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Methylmalonic Acid, a Biochemical Hallmark of Methylmalonic Acidurias but No Inhibitor of Mitochondrial Respiratory Chain*

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Methylmalonic acidurias are biochemically characterized by an accumulation of methylmalonic acid and alternative metabolites. An impairment of energy metabolism plays a key role in the pathophysiology of this disease, resulting in neurodegeneration of the basal ganglia and renal failure. It has become the subject of intense debates whether methylmalonic acid is the major toxin, inhibiting respiratory chain complex II. To elucidate whether methylmalonic acid is a respiratory chain inhibitor, we used spectrophotometric analysis of complex II activity in submitochondrial particles from bovine heart, radiometric analysis of ^{14}C-labeled substrates (pyruvate, malate, succinate), and analysis of ATP production in muscle from mice. Methylmalonic acid revealed no direct effects on the respiratory chain function, *i.e.* on single electron transferring complexes I-IV, ATPase, and mitochondrial transporters. However, we identified a variety of variables that must be carefully controlled to avoid an artificial inhibition of complex II activity. In summary, the study verifies our hypothesis that methylmalonic acid is not the major toxic metabolite in methylmalonic acidurias. Inhibition of respiratory chain and tricarboxylic acid cycle is most likely induced by synergistically acting alternative metabolites, in particular 2-methylcitric acid, malonic acid, and propionyl-CoA.

Methylmalonic acidurias are biochemically characterized by an accumulation of methylmalonic acid in tissues and body fluids (1). They are caused by an inherited deficiency of the mitochondrial enzyme methylmalonyl-CoA mutase (EC 5.4.99.2) or by defects in the synthesis of 5′-deoxyadenosylcobalamin, the cofactor of methylmalonyl-CoA mutase (2, 3). Although the etiology of methylmalonic acidurias 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. In addition, impaired psychomotor development is an important sequel. Despite improvement of treatment 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 (4) mostly affecting the globus pallidus (5, 6). It has been suggested that these pathological changes are caused by "metabolic stroke" due to accumulating toxic organic acids (7). A recent study has supported this hypothesis, demonstrating restricted diffusion and elevated amounts of lactate in the globus pallidus of affected patients signaling mitochondrial dysfunction (8). Notably, symmetrical lesions in globus pallidus are also found in patients with inherited complex II deficiency and other respiratory chain disorders (9).

Although a contribution of toxic organic acids to the neuropathogenesis of methylmalonic acidurias was suggested more than a decade ago, it remains unclear which is the main neurotoxic metabolite in this condition. MMA,[1] which reaches millimolar concentrations in body fluids and brain tissue during acute metabolic crises, was first suggested to act as an endogenous toxic metabolite, mediating neuronal damage via inhibition of mitochondrial energy metabolism (10, 11). It has been hypothesized that MMA induced inhibition of complex II (synonym, succinate:ubiquinone oxidoreductase), a multiprotein assembly imparted in the tricarboxylic acid cycle and the mitochondrial respiratory chain, has become a focus of interest (12). MMA induced cell damage in different neuronal culture systems (13–15) and evokes rotational behavior, seizures, and striatal lesions in rats after intrastriatal administration (16, 17). MMA-induced changes were prevented by succinate, antagonists of ionotropic glutamate receptors, and antioxidants (15, 16, 18). Consequently, MMA has been suggested to induce so-called "secondary" or "weak" excitotoxicity (12, 19).

However, recently we have demonstrated in striatal neuronal cultures from rat embryos that MMA-induced neuronal damage involves intracellular formation of the competitive complex II inhibitor malonate (MA) and 2-methylcitrate, a compound with multiple inhibitory properties on the tricarboxylic acid cycle (15). Therefore, we suggest that neuronal damage is mainly driven via metabolites that derive from alterna-

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[1] The abbreviations used are: MMA, methylmalonic acid; MA, malonic acid; CAPS, 3-(cyclo-hexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; MES, 4-morpholinepropanesulfonic acid; MMA, methylmalonic acid; MOPS, 4-morpholinepropanesulfonic acid; SMP, submitochondrial particle from bovine heart.

TABLE I

Experimental protocol for the radiometric analysis of [1-^{14}C]pyruvate, [U-^{14}C]malate and [1,4-^{14}C]succinate in 600 × g supernatants from muscle tissue of adult C57Bl/6 mice

CCCP, carbonyl cyanide *p*-chloro phenyl hydrazone.

	Without MMA	With MMA (1 mM)
Pyruvate oxidation		
[1-^{14}C]Pyruvate	+ Malate	+ Malate
	+ Carnitine	+ Carnitine
	+ Malate − ADP	+ Malate − ADP
	+ Malate − ADP + CCCP	+ Malate − ADP + CCCP
	+ Malate − ADP + atractyloside	
Malate oxidation		
[U-^{14}C] Malate	+ Pyruvate	+ Pyruvate
	+ Pyruvate + malonate	
	+ Acetylcarnitine	+ Acetylcarnitine
	+ Acetylcarnitine + malonate	
	+ Acetylcarnitine + arsenite	
Succinate oxidation		
[1,4-^{14}C]Succinate	+ Acetylcarnitine	+ Acetylcarnitine
	+ Acetylcarnitine + malonate	

tive oxidation pathways of propionyl-CoA rather than by MMA itself. In the present study we investigated whether MMA exerted any direct effects on mitochondrial energy metabolism and have investigated in detail the susceptibility of respiratory activity measurements to distinct artifacts. By this approach we can provide further evidence for our previous hypothesis that inhibition of the respiratory chain is not directly induced by MMA.

MATERIALS AND METHODS

Spectrophotometric Assay for Complex II Activity in Submitochondrial Particles—Submitochondrial particles from bovine heart were prepared as previously described (20). Protein was determined according to Lowry *et al.* (21) with modifications of Helenius and Simons (22) using bovine serum albumin as the standard. The catalytic activity of respiratory chain complex II was investigated in submitochondrial particles (SMPs) from bovine heart as previously described using decylubiquinone as the electron mediator (15, 23). In brief, steady state activities of mitochondrial complex II were recorded using a computer tunable spectrophotometer (SPECTRAmax Plus 384 Microplate Reader, Molecular Devices, Sunnyvale, CA) operating in the dual wavelength mode. Reduction of 2,6-dichlorophenolindophenol was detected at 610–750 nm (ϵ = 22.0 mmol/liter^{-1} × cm^{-1}) in thermostatted 96-well plates in a final volume of 300 μl (n = 8–16 experiments). Measurements were performed at standard conditions, which are defined as follows: SMPs were diluted to a final protein concentration of 2.5 mg/ml in 250 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 20 mM TRIS/HCl (pH 7.4), 2 mM NaN_3, and 20 mM sodium succinate. SMP dilution was incubated for 10 min at 37 °C. Thereafter, 15 μg of SMPs were added into each well. The reaction was started by the addition of a 300-μl reaction mixture containing 50 mM TRIS/HCl (adjusted to pH 7.4), 20 mM sodium succinate, 2 mM NaN_3, 60 mM 2,6-dichlorophenolindophenol with 0.01% Triton X-100, and 40 μM decylubiquinone. SMP dilution was kept on ice between measurements. In each experimental series, the specificity of the measured complex II activity was determined by using the specific inhibitor thenoyltrifluoroacetone (8 mM).

In analogy, the effects of the competitive complex II inhibitor malonate (MA) and the structurally related compound MMA were investigated using concentrations of up to 10 mM. To examine competition between these compounds with succinate at complex II, MA and MMA were incubated with different succinate concentrations (up to 20 mM) in the reaction mixture. Furthermore, we compared the effect of MMA on complex II activity after a 30-min incubation with 3-nitropropionic acid, a well known "suicide" inhibitor of complex II (24).

Complex II activity is modulated by a variety of variables, probably resulting in an artificial inhibition of this mitochondrial complex under non-standardized conditions. Because oxaloacetate inhibits complex II, we investigated the effects of "aging" on complex II activity in our assay, determining complex II activity during a period of up to 140 min. The effects of non-standardized conditions were tested by varying succinate concentrations, time points of measurement, and temperature at which SMP dilution was kept. Furthermore, we investigated the pH dependence of complex II activity at pH 5–10, replacing TRIS/HCl by 50 mM concentrations of a multi-buffer mixture, containing 10 mM of CAPS, MES, CHES, EPPS, and MOPS. pH optimum was determined according to Brandt and Okun (25). Furthermore, the effects of buffered and unbuffered MMA on complex II activity were tested.

Preparation of 600 × g Supernatants from Muscle—Adult C57Bl/6 mice of both sexes (n = 6) were sacrificed for the experiments. Immediately after death, muscle specimens (muscle quadriceps) were removed and homogenized (10% w/v). 600 × g supernatants were prepared according to Fisher *et al.* (26). Immediately after centrifugation, oxidation rates of [1-^{14}C]pyruvate, [U-^{14}C]malate, and [1,4-^{14}C]succinate and ATP production of unlabeled pyruvate, malate, and succinate were determined (27, 28). Aliquots of the supernatants were frozen and kept at −80 °C until measurement of protein concentrations (21, 22) and citrate synthase activity (29). Animal care followed the official governmental guidelines and was approved by the government ethics committee.

Radiometric Analysis of Mitochondrial Oxidative Phosphorylation—Analysis of single respiratory chain complexes does not allow detection effects of toxic compounds on associated proteins and transporters of the mitochondrial respiratory chain but can be detected by radiometric analysis of ^{14}C-labeled substrates. Radiometric analysis of oxidation rates of [1-^{14}C]pyruvate, [U-^{14}C]malate, and [1,4-^{14}C]succinate were determined as previously described (27, 28). We investigated whether mitochondrial respiration and oxidative phosphorylation was influenced by MMA (1 mM). An overview on the experiments performed is given in Table I.

ATP Production—ATP production was determined with unlabeled pyruvate, malate, and succinate as described previously (30). Spectrophotometric measurement of ATP production is coupled to the formation of NADPH (λ = 320–400 nm, 25 °C). The test principle consists of two enzyme reactions. In the first step, glucose (30.3 mM) and ATP are catalyzed to glucose 6-phosphate and ADP by hexokinase. Subsequently, glucose 6-phosphate dehydrogenase catalyzes glucose 6-phosphate and $NADP^+$ to 6-phosphoglucolacton and NADPH. The production of NADPH was normalized to the protein concentration and citrate synthase activity of each sample. An overview on the experiments performed is shown in Table II.

Data Analysis—Data were normalized to citrate synthase activity and protein concentrations of the same sample. If not explicitly mentioned, MMA-induced effects on complex II activity were normalized to simultaneously measured controls. This procedure was compared with a different normalization procedure using a control group that was measured at the beginning of each experimental series (see Fig. 4*A*). Data were expressed as the mean ± S.E. Experiments were performed 6–8-fold. One-way analysis of variance 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 Psiplot software 5.02a.

RESULTS

Methylmalonic Acid Does Not Inhibit Complex II Activity in SMPs—In SMP complex II revealed a high activity (V_{max}, 1.02 units/mg of protein, n = 8), a high affinity for the substrate succinate (K_m: 40 μM, n = 8), and a good inhibitory response to

TABLE II

Experimental protocol for the analysis of pyruvate- and succinate-stimulated ATP production in 600 × g supernatants from muscle tissue of adult C57Bl/6 mice

DQA, 2-*n*-decylquinoline-4-yl-amine; CCCP, carbonyl cyanidp *p*-chlorohydrazone.

	Without MMA	With MMA (1 mM)
Pyruvate		
Pyruvate	+ Malate	+ malate
	+ Malate + arsenite	
	+ Malate + CCCP	+ malate + CCCP
Succinate		
Succinate	+ Acetylcarnitine + DQA + antimycin A	
	+ Acetylcamitine + DQA − antimycin A	+ acetylcarnitine + DQA − antimycin A
		+ acetylcamitine − antimycin A

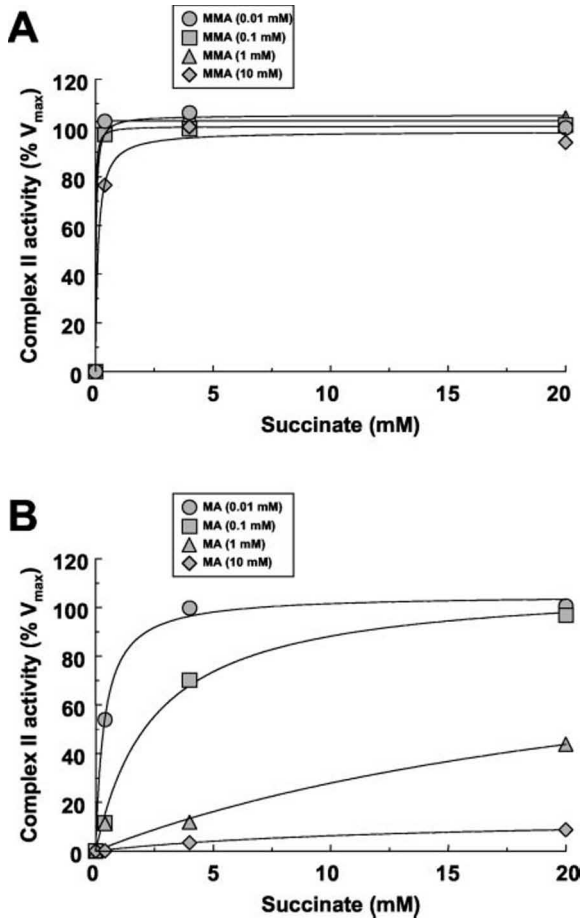

FIG. 1. **Comparison of MMA (*A*) and MA (*B*) on complex II activity in SMPs using decylubiquinone as electron mediator.** For each combination of different succinate and MMA or MA concentrations, eight experiments have been performed. Concentrations of MMA and MA were varied from 0.01 to 10 mM, and those of succinate were varied from 0 to 20 mM. The K_m (succinate) was 40 μM. Data are the mean. Notably, MMA exerted no inhibitory effect on complex II activity, whereas MA showed competitive inhibition.

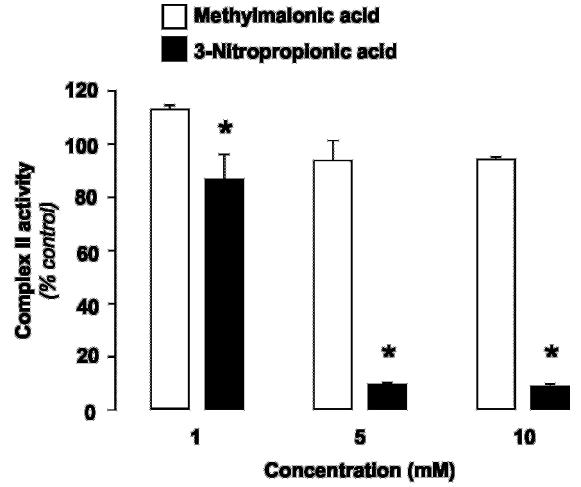

FIG. 2. **Comparison of MMA- and 3-nitropropionic acid-induced effects on complex II activity in SMPs after an incubation period of 30 min (n = 8).** Whereas 3-nitropropionic acid revealed a strong inhibition of complex II activity, MMA failed to do the same. Prolongation of the incubation period showed similar results for MMA (data not shown). *, $p < 0.05$ *versus* MMA (one-way analysis of variance followed by Scheffe's test).

the standard inhibitor thenoyltrifluoroacetone (8 mM; 10% of control activity, n = 8). Varying the absolute and relative concentrations of MMA (0, 0.01, 0.1, 1, 10 mM) and succinate (0, 0.04, 0.4, 4, 20 mM), no inhibitory effect of MMA on complex II activity was detected (n = 8, Fig. 1*A*). In addition, we could exclude any inhibitory effect of MMA (concentration range, 0.01–10 mM) on the electron-transferring complexes I, III, and IV as well as on ATP synthase (data not shown). Furthermore, we found no effect of propionic acid (up to 10 mM), the decarboxylation product of MMA, on complex II activity. In contrast, the competitive complex II inhibitor MA (0, 0.01, 0.1, 1, 10 mM) revealed an inhibition of complex II activity at varying succinate concentrations (0, 0.04, 0.4, 4, 20 mM), confirming the reliability of our assay (n = 8, Fig. 1*B*).

Next, we investigated whether prolonged incubation with MMA would induce complex II inhibition, in analogy to the suicide inhibitor 3-nitropropionic acid inactivating complex II by covalent binding. However, incubation with MMA (1–10 mM) for 30 min did not decrease complex II activity, whereas 3-nitropropionic acid induced a strong inhibition of this enzyme (n = 8, Fig. 2). A further prolongation of the incubation period (up to 120 min) showed similar results for MMA (data not shown).

Evaluation of Artificial Inhibition of Complex II Activity; Relevance of pH Effects and Aging—The pH optimum for complex II activity was determined in SMPs at a range from pH 5 to pH 10 (n = 8). Complex II activity reached a maximum at pH 7.4, whereas pH changes dramatically reduced V_{max} (Fig. 3*A*). The pH dependence of complex II activity was described according to Brandt and Okun (25), revealing a pK_A of 6.7 and a pK_B of 7.9 (Fig. 3*B*). Simultaneous experiments with buffered MMA (adjusted to pH 7.4) and unbuffered MMA (0.01 mM (pH 7.4), 0.1 mM (pH 7.4), 1 mM (pH 7.2), 5 mM (pH 5.7), 10 mM (pH 5.1)) revealed a strong decrease in complex II activity at concentrations of 1–10 mM due to a concentration-dependent pH shift (n = 8, Fig. 3*C*).

The concentrations of succinate and oxaloacetate in the test system is of relevance for V_{max}. Whereas increasing succinate concentrations have been shown to increase V_{max} (see also Fig. 1), oxaloacetate has the opposite effect. Because the relation of these two compounds changes in the SMP dilution in a time-

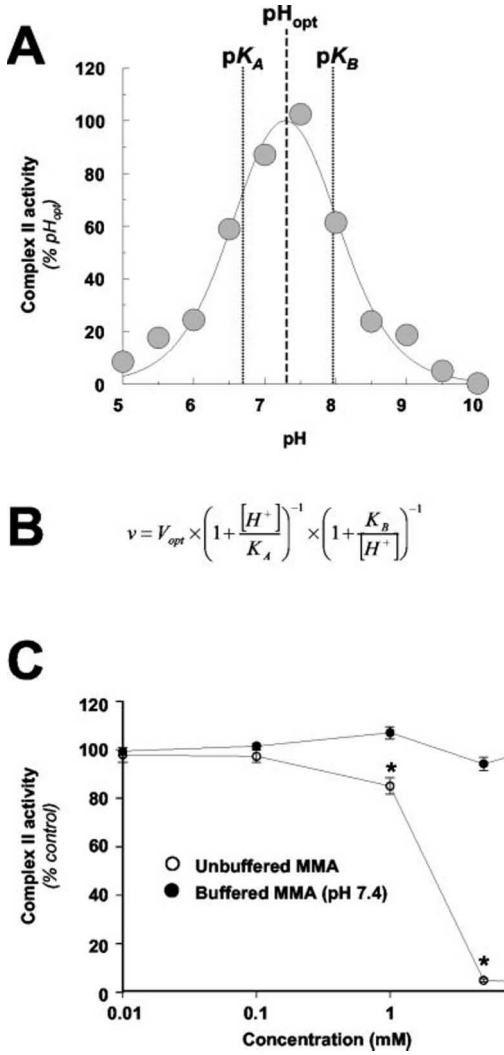

FIG. 3. **Determination of the pH optimum for complex II activity in SMPs.** *A*, the pH optimum was determined using a pH range from pH 5 to 10 ($n = 8$). *B*, following the given equation, the pH optimum and p*K* values were calculated (pH 7.4, pK_A 6.7, pK_B 7.9). *C*, using the same concentrations of buffered (pH 7.4) and unbuffered MMA in simultaneous experiments ($n = 8$), we showed that only unbuffered MMA inhibited complex II activity at millimolar concentrations, in parallel with a decrease in pH, whereas buffered MMA did not reveal any inhibition of complex II. *, $p < 0.05$ *versus* buffered MMA (one-way analysis of variance followed by Scheffe's test).

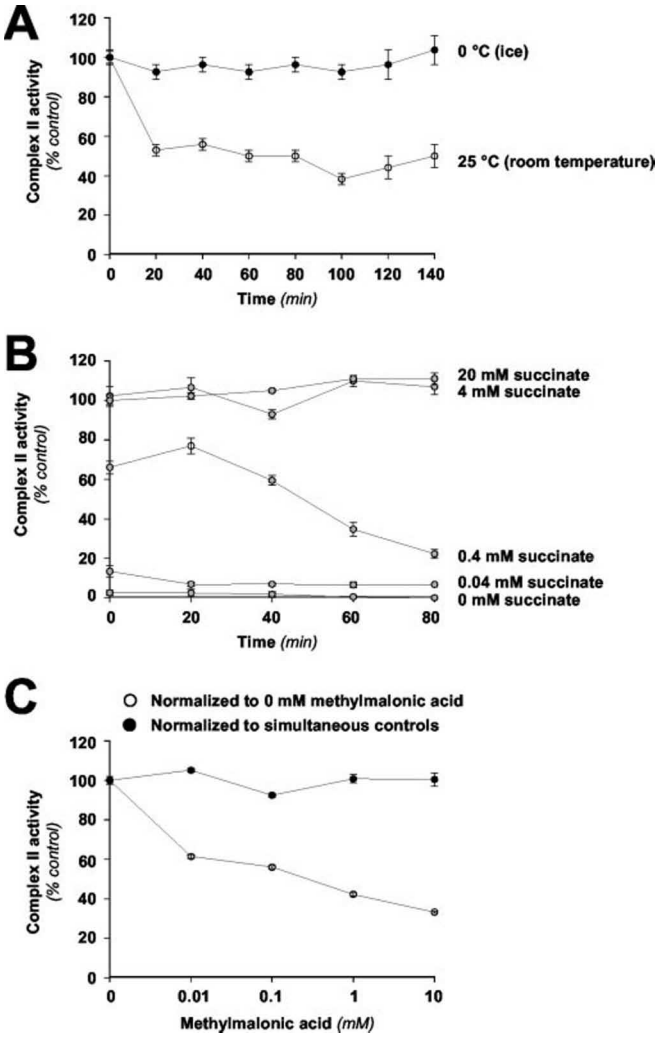

FIG. 4. **Effects of time, succinate concentrations, temperature, and normalization procedures on complex II activity in SMPs.** *A*, comparison of time-dependent and temperature-dependent effects on complex II activity ($n = 8$). If SMP dilution was kept at room temperature, complex II activity decreased in a time-dependent manner but was stable if kept on ice (*i.e.* standard conditions). *B*, effects of different succinate concentrations on complex II activity ($n = 8$). At low succinate concentrations (0.04–0.4 mM) we found a time-dependent decrease in complex II activity, whereas complex II activity remained stable at saturating concentrations of succinate (4–20 mM). *C*, comparison of different normalization procedures for complex II activity using the same set of data ($n = 8$). If normalized to synchronized controls, MMA did not induce any inhibitory effect at low succinate concentrations (0.4 mM succinate). In contrast, normalization to controls determined only once at the beginning of each experimental series falsely suggested an inhibitory effect of MMA on complex II activity. The latter normalization procedure did not mention time-dependent decrease in complex II activity and, thus, led to a misinterpretation of the data.

dependent fashion, we investigated whether aging effects would result in a decrease of V_{max}. In fact, we found a decrease in complex II activity if the SMP dilution was kept at room temperature for a period of 140 min ($n = 8$, Fig. 4*A*). If kept on ice (0 °C, *i.e.* our standard condition), complex II activity remained stable during this time period ($n = 8$, Fig. 4*A*).

Because complex II activity correlates with the concentrations of its substrate succinate, we investigated the influence of different succinate concentrations (SMP dilutions kept on ice). At 4 and 20 mM succinate (*i.e.* 100- and 500-fold K_m), complex II activity remained stable for 80 min, whereas at 0.04 and 0.4 mM succinate it rapidly decreased, most likely due to a decrease in succinate and an increase in oxaloacetate concentrations ($n = 8$, Fig. 4*B*). Because time-dependent changes in V_{max} necessitate a simultaneous measurement of controls, we investigated whether a normalization of complex II activity to (*a*) simultaneous controls or to (*b*) non-simultaneous controls (determined at the beginning of each experimental series of 3 h) would result in a misinterpretation of complex II activity measurements at low succinate concentrations. If normalized to simultaneous controls, MMA revealed no inhibitory effect on complex II activity using 0.4 mM succinate ($n = 8$, Fig. 4*c*). In contrast, using the same set of data normalization to a non-simultaneous control group, which would not detect aging effects of the control group, resulted in an apparent concentration-dependent decrease of complex II activity by MMA ($n = 8$, Fig. 4*C*).

Methylmalonic Acid Does Not Affect Mitochondrial Respiration and Oxidative Phosphorylation—Activity measurement of single respiratory chain complexes could not exclude that MMA has an inhibitory effect on associated proteins of the mitochondrial respiratory chain, *e.g.* on transporter systems. Such effects could be determined by radiometric analysis of the oxidation rate of ^{14}C-labeled substrates and of pyruvate- and

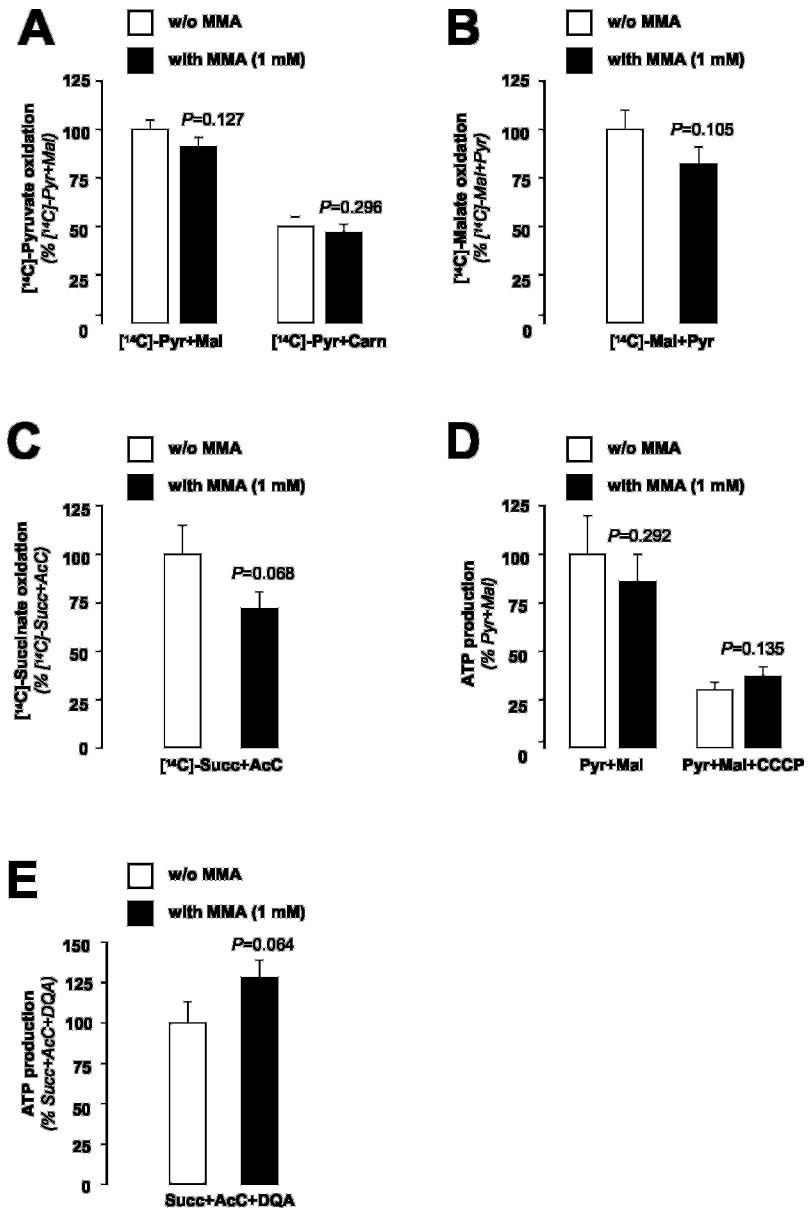

FIG. 5. **Evaluation of MMA-induced effects on oxidation rates of [1-^{14}C]pyruvate, [U-^{14}C]-malate, and [1,4-^{14}C]succinate and on pyruvate- and succinate-stimulated ATP production in 600 × *g* supernatants from muscle tissue of adult C57Bl/6 mice (*n* = 6).** MMA (1 mM) had no significant effect on pyruvate oxidation (*A*), malate oxidation (*B*), succinate oxidation (*C*), pyruvate-stimulated ATP production (*D*), or succinate-stimulated ATP production (*E*). Statistical analysis was performed using *t* test (w/o *versus* with MMA). *AcC*, acetylcarnitine; *Carn*, carnitine; *DQA*, 2-*n*-decylquinazoline-4-yl-amine; *Mal*, malate; *Pyr*, pyruvate; *Succ*, succinate; *CCCP*, carbonyl cyanide *p*-chlorophenylhydrazone.

succinate-induced ATP production in coupled mitochondria. For this purpose we used 600 × *g* supernatants from muscle tissue of adult C57Bl/6 mice ($n = 6$). MMA (1 mM) revealed no effect on the mitochondrial respiration of [1-^{14}C]pyruvate (Fig. 5*A*), [U-^{14}C]malate (Fig. 5*B*), or [1,4-^{14}C]succinate (Fig. 5*C*). Furthermore, ATP production stimulated by pyruvate (Fig. 5*D*) or succinate (Fig. 5*E*) was not affected by MMA (1 mM). The same system revealed a good inhibitory response to standard inhibitors of respiratory chain (2-*n*-decylquinazoline-4-yl-amine, antimycin A, malonate, arsenite, atractyloside) and carbonyl cyanide *p*-chlorophenylhydrazone (data not shown), confirming the reliability of our data.

DISCUSSION

The biochemical hallmark of methylmalonic acidurias is an accumulation and increased urinary excretion of the organic acid MMA. MMA is structurally similar to the competitive complex II inhibitor MA (31). Neurodegeneration in methylmalonic acidurias in particular affects the globus pallidus, which is also affected in inherited or acquired inhibition of complex II (9, 24, 31). Thus, it seemed reasonable to suggest MMA as an endogenous toxic metabolite inducing neurodegenerative changes via impairment of energy metabolism (7, 12). In fact, administration of MMA *in vitro* and *in vivo* induced neuronal cell damage involving ionotropic glutamate receptors and oxidative stress (13–17). Although two previous studies from the same group demonstrated inhibition of complex II activity by MMA (10, 11), we could not confirm this finding in a recent study (15). In contrast, we showed that MMA-induced effects are likely to be indirect, involving the intracellular formation of the competitive complex II inhibitor MA and 2-methylcitric acid, a compound with multiple inhibitory effects on the tricarboxylic acid cycle (32). Thus, we suggest that MMA-induced inhibition of complex II activity in the previous studies might have been the result of intracellular formation of MA and 2-methylcitrate.

To confirm our hypothesis that MMA has no direct effects on the mitochondrial respiratory chain, we investigated MMA using spectrophotometric analysis of complex II activity in SMPs and radiometric analysis of the mitochondrial respiration of ^{14}C-labeled substrates (pyruvate, malate, succinate). Furthermore, we studied under which experimental conditions complex II inhibition by MMA could be artificially induced. In fact, we could exclude any direct effects of MMA on single complex II activity and [1,4-^{14}C]succinate respiration. Furthermore, suc-

cinate-stimulated ATP production was not affected by MMA. Thus, these results exclude any relevant effects of MMA on complex II activity. It has been suggested that MMA inhibited the transmitochondrial malate carrier at millimolar concentrations in rat liver mitochondria (33). In this study, radiometric analysis of [U-^{14}C]malate respiration in 600 $\times$ *g* supernatants from mice muscle revealed only a small, but insignificant decrease by 1 mM MMA. An effect on the malate carrier at higher concentrations might be theoretically interesting; however, it is questionable whether this effect has any relevance for the pathophysiology of methylmalonic acidurias. Apart from this, MMA was shown to inhibit pyruvate carboxylase (34) and Na^+/K^+-ATPases (35).

MMA has no inhibitory effect on the respiratory chain function, in particular complex II activity, under the standardized conditions used in this study. However, we could identify some important variables of this enzymatic assay that must be carefully controlled to avoid false-positive inhibitory responses. First of all, the pH should be kept at the optimum for this method (pH 7.4), and all compounds tested should be adequately buffered to avoid pH shifts and, concomitantly, changes in complex II activity. Furthermore, simultaneous measurements of controls and subsequent normalization of the data to these control groups must be performed during the experimental series, in particular if low succinate concentrations are used. To prevent a time-dependent decrease in V_{max} during the experiments, saturating concentrations of succinate (4–20 mM) should be used, and the SMP dilution should be kept on ice. It cannot be excluded that additional variables have an important influence on complex II activity. However, if complex II activity is standardized using the standard procedure described above, the reliability of this assay is very high.

In conclusion, we could confirm the hypothesis that MMA exerts no direct effects on the mitochondrial respiratory chain and seemingly is not the major toxic metabolite in methylmalonic acidurias. The recent study stresses our previous concept of a synergistic inhibition of respiratory chain and tricarboxylic acid cycle by propionyl-CoA and its alternative products, in particular 2-methylcitrate and MA (15, 32).

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