Neurodegeneration in Methylmalonic Aciduria Involves Inhibition of Complex II and the Tricarboxylic Acid Cycle, and Synergistically Acting Excitotoxicity*

Jürgen G. Okun‡§%, Friederike Hörster‡%, Lilla M. Farkas**, Patrik Feyh‡, Angela Hinz‡, Sven Sauer‡, Georg F. Hoffmann‡, Klaus Unsicker**, Ertan Mayatepek‡, and Stefan Kölker‡‡

From the ‡Department of Pediatrics, Division of Metabolic and Endocrine Diseases, Im Neuenheimer Feld 150, Federal Republic of Germany and the **Department of Neuroanatomy and Interdisciplinary Center for Neurosciences, Im Neuenheimer Feld 307, University of Heidelberg, D-69120 Heidelberg, Federal Republic of Germany

Received for publication, January 30, 2002, and in revised form, February 13, 2002
Published, JBC Papers in Press, February 14, 2002, DOI 10.1074/jbc.M200997200

Methylmalonic acidurias are biochemically characterized by an accumulation of methylmalonate (MMA) and alternative metabolites. There is growing evidence for basal ganglia degeneration in these patients. The pathomechanisms involved are still unknown, a contribution of toxic organic acids, in particular MMA, has been suggested. Here we report that MMA induces neuronal damage in cultures of embryonic rat striatal cells at a concentration range encountered in affected patients. MMA-induced cell damage was reduced by ionotropic glutamate receptor antagonists, antioxidants, and succinate. These results suggest the involvement of secondary excitotoxic mechanisms in MMA-induced cell damage. MMA has been implicated in inhibition of respiratory chain complex II. However, MMA failed to inhibit complex II activity in submitochondrial particles from bovine heart. To unravel the mechanism underlying neuronal MMA toxicity, we investigated the formation of intracellular metabolites in MMA-loaded striatal neurons. There was a time-dependent intracellular increase in malonate, an inhibitor of complex II, and 2-methylcitrate, a compound with multiple inhibitory effects on the tricarboxylic acid cycle, suggesting their putative implication in MMA neurotoxicity. We propose that neuropathogenesis of methylmalonic aciduria may involve an inhibition of complex II and the tricarboxylic acid cycle by accumulating toxic organic acids, and synergistic secondary excitotoxic mechanisms.

Methylmalonic acidurias are biochemically characterized by an accumulation of methylmalonate (MMA)1 in tissue and body fluids. They are caused by an inherited deficiency of the mitochondrial enzyme methylmalonyl-CoA mutase (MCM, EC 5.4.99.2) or by defects in the synthesis of 5′-deoxyadenosylcobalamin, the cofactor of MCM (2, 3). Deficient MCM, which physiologically catalyzes the reaction of methylmalonyl-CoA to succinyl-CoA, results in an accumulation of MMA and, due to alternative pathways, propionate, 3-hydroxypropionate, and 2-methylcitrate (1).

Although the etiology of methylmalonic aciduria is heterogeneous, the clinical presentation of affected patients is similar. At disease onset, lethargy, failure to thrive, recurrent vomiting, dehydration, respiratory distress, muscular hypotonia, hepatomegaly, and coma are common clinical features, an impaired psychomotor development an important sequel. Frequent laboratory findings are metabolic acidosis, ketonuria/uria, hyperammonemia, hyperglycinemia/uria, and hypoglycemia (4). Despite the improvement of therapy during the last 20 years, the overall outcome of these patients remains disappointing, e.g. there is growing evidence for the development of long-term neurological deficits (5). Neuroimaging has revealed a symmetric degeneration of the basal ganglia, in particular in globus pallidus (6). Histopathology shows severe necrosis in the globus pallidus as well as a mild spongiosis of the subthalamic nucleus, mammillary bodies, and internal capsule (7). It has been suggested that these pathological changes are caused by “metabolic stroke” due to accumulation of toxic organic acids (8). A recent study has supported this hypothesis, demonstrating restricted diffusion and elevated amounts of lactate in globus pallidus of affected patients signaling mitochondrial dysfunction (9). Notably, symmetrical lesion in the basal ganglia are also found in patients with inherited complex II deficiency (10).

The neuropathogenesis of methylmalonic acidurias remains unclear but MMA, which reaches millimolar concentrations in body fluids and brain tissue during acute metabolic crises, was recently suggested to act as an endogenous toxic metabolite, mediating neuronal damage via inhibition of mitochondrial energy metabolism (11). In particular, MMA-induced inhibition of complex II (succinate dehydrogenase, EC 1.3.99.1), which is imparted in the TCA cycle and the mitochondrial respiratory chain, has become a focus of interest (12). MMA induces cell damage in different neuronal culture systems (13, 14). Furthermore, intrastriatal injections of MMA evokes rotational behavior, seizures, and striatal lesions in rats (15, 16). MMA-induced

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Both authors contributed equally to the study.

‡ To whom correspondence should be addressed: University Children’s Hospital, Div. of Metabolic and Endocrine Diseases, Im Neuenheimer Feld 150, D-69120 Heidelberg, Germany. Tel.: 49-6221-561716; Fax: 49-6221-565565; E-mail: Juergen_Okun@med.uni-heidelberg.de.

§ Supported by University of Heidelberg Junior Grant 12/2001.

¶ Supported by Deutsche Forschungsgemeinschaft Grant KO 2010/1-1.

The abbreviations used are: MMA, methylmalonate; MCM, methylmalonyl-CoA mutase; TCA, tricarboxylic acid cycle; NMDA, N-methyl-D-aspartate; MA, malonate; DIV, day in vitro; GluR, glutamate receptor; SMFs, submitochondrial particles from bovine heart; PMS, phenazine methosulfate; DBQ, decylubiquinone; GC-MS, gas chromatography-mass spectrometry; ANOVA, analysis of variance; TTF, theonyltrifluoroacetone; PA, propionate; MCA, 2-methylcitrate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt; CAPS, 3-cyclo-hexylamino)propanesulfonic acid; MES, 4-morpholinepropanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EPPS, 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.
changes can be prevented by succinate, N-methyl-D-aspartate (NMDA) receptor blockade, and antioxidants (15, 17). Consequently, MMA has been suggested to induce secondary (indirect) excitotoxicity (18), in analogy to the complex II inhibitors malonate (MA) (19) and 3-nitropropionate (20).

However, previous studies have failed to clarify whether MMA inhibits complex II directly or indirectly via intracellular formation of other metabolites. In the present study, we provide evidence that neuropathogenesis of methylmalonic aciduria may involve an inhibition of complex II and the TCA cycle by accumulating toxic organic acids, and synergistic secondary excitotoxic mechanisms.

**EXPERIMENTAL PROCEDURES**

**Primary Striatal Cell Cultures from Embryonic (E18) Rats—**Primary striatal cell cultures were prepared from embryonic (E18) rats as previously described (21). Briefly, pregnant Han-Wistar rats were killed by CO2 asphyxia and embryos were collected in ice-cold Ca2+- and Mg2+-free Hanks’ balanced salt solution (Invitrogen, Eggenstein, Germany). Striatata were dissociated by 0.25% trypsin and subsequently triturated with fire-polished pasteur pipettes. Cells in the supernatant were collected by centrifugation and resuspended in serum-free Neurobasal medium containing the B27 supplement (22), 100 units/ml penicillin, streptomycin, and 2 mM glutamine (all obtained from Invitrogen). Cells were seeded at a density of 150,000 cells/cm2 onto 24-well plates (Costar, Bödenheim, Germany) coated with polyornithine and laminin (Invitrogen). Cultures were maintained in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. On the 2nd day in vitro (DIV), cultures were treated with 1 μM cytosine β-arabinofuranosid (Sigma, Deisenhofen, Germany) for 24 h to inhibit glial proliferation. Animal care followed the official governmental guidelines and was approved by the government ethics committee.

**Treatment Protocol—**Vulnerability to NMDA and MA during culture period was investigated at DIV 3, 7, 10, and 14. Neurons were incubated for 1 h with NMDA in Mg2+-free HEPES-buffered saline, containing 140 mM NaCl, 5.4 mM CaCl2, 5 mM KCl, 10 mM d-glucose, 10 mM HEPES, and 10 μM glycine in deionized water (adjusted to pH 7.4). Thereafter, HEPES-buffered saline was removed and cells were grown in B27-free Neurobasal medium for another 23 h. In sister cultures, neurons were incubated with 1 mM MA (in B27-free Neurobasal medium) for 24 h. Based on these data (Fig. LA), all subsequent experiments were performed at DIV 10. Next, striatal neurons were exposed to 0.1, 0.5, 1.0, and 2.0 mM MA (in B27-free Neurobasal medium) for 24 h in sister cultures. Further experiments, MMA (10 μM) was co-incubated with the NMDA receptor antagonist MK-801 (10 μM; Tocris, Bristol, UK), the non-NMDA receptor antagonist CNQX (50 μM; Tocris), the metabolotropic GluR antagonist L-AP3 (50 μM; Tocris), succinate (0.1–10 mM; Sigma), B27 supplement or glutamate (0.01–1 μM; Sigma) for 24 h.

**Cell Viability Assay—**Cell viability of striatal neurons was determined by the trypan blue (0.5%) or by in phosphate-buffred saline; Invitrogen) exclusion method after an incubation of 24 h with MMA or MA or 23 h after the incubation with NMDA as previously described (23). Briefly, the number of stained (non-viable) and unstained (viable) neurons were counted under a microscope (1,000 ×) and the supernatants were filtered using a syringe-driven filter unit (Millex-GV, 22 μm; Millipore, Eschborn, Germany). Subsequently, ethanol was vaporized at 65 °C by a nitrogen flow. The dried residues were dissolved by the addition of 50 μl of acetonitrile containing 0.1% trifluoroacetic acid. Subsequently, acetonitrile was evaporated at 65 °C.

**Spectrophotometric Measurements of Single Respiratory Chain Complexes**—Spectrophotometric analysis of single respiratory chain complexes I and III–V were determined in SMPs as previously described (27). Briefly, fibroblast cultures from healthy volunteers (n = 10) were maintained in Dulbecco’s modified Eagle’s medium supplemented with L-glutamine and 10% fetal calf serum (all from Invitrogen). After reaching confluence, cultures were incubated in liquid nitrogen for lyophilization. After removing the water, the white solid residue was suspended in 250 μl of ethanol. After centrifugation at 5,000 × g for 10 min, the supernatants were filtered using a syringe-driven filter unit (Millex-GV, 22 μm; Millipore, Eschborn, Germany). Subsequently, ethanol was vaporized at 65 °C by a nitrogen flow. The dried residues were dissolved by the addition of 50 μl of acetonitrile containing 0.1% trifluoroacetic acid. Subsequently, acetonitrile was evaporated at 65 °C. Subsequently, the isolated samples (1 μl) were injected into the GC-MS unit. Organic acid were quantitated by use of the applied standards.

**Mitochondrial β-Oxidation of Fatty Acids—**To investigate MMA-induced effects on the mitochondrial β-oxidation of fatty acids, human skin fibroblast cultures were prepared and the differentiation of the acylcarnitines profile was followed by tandem mass spectrometry was performed as previously described (27). Briefly, fibroblast cultures from healthy volunteers (n = 10) were maintained in Dulbecco’s modified Eagle’s medium supplemented with L-glutamine and 10% fetal calf serum (all from Invitrogen). After reaching confluence, cultures were incubated with unlabeled palmitic acid, L-carnitine, and bovine serum albumin (all from Sigma) as substrates as well as with MMA (1–10 mM) or vehicle for 96 h. Thereafter, the acylcarnitine pattern of the culture media was determined using an electrospray ionization triple quadrupole mass spectrometer (PE SCIEX API 365 LC/MS/MS system; MDS Sciex, Concord, Canada) as previously described (27).

**Data Analysis—**Data are expressed as mean ± S.E. Experiments were performed at least in triplicate, except for GC-MS analysis, which was performed in duplicate. One-way analysis of variance (ANOVA) followed by Scheffe’s test (for three or more groups) or Student’s t test (for two groups) were calculated using SPSS for Windows 10.0 software. p < 0.05 was considered significant. pH dependence of complex II activity was analyzed using the Pipslot software 5.02a.

**RESULTS**

**Vulnerability of Striatal Neurons to NMDA, Malonate, and Methylmalonate—**We first established a time course in the vulnerability of cultured striatal neurons by exposing cultures at different time points (DIV 3, 7, 10, and 14) with 1 mM MMA for 1 h or with 1 mM MA for 24 h (Fig. LA). MMA-induced cell damage determined by trypan blue exclusion increased from...
DIV 3 to DIV 10 (5–60% cell damage). Cell numbers remained stable between DIV 10 and DIV 14 (60–68%). Similarly, MA-induced neuronal damage increased from DIV 3 to DIV 14 (24–58%). However, this increase was not as pronounced as for NMDA (Fig. 1A). All subsequent experiments were performed at DIV 10. Next, we determined the effects of MMA and MA (0.1–10 mM) for 24 h in B27-free Neurobasal medium induced a concentration-dependent decrease in cell viability for both organic acids, MA revealing a more pronounced neurotoxic effect than MMA. *, p < 0.001 versus control (one-way ANOVA followed by Scheffe’s test). B, incubation of striatal rat neurons with MA or MMA (both 0.1–10 mM) for 24 h in B27-free Neurobasal medium induced a concentration-dependent decrease in cell viability for both organic acids, MA revealing a more pronounced neurotoxic effect than MMA. *, p < 0.001 versus control (one-way ANOVA followed by Scheffe’s test; n = 10); #, p < 0.05 versus malonate (Student’s t test; n = 10).

To begin to address the underlying mechanisms of MMA neurotoxicity, we studied the effects of GluR antagonists, antioxidants, and succinate. MMA (10 mM)-induced cell damage (50%, p < 0.001 versus 10 mM MMA) was reduced by MK-801 (10 μM; 15% cell damage, p < 0.001) and CNQX (50 μM; 23%, p < 0.001), but not L-AP3 (50 μM; 55%), suggesting a role for ionotropic GluRs (Fig. 2A). Antioxidant-containing B27 supplement (22) also reduced MMA-induced neuronal damage (17%, p < 0.001), indicating a contribution of reactive oxygen species (Fig. 2B). Since MMA has previously been shown to inhibit complex II activity in a competitive manner (12), we co-incubated MMA (10 mM) with succinate (0.1–10 mM) for 24 h. As shown in Fig. 2B, succinate protected against MMA toxicity at concentrations of 1–10 mM (18–11% cell damage, p < 0.001).

**Methylmalonate Does Not Directly Inhibit Complex II Activity**—To investigate whether MMA, similar to MA, directly inhibits complex II, we determined complex II activity in SMPs using DBQ or PMS as electron mediators (Table I). Complex II activity decreased slowly from DIV 3 to 10 but decreased more rapidly between DIV 10 and 14. Thus, DIV 10 was delineated as a favorable time point for the investigation of neuronal damage induced by overstimulation of ionotropic GluRs or inhibition of the respiratory chain. *, p < 0.001 versus control (one-way ANOVA followed by Scheffe’s test; n = 10).
Methylmalonate Induces Synergistic Inhibition of Energy Metabolism

Influence of succinate concentrations on complex II inhibition

Competitive inhibition of MMA, MA, and PA was investigated in the presence of different succinate concentrations in the test mixture: SMPs were activated by succinate (20 mM) before activity measurement to avoid inhibition of complex II by oxaloacetate. Note that succinate revealed a different affinity in the DBQ (K_s: 40 μM) and the PMS system (K_s: 160 μM). Neither MMA nor PA inhibited complex II at low succinate concentrations using DBQ or PMS as electron transporter, excluding a competitive inhibition of complex II by these organic acids. In contrast, MA showed a competitive mode of inhibition as previously described.

activity was not different in the presence of DBQ (V_max: 1.02 units/mg protein) or PMS (V_max: 0.95 units/mg protein). However, the DBQ system revealed a higher affinity for succinate (K_m: 57 ± 2 μM) and an enhanced inhibitory response to TTPFA (8 mM; 11 ± 1% of control) compared with PMS (K_m: 171 ± 8 μM; TTPFA: 32 ± 0.5% of control). Thus, the DBQ system is apparently more reliable to investigate complex II activity. Notably, it is accepted that DBQ resembles the natural ubiquinone chemistry in mitochondria (30).

Surprisingly, we found no evidence for an inhibition of complex II at concentrations of up to 20 mM MMA neither in the DBQ (0.5–20 mM: 107–111% of control) nor PMS systems (109–115% of control; Table I). In contrast, DBQ and PMS responded to the competitive complex II inhibitor MA, revealing a slightly better inhibitory response of the DBQ (0.5–20 mM MA: 51–3% of control activity; I_{SO} = 0.5 mM MA) than the PMS system (0.5–20 mM MA: 72–2% of control; I_{SO} = 0.75 mM MA). Decreasing the succinate concentrations (0.04–20 mM: 108–98% of control) did not unmask a competitive inhibitory effect of MMA, which might have been overseen at high succinate concentrations (Table II).

Since our data did not confirm the results of a previous study using PMS (12), we systematically investigated modulatory factors of complex II activity. Complex II activity reached a maximum at pH 7.4 (V_max of mean of n = 8 experiments): 1.05 units/mg protein), whereas pH changes dramatically reduced V_max (given as units/mg protein: pH 5, 0.03; pH 6, 0.28; pH 7, 1.02; pH 8, 0.7; pH 9, 0.11; pH 10, 0.01). pH dependence was described according to Brandt and Okun (28), revealing the following pH values: pK_A = 6.73 and pK_B = 7.85 (with DBQ as an electron mediator). To exclude that MMA solutions (adjusted to pH 7.4) caused a pH shift, we measured the pH in the test mixture before and after administration of buffered MMA (up to 20 mmol/liter). However, MMA-induced pH changes were only marginal (≤0.05) and did not influence V_max. In addition, we excluded by GC-MS that MMA spontaneously desintegrated in the test mixture (data not shown). Furthermore, the decarboxylation product of MMA, propionate (PA), did not influence complex II activity at concentrations of 1–10 mM (101–103% of control; Table I). Together, our data suggest reliability of the DBQ system, but no direct effect of MMA on complex II activity.

Spectrophotometric analysis revealed no inhibitory effect of MMA on complexes I, III, and IV. However, we found a weak inhibition of complex V by MMA (K_i: 19 mmol/liter MMA) (Table III). Furthermore, MMA (1–10 mM) did not affect the mitochondrial β-oxidation of fatty acids in skin fibroblast cultures (data not shown).

Intracellular Formation of 2-Methylcitrate and Malonate—Methylmalonoyl-CoA is predominantly metabolized to succinyl-CoA, which subsequently enters the TCA cycle, if MCM activity is intact. However, intracellular MMA accumulation like in methylmalonic aciduria was shown to let alternative catabolic pathways become more prominent, e.g. the formation of acetyl-CoA via propionyl-CoA (1). Therefore, we investigated whether MMA-induced complex II inhibition in striatal neurons was explained by intracellular formation of alternative metabolites. In fact, loading of striatal cultures with MMA (10 mM) induced an intracellular increase in MMA (0–8 h incubation: 5–111 nmol/mg of protein) and the complex II inhibitor MA (0–8 h incubation: 0.05–5 nmol/mg of protein; Fig. 3). Furthermore, we found 2-methylcitrate (MCA; 0–8 h incubation: 0–93 nmol/mg of protein; Fig. 3), an organic acid frequently found in methylmalonic and propionic acidurias (1). MCA is a condensation product of propionyl-CoA and oxaloacetate (31). Propionyl-CoA cannot be detected by GC-MS methods. We excluded that MCA inhibited complex II activity in SMPs using the above described activity measurement (1 mM MCA: 102 ± 2% of control; n = 14). Together, our results suggest that neuropathogenesis of methylmalonic aciduria may involve an inhibition of complex II and the TCA cycle by accumulating toxic organic acids.

---

### Table I

| Succinate (mM) | MA (3 mM) | MMA (10 mM) | PA (10 mM) |
|---------------|----------|-------------|------------|
| 0             | 11 ± 1   | 105 ± 1     | 105 ± 1    |
| 0.04          | 10 ± 1   | 114 ± 1     | 109 ± 1    |
| 0.16          | 15 ± 1   | 100 ± 1     | 102 ± 2    |
| 1.6           | 18 ± 1   | 95 ± 1      | 99 ± 1     |
| 20            | 25 ± 1   | 99 ± 1      | 101 ± 1    |
| 1.5 mM MA: 47 ± 1% of V_max. |
| 1.5 mM MA: 43 ± 1% of V_max. |

---

### Table II

| Succinate (mM) | MA (3 mM) | MMA (10 mM) | PA (10 mM) |
|---------------|----------|-------------|------------|
| 0             | 11 ± 1   | 105 ± 1     | 105 ± 1    |
| 0.04          | 10 ± 1   | 114 ± 1     | 109 ± 1    |
| 0.16          | 15 ± 1   | 100 ± 1     | 102 ± 2    |
| 1.6           | 18 ± 1   | 95 ± 1      | 99 ± 1     |
| 20            | 25 ± 1   | 99 ± 1      | 101 ± 1    |
| 1.5 mM MA: 47 ± 1% of V_max. |
| 1.5 mM MA: 43 ± 1% of V_max. |

---

### Table III

| Succinate (mM) | MA (3 mM) | MMA (10 mM) | PA (10 mM) |
|---------------|----------|-------------|------------|
| 0             | 11 ± 1   | 105 ± 1     | 105 ± 1    |
| 0.04          | 10 ± 1   | 114 ± 1     | 109 ± 1    |
| 0.16          | 15 ± 1   | 100 ± 1     | 102 ± 2    |
| 1.6           | 18 ± 1   | 95 ± 1      | 99 ± 1     |
| 20            | 25 ± 1   | 99 ± 1      | 101 ± 1    |
| 1.5 mM MA: 47 ± 1% of V_max. |
| 1.5 mM MA: 43 ± 1% of V_max. |
DISCUSSION

In the present study, we demonstrated: 1) that MMA induces cell damage in striatal neurons involving secondary excitotoxic mechanisms; 2) that MMA, unlike MA, does not directly inhibit complex II; and 3) that MMA neurotoxicity is mediated by intracellular formation of the competitive complex II inhibitor MA and the TCA cycle inhibitor MCA.

Is Methylmalonate an Inhibitor of Respiratory Chain Complex II?—It has previously been reported that MMA induces neuronal damage, involving ionotropic GluRs and oxidative stress (13–17). These results were confirmed in the present study. Furthermore, it has been shown that MMA impairs energy metabolism by inhibition of β-hydroxybutyrate dehydrogenase (12), mitochondrial malate shuttle (32), pyruvate carboxylase (33) and, most important, respiratory chain complex II (11, 12). Surprisingly, we found no direct inhibitory effect of MMA on complex II activity. In contrast, we demonstrated a reliable inhibition of our test system using the
well characterized complex II inhibitors malonate and TTFA. Since previous studies did not exclude that MMA might induce complex II inhibition indirectly via formation of alternative toxic metabolites, we investigated the time-dependent increase in intracellular organic acids using GC-MS. In fact, we found that MMA loading of striatal rat neurons induced an intracellular accumulation of MA and MCA. The previous demonstrations that MA competitively inhibits complex II (19) and that MCA blocks the TCA cycle (34) corroborate our notion MMA-induced cell damage is mediated via intracellular formation of these two compounds rather than by direct inactivation of complex II by MMA. This is further supported by the fact that PMS, which has been used as electron mediator in the above mentioned studies (11, 12), is more reactive than DBQ, being oxidized by O2 and H2O2 to pyocyanine (35). Thus, PMS is at risk to be unspecifically inactivated as electron mediator under certain conditions.

Methylmalonate Loading Increases Malonate and 2-Methylcitrate Intracellularly—Intracellular formation of MA after MMA loading has been suggested but not proven in a previous study (13). In fact, MMA loading was used to investigate MA toxicity in this study. The same authors speculated that the methyl group of MMA was cleaved by intracellular esterases, in analogy to the hydrolysis of acetoxymethyl esters from Ca2+-selective chelators (36). However, since esterases are able to hydrolyze methoxyl esters but are unable to cleave single methyl groups, this mechanism of MA formation seems very unlikely. In the present study, MMA concentrations increased within the first 4 h of incubation and remained more or less stable between 4 and 8 h, which would be inconsistent with the above mentioned mechanism. In contrast, we propose that MA is formed by two pathways: 1) malonyl-CoA is an intermediate in the alternative oxidation pathway of propionyl-CoA to pyruvate, which is prominent in methylmalonic and propionic acidurias (37, 38). 2) Malonyl-CoA is also formed by carboxylation of acetyl-CoA catalyzed by acetyl-CoA carboxylase, the key enzyme in endogenous fatty acid synthesis. This reaction is facilitated by the inhibition of the TCA cycle, resulting in an accumulation of acetyl-CoA, and a concomitant activation of acetyl-CoA carboxylase by MCA (34). Intracellular esterases may then hydrolyze malonyl-CoA to MA. However, since MA is detectable but only moderately elevated, we doubt that MMA-induced neurotoxicity is completely explained by intracellular formation of MA.

MCA is frequently detected in methylmalonic and propionic acidurias (39). (2S,3S)-MCA is formed by condensation of propionyl-CoA and oxaloacetate, catalyzed by citrate synthase, in analogy to the formation of citrate in the TCA cycle (31, 40).
MCA exerts several inhibitory effects on TCA cycle enzymes and on the mitochondrial citrate transporter, facilitating mitochondrial accumulation of MCA (34). Furthermore, MCA mainly contributes to propionate sensitivity of bacteria lacking the MCA cycle as detoxifying mechanism (40).

Impairment of energy metabolism can interfere with the ability of neurons to maintain normal resting membrane potential due to ATP depletion and can decrease the activity of Na+/K+-ATPases (41). It has been demonstrated that MCA loading decreases the ATP/ADP ratio in neuronal rat cultures and, concomitantly, the resting membrane potential (13). Furthermore, it has been shown that MCA decreases Na+/K+-ATPase activity (42). Consequently, membrane depolarization in general and the removal of the voltage-dependent Mg2+-block of NMDA receptors in particular, results in an unimpeaded influx of Ca2+ and Na+ into neurons in general (43, 44), and after MCA loading in particular (13). Furthermore, increased [Na+] has been suggested to up-regulate NMDA receptor activity via an enhancement of Src kinase activity (45).

It should also be considered that MCA-induced neurotoxicity might involve an increase formation of ammonia. Notably, ureagenesis in methylmalonic and propionic aciduria is disturbed due to propionyl-CoA-induced inhibition of N-acetylglutamate synthase, resulting in decreased N-acetylglutamate, the required allosteric activator of carboxymethylphosphate synthase I (46, 47). In line with this, hyperammonemia is a frequent finding in methylmalonic aciduria (1).

Neurodegeneration in Methylmalonic Aciduria Involves Inhibition of Complex II and the Tricarboxylic Acid Cycle, and Synergistically Acting Excitotoxicity, a Unifying Hypothesis—In current concepts of methylmalonic aciduria, MCA is the focus of neuropathogenesis (12), suggesting inhibition of brain energy metabolism as a central mechanism. In contrast, we demonstrated in the present study that most of these effects are likely to be indirect, involving the formation of MA and MCA. Despite these discrepancies, inhibition of complex II is still of pathogenetic interest, since MA is a classic inhibitor of this respiratory chain complex. In addition, complex II activity might be reduced by the lack of succinate in methylmalonic aciduria:succinate formation is reduced due to: 1) inherited MCM deficiency, resulting in reduced formation of succinyl-CoA; and 2) the MCA-induced decreased flux through the TCA cycle. In line with this, treatment with sodium succinate was neuroprotective in the present study and in vivo (15).

MCA has been shown to inhibit the TCA cycle (34). This effect is enhanced by the inhibitory effects of MCA on the trans mitochondrial malate shuttle (32) and pyruvate carboxylase (33), resulting in a reduced regeneration of oxaloacetate. An inhibition of the TCA cycle and a reduced formation of oxaloacetate facilitates the formation of ketone bodies from acetyl-CoA and the development of hypoglycemia due to an impairment of gluconeogenesis. In fact, ketonemia/ketonuria and hypoglycemia are characteristic laboratory findings in patients with methylmalonic aciduria (1). A synopsis is given in Fig. 4. In conclusion, impairment of energy metabolism in methylmalonic aciduria is most likely mediated by a synergistic inhibition of MCA, MA, and MCA on the TCA cycle and the mitochondrial respiratory chain.

Acknowledgments—We are grateful to U. Brandt (Department of Biochemistry I, Molecular Bioenergetics, University of Frankfurt, Germany) for the kind gift of the complex I inhibitor 2-n-decyloxylinolin-4-yl-amine. We thank J. Fey and S. Exner-Camps for excellent technical assistance, and P. Schadewaldt (German Diabetes Research Institute, Düsseldorf, Germany) for the kind gift of 2-methylcitrate.

REFERENCES

1. Fenton, W. A., Gravel, R. A., and Rosenblatt, D. S. (2001) in The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Valle,