



# Effects of Biotin Deficiency on the Urinary Excretion of B-Group Vitamins in Mice

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### Abstract

**Introduction:** The eight kinds of B-group vitamins are involved with numerous metabolisms in harmony. Therefore, deprivation of one vitamin is likely to affect the pharmacokinetics and requirements of the other vitamins. Biotin has important roles, such as fatty acid synthesis, branched-chain amino acid catabolism, odd-chain fatty acid catabolism, and gluconeogenesis. We previously revealed that the urinary excretion rates of B-group vitamins decline when demand in the body increases. In the present study, we investigated the effects of biotin deficiency on the urinary excretion rates ((urinary excretion amount/intake amount) × 100) of other B-group vitamins.

**Methods:** ICR female mice were fed a 30% egg white diet with or without biotin. After feeding for 21 days, 24h urine samples were collected, and B-group vitamins were measured.

**Results and conclusion:** The urinary excretion rate of vitamin B<sub>1</sub> was markedly lower in the biotin-deficient mice than in the control mice. These results suggest that biotin deficiency increased the requirement of vitamin B<sub>1</sub> but did not affect the other six B-group vitamins.

### Keywords

Biotin; Deficiency; Vitamin B<sub>1</sub>; Urine; Mice

**Abbreviations:** 4-PIC: 4-Pyridoxic Acid; MNA: N<sup>1</sup>-Methylnicotinamide; 2-Py: N<sup>1</sup>-Methyl-2-Pyridone-5-Carboxamide; 4-Py: N<sup>1</sup>-Methyl-4-Pyridone-3-Carboxamide; Nam: Nicotinamide; ThDP: Thiamin Diphosphate; PC: Pyruvate Carboxylase; ACC: Acetyl-CoA Carboxylase; PCC: Propionyl-CoA Carboxylase; MCC: β-Methylcrotonyl-CoA Carboxylase; PDH: Pyruvate Dehydrogenase

### Introduction

B-group vitamins function as coenzymes and gene expression regulation factors [1,2] in biogenics. Therefore, the intake of water-soluble vitamins affects by disease [3,4], metabolism, and requirements for nutrition [5-8].

We have started to investigate the effect of one vitamin deficiency on other vitamin requirements and metabolism. Biotin is a water-soluble vitamin classified among the B-group of vitamins. Biotin serves as a coenzyme for carboxylases. These carboxylases have important roles in gluconeogenesis, fatty acid synthesis, and branched-chain

amino acid catabolism. It is difficult to cause biotin deficiency in people with a general dietary habit because biotin is abundant in various foods [9,10]. However, biotin deficiency has been reported in a substantial number of patients with chronic alcoholism [11-13], patients receiving long-term therapy with anticonvulsant agents [14-16], individuals born with errors in biotin metabolism [17], and infants who are taking special infant formulas in Japan (for example, amino acid formulas) [18]. It has been reported that biotin deficiency affects folacin metabolism [19-21]. Puddu et al. [22] reported that the urinary excretion of vitamin B<sub>12</sub> was not affected in rats fed a biotin-deficient diet for 60 days. To our knowledge, however, there are no other reports on the effect of a biotin-deficient diet on other B-group vitamins.

In the present study, we investigated how B-group vitamins are affected by biotin-deficient status.

### Materials and Methods

#### Chemicals

Egg white solids and sucrose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was obtained from Ajinomoto (Tokyo, Japan). A mineral mixture (AIN-93-G-MX) [23], biotin-free vitamin mixture (AIN-93) [23], dextrin, and cellulose were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Thiamin hydrochloride (vitamin B<sub>1</sub>), thiamin diphosphate (ThDP), riboflavin (vitamin B<sub>2</sub>), nicotinamide, calcium pantothenate, pteroylglutamic acid (folic acid, used as folacin), cyanocobalamin (vitamin B<sub>12</sub>), and biotin were purchased from Wako Pure Chemical Industries, Ltd. 4-Pyridoxic acid (4-PIC, a catabolite of vitamin B<sub>6</sub>) manufactured by ICN Pharmaceuticals (Costa Mesa, CA, USA) was obtained from Wako Pure Chemical Industries. N<sup>1</sup>-Methylnicotinamide (MNA) (a catabolite of nicotinamide) chloride was from Tokyo Chemical Industry (Tokyo, Japan). N<sup>1</sup>-Methyl-2-pyridone-5-carboxamide (2-Py, a catabolite of nicotinamide) [24] and N<sup>1</sup>-methyl-4-pyridone-3-carboxamide (4-Py, a catabolite of nicotinamide) [25] were synthesized as described. All other chemicals were of the highest purity available from commercial sources.

#### Mice and diets

**Deficiency experiment:** Female ICR mice (3 weeks old) were obtained from Charles River Laboratories (Tokyo, Japan), immediately divided into two groups (control group, n=6; biotin-deficient group, n=5), and housed in metabolic cages (LC-0335; CLEA Japan, Tokyo, Japan). The control mice were fed a 30% egg white diet as the control diet and the biotin-deficient mice were fed the biotin-deficient diet (Table 1) for 21 days. The 24 h urine samples were collected from 09:00-09:00 during the last days in amber bottles containing 1 mL of 1 mol/L HCL and were stored at -20°C until needed.

Animals were allowed free access to food and water, and body weight was measured every two days. Food intake of mice was measured daily. Temperature was maintained at approximately 20°C with 60% humidity and a 12 h light/dark cycle (lights on at 6:00 and off at 18:00). The care and treatment of the experimental animals conformed to the guidelines for the ethical treatment of laboratory animals set by the University of Shiga Prefecture (Shiga, Japan) (Approval No. 26-3).

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**Table 1:** Diet compositions [23].

	Control diet (g/kg diet)	Biotin-deficient diet (g/kg diet)
Egg white solids	300	300
Gelatinized cornstarch	309.3	309.3
Sucrose	154.2	154.2
Corn oil	80	80
Dextrin	50	50
Cellulose	50	50
Choline bitartrate	2.5	2.5
Mineral mixture (AIN-93-G-MX) <sup>1</sup>	42	42
Biotin-free vitamin mixture (AIN-93) <sup>1</sup>	12	12
Biotin	0.004	-

### Measurement of B-group vitamins in urine

**Vitamin B<sub>1</sub>, vitamin B<sub>2</sub>, vitamin B<sub>12</sub>, pantothenic acid, folacin, and biotin:** Preparation and measurement of the extracts of vitamin B<sub>1</sub>, vitamin B<sub>2</sub>, vitamin B<sub>12</sub>, pantothenic acid, folacin, and biotin from urine were done as previously described [6]. Vitamin B<sub>1</sub> was measured as thiamin and vitamin B<sub>2</sub> was measured as riboflavin.

**Vitamin B<sub>6</sub>:** Vitamin B<sub>6</sub> is excreted in urine as 4-PIC. The 4-PIC in urine was measured by high performance liquid chromatography (HPLC) using a previously described method [6].

**Nicotinamide and its metabolites:** Nicotinamide (Nam) [26], MNA [27], 2-Py [26], 4-Py [26], and Nam *N*-oxide [26] were described previously.

### Calculation of urinary excretion rate of vitamins

Urinary excretion percentage of each vitamin was calculation by the following formula:

Urinary excretion of rate (%) = urinary excretion of each vitamin (nmol/d) / each vitamin intake (nmol/d) × 100.

Each vitamin intake was calculated by food intake on day 21 and each vitamin's content in the vitamin mixture. Vitamin B<sub>2</sub> is contained in egg white solids [28]. So, we calculated the urinary excretion percentage of vitamin B<sub>2</sub> considering vitamin B<sub>2</sub> coming from the egg white solids as 3.25 μmol riboflavin/100 g egg white solids [28].

### Liver

Female ICR mice (3 weeks old) were obtained from Charles River Laboratories (Tokyo, Japan) and immediately divided into two groups (control group, n=5; biotin-deficient group, n=5) and housed in plastic cages (5/cage). The control mice were fed the 30% egg white diet as the control diet and the biotin-deficient mice were fed the biotin-deficient diet for 21 days (Table 1). On day 22, the mice were sacrificed and their livers were removed.

**Measurement of biotin in liver:** Frozen liver samples, approximately 0.5 g, were thawed, minced, and then added to 2 volumes of 2.25 mol/L H<sub>2</sub>SO<sub>4</sub> and homogenized with a Teflon-glass homogenizer. The suspension was autoclaved at 121°C for 1 h to convert the bound form of biotin to the free form of biotin. After being cooled, the suspension was centrifuged at 10,000 × g for 10 min at 4°C, and supernatant was used for measuring biotin in the microbioassay method [6].

### Measurement of vitamin B<sub>1</sub> in liver

Liver contains conjugated forms of vitamin B<sub>1</sub> such as the free form of vitamin B<sub>1</sub> and ThDP. We measured the free form of thiamin and ThDP in the liver by HPLC as follows [29] with slight modification.

Frozen liver samples, approximately 0.5 g, were thawed, minced, and then added to 10 volumes of 5% trichloroacetic acid and homogenized with a Teflon-glass homogenizer. The acidified homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant tissues (200 μL) were added to 40 μL of 1% cyanogen bromide and 80 μL of 5% NaOH. After 10 min at room temperature, 80 μL of 1.5 mol/L HCl was added for neutralization. The resulting supernatant was passed through a 0.45-μm Hydrophilic Durapore™ filter (PVDF; Millipore, Bedford, MA, USA). The filtrate (20 μL) was directly injected into an HPLC system to measure the level of thiochrome (thiamin-derived fluorescent compound) and thiochrome-diphosphate (ThDP-derived fluorescent compound). A Tosoh ODS-100S (15 × 3.2 mm, I.D., average particle size: 5 μm) column was used as a pre-column and a Tosoh ODS-100S (250 × 4.6 mm, I.D., average particle size: 5 μm) column was used as an analytical column. Thiochrome in samples was separated using the following mobile phase: 0.05 mol/L KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0) containing 3% acetonitrile. Thiochrome-diphosphate in samples was separated by using following mobile phase: 0.05 mol/L KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub> buffer (pH 7.0) containing 1.5% acetonitrile. Flow speed was set at 1.0 mL/min. Fluorescence intensities were measured with an excitation wavelength of 375 nm and emission wavelength of 430 nm.

### Statistical analyses

All data are indicated as average ± SE. The change in urinary excretion of vitamin B<sub>1</sub> was analyzed by two-way ANOVA. Other data were analyzed by Student's *t*-test. *P*<0.05 was considered significant. All statistical analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

### Results

#### Effects of biotin deficiency on body weight and food intake

Changes in animal body weight (Figure 1) and food intake (Table 2) on the last day of the experiment did not differ markedly between the control and biotin-deficient groups. Liver weights in the biotin-deficient group were not affected by feeding on the biotin-deficient diet (Table 2).

#### Effect of biotin deficiency on the urinary excretion of B-group vitamins

Table 3 shows the effects of biotin deficiency on the urinary excretion amounts of vitamin B<sub>1</sub>, vitamin B<sub>2</sub> and biotin. Figure 2 shows the changes in urinary excretion amounts of vitamin B<sub>1</sub> during the experiment.

Urinary excretion of vitamin B<sub>1</sub> was 42% lower in the biotin-deficient group than in the control group on day 21 (Table 3).

Figure 3 shows the urinary excretion rates of B-group vitamins when the animals were in biotin-deficient status. The urinary excretion rate of vitamin B<sub>1</sub> was 32% lower in the biotin-deficient group than in the control group on day 21 (Figure 3A).

Urinary excretion of vitamin B<sub>2</sub> was 11% lower in the biotin-deficient group than in the control group on day 21 (Table 3);

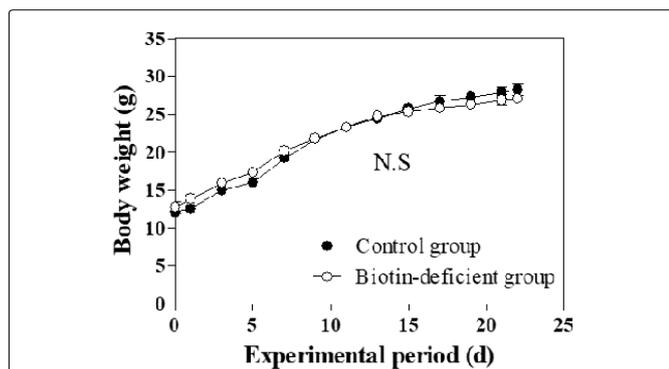


Figure 1: Effect of biotin deficiency on mice growth.

(A) Body weights during the experimental period. Values are mean ± SE; control, n=6; biotin-deficient n=5. The significance of the differences was tested using Student's *t*-test for each day. Animal body weights during the experimental period did not differ markedly between the control and biotin-deficient groups.

Table 2: Effect of biotin-free diet on body weight, food intake, and liver weight in mice.

	Control diet	Biotin-deficient diet
Final body weight (g)	28.5 ± 1.2	27.5 ± 0.5
Food intake during the experiment (g/21 d)	4.0 ± 0.4	3.9 ± 0.2

Values are mean ± SE; control, n=6; biotin-deficient group, n=5.

Table 3: Urinary excretion amounts of B-group vitamins on day 21 in mice.

	Control group	Biotin-deficient group
Vitamin B <sub>1</sub> (nmol/d)	35.0 ± 2.1	20.2 ± 2.9*
Vitamin B <sub>2</sub> (nmol/d)	117 ± 3	105 ± 3*
4-PIC, a catabolite of vitamin B <sub>6</sub> (nmol/d)	11.5 ± 2.6	19.8 ± 6.1
Vitamin B <sub>12</sub> (pmol/d)	2.9 ± 0.4	3.0 ± 0.3
Sum of Nam and its catabolites (µmol/d)	2.29 ± 0.22	2.15 ± 0.39
Pantothenic acid (nmol/d)	310 ± 49	247 ± 54
Folacin (nmol/d)	5.2 ± 0.6	4.0 ± 1.0
Biotin (nmol/d)	14.5 ± 2.7	0.05 ± 0.01***

Values are mean ± SE; control, n=6; biotin-deficient group, n=5. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001; the significance of the differences was tested using Student's *t*-test

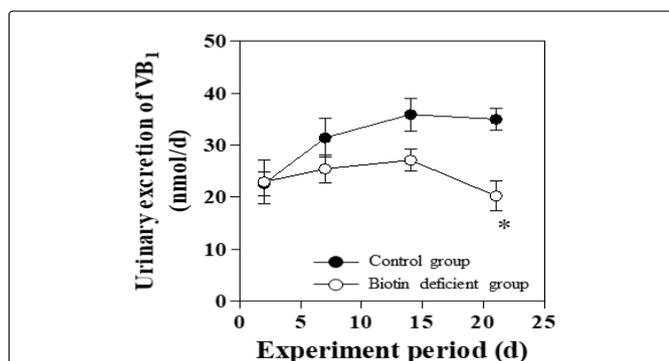


Figure 2: Change in urinary excretion of vitamin B<sub>1</sub> during experimental term.

The urinary excretion of vitamin B<sub>1</sub> on days 2,7,14 and 21. Control, n=6; biotin-deficient group, n=5. Values are mean ± SE. \**P*<0.05; the significance of the differences was tested using two-way ANOVA.

however, the urinary excretion percentage of vitamin B<sub>2</sub> did not differ between the control and biotin-deficient groups (Figure 3B).

The urinary excretion amounts of 4-PIC (a catabolite of vitamin B<sub>6</sub>), vitamin B<sub>12</sub>, sum of Nam and its catabolites such as MNA, 2-Py, and 4-Py, pantothenic acid, and folacin were not affected nor were the urinary excretion percentages affected (Table 3, Figures 3C-3G).

### Vitamin B<sub>1</sub> content in liver

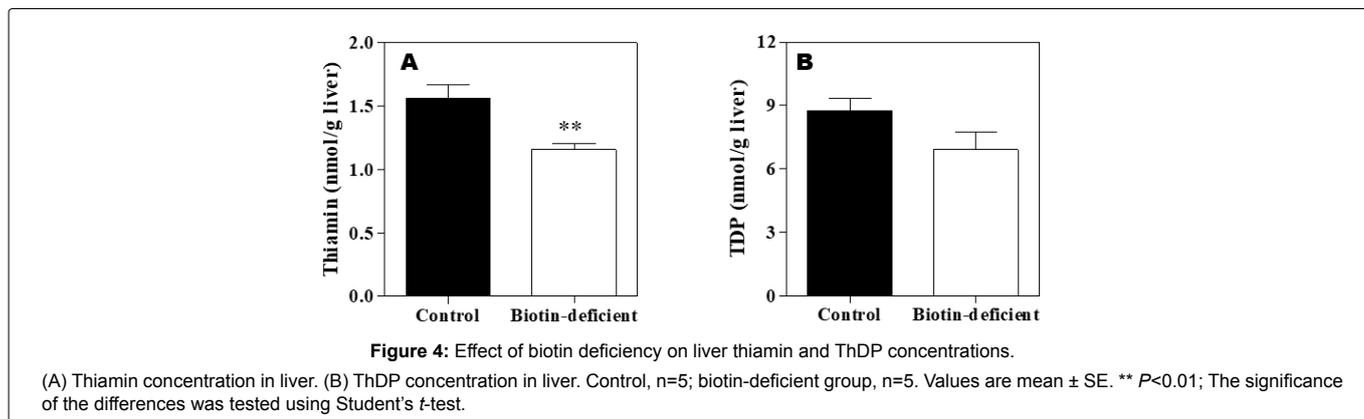
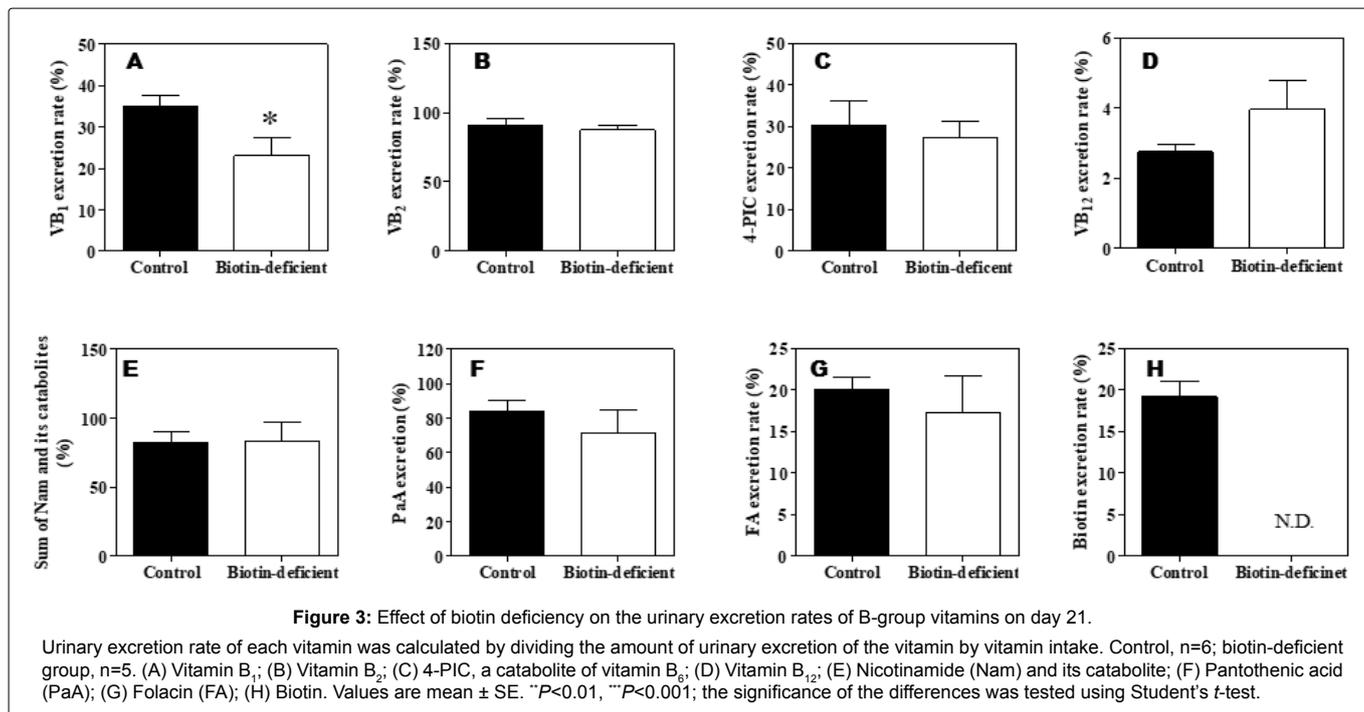
The concentration of the free form of thiamin was lower in the biotin-deficient group than in the control group (Figure 4A). ThDP, which is the coenzyme form of vitamin B<sub>1</sub>, did not differ between the control mice and biotin-deficient mice (Figure 4B).

### Discussion

Eight kinds of B-group vitamins are involved in numerous metabolisms in harmony. Therefore, deprivation of one vitamin from the eight is likely to affect the pharmacokinetics and requirements of the other vitamins. Biotin has important roles, such as fatty acid synthesis, branched-chain amino acid catabolism, odd-chain fatty acid catabolism, and gluconeogenesis. We previously reported that the urinary excretion rates of B-group vitamins declined when demand in the body increased [30,31]. This study investigated the effects of biotin deficiency on the urinary excretion rates of other B-group vitamins.

We demonstrated that feeding the biotin-deficient diet for 21 days markedly decreased the urinary excretion rate of vitamin B<sub>1</sub> in ICR mice. The concentration of the free form of vitamin B<sub>1</sub> in the liver was lower in the biotin-deficient mice than in the control mice. Nevertheless, the concentration of ThDP, a coenzyme of vitamin B<sub>1</sub>, was not different between the biotin-deficient and control mice. Generally, the free form of thiamin in the body reflects the amount of surplus of vitamin B<sub>1</sub> [31,32]. The urinary excretion amounts of vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, pantothenic acid, folacin, and niacin were not affected by the biotin deficiency. These results suggest that biotin deficiency increased the vitamin B<sub>1</sub> requirement. A prolonged biotin-deficient status will induce vitamin B<sub>1</sub> deficiency and ketosis (Figure 5).

Biotin is coenzyme of four kinds of carboxylases: pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), and β-methylcrotonyl-CoA carboxylase (MCC). These four carboxylase activities decrease in biotin-deficient animals [33]. As a result, gluconeogenesis, fatty acid synthesis, branched-chain amino acid catabolism and odd-chain fatty acid catabolism are disordered in biotin-deficient animals (Figure 5). For reactions involving vitamin B<sub>1</sub>, many kinds of 2-oxo acids dehydrogenases and transketolase are well known. The metabolism of pyruvate is the sole reaction that concerns both biotin and vitamin B<sub>1</sub>. PC is an enzyme that metabolizes pyruvate to oxaloacetate and is a key enzyme in gluconeogenesis, as well as being an important anaplerotic reaction for maintenance of an adequate supply of oxaloacetate for citrate cycle activity. In biotin-deficient animals, PC activity decreases in the liver and pyruvate accumulates [34,35]. The accumulated pyruvate should be metabolized to oxaloacetate, acetyl CoA, or lactate. In biotin-deficient animals, the PC reaction is decreased, so that much more pyruvate is metabolized to acetyl-CoA or lactate. Pyruvate dehydrogenase (PDH) is the enzyme that can metabolize pyruvate to acetyl CoA. PDH needs vitamin B<sub>2</sub>, niacin, and pantothenic acid as well as vitamin B<sub>1</sub> as coenzymes. But, the urinary excretion



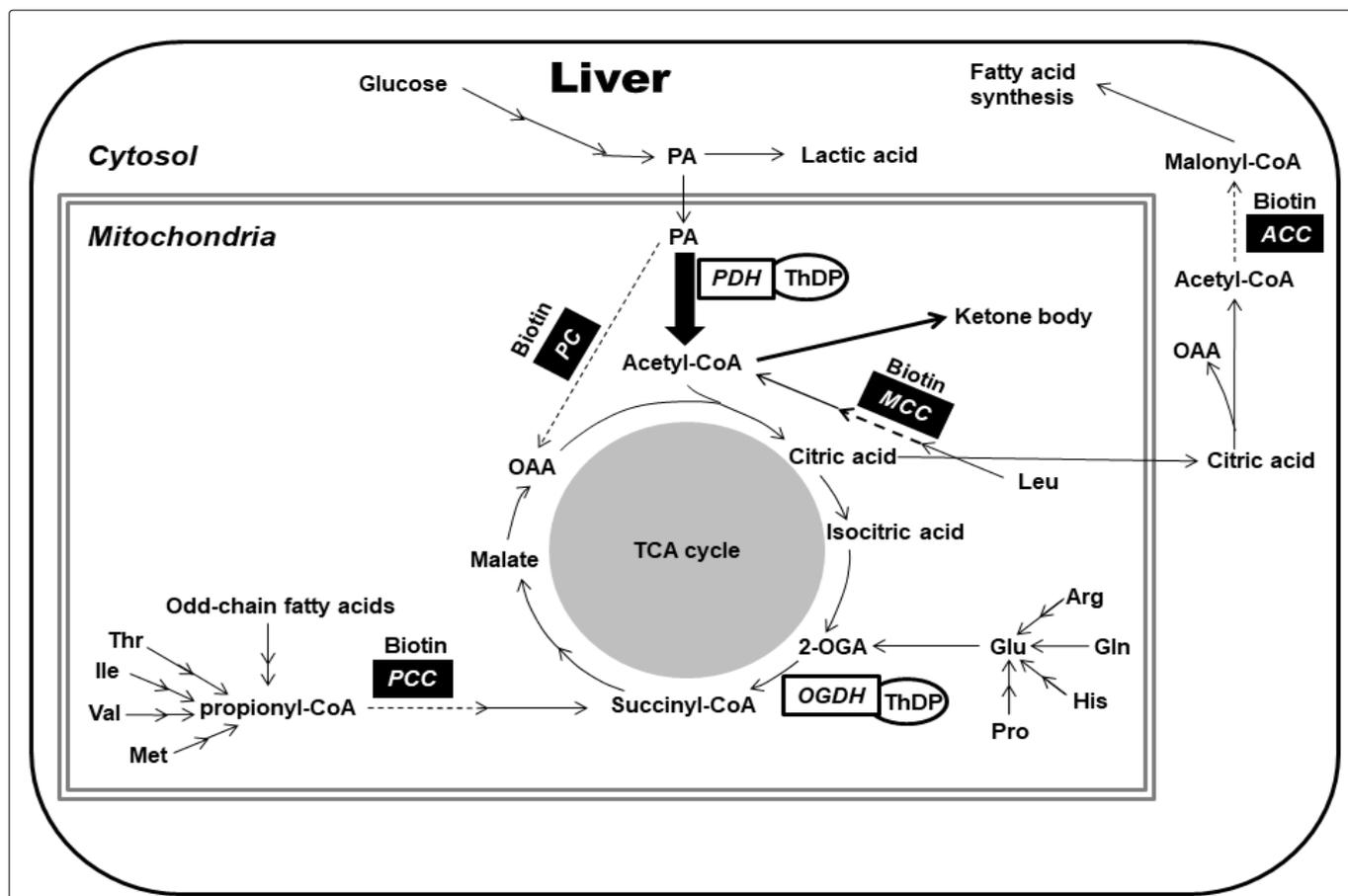
amounts of vitamin B<sub>2</sub>, pantothenic acid, and niacin were not affected by biotin deficiency. The present study suggests that the pyruvate decarboxylase reaction, which needs ThDP as a coenzyme, is the rate-limiting step in whole PDH reaction. Thus, the demand of vitamin B<sub>1</sub> as cocarboxylase must increase in a biotin-deficient state. The excess acetyl-CoA is converted to ketone bodies.

Although vitamins B<sub>2</sub>, pantothenic acid, niacin are associated with whole PDH reaction, urinary excretion percentage of these vitamins did not change in the biotin deficient group in our study. Vitamin B<sub>1</sub> is a vitamin that is more easier to deficiency than other vitamins because vitamin B<sub>1</sub> pool is small [30,36-39]. Accumulations of vitamin B<sub>2</sub>, pantothenic acid and niacin in liver are 3 to 50 times higher than vitamin B<sub>1</sub> [30,40]. Therefore, we considered that the impact of biotin deficiency did not appear markedly for the nutrient of vitamin B<sub>2</sub>, pantothenic acid and niacin. It has been reported that urinary excretion of folic acid was decreased in the biotin deficient rats were fed the biotin free diet for approximately 60 days [19-21]. In our study, folic acid level on urine was not affected by the biotin deficiency. We fed the biotin

deficient diet to mice for 21 days. We considered that urinary folic acid level did not decrease in the biotin deficient group because the experiment term was short. In actuality, the urinary excretion percentage of folic acid was decreased in mice fed biotin deficient diet for 35 days (control group: 21.6% ± 1.6%, biotin deficient group: 12.2% ± 3.3%, P<0.05).

### Conclusion

Biotin deficiency has been reported in a substantial number of patients with chronic alcoholism [11-13], patients on long-term parenteral nutrition, patients receiving long-term therapy with anticonvulsant agents [14-16], individuals born with errors in biotin metabolism [17], and infants who are taking special infant formulas in Japan (for example amino acid formulas [18]). These people might have a vitamin B<sub>1</sub> deficiency. PDH activity is markedly lower in the vitamin B<sub>1</sub> deficient animal [36], so pyruvate accumulates in the body. Therefore, vitamin B<sub>1</sub> deficiency might be hidden by biotin deficiency, and biotin deficiency might be exacerbated by vitamin B<sub>1</sub> deficiency.



**Figure 5:** Pyruvate metabolism with insufficient nutritional status of biotin in liver.

PA: Pyruvate; PDH: Pyruvate Dehydrogenase; ThDP: Thiamin Diphosphate; PC: Pyruvate Carboxylase; PEP: Phosphoenolpyruvate; ACC: Acetyl-CoA Carboxylase; PCC: Propionyl-CoA Carboxylase; MCC: β-Methylcrotonyl-CoA Carboxylase; 2-OGA: 2-Oxoglutarate; OGDH: 2-Oxoglutarate Dehydrogenase; OAA: Oxaloacetate; Leu: Leucine; Arg: Arginine; Gln: Glutamine; Glu: Glutamic acid; His: Histidine; Pro: Proline; Thr: Threonine; Ile: Isoleucine; Val: Valine; Met: Methionine.

**Authors’ Disclosure**

The authors have no conflicts of interest to declare.

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**Authors’ Contributions**

AT and KS designed the study. AT and KS drafted the manuscript. AT performed the experiments. All authors read and approved the final manuscript.

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