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ORIGINAL PAPER

Vitamin B6 deficiency augments endogenous oxalogenesis after intravenous L-hydroxyproline loading in rats

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Abstract The effects of an intravenous hydroxyproline load on endogenous oxalogenesis were compared in rats fed a standard diet or a vitamin B6-deficient diet. Twelve male Wistar rats were randomized to two groups and were fed either a standard diet (control group) or a vitamin B6-deficient diet for 3 weeks. Then the animals were intravenously administered 100 mg (762.6 μmol)/ml hydroxyproline. In the control group, infusion of hydroxyproline increased the 5-h urinary oxalate and glycolate excretion above baseline to 0.27% (2.02 ± 1.11 μmol) and 0.32% (2.43 ± 1.60 μmol) of the administered dose (mol/mol), while it was respectively 2.01% (15.24 ± 2.13 μmol) and 0.00% (−0.02 ± 0.19 μmol) of the dose in the vitamin B6-deficient group. Therefore, vitamin B6 deficiency augmented endogenous synthesis of oxalate from hydroxyproline by 7.56-fold (15.24/2.02) compared with that in the control group. Urinary citrate excretion was significantly lower at baseline and all other times in the vitamin B6-deficient group compared with the control group. In conclusions, L-hydroxyproline loading augmented endogenous oxalogenesis in the vitamin B6-deficient group without causing hyperglycolic aciduria, and also led to significant hypocitraturia. These findings suggest that hydroxyproline is not metabolized to oxalate via glycolate, but rather via the 4-hydroxyglutamate to glyoxylate pathway (usually requiring vitamin B6-dependent enzymes) even in the presence of vitamin B6 deficiency.

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Introduction

In humans and animals the chief immediate metabolic precursor of oxalate is glyoxylate, which is formed from glycolate in the peroxisomes and from hydroxyproline in the mitochondria. The glyoxylate pathway is associated with gluconeogenesis, and it is believed to be the major metabolic pathway for the synthesis of oxalate, particularly in persons with primary hyperoxaluria [1]. Among the various precursors of oxalate, glycolate and glyoxylate appear to be the most important substances that promote oxalogenesis [2, 3], whereas administration of either glycine or ascorbate does not increase urinary oxalate excretion in rats [4–7]. A number of experimental models have been developed to study calcium oxalate urolithiasis, and virtually all of them employ hyperoxaluria as the basic abnormality. In all the experimental models renal injury is associated with crystal deposition mainly on the intraluminal side of renal tubules and preferentially attached to renal papillary tips and fornices. And rat renal calcium oxalate urolithiasis is similar to calcium oxalate stone disease in human [8]. A recent study of pigs fed a 10% hydroxyproline diet revealed the development of hyperoxaluria and calcium oxalate crystalluria, suggesting that omnivores or herbivores (which usually do not have a good glyoxylate-detoxifying system) may be suitable as a model of hyperoxaluria after a hydroxyproline load [9, 10]. We previously reported that intravenous infusion of hydroxyproline increased

urinary oxalate in close relation with urinary glycolate in rats [11, 12]. In addition, Ribaya and Gershoff [13–15] reported that urinary oxalate excretion was increased in vitamin B6-deficient rats or control rats fed with a diet containing 5.2% hydroxyproline, while a diet containing 3% glycine and 5.2% hydroxyproline caused an increase of renal oxalate and glyoxylate levels, but decreased the glycolate level, in vitamin B6-deficient rats compared with controls. These findings prompted us to study the urinary excretion of oxalate and glycolate after administration of L-hydroxyproline to vitamin B6-deficient rats.

Materials and methods

Twelve male Wistar rats weighing 174.95 ± 5.13 g (mean ± SD) were acclimatized at the Animal Center, and then were randomized to two groups of six animals each. One group was fed a standard (CE-2) diet (controls), while the other rats were fed a vitamin B6-deficient diet for 3 weeks. Both groups of rats had free access to drinking water. After 3 weeks, the rats were anesthetized with an intraperitoneal injection of urethane (1.2 g/kg body weight) and were hydrated with physiological saline via the femoral vein at a rate of 2.5–3.5 ml/h. Then the animals in both groups were administered 1 ml of a 100 mg/ml hydroxyproline solution as a slow intravenous infusion over 10 min. Infusion of saline was continued at a rate of 3.5 ml/h throughout the experiment.

The hydroxyproline solution was prepared by dissolving 100 mg (762.6 μmol) of hydroxyproline (molecular weight: 131.13; Wako Pure Chemicals, Osaka, Japan) in 1 ml of pure water. The bladder was emptied 1 h before hydroxyproline infusion and hourly urine specimens were collected by bladder puncture at baseline and every hour until 5 h after infusion. Measurement of the urine volume was done at each time of collection. The urine specimens were immediately stored at −80°C until assay.

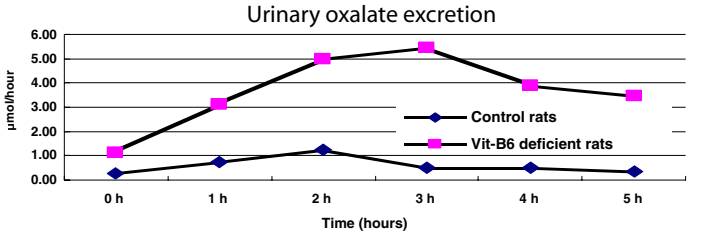
Thawed urine specimens were filtered through a disposable 0.2 μm filter (Millex-LG syringe-driven unit, Millipore, Bedford, MA, USA), were diluted 20- to 40-fold with Milli-Q level pure water that was obtained using a water purification system (Millipore), and were injected into a capillary tube at 50 mbar (5,000 Pa) for 4 s (approximately 20 nl). Then measurement of the urinary glycolate and citrate levels was done by capillary electrophoresis (Agilent CE, Germany) using an organic acids buffer (pH 5.6) for high performance capillary electrophoresis (HPCE) that contained 5 mM 2,6-pyridinedicarboxylic acid and 0.5 mM cetyltrimethylammonium bromide (CTAB) (Agilent Technologies, Germany) [16, 17]. In addition, aliquots of the thawed urine specimens were acidified to <pH 2 with 6 N HCl and then were diluted with water in the same fashion to measure urinary oxalate by capillary electrophoresis with a pyromellitic acid electrolyte buffer (pH 7.7) for anion HPCE (Fluka, Switzerland) [16, 17].

Urinary levels of oxalate, glycolate, and citrate at 0 h were defined as the baseline values for excretion of each substance. The cumulative increment of urinary oxalate and glycolate excretion above baseline (recovery of excretion) after infusion of hydroxyproline was calculated for each group by subtracting the baseline oxalate and glycolate values. Hourly urinary excretion levels for oxalate, glycolate, and citrate were compared with the respective baseline values using the Wilcoxon signed ranks test (2-tailed), while the hourly and total urinary excretion values were compared between groups using the Mann–Whitney *U* test. Data are reported as the mean ± SD and statistical significance was set at $P < 0.05$ for all comparisons.

Results

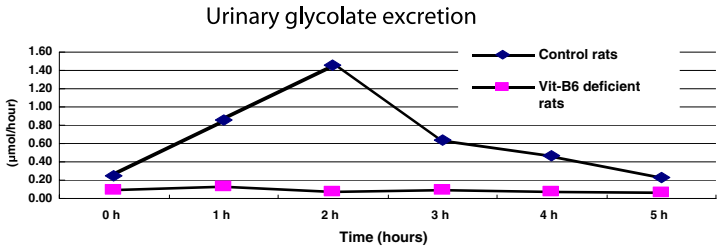
The rats showed significant weight gain over time from 176.33 ± 5.28 g (mean ± SD) and 173.56 ± 5.04 g at baseline to 394.38 ± 8.40 g and 309.10 ± 15.61 g after 3 weeks in the control and vitamin B6-deficient groups, respectively ($P < 0.05$ for baseline vs. 3 weeks and between the two groups at 3 weeks). Weight gain in the vitamin B6-deficient group occurred at a rate of approximately 6.45 g/day, which was lower than in the control group (approximately 10.38 g/day), and this difference was statistically significant ($P < 0.01$).

Baseline urinary oxalate excretion was significantly higher in the vitamin B6-deficient group (1.14 ± 0.27 μmol) than in the control group (0.27 ± 0.09 μmol) ($P < 0.01$) (Figs. 1, 2). Hourly urinary oxalate excretion peaked within 2–3 h after hydroxyproline infusion in both groups (Fig. 1). In the vitamin B6-deficient group, hourly urinary oxalate excretion was significantly higher than baseline from 1 to 5 h. In the control group, it was higher from 1 to 3 h after hydroxyproline infusion ($P < 0.05$) (Fig. 1). The total (0–5 h) urinary oxalate excretion was significantly higher in the vitamin B6-deficient group (22.07 ± 1.85 μmol) than in the control group (3.65 ± 0.74 μmol) ($P < 0.01$, between groups). The 5-h cumulative increment of urinary oxalate excretion above baseline (recovery rate) accounted for 0.27% (mol/mol) (2.02 ± 1.11 μmol) versus 2.01% (15.24 ± 2.13 μmol) of the administered dose of hydroxyproline in the control group and the



Hourly Ox excretion	0	1	2	3	4	5 hours	Total	Increment
Control group (μmol)	0.27± 0.09	0.74± 0.12	1.25± 0.54	0.50± 0.23	0.52± 0.23	0.37± 0.07	3.65± 0.74	2.02± 1.11
Comparison vs baseline (p value)		0.028	0.028	0.046	0.075	0.075		
Between groups (p value)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Vitamin B6 (−) group (μmol)	1.14± 0.27	3.13± 0.68	4.98± 0.68	5.47± 1.13	3.88± 0.39	3.47± 1.20	22.07± 1.85	15.24± 2.13
Comparison vs baseline (p value)		0.028	0.028	0.028	0.028	0.028		

Fig. 1 Urinary oxalate (Ox) excretion after administration of L-hydroxyproline to vitamin B6-deficient rats and control rats. Total = total cumulative urinary oxalate excretion from 0 to 5 h. Increment = cumulative increment of urinary oxalate excretion above baseline from 1 to 5 h after hydroxyproline loading



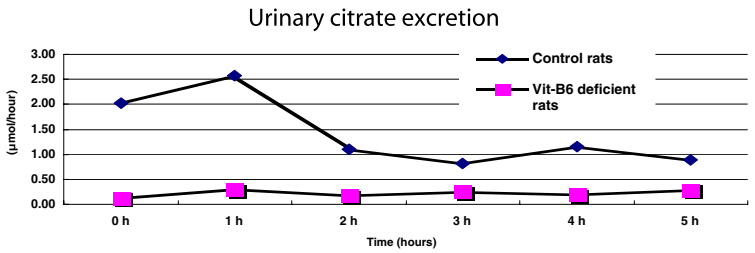
Hourly Glc excretion	0	1	2	3	4	5 hours	Total	Increment
Control group (μmol)	0.25± 0.07	0.85± 0.26	1.46± 0.69	0.64± 0.54	0.47± 0.17	0.23± 0.10	3.90± 1.56	2.43± 1.60
Comparison vs baseline (p value)		0.028	0.028	0.028	0.046	0.917		
Between groups (p value)	0.002	< 0.001	< 0.001	0.034	< 0.001	0.003	< 0.001	< 0.001
Vitamin B6 (−) group (μmol)	0.09± 0.06	0.13± 0.06	0.08± 0.04	0.09± 0.04	0.08± 0.05	0.06± 0.04	0.54± 0.22	−0.02± 0.19
Comparison vs baseline (p value)		0.075	0.345	0.345	0.463	0.345		

Fig. 2 Urinary glycolate (Glc) excretion after administration of L-hydroxyproline to vitamin B6-deficient rats and control rats. Total = total cumulative urinary glycolate excretion from 0 to 5 h. Increment = cumulative increment of urinary glycolate excretion above baseline from 1 to 5 h after hydroxyproline loading

vitamin B6-deficient group, respectively ($P < 0.01$, between groups). In the vitamin B6-deficient group, total urinary oxalate excretion was increased by approximately 6.04-fold (22.07 vs. 3.65) and the 5-h increment above baseline was 7.56-fold greater than in the control group (15.24 vs. 2.02) (Fig. 1).

Baseline urinary glycolate excretion was significantly higher in the control group (0.25 ± 0.07 μmol) than in the vitamin B6-deficient group (0.09 ± 0.06 μmol) ($P < 0.01$). Hourly urinary glycolate excretion peaked at 2 h in the control group, but remained low without any change in vitamin B6-deficient group. In the control group, urinary glycolate excretion was significantly higher than baseline from 1 to 4 h ($P < 0.05$), but it remained low from 1 to 5 h in the vitamin B6-deficient group (Fig. 2). Infusion of hydroxyproline increased urinary glycolate excretion to 0.32% (2.43 ± 1.60 μmol) versus 0.00% (0.02 ± 0.19 μmol) of the administered dose in the control and vitamin B6-deficient groups, respectively ($P < 0.01$, between groups). The 5-h cumulative increment of urinary glycolate excretion above baseline (recovery rate) accounted for 0.32% (2.43 ± 1.60 μmol) of the administered dose of hydroxyproline in the control group. Total urinary glycolate excretion was significantly lower in the vitamin B6-deficient group (0.54 ± 0.22 μmol) than in the control group (3.90 ± 1.56 μmol) ($P < 0.01$, between groups).

Baseline urinary citrate excretion was significantly lower in the vitamin B6-deficient group (0.13 ± 0.07 μmol) than in the control group (2.03 ± 0.52 μmol) ($P < 0.01$). In the control group, hourly urinary citrate excretion peaked at 1 h and was significantly lower than baseline at 2, 3, and 5 h after hydroxyproline infusion ($P < 0.05$) (Fig. 3). In the vitamin B6-deficient group, however, it remained low at all times and only



Hourly Cit excretion	0	1	2	3	4	5 hours	Total	Increment
Control group (μmol)	2.03± 0.52	2.57± 0.88	1.09± 0.57	0.82± 0.60	1.15± 0.74	0.89± 0.69	8.55± 2.85	−3.61± 4.35
Comparison vs baseline (p value)		0.116	0.046	0.028	0.116	0.046		
Between groups (p value)	< 0.001	< 0.001	0.003	0.048	0.011	0.077	< 0.001	0.042
Vitamin B6 (−) group (μmol)	0.13± 0.07	0.30± 0.23	0.18± 0.09	0.25± 0.13	0.20± 0.14	0.29± 0.29	1.35± 0.85	0.57± 0.57
Comparison vs baseline (p value)		0.028	0.028	0.028	0.173	0.173		

Fig. 3 Urinary citrate (Cit) excretion after administration of L-hydroxyproline to vitamin B6-deficient rats and control rats. Total = total cumulative urinary citrate excretion from 0 to 5 h. Increment = cumulative increment of urinary citrate excretion above baseline from 1 to 5 h after hydroxyproline loading

showed a slight increase at 1–3 h after hydroxyproline infusion ($P < 0.05$) (Fig. 3). Total cumulative urinary citrate excretion and the 5-h cumulative increment of citrate excretion was 8.55 ± 2.85 and -3.61 ± 4.35 μmol versus 1.35 ± 0.85 and 0.57 ± 0.57 μmol in the control group and the vitamin B6-deficient group, respectively ($P < 0.05$, between groups).

Discussion

Glyoxylate, glycolate, and hydroxyproline are important precursors of oxalate that are mainly metabolized in the liver, while ascorbate is another precursor that produces oxalate under special conditions [7]. In herbivores, a major endogenous source of glyoxylate is oxidation of glycolate by glycolate oxidase in the peroxisomes, the peroxisomal localization of serine:pyruvate/alanine:glyoxylate aminotransferase (SPT/AGT or AGT-1) may be important for removal of glycolate-derived glyoxylate. Two forms of alanine:glyoxylate aminotransferase (AGT-1 and AGT-2) have been reported in the mitochondria of rat liver but we do not know how AGT-1 and AGT-2 share the role of metabolizing glyoxylate in mitochondria. In carnivores, the largely mitochondrial localization of SPT/AGT is important for the formation of glyoxylate, which is synthesized directly from 4-hydroxyproline-2-ketoglutarate in the mitochondria [10, 11]. The intermediate step involves transamination of 4-hydroxyglutamate to 4-hydroxy-2-ketoglutarate and this enzymatic reaction requires aspartate aminotransferase (AspAT) and vitamin B6 as a cofactor [13, 14, 18]. Despite being a vitamin B6-dependent reaction, the conversion of hydroxyproline to oxalate (via glyoxylate) was prominent in our vitamin B6-deficient animals, so further study of vitamin B6-dependent AspAT is warranted.

Urinary oxalate excretion was reported to be high in rats given glyoxylate, glycolate, hydroxypyruvate, and hydroxyproline, showing an increase in this order [2, 3]. In rats, the urinary oxalate excretion as a percentage of the administered dose (mol/mol: recovery rate) was approximately 22% for glyoxylate, 6.1% for glycolate, 0.49% for ethylene glycol, 0.4% for hydroxypyruvate, 0.26% for hydroxyproline, and 0.02% for pyruvate [12]. Little or no increment of oxalate excretion was observed in rats given glycine, xylitol, or ascorbate [12]. Urinary glycolate excretion as a percentage of the administered dose (mol/mol: recovery rate) was approximately 2.92% for glyoxylate, 8.9% for glycolate, 0.66% for ethylene glycol, 0.32% for hydroxypyruvate, 0.2% for hydroxyproline, 0.017% for glycine, and 0.005% for xylitol [12]. No increase of glycolate was observed after dosing with sodium pyruvate or ascorbate [12]. Pyruvate was previously identified by Chow et al. [19] as preventing the deposition of calcium oxalate in the kidneys. They also reported that pyruvate decreases urinary oxalate excretion in an experimental model of urolithiasis and suggested that pyruvate could prevent urolithiasis by decreasing oxalate synthesis [19]. We subsequently concluded that pyruvate salts inhibit calcium oxalate crystal formation in the kidneys by increasing the urinary citrate concentration rather than by decreasing oxalate synthesis [20], and we showed that pyruvate administration led to a minimal increase of oxalate that was unrelated to glycolate [12].

Vitamin B6 deficiency results in increased excretion of glycolate as well as oxalate in cats and rats [21–23]. Hydroxyproline has been suggested to cause the formation of calcium oxalate stones in rats [24], or to

promote excessive urinary oxalate excretion in vitamin B6-deficient rats [14, 25]. Hydroxyproline-induced hyperoxaluria was also demonstrated in 16 infants who were given 1 g of hydroxyproline [26]. We previously reported that the increase of urinary oxalate and glycolate after administration of L-hydroxyproline (630 mg) was 0.24 and 0.06% of the dose, respectively [11]. Then we confirmed similar results and showed that L-hydroxyproline caused an increase of urinary oxalate along with urinary glycolate in normal rats [12]. In vitamin B6-deficient rats, hyperoxaluria is both due to decreased transamination by alanine:glyoxylate aminotransferase (AGT) and enhancement of glycolate oxidase activity [27–29].

As reported previously, the urinary glycolate level corrected for creatinine was lower at baseline in the control rats than the rats fed a vitamin B6-deficient diet for 4 weeks [29]. Low serum creatinine levels and high urinary creatinine levels along with less weight gain suggested mild malnutrition, dehydration, and cachexia due to 4 weeks of vitamin B6 deficiency. However, it is unclear whether changes of catabolic, acidotic, or glycolytic metabolism lead to altered glycolate production. Although urinary glycolate excretion at baseline shows slight increase in relation to weight gain and the duration of vitamin B6 deficiency (1, 2, or 3 weeks), these changes were very subtle (unpublished data) and could be too small to be worth studying. In addition, we do not know anything about the possible induction of glycolate-related enzymes over time due to vitamin B6 deficiency. Therefore, we shortened the feeding period to 3 weeks for this study, but growth retardation was still not negligible. The mean weight of the animals in this study differed between the groups and we compared hourly glycolate excretion, while creatinine-corrected urinary glycolate excretion was assessed in the previous study [29]. There are no definite factors that explain the discrepant or conflicting results, and we are not sure whether these minor differences are worth exploring further.

On the other hand, glycolate excretion was increased significantly over time by hydroxyproline administration in the controls, but it did not increase at all in the vitamin B6-deficient animals. The immediate metabolic precursors of glycolate are glycolaldehyde (from hydroxypyruvate) and glyoxylate, so both substances are potential sources for production of glycolate [30]. In normal rats, glycolate is either derived from glycolaldehyde (hydroxypyruvate) or from glyoxylate via glyoxylate reductase. This enzyme is dependent on NADH rather than pyridoxal, but its redox status and activity may be altered by changes of other pyridoxal-dependent enzymes. Mitochondrial glycolate

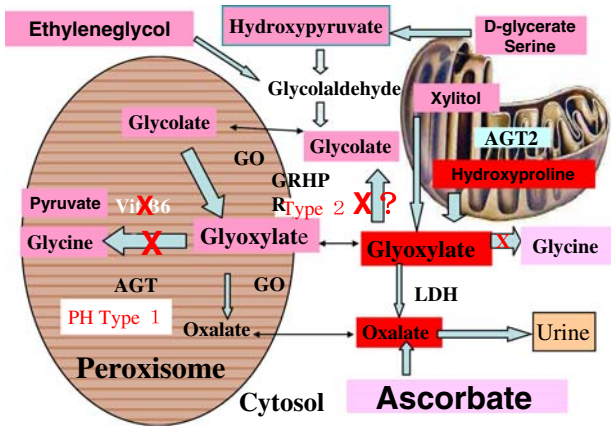


Fig. 4 Proposed pathways of glyoxylate metabolism in vitamin B6 deficiency

production could be the principal source of hydroxyproline for oxalate synthesis in primary hyperoxaluria 1 [31]. Glycolate was reported to be largely derived from metabolism after meals [32], but the source was not indicated clearly. The authors may have meant that glycolate was not directly ingested, but was derived from glyoxylate or other precursors. However, better evidence based on loading with both isotopic substances is required. So far, we know that two substances which increase urinary glycolate excretion in vitamin B6-deficient rats are hydroxypyruvate and ethylene glycol (unpublished data). Peroxisomal glycolate production could be the source of these substances for oxalate synthesis.

Metabolism of hydroxyproline to oxalate does not involve the peroxisomal glycolate pathway and glyoxylate is produced in the mitochondria. The reverse reaction that transfers glyoxylate to glycolate is catalyzed by cytosolic glyoxylate reductase in normal rats. The association of hyperoxaluria with hypoglycolic aciduria in vitamin B6-deficient rats may suggest that glyoxylate reductase (which usually converts glyoxylate to glycolate in the liver) also malfunctions due to B6 deficiency, so that glyoxylate produced from hydroxyproline cannot be detoxified to glycolate by this enzyme and therefore is metabolized to oxalate by LDH. Vitamin B6 deficiency appears to produce a combination of AGT and glyoxylate reductase/hydroxypyruvate reductase (GRHPR) knockout or knockdown, but this needs further investigation (Fig. 4).

The intake of animal protein is associated with a significant increase of urinary oxalate excretion in humans [33–35]. There are two explanations for this hyperoxaluria associated with meat intake. The intake of oxalogenic precursors in meat and increased dietary oxalate absorption by the presence of animal fat in the

bowel are implicated in the genesis of hyperoxaluria [36, 37]. Collagen accounts for approximately 7% of all animal protein (on a weight basis) [38] and the hydroxyproline content of collagen is as high as 13%. Thus, the hydroxyproline content of 100 g meat is approximately 1 g. If our present results obtained in rats can be applied to humans, the absorption of about 30% of dietary hydroxyproline (300 mg = 2.3 mmol) may result in conversion to 4 mg of oxalate ($2.3 \times 0.02 \times 90$) in patients with vitamin B6 deficiency. Therefore, dietary protein intake may contribute to hyperoxaluria in humans due to hydroxyproline-associated endogenous oxalogenesis and fat-associated hyperabsorption of exogenous oxalate.

Hypocitraturia is another important finding associated with vitamin B6 deficiency [29] and this was confirmed in the present study. Chronic metabolic acidosis causes hypocitraturia and was reported to increase renal cortical ATP citrate lyase activity in rats [39]. Adipose tissue and liver ATP citrate lyase activity was reported to be decreased by vitamin B6 deficiency [40]. Tosukhong et al. [41, 42] reported an inverse relationship between the activity of leukocyte ATP citrate lyase (a potential predictor of enzymatic activity in renal tubular cells) and urinary citrate excretion.

If this holds true, low ATP citrate lyase activity associated with vitamin B6 deficiency should lead to hypercitraturia. In reality, however, this may occur the other way around. That is, vitamin B6 deficiency may impair the TCA cycle and decrease citrate production, so that the citrate level remains low in blood and renal tissue despite a decrease of ATP citrate lyase activity, but this point needs further investigation.

In conclusion, the infusion of L-hydroxyproline increased urinary oxalate excretion in normal rats, and caused an exaggerated increase (7.5-fold) in the increment of urinary oxalate excretion that persisted for more than 5 h without hyperglycolic aciduria in vitamin B6-deficient rats, suggesting that endogenous oxalogenesis occurred via mitochondrial glyoxylate rather than via peroxisomal glycolate. Vitamin B6 deficiency was also associated with hypocitraturia. Therefore, the risk of increased oxalate excretion associated with hypocitraturia must be kept in mind after intake of meat by persons with low vitamin B6 levels due to malnutrition, long-term antibiotic therapy, hyperalimentation, or bowel disease, among other causes.

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