OBSERVATIONS OF THE CONCENTRATION OF ZINC AND IRON IN TISSUES OF VITAMIN B₆-DEFICIENT GERM-FREE RATS

Masamichi Ikeda, Tokuji Hosotani, Takashi Ueda, Yahito Kotake, and Bunsaku Sakakibara

Faculty of Nutrition, Kobe-Gakuin University, Tarumi, Kobe 673, Japan
(Received September 29, 1978)

Summary The transition of zinc and iron metabolism in vitamin B₆ deficiency was investigated using germ-free and conventional rats. In contrast to previous reports, a decrease in zinc content was not observed in the liver, pancreas, kindney, spleen, lung or testes of vitamin B₆-deficient conventional and germ-free rats, but we found an increase in zinc content in the kidney of conventional rats and in the liver and spleen of germ-free rats. Vitamin B₆-deficient conventional and germ-free rats retained more iron in their tissues than the control animals did, except for the spleen of germ-free rats. The deposit of iron was more evident in vitamin B₆-deficient germ-free rats than in vitamin B₆-deficient conventional rats, and is possibly proportional to the degree of vitamin B₆ deficiency. It is possible that the deposit of iron in the organs had some influence on metabolic disorders in vitamin B₆-deficient rats.

Keywords vitamin B₆ deficiency, zinc, iron, germ-free rat

A reduction of insulin activity and microcytic anemia are known to occur in vitamin B₆ deficient animals (1–6).

Hsu reported that the zinc content in the liver, plasma and pancreas of vitamin B₆ deficient rats was significantly lower than that of control rats (7). Reddy et al. reported that there was no significant difference between germ-free and conventional rats in the absorption and net retention of zinc (8). However, Smith et al. suggested that intestinal microflora had some effect on zinc metabolism (9). On the other hand, enhanced iron absorption has been reported in pyridoxine-deficient

1 Part of this report was presented at the Annual Meeting of the Japanese Society of Food and Nutrition at Nakamura-Gakuen College on May, 1978.
2 池田雅充，細谷徳治，上田隆史，古武弥人，細原文作
swine (3) and rats (10) despite replete body stores. Reddy et al. showed that a lack of intestinal microflora resulted in a lower rate of metabolism of iron (8), whereas germ-free rabbits utilized natural sources of iron more efficiently than conventional animals did (11). These findings suggest that the availability of zinc and iron in germ-free animals must have been influenced by the absence of intestinal microflora. However, little is known regarding the relationship of vitamin B₆ deficiency to zinc or iron metabolism and intestinal microflora.

The present studies using germ-free rats were conducted to investigate the transition of zinc and iron metabolism in rats with vitamin B₆ deficiency.

EXPERIMENTAL

Ten male Sprague-Dawley germ-free rats, weighing 100 to 108 g were caged individually in screen-bottomed, stainless steel cages kept in an isolator maintained at 22 to 25°C and 15 male Sprague-Dawley conventional rats weighing 100 to 109 g were also caged individually in screen-bottomed, stainless steel cages kept in a room maintained at 22 to 25°C. Lighting was regulated automatically to provide constant periods of alternating light and darkness (the light was on from 8:00 to 18:00). The germ-free status of the animals was routinely verified by standard microbiological procedure.

The composition of the control diet used in this experiment is given in Table 1. The deficient diet had the same composition as the control one. Pyridoxine was completely removed from the deficient diet. Both diets were autoclaved at 126°C for 30 min prior to entering the germ-free isolator. Vitamins were supplied in sufficient amounts, because some portion of the vitamins decomposed during sterilization. A portion of each batch of autoclaved diet was removed from the isolator and stored at 5°C for feeding to the conventional rats.

Animals were divided into three conventional and two germ-free groups. Each

| Ingredient           | Percentage (%) |
|----------------------|----------------|
| Casein (vitamin free)| 31.4           |
| Sucrose              | 50.0           |
| Corn oil             | 10.0           |
| Cellulose            | 4.0            |
| Salt mixture 1       | 4.0            |
| Vitamin mixture 2    | 0.3            |
| L-Tryptophan         | 0.3            |

1. The salt mixture of Hegsted et al. (1941), J. Biol. Chem., 138, 459.
2. The vitamins supplied the following: (mg/kg diet) retinyl acetate, 4.4; ergocalciferol, 4.5; 2-methyl-naphthoquinone, 4.2; thiamine HCl, 231; riboflavin, 48; pyridoxine HCl, 12; calcium pantothenate, 415; nicotinic acid, 124; inositol, 242; p-amino-benzoic acid, 82.5; folic acid, 6.0; biotin, 1.0; vitamin B₁₂, 0.1; choline HCl, 1,100.
group consisted of 5 rats. Each group of germ-free and conventional rats was fed a deficient diet *ad libitum*, one germ-free group was fed 15 g of control diet per day and two groups of conventional rats were fed different amounts of the control diet, one 15 g and the other 10 g per day, respectively. The daily intake of each group was recorded.

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

The tissues were homogenized in 20 volumes of ice-cold water, and 1 ml aliquots of each homogenate were ashed with concentrated sulfuric and nitric acids.

Zinc concentration was determined from the ashed samples with an atomic absorption spectrophotometer by the internal standard method (12).

Iron concentration was measured by the use of bathophenanthroline sulfonic acid from the ashed samples (13).

Protein concentration was determined by the biuret reaction (14).

RESULTS

Zinc levels per g of wet weight were determined in tissues of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet, as shown in Table 2. In conventional rats, there was no significant difference in the liver, pancreas, spleen, lung or testes between the control and deficient groups, but a significant increase was observed only in the kidney of the deficient group. Although a significant difference was not observed in the pancreas, kidney, lung or testes of germ-free rats between the control and deficient groups, the zinc levels tended to increase in the liver, kidney and lung, and especially in the spleen.

| Tissues | Conventional | Germ-free |
|---------|--------------|-----------|
|         | Control (a)  | Control   | Deficient |
| Liver   | 34.1±5.1     | 31.8±2.6  | 30.6±2.8  |
| Pancreas| 23.7±7.2     | 25.2±2.8  | 27.2±2.9  |
| Kidney  | 26.7±3.0     | 30.7±2.0  | 47.6±3.3*** |
| Spleen  | 23.8±1.2     | 26.6±2.4  | 23.5±3.1  |
| Lung    | 16.9±1.0     | 20.2±1.2  | 18.8±0.8  |
| Testes  | 29.7±3.8     | 28.9±3.1  | 30.7±2.2  |

Zn concentrations are expressed as µg/g wet weight for each tissue. Values are means ± SD; * p<0.05; ** p<0.01; *** p<0.001. Control (a) were nearly pair-fed controls, and cupped rats were used.
Zinc levels per mg of protein were also determined in tissues of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet, as shown in Table 3. The difference in zinc levels between the control and deficient groups were the same as those determined per g of wet weight except for the liver and kidney of germ-free rats.

Iron levels per g of wet weight were determined in tissues of conventional and germ-free rats fed the control diet and the vitamin B₆-deficient diet, as shown in Table 4. The iron levels in the liver and kidney of conventional vitamin B₆-deficient rats significantly increased in comparison with the control group. The levels in the pancreas, spleen, lung and testes also increased, but not significantly. In germ-free rats, the iron levels of the liver, kidney, spleen, lung and testes significantly increased in the deficient group as compared with the control group, and the level of the pancreas tended to increase in the deficient group.

| Table 3. Zn levels in tissues of conventional and germ-free rats fed a control diet and a vitamin B₆ deficient diet. |
|---|---|---|---|
| Tissues | Conventional | | Germ-free |
| | Control (a) | Control | Deficient | Control | Deficient |
| Liver | 0.150 ± 0.010 | 0.125 ± 0.010 | 0.117 ± 0.005 | 0.128 ± 0.011 | 0.118 ± 0.026 |
| Pancreas | 0.130 ± 0.027 | 0.162 ± 0.020 | 0.164 ± 0.016 | 0.160 ± 0.008 | 0.168 ± 0.018 |
| Kidney | 0.190 ± 0.014 | 0.172 ± 0.006 | 0.276 ± 0.033* | 0.189 ± 0.007 | 0.344 ± 0.087* |
| Spleen | 0.137 ± 0.017 | 0.146 ± 0.017 | 0.130 ± 0.016 | 0.128 ± 0.013 | 0.162 ± 0.007** |
| Lung | 0.139 ± 0.017 | 0.137 ± 0.019 | 0.133 ± 0.005 | 0.133 ± 0.015 | 0.123 ± 0.019 |
| Testes | 0.359 ± 0.045 | 0.319 ± 0.011 | 0.348 ± 0.022 | 0.345 ± 0.077 | 0.314 ± 0.020 |

Zn concentrations are expressed as µg/mg protein for each tissue. Values are means ± SD; * p < 0.05; ** p < 0.01.
Control (a) were nearly pair-fed controls, and cupped rats were used.

| Table 4. Fe levels in tissues of conventional and germ-free rats fed a control diet and a vitamin B₆ deficient diet. |
|---|---|---|---|
| Tissues | Conventional | | Germ-free |
| | Control | Deficient | Control | Deficient |
| Liver | 130.7 ± 34.2 | 198.5* ± 24.3 | 225.6 ± 28.3 | 665.5*** ± 82.6 |
| Pancreas | 29.1 ± 6.1 | 41.8 ± 9.8 | 54.3 ± 8.5 | 66.9 ± 6.0 |
| Kidney | 89.8 ± 5.9 | 100.9* ± 8.0 | 88.2 ± 3.0 | 100.5* ± 7.0 |
| Spleen | 440.5 ± 84.0 | 539.5 ± 119.7 | 668.0 ± 54.1 | 349.0** ± 85.5 |
| Lung | 110.4 ± 21.8 | 125.6 ± 32.7 | 72.8 ± 12.0 | 176.5*** ± 12.2 |
| Testes | 28.5 ± 2.0 | 42.9 ± 8.2 | 32.6 ± 8.1 | 45.5* ± 5.1 |

Fe concentrations are expressed as µg/g wet weight for each tissue. Values are means ± SD; * p < 0.05; ** p < 0.01; ***p < 0.001.
Iron levels per mg of protein were also determined in tissues of conventional and germ-free rats fed the control diet and the vitamin B6-deficient diet, as shown in Table 5. In conventional rats, a significant increase in iron were observed in the kidney of the deficient group. In the case of germ-free rats, a significant increase in iron was observed in the kidney and lung of the deficient group. However, the iron level in the spleen per g wet weight and mg of protein markedly decreased in vitamin B6-deficient germ-free and conventional rats.

**DISCUSSION**

Reddy et al. reported that there was no significant difference between germ-free and conventional rats in the absorption and net retention of zinc (8). On the other hand, it has been shown that bacterial endotoxin results in a rapid decrease in plasma zinc concentration in both humans and animals (15), and the presence of certain microorganisms results in an increased dietary zinc requirement (9). Moreover, Smith et al. showed that the visible signs of zinc deficiency were delayed and less severe in germ-free rats as compared to conventional animals raised on zinc-deficient diets, suggesting a microfloral effect in precipitating deficiency conditions (9). Our present data were consistent with Reddy’s report on control groups, but not on vitamin B6-deficient groups.

Hsu reported that pyridoxine deficiency in rats resulted in a decrease in zinc contents in the plasma, liver, pancreas and heart tissues and an increase on Zn-65 uptake in the plasma and liver after an intramuscular injection of radiozinc (7). In our studies, however, no significant change was observed in the zinc content of various tissues of conventional and germ-free rats fed the vitamin B6-deficient diet, and moreover the increase in zinc content was observed in the kidney of conventional rats and in the liver, kidney, lung and spleen of germ-free rats fed the deficient diet.
The factors affecting the different results could be thought to be as follows: i) diet composition, ii) food utilization and iii) the severity of vitamin B₆-deficiency for the experimental animals. Our diet composition (Table 1) was similar to Hsu’s. The vitamin B₆ contamination originated in our diet seems to be less than that of Hsu’s, because the diet used in this experiment was sterilized by steam at 126°C for 30 min and germ-free rats were used in our experiments.

Quarterman et al. reported that zinc-deficient rats had less insulin sensitivity than their controls (1). It has been reported by Huber et al. that insulin activity was reduced in vitamin B₆-deficient rats (2), although whether vitamin B₆ deficiency causes reduced insulin activity directly or indirectly is not clear.

Murakami found that xanthurenic acid and insulin readily combined in vitro (16), and Kotake et al. reported that the xanthurenic acid-insulin complex showed less hormonal activity on glucose metabolism in adipose tissues than native Zn-insulin in rats (17). In another study (18), it was observed that rats fed a vitamin B₆-deficient diet with deoxypyridoxine injections increased urinary excretion of zinc combined with xanthurenic acid compared to rats which were only fed a vitamin B₆-deficient diet. Therefore, these findings suggest that a more severe vitamin B₆ deficiency caused by deoxypyridoxine injections may give rise to a decrease in zinc content in tissues.

On the other hand, a high iron content was observed in all tissues of vitamin B₆-deficient conventional and germ-free rats, except for the spleen of germ-free rats. Especially, the iron content in the liver and lung of germ-free rats was higher than that of control animals. It has been reported that body iron, serum iron and liver and spleen levels were significantly increased in the vitamin B₆-deficient group compared to the control group (5), and that vitamin B₆ deficiency did not enhance the absorption of iron when a single dose of 0.1 mg of iron was given by stomach tube or under conditions of iron intake considered to be physiological (50 to 100 g), whereas with a daily iron intake of about 1 mg, vitamin B₆-deficient animals showed a significantly greater iron absorption (19). Our present report appears to agree with this observation, since iron intake in our experiments was 1.3 mg for deficient groups and 2.4 mg for the control groups in both conventional and germ-free rats.

Reddy et al. and Wostman et al. reported that germ-free rats with an absence of viable intestinal microflora had markedly decreased absorption and net retention of iron (8, 20), but a decrease in iron levels in germ-free rats was not observed in this study.

Microcytic anemia has been reported in experimental animals and human subjects during vitamin B₆ deficiency (3–6, 16–18). Vitamin B₆-deficient anemia might be attributed to a defect of heme biosynthesis and associated with high serum iron concentrations. Cortwright et al. reported that a deficiency in dietary intake of iron prevented hemosiderosis of tissues in vitamin B₆-deficient animals (3). In liver diseases, high serum ferritin levels are commonly found. Prieto et al. suggested that the level of serum ferritin depends on both the degree of hepatocellular damage and the liver iron stores (21). Excess iron stores in the liver induced hemocho-
ZINC AND IRON IN V. B₆ DEFICIENCY

matosis together with several metabolic disorders, but the reason for this is not clear. However, we assumed that a symptom arising from vitamin B₆ deficiency may be due to excess iron deposits in the liver. Generally, the liver and spleen store iron, but our results indicate that the spleen of vitamin B₆-deficient germ-free rats show a marked decrease in the iron level.

The present observations suggest that the iron in the spleen is easily transported into the blood stream in severe vitamin B₆ deficiency or that the iron metabolism of germ-free rats is intrinsically different than in conventional rats.

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

REFERENCES

1) QUARTERMAN, J., MILLS, C. F., and HUMPHRIES, W. H. (1966): The reduced secretion of, and sensitivity to insulin in zinc-deficient rats. Biochem. Biophys. Res. Commun., 25, 354-358.
2) HUBER, A. M., GERSHOF, S. N., and HEGSTED, D. M. (1964): Carbohydrate and fat metabolism and response to insulin in vitamin B₆ deficient rats. J. Nutr., 82, 371-378.
3) CORTWRIGHT, G. E., WINTROBE, M. M., and HUMPHREYS, S. (1944): Studies on anemia in swine due to pyridoxine deficiency together with data on phenylhydrazine anemia. J. Biol. Chem., 153, 171-182.
4) HARRIS, J. W., WHITTINGTON, R. M., WEISMAN, R., Jr., and HARRIGAN, D. L. (1956): Pyridoxine responsive anemia in the human adult. Proc. Soc. Exp. Biol. Med., 91, 427-432.
5) MCKIBBIN, J. M., SCHAEPER, A. E., FROST, D. V., and ELVEHEJEM, C. A. (1942): Studies on anemia in dogs due to pyridoxine deficiency. J. Biol. Chem., 142, 77-84.
6) STREET, H. R., COWGILL, G. R., and ZIMMERMAN, H. M. (1941): Some observations of vitamin B₆ deficiency in the dog. J. Nutr., 21, 275-290.
7) HSU, J. M. (1965): Zinc content in tissues of pyridoxine deficient rats. Proc. Soc. Exp. Biol. Med., 119, 177-180.
8) REDDY, B. S., PLEASANTS, J. R., and WOSTMANN, B. S. (1972): Effect of intestinal microflora on iron and zinc metabolism and on activities of metalloenzymes in rats. J. Nutr., 102, 101-108.
9) SMITH, J. C., MCDANIEL, E. G., Jr., MCBEE, L. D., DOFT, F. S., and HALSTED, J. A. (1972): Effect of microorganisms upon zinc metabolism using germfree and conventional rats. J. Nutr., 102, 711-720.
10) GUBLER, C. J., CARTWRIGHT, G. E., and WINTROBE, M. M. (1949): The effect of pyridoxine deficiency on the absorption of iron by the rat. J. Biol. Chem., 178, 989-996.
11) REDDY, B. S., PLEASANTS, J. R., ZIMMERMAN, D. R., and WOSTMANN, B. S. (1965): Iron and copper utilization in rabbits as affected by diet and germfree status. J. Nutr., 87, 189-196.
12) ROGERS, G. R. (1968): Collaborative study of atomic absorption spectrophotometric method for determining zinc in foods. J.A.O.A.C., 51, 1042-1045.
13) MATSUBARA, T. (1961): Studies on the method for determination of iron in biological materials, especially serum and whole blood—A new proposal for the standardization of the method—, Acta Haem. Jpn., 24, 434-452.
14) GORNALL, A. G., BARDDEWILL, C. S., and DAVID, M. M. (1949): Determination of serum protein by means of the biuret reaction. J. Biol. Chem., 177, 751-766.
15) PΕKEREK, R. S., and ΒΕSELI, W. R. (1969): Effect of endotoxin upon serum zinc concentrations in the rat. Appl. Microbiol., 18, 482-484.
16) MURAKAMI, E. (1968): Studies on the xanthurenic acid-insulin complex. J. Biochem., 63, 573-577.
17) ΚΟΤΑΚΕ, Y., UEDA, T., ΜΟΡΙ, T., MURAKAMI, E., and HATTORI, M. (1975): The physiological
significance of the xanthurenic acid-insulin complex. J. Biochem., 77, 685–687.

18) UEBA, F., KOTAKE, Y., UEDA, T., GODA, K. (1976): unpublished.

19) NEAL, R. A., and PEARSON, W. N. (1962): Effect of pyridoxine deficiency on iron absorption in the rat. J. Nutr., 78, 215–218.

20) WOSTMANN, B. S., and BRUCKNER-KARDOS, E. (1965): Oxidation-reduction potentials in cecal contents of germfree and conventional rats. Proc. Soc. Exp. Biol. Med., 121, 1111–1114.

21) PRIETO, J., BARRY, M., and SHERLOCK, S. (1975): Serum ferritin in patients with iron overload and with acute and chronic liver diseases. Gastroenterology, 68, 525–533.