EFFECT OF VITAMIN B₆ DEFICIENCY ON THE LEVELS OF SEVERAL WATER-SOLUBLE VITAMINS IN TISSUES OF GERM-FREE AND CONVENTIONAL RATS¹

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(Received September 29, 1978)

Summary The influence of vitamin B₆ deficiency on the levels of several water-soluble vitamins and on acetyl-coenzyme A carboxylase activity was investigated using of germ-free and conventional rats.

Judging from the vitamin B₆ levels in tissues and the percent of decrease, the degree of vitamin B₆ deficiency was more severe in the tissues of deficient germ-free rats than in deficient conventional rats. Nicotinic acid, pantothenic acid and biotin levels per wet weight significantly decreased in the liver of vitamin B₆-deficient germ-free rats, and nicotinic acid levels per wet weight significantly decreased in the liver of deficient conventional rats. In the kidney of vitamin B₆-deficient germ-free rats, a significant decrease in riboflavin and biotin levels was observed, although there was no observable difference in riboflavin, nicotinic acid, biotin and pantothenic acid levels in the kidney of deficient conventional rats. From an enzymatic standpoint, acetyl-coenzyme A carboxylase activity was especially significantly decreased in both germ-free and conventional rats fed a vitamin B₆-deficient diet, and the percent of decrease was more in germ-free rats than in conventional ones.

These findings suggest that vitamin B₆ deficiency had stronger effects on the levels of water-soluble vitamins in germ-free rats compared with conventional rats.

Keywords vitamin B₆ deficiency, riboflavin, nicotinic acid, pantothenic acid, biotin, germ-free rat

¹ Part of this report was presented at the Annual Meeting of the Vitamin Society of Japan at Tokushima on May, 1978.

This work was supported in part by a grant-in-aid (to M.I.) for scientific research from the Ministry of Education, Science and Culture of Japan.

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It has been known that there is some relationship between vitamin B₆ and the other water-soluble vitamins (1-8).

It has been reported that experimentally induced vitamin B₆ deficiency mimicked symptoms of deficiency of other B-complex vitamins (1), and that when a concomitant deficiency of the monkey antianemia factor occurred under the influence of vitamin B₆ deficiency, the addition of liver substance to the control diet was necessary for optimum growth and blood regeneration (2). Then, there are some reports about deprivation of other B-complex vitamins under the influence of vitamin B₆ deficiency (3-7). These reports indicate that vitamin B₆ deficiency perhaps has some influence on the progress of the other vitamin metabolism, which might induce the changes of enzymatic activities.

The present studies were conducted to investigate the influence of vitamin B₆ deficiency on riboflavin, nicotinic acid, pantothenic acid and biotin levels in tissues using germ-free rats in which it is easier to produce short-term vitamin B₆ deficiency than in conventional rats, and also to obtain evidence of changes in enzyme activities.

EXPERIMENTAL

Male Sprage-Dawley germ-free and conventional rats were purchased from Clea Japan, Inc. Pyridoxal phosphate (PLP) and acetyl-CoA were purchased from Kyowa Brewery Co. (Japan). ATP, glutathion and crystalline bovine serum albumin were products of Sigma Chemicals Co. (St. Louis, USA). KH¹⁴CO₃ was purchased from the Radiochemical Center (Amersham, England). All other chemicals were of analytical grade.

Animals and diet. The composition of the diet and animal maintenance and care used in this experiment have been described in a previous paper (17). Ten germ-free rats, weighing 100 to 108 g and 15 conventional rats, weighing 100 to 109 g were used. The animals were divided into three conventional and two germ-free groups. Each group consisted of 5 rats. One group of germ-free and one of conventional rats were fed a vitamin B₆-deficient diet *ad libitum*, one group of germ-free and one of conventional rats were fed a control diet of 15 g per day (control group), and another group of conventional rats was fed a control diet of 10 g per day (control (a) group). The dietary amount of the control (a) group, 10 g per day, was nearly equal to that of the vitamin B₆-deficient groups, 7-10 g per day, so the control (a) group was used as nearly pair-fed controls in this report. Then, only control (a)'s were used in the present experiment, and these rats were cupped to prevent coprophagy.

After 48 days and following a fasting period of about 16 hr, the animals were killed by exsanguination through the abdominal aorta under light ether anesthesia. The liver and kidney were dissected, rinsed with physiological saline, blotted dry on filter paper, weighed, and stored at -20°C until analysis.

Determination of vitamins. Total vitamin B₆ levels of tissues were measured by a microbiological method using a *Saccharomyces carlsbergensis* 4228, ATCC 9080 (9),

after hydrolysis of the samples in 1/11 N of H₂SO₄ at 121°C for one hour. Riboflavin levels of tissues were also measured by a microbiological assay method using a *Lactobacillus casei*, ATCC 7469 (10). Nicotinic acid, pantothenic acid and biotin levels of tissues were also determined by the same assay method using a *Lactobacillus plantarum*, ATCC 8014 (11-13).

Determination of acetyl-CoA carboxylase activity. Acetyl-CoA carboxylase [EC 6.4.1.2] activity was determined by the H¹⁴CO₃⁻ fixation assay according to the method of NAKANISHI and NUMA (14). Protein concentration was determined by the biuret reaction with crystalline bovine serum albumin as the standard (15).

RESULTS

Vitamin B₆ levels in tissues

Vitamin B₆ levels in the tissues of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet are shown in Table 1. Vitamin B₆ levels were lower in deficient germ-free rats than in deficient conventional ones except for the liver levels per g of wet weight. Moreover, the percent ratio of deficiency per control was also lower in germ-free rats than in conventional ones in all cases.

Riboflavin levels in tissues

Riboflavin levels in the liver, kidney and plasma of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet are shown in Table 2. There was no decrease in riboflavin levels in the tissues on conventional rats. However, the level of riboflavin in the liver of germ-free rats significantly decreased on a scale of ng per mg of protein and tended to decrease in µg per g of wet weight. A significant

Table 1. Vitamin B₆ levels in the liver, kidney and plasma of conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Tissues	Units	Conventional				Germ-free			
		Control (a)	Control	Deficient	<i>p</i>	Control	Deficient	<i>p</i>	
Liver	A	8.31 ± 1.42	7.08 ± 1.54	2.17 ± 0.65	***	8.86 ± 1.73	2.25 ± 0.84	***	
	B	29.4 ± 5.4	27.8 ± 5.6	8.4 ± 2.9	***	32.7 ± 7.1	5.7 ± 3.5	***	
Kidney	A	7.43 ± 1.33	6.16 ± 1.12	2.68 ± 1.19	***	9.74 ± 1.81	1.11 ± 0.29	***	
	B	31.3 ± 6.4	34.4 ± 5.1	12.8 ± 8.2	***	57.5 ± 10.4	8.9 ± 4.0	***	
Plasma	C	59.6 ± 13.9	68.8 ± 21.5	10.3 ± 2.6	***	47.3 ± 7.6	5.8 ± 1.3	***	
	B	0.97 ± 0.22	1.07 ± 0.34	0.17 ± 0.03	***	0.83 ± 0.14	0.09 ± 0.03	***	

Values are means ± SD. *** *p* < 0.001.

Control (a) is nearly pair-fed controls, and cupped rats were used.

Vitamin B₆ concentrations are expressed as follows: A, µg/g wet weight; B, ng/mg protein; C, ng/ml.

Table 2. Riboflavin levels in the liver, kidney and plasma of conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Tissues	Units	Conventional				Germ-free		
		Control (a)	Control	Deficient	<i>p</i>	Control	Deficient	<i>p</i>
Liver	A	30.3 ± 4.5	26.9 ± 2.3	28.5 ± 3.1	NS	30.4 ± 2.5	25.6 ± 2.2	NS
	B	108 ± 13	99 ± 8	110 ± 16	NS	112 ± 11	63 ± 23	*
Kidney	A	30.5 ± 3.7	32.0 ± 3.6	32.7 ± 3.7	NS	28.3 ± 3.6	17.8 ± 1.5	*
	B	164 ± 19	180 ± 22	189 ± 14	NS	168 ± 25	143 ± 11	NS
Plasma	C	151 ± 36	178 ± 47	157 ± 18	NS	148 ± 6	294 ± 167	NS
	B	2.65 ± 0.42	2.73 ± 0.58	2.59 ± 0.36	NS	2.60 ± 0.11	2.73 ± 0.07	NS

Values are means ± SD. * *p* < 0.05. NS = not significant.

Control (a) is nearly pair-fed controls, and cupped rats were used.

Riboflavin concentrations are expressed as follows: A, µg/g wet weight; B, ng/mg protein; C, ng/ml.

Table 3. Nicotinic acid levels in the liver, kidney and plasma of conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Tissues	Units	Conventional				Germ-free		
		Control (a)	Control	Deficient	<i>p</i>	Control	Deficient	<i>p</i>
Liver	A	243 ± 29	253 ± 20	200 ± 26	*	256 ± 32	183 ± 30	*
	B	985 ± 53	974 ± 45	773 ± 119	NS	941 ± 124	424 ± 77	***
Kidney	A	513 ± 98	480 ± 131	636 ± 77	NS	447 ± 116	263 ± 144	NS
	B	302 ± 57	272 ± 84	368 ± 41	NS	260 ± 60	215 ± 11	NS
Plasma	C	484 ± 96	516 ± 135	379 ± 30	NS	731 ± 115	335	—
	B	7.9 ± 1.8	8.2 ± 2.6	6.4 ± 0.5	NS	12.8 ± 2.2	5.0	—

Values are means ± SD. * *p* < 0.05; *** *p* < 0.001.

NS = not significant.

Control (a) is nearly pair-fed controls, and cupped rats were used.

Nicotinic acid concentrations are expressed as follows: A, µg/g wet weight; B, ng/mg protein; C, ng/ml.

decrease in riboflavin levels was also observed, when it was measured by µg per g of wet weight of germ-free rat kidney.

Nicotinic acid levels in tissues

Nicotinic acid levels in the liver, kidney and plasma of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet are shown in Table 3.

In the liver, a significant decrease in nicotinic acid levels was also observed by μg per g of wet weight of conventional rats, and by μg per g of wet weight and ng per mg of protein of germ-free rats. A slight decrease in nicotinic acid levels was observed in conventional rat liver measured by ng per mg of protein. In the plasma the level of nicotinic acid tended to decrease in deficient germ-free rats.

Pantothenic acid levels in tissues

Pantothenic acid levels in the liver and kidney of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet are shown in Table 4. In the liver, a significant decrease was observed in germ-free rats, but not in conventional rats. There was not a significant decrease in pantothenic acid in the kidney of vitamin B₆-deficient conventional or germ-free rats.

Biotin levels in tissues

Biotin levels in the liver, kidney and plasma of conventional and germ-free rats

Table 4. Pantothenic acid levels in the liver of conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Tissues	Conventional				Germ-free		
	Control (a)	Control ($\mu\text{g/g}$ wet weight)	Deficient	<i>p</i>	Control ($\mu\text{g/g}$ wet weight)	Deficient	<i>p</i>
Liver	74.6 $\pm$ 14.1	79.5 $\pm$ 12.8	71.6 $\pm$ 8.9	NS	81.6 $\pm$ 15.4	43.0 $\pm$ 19.3	*
Kidney	61.9 $\pm$ 10.8	59.6 $\pm$ 10.3	57.6 $\pm$ 7.8	NS	64.3 $\pm$ 12.5	58.4 $\pm$ 10.2	NS

Values are means $\pm$ SD. * $p < 0.05$. NS = not significant.

Control (a) is nearly pair-fed controls, and cupped rats were used.

Table 5. Biotin levels in the liver, kidney and plasma of conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Tissues	Units	Conventional				Germ-free		
		Control (a)	Control	Deficient	<i>p</i>	Control	Deficient	<i>p</i>
Liver	A	1.69 $\pm$ 0.22	1.78 $\pm$ 0.17	1.47 $\pm$ 0.22	NS	1.83 $\pm$ 0.33	0.89 $\pm$ 0.02	**
	B	6.84 $\pm$ 1.39	7.02 $\pm$ 0.63	5.71 $\pm$ 1.26	NS	6.76 $\pm$ 1.45	2.14 $\pm$ 0.61	**
Kidney	A	0.944 $\pm$ 0.095	0.865 $\pm$ 0.078	0.927 $\pm$ 0.046	NS	0.962 $\pm$ 0.083	0.612 $\pm$ 0.143	*
	B	5.46 $\pm$ 0.73	4.85 $\pm$ 0.28	5.36 $\pm$ 0.05	NS	5.70 $\pm$ 0.64	4.93 $\pm$ 0.89	NS
Plasma	C	4.25 $\pm$ 1.47	3.76 $\pm$ 1.27	3.07 $\pm$ 0.65	NS	5.43 $\pm$ 1.69	2.65 $\pm$ 0.69	NS
	B	0.083 $\pm$ 0.041	0.059 $\pm$ 0.022	0.050 $\pm$ 0.010	NS	0.095 $\pm$ 0.028	0.040 $\pm$ 0.010	*

Values are means $\pm$ SD. * $p < 0.05$; ** $p < 0.01$. NS = not significant.

Control (a) is nearly pair-fed controls, and cupped rats were used.

Biotin concentrations are expressed as follows: A, $\mu\text{g/g}$ wet weight; B, ng/mg protein; C, ng/ml.

Table 6. Acetyl-CoA carboxylase activity in the liver of conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Rats	Control (dpm)	Deficient (dpm)	<i>p</i>
Conventional	4,049 ± 680 (5)	2,120 ± 599 (5)	*
Germ-free	3,148 ± 892 (5)	641 ± 143 (5)	**

Values are means ± SD. * $p < 0.02$; ** $p < 0.01$.

Enzyme activity was determined at 37°C by the H¹⁴CO₃⁻ fixation method (14), and ¹⁴C activity was determined with the use of a liquid scintillation spectrometer. The number of experiments is shown in parentheses.

fed the control diet and the vitamin B₆-deficient diet are shown in Table 5. In the liver, a significant decrease in biotin levels was observed in germ-free rats but a slight decrease was found in conventional rats. In the kidney, there was no decrease in conventional rats, but a significant decrease was observed in germ-free rats shown by µg per g of wet weight. In the case of plasma, a significant decrease in biotin levels per mg of protein was observed in germ-free rats, but not in conventional ones.

Acetyl-CoA carboxylase activity in the liver

To determine the influence of vitamin B₆ deficiency on one of the biotin-dependent enzymes, acetyl-CoA carboxylase activity in the liver of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet was assayed, as shown in Table 6. In conventional rats, acetyl-CoA carboxylase activity was 47.7% less in the vitamin B₆-deficient group as compared to the control group. In germ-free rats, enzyme activity was 79.6% less than in the vitamin B₆-deficient group than in the control group.

DISCUSSION

In a previous report (17), we demonstrated that a more severe vitamin B₆ deficiency was obtained in germ-free rats than in conventional ones, judging from body weight gain and xanthurenic acid excretion. Then, the vitamin B₆ levels of the liver, kidney and plasma were also less in deficient germ-free rats than in deficient conventional ones (Table 1). These findings agree with the report (16) that the presence of intestinal microflora made some contribution to improvement of vitamin B₆ deficiency.

TERROINE and ADRIAN have reported that vitamin B₆ deficiency caused a decrease in the concentrations of riboflavin, nicotinic acid and pantothenic acid in rat tissues (7). HENDERSON *et al.* reported that vitamin B₆-deficient animals excreted much less nicotinic acid and quinolinic acid than corresponding control animals (23). Furthermore, many observations have been made that vitamin B₆ deficiency caused a reduction in concentrations of folic acid and citrovorum factor

in rat liver (4), and that pyridoxine deficiency results in an impaired absorption of orally-administered vitamin B₁₂ (3). In the present study, riboflavin levels significantly decreased in the liver as shown by ng per mg of protein and in the kidney as shown by μ g per g of wet weight in germ-free rats, but did not in conventional rats. Since it has been reported that riboflavin is required for the conversion of pyridoxine to pyridoxal phosphate and this conversion is low in riboflavin deficiency (8, 18, 20), it can be speculated that the decrease in riboflavin levels causes a more severe vitamin B₆ deficiency in germ-free rats than in conventional ones fed a vitamin B₆-deficient diet.

In the liver, a 20% decrease in nicotinic acid levels in conventional rats and a 28% decrease in nicotinic acid levels in germ-free ones were observed, as shown in Table 3, whereas TULPULÉ (6) and TERROINE *et al.* (7) have reported that vitamin B₆ deficiency caused a 15% decrease in the concentration of nicotinic acid in the liver. UEDA *et al.* have reported that NAD content in the liver decreased in vitamin B₆-deficient rats (19). Nicotinic acid is synthesized *de novo* from tryptophan in the liver, and several vitamin B₆-dependent enzymes are involved in this pathway. Therefore, the decrease in nicotinic acid levels in the liver may, in part, induce a hindrance of this pathway due to the decrease of vitamin B₆-dependent enzymes.

TERROINE *et al.* have reported that vitamin B₆ deficiency caused a 70% decrease in the concentration of pantothenic acid in rat liver (7). In this report, a significant decrease in the level of pantothenic acid was found in germ-free rats, but not in conventional ones.

There have been no reports on the influence of vitamin B₆ deficiency on biotin metabolism. In the liver of vitamin B₆-deficient germ-free rats, biotin levels decreased in one-half, by μ g per g of wet weight and in one-third by ng per mg of protein, and biotin levels significantly decreased in the kidney as shown by μ g per g of wet weight and in the plasma by ng per ml. It seems that the decrease in tissue biotin levels was remarkable among the vitamins tested. Therefore, we intended to demonstrate acetyl-CoA carboxylase activity in the liver to observe the influence of biotin level on enzyme activity, as shown in Table 6. Enzyme activity showed a 79.6% decrease in germ-free rats and a 47.7% decrease in conventional rats. It is known that the activity of biotin-dependent carboxylase is reduced in biotin-deficient animals (24, 25). However, there might not be a direct relationship between the decrease in biotin levels and acetyl-CoA carboxylase activity in the liver, because the biotin levels in the liver showed a 68.4% decrease in germ-free rats and an 18.7% decrease in conventional ones.

The mechanism of this decrease in enzyme activity is not obscure, but another possibility should also be considered. Tryptophan metabolites, xanthurenic acid, might inhibit enzyme activity, since HASHIMOTO *et al.* have found that some of the tryptophan metabolites, kynurenate and xanthurenic acid, were potent inhibitors of acetyl-CoA carboxylase (21, 22), and we have reported that vitamin B₆-deficient germ-free rats excreted more xanthurenic acid than deficient conventional ones did (17).

The physiological significance and mechanism of the decrease in these water-

Table 7. The levels of plasma protein in conventional and germ-free rats fed a control diet and a vitamin B₆-deficient diet.

Rats	Control (mg/ml)	Deficient (mg/ml)	<i>p</i>
Conventional	61.6 ± 3.4	64.7 ± 4.7	NS
Germ-free	64.3 ± 3.9	57.2 ± 2.3	*

Values are means ± SD. * $p < 0.05$. NS = not significant.

soluble vitamins have not yet been determined, but one might speculate on this phenomenon in the following fashion. It is insisted on by many investigators that vitamins which are synthesized by some intestinal microflora are utilized by coprophagy. Therefore, cupped conventional rats which were prevented from coprophagy are not able to utilize vitamins synthesized by intestinal microflora. The control (a) values from the present data were little different from the control values as shown in Tables 2 to 5. This may mean that the amount of vitamins supplied by coprophagy was not large enough in comparison with vitamins supplied by the control diet, because a sufficient amount of vitamins was added to the control diet. Therefore, it is reasonable to speculate that the decrease in riboflavin, nicotinic acid, pantothenic acid and biotin levels was not due to an absence of intestinal microflora in the case of germ-free rats.

It has been reported that fatty liver was caused in vitamin B₆-deficient rats fed a high-protein diet (26, 27). The plasma protein levels significantly decreased in vitamin B₆-deficient germ-free rats, but not in vitamin B₆-deficient conventional ones (Table 7). This result suggests that a liver disorder exists in vitamin B₆-deficient germ-free rats. Therefore, the decrease in riboflavin, nicotinic acid, pantothenic acid and biotin levels may be thought to be due to the decrease in storage and utilization, or to the increase of excretion of vitamins accompanied by the liver disfunction under the influence of vitamin B₆ deficiency.

The authors would like to thank Mrs. K. Kurimoto and Mr. T. Mori for their excellent technical assistance.

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