for the general mechanisms of hypo-

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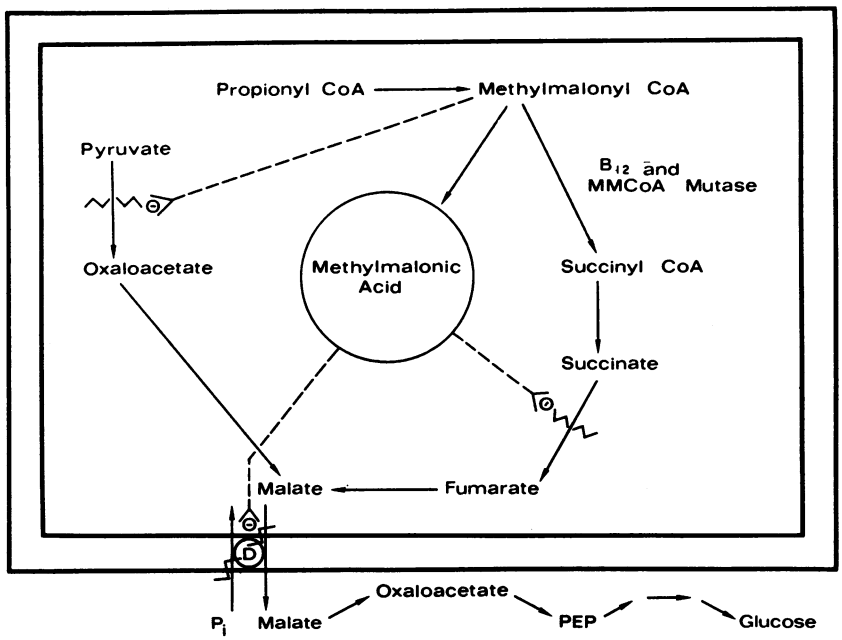
[1] *Abbreviations used in this paper:* MMA, methylmalonic acid; MMCoA, methylmalonyl coenzyme A; EGTA, ethylene glycol bis(β-aminoethyl ether)-tetraacetic acid; FCCP, carbonyl-cyanide-p-trifluoro-methoxyphenyl hydrazine.



SCHEME 1 Possible sites of inhibition of gluconeogenesis when methylmalonyl CoA mutase activity is impaired. The rectangular box represents a liver mitochondrion suspended in cytoplasm, and the circle labeled "D" between membranes represents the dicarboxylate transport system. Sites of inhibition are indicated by a wavy line. The series of arrows after PEP (phosphoenolpyruvate) indicates that a number of reactions is required for the formation of glucose. Other abbreviations include "B_{12}," for the cobamide coenzyme which is a derivative of vitamin B_{12}, and MMCoA mutase for methylmalonyl CoA mutase. The racemase which converts MMCoA ("a" form) to MMCoA ("b" form) is not shown in the scheme, since available evidence indicates no regulatory control at this site (1,7).

glycemia associated with a deficiency of MMCoA mutase activity.

METHODS

Rats (150 g Wistar, obtained from Microbiological Associates, Inc, Bethesda, Md.) were allowed free access to Purina Rat Chow before sacrifice. Liver mitochondria were isolated by conventional methods (19) in a medium containing 0.25 M sucrose, 5.0 mM Tris-HCl, and 1 mM ethylene glycol bis(β-aminoethyl ether)-tetraacetic acid (EGTA), pH 7.4. The preparation was resuspended to give a final protein concentration of 20 mg/ml. Mitochondrial protein was determined colorimetrically (20). Concentrations of agents used in all studies appear in figures or text.

Respiration was measured polarigraphically (21). The ammonium swelling studies were performed as described by Chappell and Haarhoff (22), and the methods of Robinson et al. (19) were employed to determine malate-^{14}C and citrate-^{14}C exchanges.

Carbonyl-cyanide-p-trifluoro-methoxyphenyl hydrazine (FCCP) was provided by Dr. G. R. Williams, Department of Biochemistry, University of Toronto. Isocitrate, oxoglutarate, citric acid, antimycin A, rotenone, and fumaric acid were obtained from Sigma Chemical Co., (St. Louis, Mo.). 2-Methylmalonic acid (MMA) and 2-pentylmalonate were obtained from K & K Labs, Inc. (Plainview, N. Y.), and L-malate from Eastman Organic Chemicals, Eastman Kodak Co, (Rochester, N. Y.).

RESULTS

Low concentrations of isocitrate or oxoglutarate do not increase mitochondrial oxygen consumption significantly unless L-malate is present (Fig. 1A) (14). With isocitrate or oxoglutarate as substrates, addition of MMA was able to substitute partially for malate (Fig. 1B). MMA did not inhibit the malate-induced stimulation of oxygen consumption by mitochondria incubated with isocitrate (Fig. 1A). In contrast, MMA inhibited the rate of malate-stimulated respiration of mitochondria incubated with oxoglutarate. This inhibition (Fig. 1A) may be analogous to the well-known inhibition of succinic dehydrogenase activity by malonate. Data in Fig. 1 suggest that MMA can enter the mitochondrion (presumably on the dicarboxylate carrier), and exchange on either the tricarboxylate or oxoglutarate carrier (Scheme 2).

The ammonium swelling technique was used to investigate the effect of MMA on dicarboxylate transport independent of oxidation (Fig. 2). Mitochondria, in-

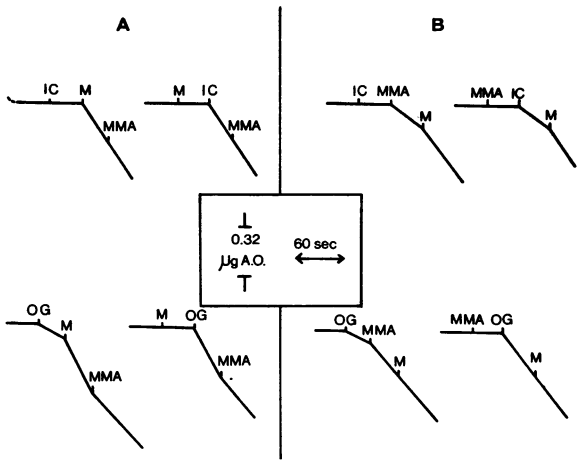
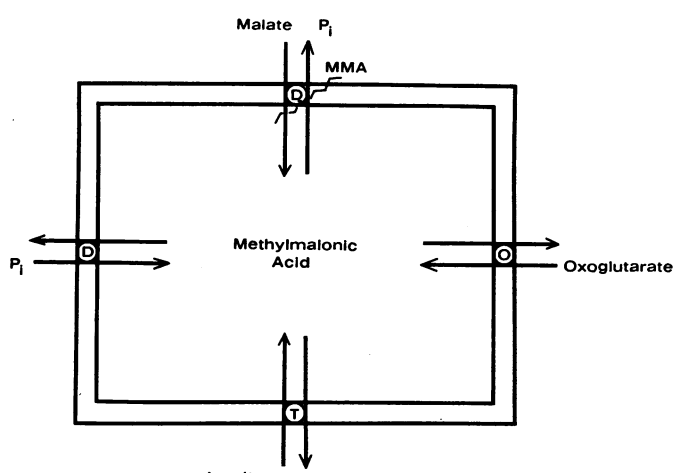


FIGURE 1 Polarigraphic measurements of oxygen consumption by rat liver mitochondria showing the stimulation of isocitrate and oxoglutarate oxidation by MMA. Rat liver mitochondria (2–5 mg protein) were suspended in 2.0 ml incubation medium containing 125 mM KCl, 20 mM Tris-HCl pH 7.4, 1.6 mM inorganic phosphate, 2.0 mM $MgCl_2$ and 1 μM FCCP at 25°C. Endogenous substrates were oxidized before additions as noted. Final concentrations of isocitrate (IC), L-malate (M), oxoglutarate (OG), and MMA added were 0.5 mM, 0.25 mM, 2.5 mM, and 2.5 mM, respectively.

cubated with rotenone to inhibit oxidation, were suspended in isosmotic ammonium salts of either fumarate (curve 1 of Fig. 2), malate (curve 2 of Fig. 2), or malate plus fumarate (curve 3 of Fig. 2). Under these conditions, the rate of mitochondrial swelling is dependent on penetration of an anion associated with the addition of inorganic phosphate (22). With malate as the anion, the swelling rate was equally rapid at concentrations of 50 or 100 mM (Fig. 2). In contrast, with fumarate as the anion, swelling was inappreciable (curve 1 of Fig. 2). These data confirm previous observations by others (22). With MMA as the anion, swelling occurred at a slower rate than in the presence of malate (curve 4 of Fig. 2), suggesting a slower rate of mitochondrial penetration of MMA than of malate on the dicarboxylate carrier. The combination of 50 mM MMA and 50 mM malate (curve 5 of Fig. 2) resulted in a significantly slower rate of phosphate-induced swelling than that seen with 100 mM L-malate, or with the combination of 50 mM L-malate and 50 mM fumarate. These data indicate that MMA inhibited the malate-phosphate exchange when MMA was present at a concentration equal to that of malate, even though both MMA and L-malate are substrates for the same transport system.

When rat liver mitochondrial NAD(P) redox changes were followed by fluorimetry (23), a rapid oxidation occurred after the addition of the uncoupling agent FCCP (Fig. 3). After addition of the respiratory inhibitor antimycin A, L-malate alone caused a small reduction of NAD(P) (Fig. 3A). After either isocitrate or oxoglutarate was added to the system under these conditions, rapid reduction of intramitochondrial NAD(P) occurred (Fig. 3A). If either isocitrate or oxoglutarate were added alone, only slight reduction



SCHEME 2 Model for effects of MMA on transport carriers for malate in rat liver mitochondria. The transport carriers are represented as circles on the membranes of a mitochondrion, with "D" standing for the dicarboxylate transport system, "T" for the tricarboxylate transporter, and "O" for the transport system which exchanges malate for oxoglutarate. Inorganic phosphate is abbreviated "P_i." The scheme illustrates that MMA can exchange on either of the three transport systems, and it inhibits the exchange of malate with P_i. For documentation, see the text.

occurred until malate was added (Fig. 3A). These findings confirm the requirement for a dicarboxylate anion as an activator of the tricarboxylate or oxoglutarate transporting system (14).

In the absence of isocitrate or oxoglutarate, addition of MMA had no effect on the redox potential (Fig. 3B). Other data presented in Fig. 3B demonstrate that MMA could substitute for L-malate as an activator of the tricarboxylate or oxoglutarate transport systems. The stimulation of isocitrate oxidation by MMA could be prevented by incubation of mitochondria with pentylmalonate before the addition of MMA. Pentylmalonate inhibited the MMA effect by blocking MMA entry into mitochondria on the dicarboxylate carrier (data not shown).

As indicated in experiments depicted in Fig. 3A, the addition of 0.25 mM L-malate alone rapidly increased the steady state concentration of NAD(P)R to a small extent. This reduction was inhibited by MMA (Fig. 4). The rapid and nearly complete reduction of NAD(P)H

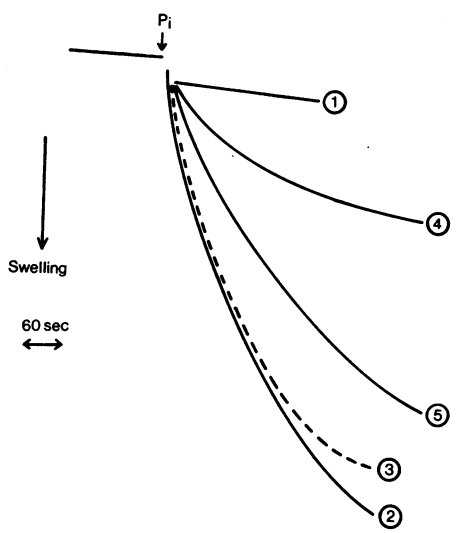


FIGURE 2 Swelling of rat liver mitochondria in isosmotic ammonium malate, fumarate, or MMA. Liver mitochondria (1–2 mg protein) were suspended in 1.0 ml of medium containing 100 mM ammonium malate, fumarate or MMA, or 50 mM concentrations of each of two of these substrates. The medium also contained 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, and 0.5 μg/ml rotenone. Addition of ammonium phosphate (P_i) was made at the indicated time to give a final concentration of 2.0 mM. Swelling was followed by recording changes in absorbance at 578 nm of the mitochondrial suspension in a 1 cm light path cell. In curve 1, 100 mM ammonium fumarate was present; in curve 2, 100 mM ammonium malate was present; in curve 3, 50 mM ammonium fumarate and 50 mM ammonium malate were present; in curve 4, 100 mM ammonium MMA was present; and in curve 5, 50 mM ammonium MMA and 50 mM ammonium malate were present in the incubation medium.

TABLE I

Effect of Methylmalonic Acid on Citrate-^{14}C Exchanges in Rat Liver Mitochondria

Additions	Supernatant fraction (corrected cpm)*	Percentage exchange‡
Malate	3,286	15.3
MMA	1,414	6.59
Malate + MMA	4,500	21.0

Mitochondria (8 mg protein) were preloaded with citrate-^{14}C and isocitrate-^{14}C according to procedures of Robinson et al. (19). Mitochondria were added to 1 ml incubation medium containing 125 mM KCl, 20 mM Tris-HCl, pH 7.4 at 10°C. Final concentrations of chemicals listed were 1 mM. After 2 min, the mitochondria were separated by centrifugation, and radioactivity was determined in the supernatant fraction and in the total supernatant plus pellet.

* Corrected counts per minute in supernatant fractions indicates counts per minute in the presence of the indicated substrate minus the counts per minute in the absence of substrate. In the experiment given, the counts per minute of citrate and isocitrate in the supernatant fraction after incubation in the absence of substrate was 4,295. Results shown are representative of five separate experiments, and counts per minute presented are means of triplicate observations from a single experiment.

‡ Percentage exchange was calculated as follows:

$$\frac{\text{Corrected counts per minute in supernatant fraction}}{\text{Total counts per minute in supernatant plus pellet}} \times 100.$$

which ordinarily followed isocitrate addition in the presence of L-malate was also decreased by MMA (Fig. 4), with a greater inhibition obtained with 5 mM MMA than with 2.5 mM MMA (compare curves 2 and 3 with curve 1 of Fig. 4).

The effects of MMA on citrate-^{14}C exchange in mitochondria incubated with antimycin A and rotenone are summarized in Table I. These data demonstrate that MMA exchanged for citrate at a rate which was 40% of that observed with malate. Since the exchange occurred in mitochondria whose respiration was inhibited, it may be concluded that MMA increased citrate transport independent of oxidation. It should be noted that MMA and malate are both substrates for the tricarboxylate carrier, and that MMA does not inhibit this transporter (Fig. 1). The additive effects of nonsaturating concentrations of the two compounds on the exit of citrate and isocitrate from preloaded mitochondria (Table I) corroborated these results.

DISCUSSION

Data presented in Fig. 2 demonstrate that malate transport into mitochondria was inhibited by MMA, and this most probably accounts for the inhibition of malate oxi-

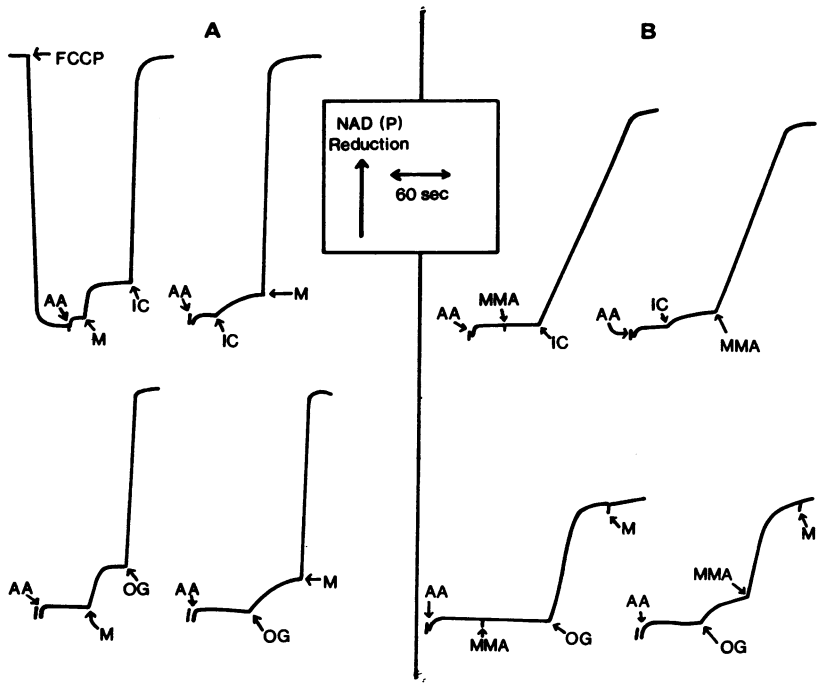


FIGURE 3 Mitochondrial redox changes showing the effects of MMA on isocitrate and oxoglutarate oxidation. Rat liver mitochondria (2 mg protein) were suspended in 1.0 ml medium at 30°C containing 125 mM KCl, 20 mM Tris-HCl at pH 7.4, 2 mM $MgCl_2$, and 2 mM inorganic phosphate. Pyridine nucleotide oxidation/reduction changes were followed fluorimetrically (23). The initial experimental manipulations shown in the upper part of experiment. A are not repeated for the other tracings, because initial procedures used were the same for the remainder of the experiments. Additions were as follows: 1 μM FCCP, 0.3 μg/ml antimycin A (AA), 0.25 mM L-malate (M), 1.0 mM isocitrate (IC), 5 mM oxoglutarate (OG), and 5 mM MMA, as indicated on the tracings.

dation by MMA depicted in Fig. 4. We assume that these findings on malatae entry are applicable to malate efflux from mitochondria, since it appears likely that the carrier properties for anion transporters are similar on both sides of the mitochondrial membrane. When we measured the efflux of L-malate-^{14}C previously loaded into mitochondria, we were unable to obtain an inhibition by MMA (data not shown). This result was anticipated because malate exchange with MMA occurs on both the tricarboxylate and oxyglutarate carriers, neither of which is inhibited by MMA (Scheme 2). Thus, even though the dicarboxylate carrier was inhibited by MMA (Fig. 2), the exit of L-malate-^{14}C readily occurred via the remaining carrier systems. In order to test the effects of MMA on malate exit via the dicarboxylate carrier from preloaded mitochondria, it would be necessary to use other inhibitors which specifically blocked only the tricarboxylate and oxyglutarate transport systems. Although a suitable inhibitor exists (1,2,3-benzene tricarboxylate) for the tricarboxylate carrier (19), none is available for the oxyglutarate carrier at this time.

The observed inhibition by MMA of the mitochondrial dicarboxylate transport system could partially account for the hypoglycemia associated with patients having methylmalonic aciduria. As indicated in Scheme 1, regulatory reactions for gluconeogenesis which could be inhibited by MMA or methylmalonyl CoA include several of the steps responsible for the conversion of intramitochondrial substrates to extramitochondrial oxaloacetate, leading to the generation of phosphoenolpyruvate and hence to glucose.

Williamson, Anderson, and Browning (17) have reported that butylmalonate inhibited gluconeogenesis from pyruvate by perfused livers, presumably by inhibiting the mitochondrial exit of malate generated from pyruvate via oxaloacetate formation. Weideman Hems, Williams, Spray, and Krebs (24) recently demonstrated that MMA (5 mM) inhibited gluconeogenesis from propionate by rat kidney cortex slices. The conversion of

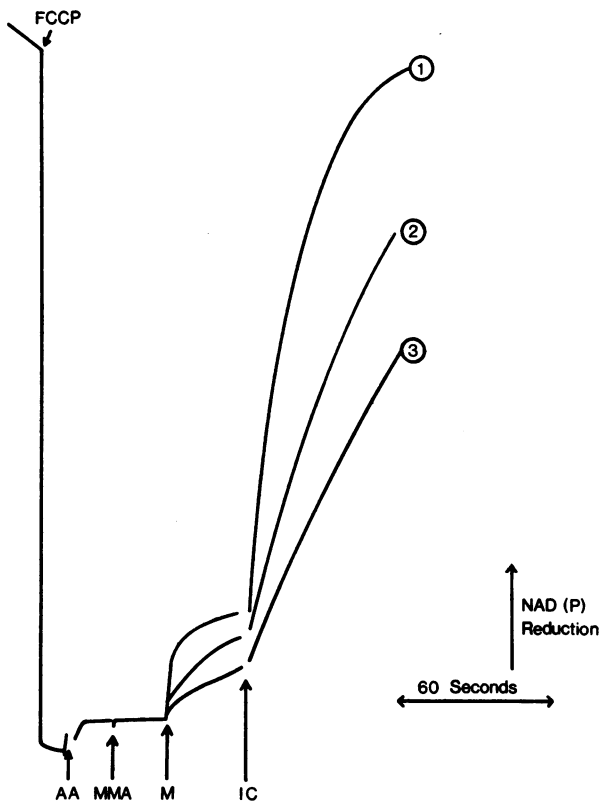


FIGURE 4 Mitochondrial redox changes showing the inhibition by MMA of malate and isocitrate oxidation. The details of incubation were the same as those described in Fig. 3. Other additions were as follows: 1 μM FCCP, 0.3 μg/ml antimycin A (AA), 0.25 mM L-malate (M), and 1.0 mM isocitrate (IC). Curve 1 represents the control incubated in the absence of MMA, whereas curves 2 and 3 show data in the presence of 2.5 and 5.0 mM MMA, respectively.

propionate to malate bypasses the pyruvate carboxylase reaction, but it remains possible that MMA could have inhibited gluconeogenesis either by blocking succinic dehydrogenase, malate exit, or both (Scheme 1). Assuming that similar effects are obtained in liver (17) as in kidney (24), it follows that accumulation of MMA in patients having methylmalonic aciduria would inhibit gluconeogenesis by inhibiting reactions shown in Scheme 1. The plasma concentration of MMA in patients is around 1–2 mM (1, 6), and it could reasonably be expected to be appreciably higher within liver cells of patients lacking MMCoA mutase atcivity. From data presented in the Results section, it appears that these levels of MMA would inhibit the malate transport system.

In patients with hypoglycemia, ketonemia, and methylmalonic aciduria, it may be predicted from data presented in Figs. 1–3 that treatment with sodium citrate plus glucose may be more beneficial than glucose alone. The citrate would enter liver mitochondria in exchange for MMA, thus allowing a more rapid escape of MMA from the mitochondria. The increased MMA levels in the cytosol would facilitate more rapid diffusion to the extracellular space and eventual excretion in the urine. This should permit a faster restoration of normal hepatic levels of MMA and MMCoA, thereby relieving the inhibition of malate exist and gluconeogenesis.

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