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Pathophysiologic Study on Methylmalonic Aciduria: Decrease in Liver High-Energy Phosphate after Propionate Loading in Rats

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ABSTRACT. Methylmalonate or propionate was i.v. infused into B_{12}-deprived and control rats. In the B_{12}-deprived rats, the plasma and liver concentrations of B_{12} decreased to 8 and 13%, respectively, of those of the control rats. The propionate loading produced a disproportionate increase in liver propionate levels; the mean ratio of methylmalonate to propionate in the liver was approximately 1.0 after methylmalonate loading, whereas it was 0.1 to 0.2 after propionate loading. The liver propionate and methylmalonate levels in the B_{12}-deprived rats were twice as high as those in the control rats. The mean ratio of β-ATP to inorganic phosphate in the liver, measured with ^{31}P-magnetic resonance spectroscopy, decreased from 0.60 to 0.48 in the B_{12}-deprived rats and from 0.78 to 0.63 in the control rats after methylmalonate loading; the ratio decreased from 0.57 to 0.37 in the B_{12}-deprived rats and from 0.76 to 0.56 in the controls after propionate loading. Statistical analysis showed that propionate loading caused a more marked decrease in ATP than did methylmalonate loading ($F = 26.33$, degree of freedom 1 and 15; $p < 0.001$), while B_{12}-deprivation caused a more marked decrease in ATP than did the control diet ($F = 92.26$, *df* 1 and 15; $p < 0.001$). The concentrations of tricarboxylic acid cycle intermediates and related organic acids in the livers of the rats suggested that propionate inhibited NAD^+-dependent enzymes in the cycle. These results indicate that propionate, which accumulates during crises in methylmalonic aciduria, contributes to the decrease in ATP levels to a greater extent than does methylmalonate, and thus impairs the ATP-dependent carboxylation of propionate itself to methylmalonate. (*Pediatr Res* 30: 5–10, 1991)

Abbreviations

MMCoA, methylmalonyl-CoA
PCoA, propionyl-CoA
MMA, methylmalonate
PA, propionate
TCA, tricarboxylic acid
PMRS, phosphorous magnetic resonance spectroscopy
pHi, intracellular pH
Pi, inorganic phosphate
B_{12}, vitamin B_{12}
ANOVA, analysis of variance

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Methylmalonic aciduria is an inborn error of metabolism that is due to a deficiency of MMCoA mutase, which catalyzes the reaction of MMCoA to succinyl-CoA (1, 2). Propionic acidemia is due to a deficiency of PCoA carboxylase, which catalyzes the reaction of PCoA to MMCoA (3–5). These two diseases are often regarded as having similar pathologic features, inasmuch as their metabolic defects are close and high plasma levels of PA are often detected during crises in both diseases (1). In addition, it is of interest that PA levels in the body fluids of patients with methylmalonic aciduria are sometimes much higher than MMA levels during crises, and that the urinary excretion of MMA increases during the convalescent stage (6, 7).

The clinical manifestations of methylmalonic aciduria and propionic acidemia during crises include lethargy, seizures, and coma, and laboratory findings include hyperammonemia, hyperglycinemia, hypoglycemia, lactic acidosis, and ketosis (1, 2). These findings suggest a disturbance of the energy production system in these diseases. *In vitro* studies have shown that PA or its metabolites can inhibit several metabolic processes (1, 2), including citrate synthesis (8, 9), pyruvate metabolism (10), the urea cycle (11), and the glycine cleavage system (1). Although several investigators have studied the *in vitro* effects of PA on selected TCA cycle intermediates or enzymes (12), amino acid metabolism (13), and tissue ATP levels (11, 13–15), few studies have examined the *in vivo* effects of PA on the TCA cycle or ATP production. *In vitro* studies have failed to give consistent results regarding the effects of PA on ATP production. This failure is probably due to the technical difficulties of sample preparation (11, 13–15).

Recently, PMRS has been used to measure high-energy phosphorous compounds and pHi of animal and human tissues or organs, noninvasively and sequentially (16). Using this technique, we measured rat liver ATP levels during PA or MMA loading tests to assess the effects of these acids on the energy production system. We used vitamin B_{12}-deprived rats, because B_{12} deficiency results in decreased MMCoA mutase activity and increased plasma and tissue PA and MMA levels (17–21). It was assumed that PA or MMA loading in the B_{12}-deprived rat would closely reflect the biochemical conditions present during an acute crisis of methylmalonic aciduria. We also measured the liver concentrations of the TCA cycle intermediates and related organic acids to investigate the *in vivo* effects of PA and MMA on the energy production system.

MATERIALS AND METHODS

Animals and their management. Weanling male Wistar rats (Clea Japan, Inc., Tokyo, Japan) were kept individually in metabolic cages with steel-mesh floors to minimize coprophagy. Two groups of 18 rats each were fed a B_{12}-deficient diet (B_{12}-deprived rats) and a routine diet (control rats). The composition of the B_{12}-deficient diet was (in g/100 g diet): vitamin-free casein, 18; sucrose, 67.6; corn oil, 8; vitamin-free cellulose, 1.5; DL-methi-

onine, 0.3; choline chloride, 0.1; vitamin mixture 0.5; salt mixture, 4. The vitamin mixture contained (per 100 g diet): vitamin A, 2,500 IU; vitamin D_3, 500 IU; vitamin B_1, 1.9 mg; vitamin B_2, 2.0 mg; vitamin B_6, 1.9 mg; vitamin C, 30.5 mg; vitamin E, 12.5 mg; vitamin K_3, 0.7 mg; biotin, 0.05 mg; folic acid, 0.25 mg; Ca-pantothenate, 10 mg; *p*-aminobenzoic acid, 12.5 mg; nicotinic acid, 6.25 mg; *myo*-inositol, 12.5 mg. The salt mixture contained the following minerals (in mg/100 g diet): K, 475; P, 440; Na, 425; Mg, 25; Fe, 23.5; Zn, 0.7; Mn, 4.8; Cu, 0.4; and I, 3.3. The diet and water were supplied *ad libitum* for 12 wk. The body weights were recorded and 12-h urine samples were collected once a week throughout the experiment.

Animal preparation. The rats were anesthesized with an intraperitoneal injection of sodium pentobarbital (5 mg/100 g body wt). The bilateral internal jugular veins were cannulated for the infusion of organic acids or saline solution and sampling of blood. The liver was exposed by abdominal incision and covered with a plastic film for PMRS analysis.

Organic acid administration. PA (10 mmol/kg/h) or MMA (10 mmol/kg/h) loading was performed in the rats. The solution, adjusted to pH 7.4 with NaOH, was infused at a rate of 5 mL/h through the jugular catheter. Heparinized blood was drawn from another catheter before and 2 h after organic acid administration. A comparable volume of saline was infused to six control rats and six B_{12}-deprived rats, and the same volume of 5% NaCl solution, the osmotic pressure of which is equivalent to the osmotic pressures of the PA and MMA solutions, was also given to five control rats to examine the hyperosmolar effects on liver ATP. Plasma was collected after centrifugation at 4°C. After the rats were killed by decapitation, the livers and brains were removed and immediately frozen with liquid nitrogen. The plasma and tissue samples were kept at −80°C until analysis.

PMRS. The spectra were recorded at 35.3 MHz with a BEM 250/80 spectrometer (Otsuka Electronics, Inc., Philadelphia, PA), equipped with a wide-bore magnet (2.0 tesla), acquired with a pulse-repetition time of 2 s. The sum of 120 scans was recorded using 23-μs radio-frequency pulses at 4-min intervals. A surface coil (20 mm diameter) was placed on the surface of the liver. Homogeneity was obtained by shimming on water in the liver, and a nonspinning line width at half height of 0.3 ppm was obtained. The β-ATP/Pi ratio was calculated by measuring the peak height of the signals in the spectra. The pHi was calculated from the chemical shift of the Pi signal relative to α-ATP (δ) using the following equation (22):

$$\mathrm{pHi} = 6.83 - \log\,[(13.21 - \delta)/(\delta - 11.10)]$$

Organic acid analysis. The tissue specimens were weighed and homogenized by hand with distilled water in a glass homogenizer. The PA concentration was determined using the solvent extraction method. To a small screw-top vial containing 0.2 mL of the homogenate or plasma, we added 0.1 g of NaCl, 0.05 mL of 6N HCl, and 0.25 mL of ethylacetate with 0.05 mg of *n*-valeric acid as an internal standard. The mixture was shaken vigorously for 1 min and centrifuged at 2500 rpm for 30 s. The organic phase was transferred to a microtube as soon as possible, and the aqueous phase was again extracted with 0.25 mL of ethylacetate. The combined organic phase was injected into a Shimadzu GC-7A gas chromatograph (Shimadzu Seisakusho Ltd., Kyoto, Japan) equipped with a 2-m glass coil column packed with 5% neopentylglycoladipate + 2% H_2PO_4 on chromosorb W (AW-DMCS; Wako Pure Chemical Industries Ltd., Osaka, Japan). The injection port temperature was 190°C and the oven temperature was programmed at 1.5°C/min from 85 to 190°C. Helium was the carrier gas, and the flow rate was 45 mL/min. A linear calibration plot for the PA standards in ethylacetate extract was obtained in the range of 0.05–20 mmol/L, and the coefficients of variation of the 0.1-, 1-, and 10-mmol/L PA standards were 11.7, 1.7, and 5.1%, respectively ($n = 7$). The production of free PA due to decarboxylation of MMA in the injection port was 0.2, 0.3, and 2.3% of the injected MMA standard in ethylacetate when the temperature of the injection port was set at 120, 190, and 240°C, respectively.

The nonvolatile organic acids were extracted with ethylacetate after the homogenate had been saturated with NaCl and acidified with HCl to pH 1.0. Dry extracts were trimethylsilylated. The polar organic acids were extracted using DEAE-Sephadex. To deproteinize the plasma and tissue homogenate, 0.2 mL of sample was mixed with 2 mL of ice-cold methanol and allowed to stand for 20 min. The mixture was centrifuged at 3500 rpm for 15 min, and the supernatant was transferred. Two mL of methanol was added to the precipitate and the procedure described above was repeated. The combined supernatant was filtered through filter paper, after which the methanol in the filtrate was evaporated under a nitrogen stream, and the liquid lyophilized. The residue was dissolved in 1 mL of distilled water, and a saturated barium hydroxide solution was added to precipitate inorganic sulphate and phosphate. After keto-acid oxime formation with hydroxylamine hydrochloride, the neutralized solution was applied to a DEAE-Sephadex column, and the organic acids were eluted with aqueous pyridinium acetate buffer after washing with water. The eluate was lyophilized and the residue was derivatized with 20 μL of pyridine and 100 μL of N,O-bis(trimethylsilyl)-trifluoroacetoamide. The organic acids were determined with a model 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a 3392A integrater. The column was an ULTRA-1 fused silica capillary column (25 m × 0.20 mm) (Hewlett-Packard). The carrier gas was helium and the column flow was 0.79 mL/min at 50°C. The injection port was set as a split mode. The oven temperature was programmed at 4.8°C/min from 50°C. Fumarate, malate, oxaloacetate, *cis*-aconitate, and 2-ketoglutarate were determined with selected ion monitoring, using a JMS DX-300 gas chromatograph/mass spectrometer and JMA-3500 mass data analysis system (JEOL, Tokyo, Japan). The column was an ULTRA-2 fused silica capillary column (25 m × 0.32 mm) (Hewlett-Packard). The temperature was programmed at 8°C/min from 50 to 210°C. The ions were monitored as follows: m/z 245 for fumarate; m/z 335 for malate; m/z 363 for oxaloacetate; m/z 375 for *cis*-aconitate; m/z 377 for 2-ketoglutarate; and m/z 296 for 2-hydroxyphenylacetate (internal standard).

Measurement of B_{12} content. Half a gram of tissue was placed in 5 mL of saline and sonicated in an ice bath for 10 min. The homogenate was centrifuged at 2000 × *g* for 30 min, with the supernatant then used for the assay. The concentration of B_{12} in the supernatant or plasma was determined with the radioisotopic kit, MAGIC Vitamin B12[Co^{57}] / Folate[^{125}I] No-Boil Radioassay (Ciba·Corning Diagnostics Corp., Walpole, MA).

Statistical analysis. Values are expressed as the mean and SD. The *t* test was used for statistical comparisons of the body weights of the rats, B_{12} content, organic acid concentration, β-ATP/Pi ratio, and pHi. Differences among the subgroup data obtained with PMRS and organic acid analysis were compared by two-way ANOVA for repeated measures, and by Tukey's multiple comparison. Statistical significance was defined as $p < 0.05$.

RESULTS

Significant growth retardation was observed in the B_{12}-deprived rats ($p < 0.001$), the average body weight of which was 298.1 ± 13.2 g after 12 wk of the deficient diet, representing a body weight approximately 20% lower than that of the control rats (367.6 ± 21.7 g). The plasma and tissue concentrations of B_{12} in the two groups at 12 wk of the experiment are shown in Table 1. The mean plasma B_{12} level of the B_{12}-deprived rats was approximately 8% of that of the control rats. The liver and brain concentrations of B_{12} were also markedly lower than those of the control rats. Urinary MMA excretion in the B_{12}-deprived rats increased progressively and reached 8.4 ± 1.2 μmol/mg creatinine after 12 wk of the diet (urinary MMA excretion was not

Table 1. *B_{12} in control and B_{12}-deprived rats*

	Plasma (pg/mL)	Liver (ng/g)	Brain (ng/g)
Control rats	918 ± 196 (n = 10)	73.4 ± 16.6 (n = 6)	28.1 ± 4.8 (n = 6)
B_{12}-deprived rats	75 ± 14* (n = 10)	9.4 ± 1.3* (n = 12)	7.7 ± 1.8* (n = 12)

* $p < 0.001$ (t test) compared to control rats.

detectable in the control rats), although there were day-to-day and individual variations.

The plasma and liver concentrations of PA and MMA are shown in Table 2. The PA and MMA levels in the plasma before the loading test and those in the liver after saline loading in the B_{12}-deprived rats were significantly ($p < 0.01$) higher than those in the control rats. The mean liver levels of PA in the B_{12}-deprived rats were 3 times higher than those of MMA. After PA or MMA infusion, the increase in the PA or MMA levels in the B_{12}-deprived rats was markedly greater than that in the control rats. After MMA loading, the mean liver concentrations of PA in the control rats and in the B_{12}-deprived rats were almost equal to those of MMA; thus, the mean ratio of MMA to PA was approximately 1.0. The mean liver concentration of PA after PA loading was higher than that of MMA after MMA loading; thus, the mean ratio of MMA to PA in the liver after PA loading was 0.2 in the control rats and 0.1 in the B_{12}-deprived rats. However, the sum of PA and MMA in the liver after PA loading was similar to that after MMA loading.

The liver β-ATP/Pi ratios and pHi before and after PA or MMA loading are shown in Table 3. The mean liver β-ATP/Pi ratio and pHi in the B_{12}-deprived rats were significantly lower than those in the control rats before the loading tests. Saline loading did not produce significant changes in the liver β-ATP/Pi ratio or pHi in either group. Five percent NaCl solution loading in the control rats did not produce significant changes in the liver ATP/Pi ratio (data not shown).

In the B_{12}-deprived rats, the β-ATP/Pi ratio and pHi fell to values lower than those in the control rats after PA or MMA loading. The relationship between the β-ATP/Pi ratio after PA or MMA loading and the liver concentrations of these organic acids in the four subgroups are shown in Figures 1 and 2. ANOVA showed that PA loading in both the B_{12}-deprived rats and the control rats caused a more marked decrease in the liver ATP/Pi ratio than did MMA loading ($F = 26.33$, df 1 and 15; $p < 0.001$), and that the B_{12}-deprivation also decreased the liver ATP/Pi ratio ($F = 92.26$, df 1 and 15; $p < 0.001$). B_{12}-deprivation seemed to have only a slight influence upon the effect of loaded compounds in decreasing the liver ATP/Pi ratio, inasmuch as the interaction of these factors (*i.e.* B_{12}-deprivation and the hepatic levels of PA or MMA) was not statistically significant ($F = 2.14$, df 1 and 15; NS)

The concentrations of the intermediates in the TCA cycle and related organic acids in the liver are shown in Table 4. The liver succinate levels were higher after PA loading than after MMA loading; thus, the ratios of succinate to fumarate increased more markedly after the former than after the latter, although the changes in the concentrations of succinate were smaller in the B_{12}-deprived rats than in the control rats. In both groups, the concentrations of malate increased significantly after PA and MMA loading. The levels of *cis*-aconitate increased more markedly after PA loading than after MMA loading, whereas the levels of 2-ketoglutarate were higher after MMA loading than after PA loading. The hepatic levels of ketone bodies were higher in the B_{12}-deprived rats than in the control rats. 3-Hydroxybutyrate showed no significant changes in either group, whereas acetoacetate increased markedly after PA or MMA loading in both groups. Thus, the ratio of 3-hydroxybutyrate to acetoacetate decreased after PA or MMA loading.

DISCUSSION

In this experiment, it was demonstrated that liver ATP, which was measured using PMRS, decreased after i.v. PA and MMA loading. The results of ANOVA showed that the decrease in liver ATP was related more to PA loading than to MMA loading, and more to B_{12}-deprivation than to normal feeding. The fact that in the B_{12}-deprived rats the liver concentrations of PA after the PA or MMA loading tests were twice as high as those of the control rats suggests that the decrease in liver ATP in the former is due to the higher concentration of liver PA in this group as compared with the control rats. It is also thought that the differences between the loading compounds were due to the differences in liver PA concentration after the loading tests. Although PA production through decarboxylation in the heated injection port has been reported when an aqueous solution containing large amounts of MMA was injected into a gas chromatograph (23), in the present study, solvent extraction with ethylacetate minimized the contribution of such decarboxylation and thus facilitated the quantitative analysis of PA concentrations. Thus, based on the relationship between the ATP/Pi ratio and the liver PA or MMA levels and the results of ANOVA, it is clear that the accumulation of PA, rather than MMA, in the liver plays the major role in the decrease in the liver ATP/Pi ratio.

We believe that these findings help elucidate the pathophysiology of methylmalonic aciduria during acute crises because the plasma levels of PA often exceed those of MMA and urinary excretion of MMA is greater during the convalescent period than during the crisis itself (6, 7). Disturbances of both ATP production and consumption can result in decreased ATP levels. The following reactions are considered to be metabolic pathways related to ATP production and consumption during PA or MMA loading: the carboxylation of PCoA, the acyl-CoA formation from free organic acid, and ATP production in the TCA cycle.

The carboxylation of PCoA catalyzed by PCoA carboxylase is: $ATP + HCO_3^- + PCoA \rightleftharpoons ADP + Pi + MMCoA$ (3–5). The apparent equilibrium constant of the reaction is reported to average 5.7 at pH 8.1 and 28°C and, based on the reported free-

Table 2. *PA and MMA levels in plasma and liver*

	PA		MMA	
	Plasma (μmol/mL)	Liver (μmol/g)	Plasma (μmol/mL)	Liver (μmol/g)
Control rats				
Before*	0.02 ± 0.001	0.03 ± 0.008	0.01 ± 0.005	0.02 ± 0.004
After PA loading	26.8 ± 8.0	8.2 ± 1.9	0.09 ± 0.01	1.49 ± 0.14
After MMA loading	15.1 ± 1.8	6.5 ± 1.1	35.6 ± 3.8	6.4 ± 0.8
B_{12}-deprived rats				
Before*	0.15 ± 0.01	0.18 ± 0.02	0.11 ± 0.02	0.06 ± 0.008
After PA loading	53.0 ± 8.6	17.7 ± 1.2	0.31 ± 0.02	1.77 ± 0.11
After MMA loading	25.5 ± 2.3	11.9 ± 0.9	50.0 ± 5.1	11.9 ± 1.3

* Liver PA and MMA were measured after saline loading. Each subgroup consisted of six rats.

Table 3. *Liver high-energy phosphate and pHi**

	PA loading		MMA loading	
	β-ATP/Pi	pHi	β-ATP/Pi	pHi
Control rats				
Before	0.76 ± 0.03	7.38 ± 0.03	0.78 ± 0.03	7.38 ± 0.03
2 h after	0.56 ± 0.06†	7.23 ± 0.02†	0.63 ± 0.05†	7.31 ± 0.06†
B_{12}-deprived rats				
Before	0.57 ± 0.03‡	7.28 ± 0.03‡	0.60 ± 0.03‡	7.29 ± 0.02‡
2 h after	0.37 ± 0.06†§	7.07 ± 0.02†§	0.48 ± 0.03†§	7.20 ± 0.03†§

* Each subgroup consisted of six rats.
† $p < 0.001$ (Tukey's multiple comparison) compared to before loading.
‡ $p < 0.001$ (Tukey's multiple comparison) compared to control rats before loading.
§ $p < 0.001$ (Tukey's multiple comparison) compared to control rats 2 h after loading.

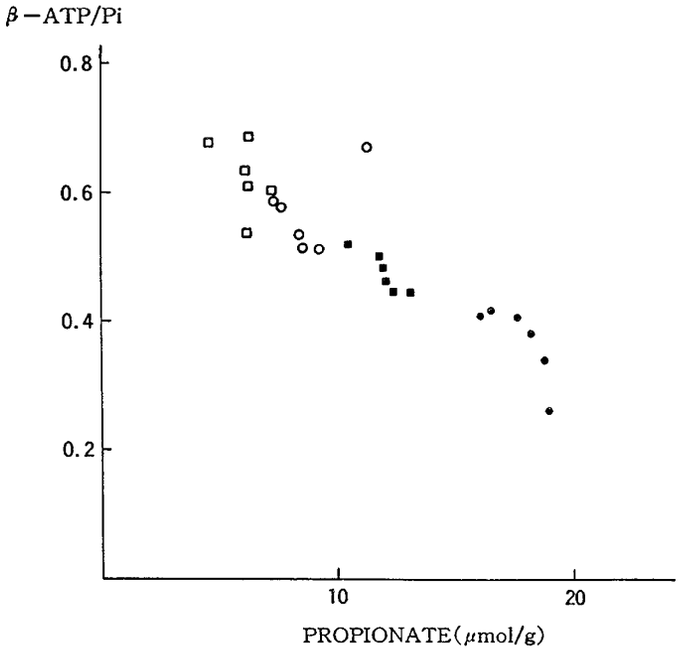

Fig. 1. Relationship between the liver concentrations of PA after organic acid loading and the ratio of β-ATP to Pi in the liver. PA loading to the B_{12}-deprived rats (*closed circles*), PA loading to the control rats (*open circles*), MMA loading to the B_{12}-deprived rats (*closed squares*), and MMA loading to the control rats (*open squares*).

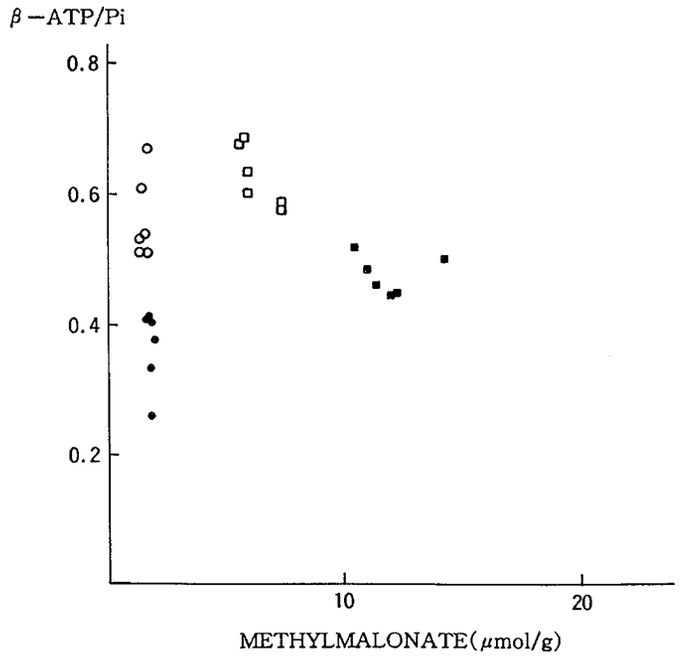

Fig. 2. Relationship between the liver concentrations of MMA after organic acid loading and the ratio of β-ATP to Pi in the liver. PA loading to the B_{12}-deprived rats (*closed circles*), PA loading to the control rats (*open circles*), MMA loading to the B_{12}-deprived rats (*closed squares*), and MMA loading to the control rats (*open squares*).

energy change of −1078 cal/mol, the reaction is thought to be readily reversible (5). Tietz and Ochoa (4) investigated this reversibility of PCoA carboxylase using purified enzyme from pig heart. They demonstrated that labeled ATP was formed from ADP and labeled Pi or from labeled ADP and Pi in the presence of MMCoA. Few reports, however, have demonstrated this reversibility or a consistent relationship between ATP and PA *in vivo*. Cathelineau *et al.* (11) reported that PA depleted ATP in isolated rat liver mitochondria, whereas Stewart and Walser (13) reported that liver ATP levels were unchanged after intraperitoneal injections of both PA and MMA. Our results showing that MMA loading led to similar levels of PA and MMA in the liver and that high concentrations of liver PA decreased liver ATP more significantly than did MMA demonstrate the reversibility of PCoA carboxylase *in vivo* and the role of accumulated PA, rather than of MMA, in the reduction of ATP levels.

These ATP changes and the impaired metabolism of PA and MMA were shown more clearly by loading of these organic acids in the B_{12}-deprived rats than in the control rats. In our experiments, the mean liver B_{12} level of the B_{12}-deprived rats was approximately 13% of that of the control rats. Scott *et al.* (20) reported that the liver B_{12} levels and total MMCoA mutase activity in B_{12}-deprived rats were 46 and 76%, respectively, of the values in control rats. Brass and Stabler (21) reported that a 74% decrease in liver B_{12} content resulted in a 21% decrease in hepatic MMCoA mutase activity. In our experiments, the liver PA and MMA levels of the B_{12}-deprived rats after loading were twice as high as those of the control rats. It is thought that this difference in concentration represents the decrease in MMCoA mutase activity *in vivo*.

Because *in vitro* studies, in which each enzyme activity is measured, do not always reflect the *in vivo* metabolism, we attempted to evaluate the *in vivo* effects of PA and MMA on the TCA cycle enzymes by assessing the substrate/product ratio, which is thought to reflect the impairment of enzyme activity under certain conditions. The following results are of interest in assessing TCA cycle enzyme activity: *1*) more marked increases in succinate levels or ratios of succinate to fumarate in both groups after PA loading than after MMA loading; *2*) increased levels of *cis*-aconitate after PA loading than after saline or MMA loading; and *3*) higher levels of malate in both groups after PA or MMA loading than after saline loading.

In both groups, the higher succinate levels after PA loading than after MMA loading suggest that PA inhibits the activity of succinate:CoA ligase (guanosine 5′-triphosphate) (12) or succinate dehydrogenase, inasmuch as MMA loading is thought to

Table 4. *Liver TCA cycle intermediates and related organic acids (μmol/g tissue)*

	Succinate	Fumarate	Malate	Oxaloacetate	Citrate	*cis*-Aconitate	2-Ketoglutarate	3-Hydroxybutyrate	Acetoacetate
Control rats									
Saline loading*	0.008 ± 0.002	0.057 ± 0.008	0.22 ± 0.04	0.018 ± 0.003	0.095 ± 0.018	0.17 ± 0.03	0.74 ± 0.08	0.19 ± 0.04	0.02 ± 0.00
PA loading	0.364 ± 0.008†‡	0.291 ± 0.059†	0.31 ± 0.05†	0.016 ± 0.003	0.084 ± 0.020	0.51 ± 0.05†‡	0.82 ± 0.17‡	0.15 ± 0.07	0.03 ± 0.01†
MMA loading	0.115 ± 0.014†	0.357 ± 0.022†	0.33 ± 0.04†	0.036 ± 0.005	0.057 ± 0.011	0.17 ± 0.04	2.17 ± 0.60†	0.17 ± 0.04	0.06 ± 0.02†
B_{12}-deprived rats									
Saline loading	0.012 ± 0.003	0.096 ± 0.019	0.19 ± 0.03	0.005 ± 0.003	0.066 ± 0.001	0.24 ± 0.03	0.16 ± 0.03	0.35 ± 0.09§	0.04 ± 0.01§
PA loading	0.062 ± 0.008†‡§	0.084 ± 0.007‡§	0.34 ± 0.11†	0.025 ± 0.003	0.042 ± 0.007‡	1.18 ± 0.06†‡§	0.49 ± 0.09†‡	0.39 ± 0.08§	0.17 ± 0.04†§
MMA loading	0.016 ± 0.006§	0.034 ± 0.004§	0.29 ± 0.07†	0.008 ± 0.001	0.126 ± 0.013†	0.41 ± 0.04†§	1.16 ± 0.34†	0.31 ± 0.05§	0.15 ± 0.04†§

* Each subgroup consisted of six rats.
† $p < 0.05$ (Tukey's multiple comparison) compared to saline loading in control rats or in B_{12}-deprived rats.
‡ $p < 0.05$ (Tukey's multiple comparison) compared to PA loading and MMA loading.
§ $p < 0.05$ (Tukey's multiple comparison) compared to control rats.

supply larger amounts of succinyl-CoA to the TCA cycle than PA loading because of the higher MMA levels found after MMA loading than after PA loading. It is possible that the lower levels of succinate and fumarate found after PA or MMA loading in the B_{12}-deprived rats, as compared to the control rats, reflect decreased activity of hepatic MMCoA mutase in the former. Increased *cis*-aconitate levels after PA loading suggest decreased aconitase or isocitrate dehydrogenase activity, which requires NAD^+. It is not clear which enzyme is involved, because we could not measure the levels of citrate and isocitrate separately. Increased malate levels after PA and MMA loading may show decreased activity of malate dehydrogenase, which also requires NAD^+. The ratio of 3-hydroxybutyrate to acetoacetate is known to reflect the mitochondrial ratio of NAD^+ to $NADH^+$ (24). Decreases in these ratios after PA and MMA loading suggest that PA and MMA have inhibitory effects on these NAD^+-dependent dehydrogenases of the TCA cycle. These findings are in accordance with those of the *in vitro* study of Rosario and Medina (25), which showed that PA stimulated ketogenesis and decreased the ratio of 3-hydroxybutyrate to acetoacetate.

The results of our analysis of *in vivo* TCA cycle intermediates suggest that PA may interfere with energy production, due to inhibition of the TCA cycle through the inhibition of some of the above-mentioned enzymes. Thus, the remarkable decrease in hepatic ATP after PA infusion is thought to be induced by the consumption of ATP by acyl-CoA synthetase and PCoA carboxylase, as well as by the inhibition of ATP production by PA. Based on our data, it is speculated that during crises of methylmalonic aciduria an accumulation of MMCoA causes an increase in PCoA through the reverse reaction of PCoA carboxylation and a subsequent increase in PA. This increase in PA creates a vicious circle, namely, increased PA inhibits the energy production system, and PCoA, which is produced through the increased catabolism of protein and lipids, cannot be metabolized to MMCoA because of ATP depletion; thus, the PA level increases further.

The results of our experiment emphasize both the importance of monitoring PA levels in methylmalonic aciduria, especially during crises, and the need for the earliest possible direct removal of PA, using procedures such as hemodialysis (26) or continuous peritoneal dialysis (27).

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