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METABOLISM AND METABOLIC STUDIES

Urinary excretion of B-group vitamins reflects the nutritional status of B-group vitamins in rats

Katsumi Shibata*, Chisa Sugita, Mitsue Sano and Tsutomu Fukuwatari

Department of Food Science and Nutrition, School of Human Cultures, The University of Shiga Prefecture, Hikone, Shiga 522-8533, Japan

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Abstract

We have reported previously that the urinary excretion of B-group vitamins reflects recent dietary intakes of these vitamins. We also proposed reference values for the urinary levels of B-group vitamins for human subjects, and used these for evaluating human nutritional status. However, the question arises as to whether the urinary excretion of B-group vitamins in animals or human subjects decreases immediately before they become B-group vitamin insufficient or when fed a diet low in vitamins. In the present study, rats were fed a vitamin-free diet for 5 d, and changes in the levels of B-group vitamins in urine and blood were monitored. Urinary excretion of vitamin B_1, vitamin B_2, 4-pyridoxic acid (a catabolite of vitamin B_6), pantothenic acid, folate and biotin steeply decreased, and all of the values reached zero within 1–2 d. With respect to blood, the concentrations of only three of the eight B-group vitamins (vitamin B_1, pyridoxal phosphate and biotin) decreased to 15 % ($P < 0{\cdot}0001$), 7 % ($P < 0{\cdot}0001$) and 2 % ($P < 0{\cdot}0001$) on day 5, respectively, compared with the values at the beginning of the experiment. The decrease was more rapid and the changes were greater in the urine samples than in the blood samples. The present data complement our previous proposal that the urinary excretion of B-group vitamins reflects the nutritional status of these vitamins.

Key words: **Vitamins: Deficiency: Urine: Blood: Rats**

The classical signs of severe deficiency are seldom observed with respect to B-group vitamins. Instead, an increase in non-specific symptoms, general morbidity and a loss of appetite related to impaired vitamin status are commonly seen. The aim of the present study was to determine a 'pre-sign' that can be observed before the appearance of an obvious vitamin deficiency such as beriberi, pellagra or ariboflavinosis. In general, vitamin deficiencies appear if vitamin intake is inadequate. However, these deficiencies can be caused by malabsorption and the reduced synthetic ability of coenzymes. Therefore, the classical assessment, which is undertaken by comparing the intakes of B-group vitamins with Dietary Reference Intakes[1–3], is of limited effectiveness. Biochemical methods may be more suitable to use for assessment of B-group vitamin nutritional status. They include measuring, in erythrocytes, the activity of transketolase for vitamin B_1 status[4], glutathione reductase for vitamin B_2 status[5], and aspartate aminotransferase and alanine aminotransferase for vitamin B_6 status[6]. Measurement of the activities of these enzymes represents a functional test of the adequacies of vitamins B_1, B_2 and B_6. Relative enhancements of the activities of these enzymes by *in vitro* saturation with thiamine diphosphate[7], flavin adenine dinucleotide[8] and pyridoxal phosphate[6] have been shown to be a sensitive and specific measurement for the detection and evaluation of these vitamins. Suitable biological methods are not available for the detection of other B-group vitamins such as niacin, pantothenic acid, folate, biotin and vitamin B_{12}.

Inadequate intake of vitamins typically causes a reduction in the concentration of vitamins in the cells and body fluid. So far, the most commonly used procedure to assess the nutritional status of B-group vitamins has been measurement of the urinary levels of these vitamins[9–13]. Our research

Abbreviation: MW, molecular weight.

* **Corresponding author:** K. Shibata, fax +81 749 28 8499, email kshibata@shc.usp.ac.jp

team[14–21] and other investigators[22–27] have shown that the urinary excretion of B-group vitamins provides an indication of the recent dietary intakes of these vitamins. However, the questions remain as to whether the urinary excretion of B-group vitamins in animals and human subjects decreases immediately before they become B-group vitamin insufficient or when fed a diet low in vitamins.

In the present study, we investigated the changes in urinary excretion of water-soluble vitamins and those of blood and liver concentrations using rats fed a vitamin-free diet. Our findings suggest that a urine sample is the biological sample most suitable to use as a surrogate indicator of B-group vitamin insufficiency.

Methods and materials

Ethical approval of study protocol

The care and treatment of experimental animals conformed to the guidelines for the ethical treatment of laboratory animals set by The University of Shiga Prefecture (Shiga, Japan).

Chemicals

Vitamin-free milk casein, sucrose and L-methionine were purchased from Wako Pure Chemical Industries. Maize oil was purchased from Ajinomoto. Gelatinised maize starch, a mineral mixture (American Institute of Nutrition (AIN)-93G mineral mixture)[28], and a vitamin mixture (nicotinic acid-free AIN-93 vitamin mixture containing 25 % choline bitartrate)[28] were obtained from Oriental Yeast Co., Ltd.

Thiamine hydrochloride ($C_{12}H_{17}ClN_4OS$-HCl; molecular weight (MW) = 337·27 Da), riboflavin ($C_{17}H_{20}N_4O_6$; MW = 376·3 Da), pyridoxine hydrochloride ($C_8H_{11}NO_3$-HCl; MW = 205·63 Da), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$; MW = 1355·40 Da), nicotinamide ($C_6H_6N_2O$; MW = 122·13 Da), calcium pantothenate ($C_{18}H_{32}N_2O_{10}$-Ca; MW = 476·54 Da), folic acid ($C_{19}H_{19}N_7O_6$; MW = 441·40 Da) and D(+)-biotin ($C_{10}H_{16}N_2O_3S$; MW = 244·31 Da) were purchased from Wako Pure Chemical Industries. 4-Pyridoxic acid ($C_8H_9NO_4$; MW = 183·16 Da) manufactured by ICN Pharmaceuticals was obtained through Wako Pure Chemical Industries.

N^1-Methylnicotinamide chloride ($C_7H_9N_2O$-HCl; MW = 159·61 Da) was purchased from Tokyo Kasei Kogyo. N^1-Methyl-2-pyridone-5-carboxamide (2-Py, $C_7H_8N_2O_2$; MW = 152·15 Da) and N^1-methyl-4-pyridone-3-carboxamide (4-Py, $C_7H_8N_2O_2$; MW = 152·15 Da) were synthesised employing the methods of Pullman & Colowick[29] and Shibata *et al.*[30], respectively. All other chemicals used were of the highest purity available from commercial sources.

Animals and treatment

The room was maintained at about 22°C and at a humidity of about 60 %. A 12 h light–12 h dark cycle (06.00–18.00 hours/18.00–06.00) was employed.

To acclimatise them to a new environment, male Wistar rats (6 weeks old; CLEA Japan) were kept in individual rat metabolism cages (CT-10; CLEA Japan) and fed a control diet *ad libitum* (Table 1) for 7 d. The rats were then divided into two groups. One group was fed a control diet and the other fed a vitamin-free diet (Table 1) for 5 d. The start of the experiment was designated as the time when the diet was changed. Each day, 24 h urine samples were collected (09.00–09.00 hours) in amber bottles containing 1 ml of 1 M-HCl and stored at −25°C until use. Rats were killed at about 09.00 hours; blood was collected and livers (not perfused) were taken to measure the contents of B-group vitamins in the urine, liver and blood. The removed livers were preserved at −25°C until use.

Table 1. Composition of the diets

	Control diet (%)	Vitamin-free diet (%)
Milk casein (vitamin-free)	20·0	20·0
L-Methionine	0·2	0·2
Gelatinised maize starch	46·9	47·9
Sucrose	23·4	23·4
Maize oil	5·0	5·0
Mineral mixture (AIN-93-MX-G)*	3·5	3·5
Vitamin mixture (AIN-93-VX containing 25 % choline bitarrate, nicotinic acid-free)*	1·0	0

*The compositions of the mineral mixture and the vitamin mixture are described by Reeves[28].

Measurement of levels of B-group vitamins in urine, liver and blood

Preparation and measurement of the extracts of B-group vitamins from urine and blood were as previously described[14,31,32].

Vitamin B_1. Frozen liver samples, about 0·5 g, were thawed, minced, added to ten volumes of cold 5 % TCA and homogenised in a blender (Waring). The acidified homogenate was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was retained and used for the measurement of the levels of vitamin B_1[33].

Vitamin B_2. Frozen liver samples, about 0·5 g, were thawed, minced, added to ten volumes of 50 mM-KH_2PO_4–K_2HPO_4 buffer (pH 7·0) and homogenised in a blender. To a 0·1 ml aliquot of the homogenate, 0·44 ml of water and 0·26 ml of 0·5 M-H_2SO_4 were added and kept at 80°C for 15 min. After cooling, 0·2 ml of 10 % TCA were added and the mixture was centrifuged at 10 000 ***g*** for 3 min at 4°C. From the supernatant obtained, 0·2 ml were withdrawn and added to 0·2 ml of 1 M-NaOH. The alkalised mixture was irradiated at a height of 20 cm from the liquid with two fluorescent lamps (20 W) for 30 min at room temperature. Then, 0·02 ml of glacial acetic acid were added to the mixture. The neutralised mixture was passed through a 0·45 μm microfilter. The filtrate was injected directly into an HPLC system for measuring lumiflavin levels[34].

Vitamin B_6. Frozen liver samples, about 0·5 g, were thawed, minced, added to 90 ml of 55 M-HCl and homogenised in a blender. The homogenate was autoclaved at 121°C for 3 h to convert vitamin B_6 coenzyme to the free form of vitamin B_6. After cooling, the mixture was adjusted to pH 5·0 with 1 M-NaOH and then brought up to 100 ml with water. The solution was filtered with qualitative filter number 2

(Advantec). The filtrate was used for measuring levels of vitamin B_6 as previously described[35].

Vitamin B_{12}. Frozen liver samples, about 0·5 g, were thawed, minced and then added to 2·5 ml of 0·57 M-acetic acid–sodium acetate buffer (pH 4·5) plus 5 ml of water and 0·1 ml of 0·05 % KCN. The suspension was homogenised in a blender. The homogenate was then put into a boiling water-bath for 5 min. After cooling, 0·15 ml of 10 % metaphosphoric acid was added and brought up to 10 ml with water. The solution was filtered with qualitative filter number 2 (Advantec). The filtrate was used for measuring levels of vitamin B_{12} as previously described[36].

Nicotinamide. Frozen liver samples, about 0·6 g, were thawed, minced and then added to five volumes of 0·1 g/ml isonicotinamide. The suspension was then homogenised in a blender. The homogenate (1 ml) was withdrawn, added to 4 ml of water, and then autoclaved at 121°C for 10 min to convert pyridine nucleotide coenzymes to nicotinamide. After cooling, the mixture was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was retained, the precipitated materials

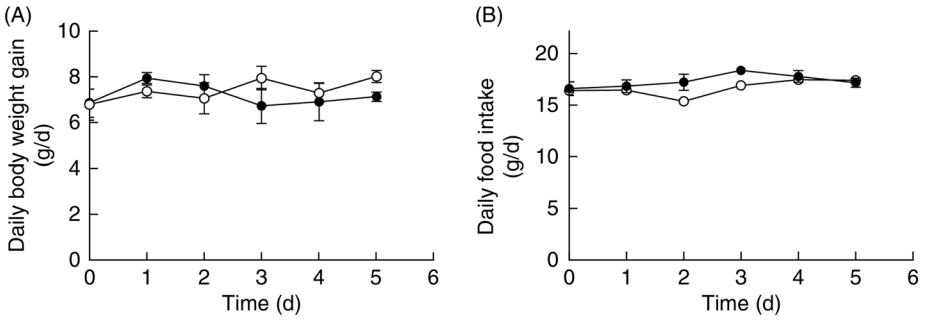

Fig. 1. Changes in body weight (A) and food intake (B) in male Wistar rats (aged 7 weeks) fed a vitamin-free diet (●) or a control diet (○) for 5 d. Values are means for five rats, with standard errors represented by vertical bars.

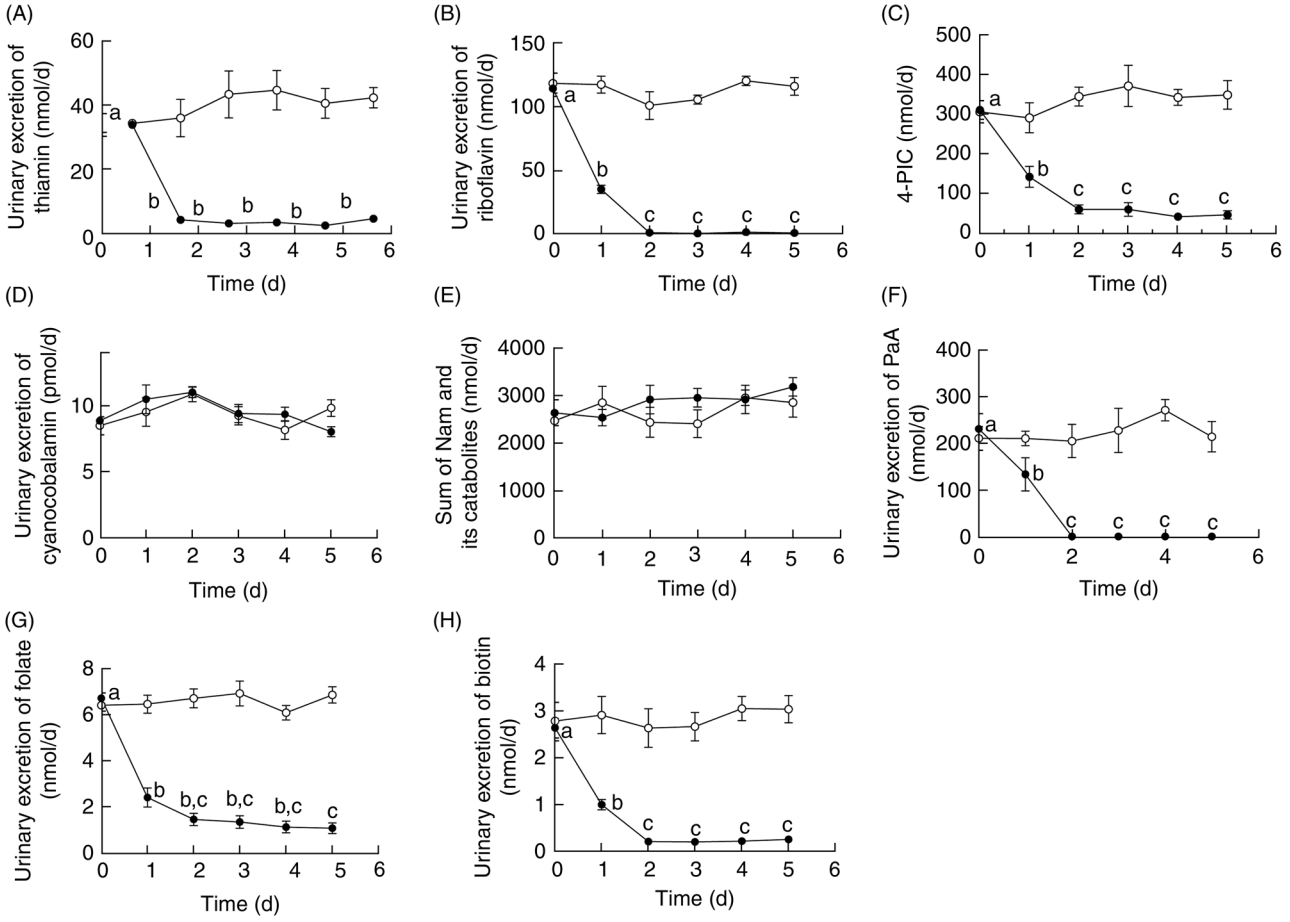

Fig. 2. Changes in the urinary excretion of thiamine (A), riboflavin (B), 4-pyridoxic acid (4-PIC) (C), vitamin B_{12} (cyanocobalamin) (D), nicotinamide (Nam) and its metabolites (E), pantothenic acid (PaA) (F), folate (G) and biotin (H) in male Wistar rats (aged 7 weeks) fed a vitamin-free diet (●) or a control diet (○) for 5 d. Each day, 24 h urine samples (09.00–09.00 hours) were collected. Values are means for five rats, with standard errors represented by vertical bars. [a,b,c] Mean values with unlike letters for the group fed the vitamin-free diet were significantly different ($P < 0·05$; one-way ANOVA followed by Tukey's multiple-comparison tests).

were extracted again with 5 ml of water, and the resulting supernatant was retained. Both retained supernatants were combined and the extract was used for measuring nicotinamide levels as previously described[30].

Pantothenic acid. Frozen liver samples, about 0·2 g, were thawed, minced and then added to ten volumes of 50 mM-KH_2PO_4–K_2HPO_4 buffer (pH 7·0). The suspension was homogenised with a Teflon/glass homogeniser. The homogenate was incubated overnight at 37°C to convert free pantothenic acid from the bound type of pantothenate compounds. The reaction was stopped by placing the mixture into a boiling water-bath for 5 min. After cooling, the mixture was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was retained, the precipitated materials were extracted again with 2 ml of water and the resulting supernatant retained. Both retained supernatants were combined, and the extract was used for measuring levels of pantothenic acid as previously described[37].

Folate. Frozen liver samples, about 0·5 g, were thawed, minced and then added to ten volumes of 0·1 M-KH_2PO_4–K_2HPO_4 buffer (pH 6·1). The suspension was homogenised in a blender. The homogenate was autoclaved at 121°C for 5 min. After cooling, 2·5 ml of proteinase MS (200 U/ml of water; Kaken Pharmaceutical Co., Ltd.) was added. The mixture was incubated at 37°C for 3 h to digest proteins and then release polyglutamated folates from the protein-bound types. The reaction was stopped by placing the mixture into a boiling water-bath for 10 min. After cooling, 0·5 ml of conjugase (extract from porcine kidney acetone powder (type II); Sigma–Aldrich) was added to the mixture and incubated overnight at 37°C to convert polyglutamated folates to monoglutamated folates. The reaction was stopped by placing the mixture in a boiling water-bath for 10 min. After cooling, the mixture was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was retained, the precipitated materials extracted again with 3 ml of water, and the resulting supernatant retained. Both the retained supernatants were combined, and the extract was used for measuring folate levels as previously described[38]. The conjugase solution was made up as follows: 60 ml of 50 mM-KH_2PO_4–K_2HPO_4 buffer (pH 7·0) was added to 20 g of porcine kidney acetone powder and stirred for 30 min at 4°C. The suspension was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was dialysed against a large amount of 50 mM-KH_2PO_4–K_2HPO_4 buffer (pH 7·0) to remove endogenous folate from the kidney acetone powder. The dialysed conjugase solution was used.

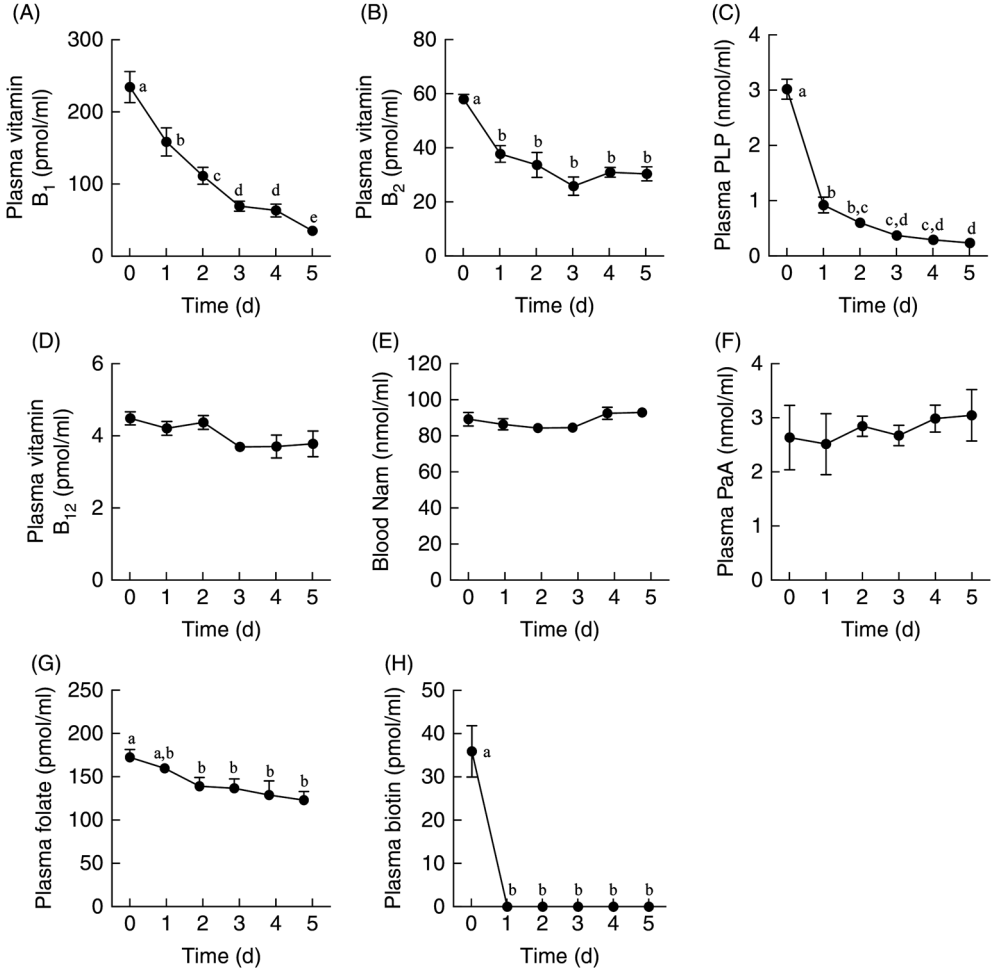

Fig. 3. Changes in the levels of vitamin B_1 (A), vitamin B_2 (B), pyridoxal phosphate (PLP) (C), vitamin B_{12} (D), nicotinamide (Nam) (E), pantothenic acid (PaA) (F), folate (G) and biotin (H) in the plasma or whole blood of male Wistar rats (aged 7 weeks) fed a vitamin-free diet for 5 d. Rats were killed each day and blood was collected. Values are means for five rats, with standard errors represented by vertical bars. ^{a,b,c,d,e} Mean values with unlike letters were significantly different ($P < 0·05$; one-way ANOVA followed by Tukey's multiple-comparison tests).

Biotin. Frozen liver samples, about 0·5 g, were thawed, minced, added to two volumes of 2·25 M-H_2SO_4 and then homogenised in a blender. The suspension was autoclaved at 121°C for 1 h to convert bound biotin to the free form of biotin. After cooling, the suspension was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was used for measuring biotin levels[39].

Statistical analyses

Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparison tests. $P < 0{\cdot}05$ was considered significant. All statistical analyses were undertaken using GraphPad Prism version 5.0 (GraphPad).

Results

Effects of feeding vitamin-free diet on food intake and gain in body weight

Male Wistar rats (aged 7 weeks) were fed the vitamin-free diet or the control diet for 5 d. No significant difference was observed in the daily gain in body weight between the control group and the vitamin-free diet group (Fig. 1(A)). No significant difference in daily food intake between the two groups was observed (Fig. 1(B)). Rats fed the vitamin-free diet for 5 d showed no abnormalities in body appearance.

Effects of feeding vitamin-free diet on the urinary excretion of B-group vitamins

Urinary excretion of each water-soluble vitamin, except for vitamin B_{12} and the sum of nicotinamide and its catabolites, decreased steeply upon feeding the vitamin-free diet (Fig. 2). Excreted levels of vitamin B_1, vitamin B_2, 4-pyridoxic acid (catabolite of vitamin B_6), pantothenic acid, folate and biotin steeply decreased and approached zero within 1–2 d. Urinary excretion of vitamin B_{12} did not decrease because the main elimination route of vitamin B_{12} is through the bile duct and not the kidney[40]. Urinary excretion of the sum of nicotinamide and its catabolites did not decrease because nicotinamide is biosynthesised from tryptophan in the vitamin-free diet.

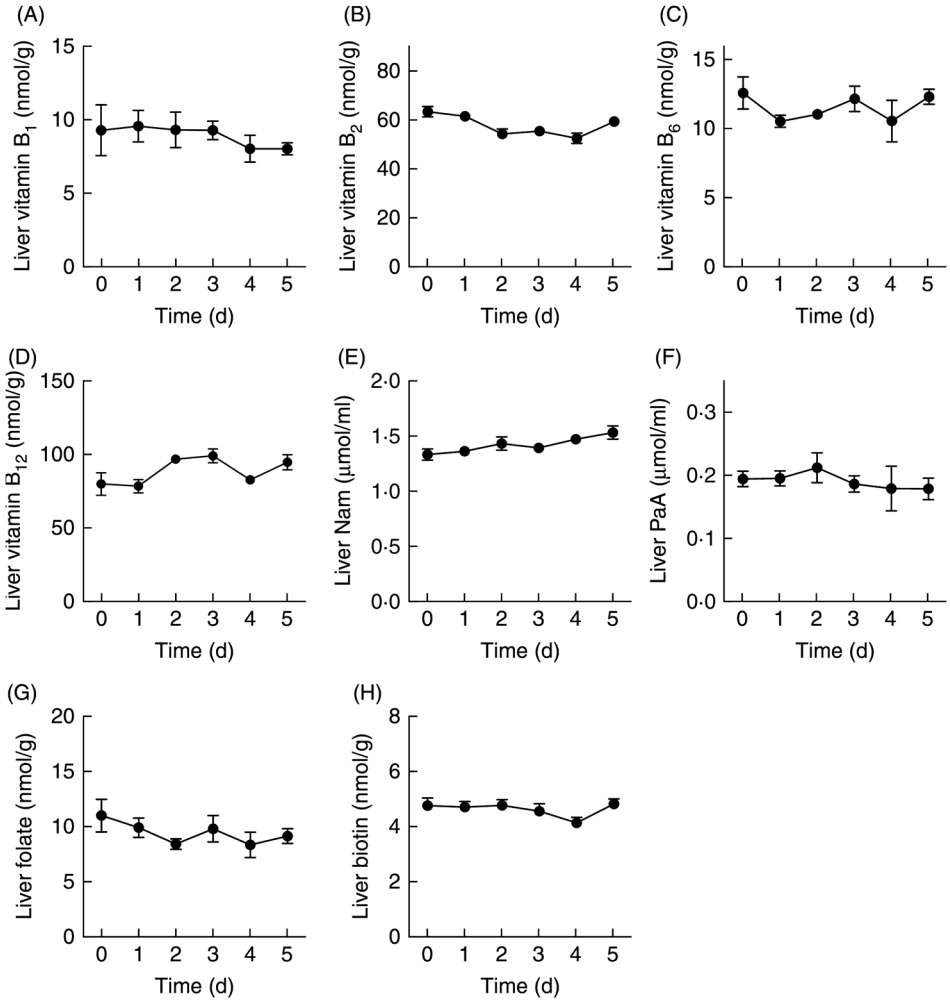

Fig. 4. Changes in the levels of vitamin B_1 (A), vitamin B_2 (B), vitamin B_6 (C), vitamin B_{12} (D), nicotinamide (Nam) (E), pantothenic acid (PaA) (F), folate (G) and biotin (H) in the livers of male Wistar rats (aged 7 weeks) fed a vitamin-free diet for 5 d. Rats were killed every day and their livers were removed. Values are means for five rats, with standard errors represented by vertical bars.

Effects of feeding vitamin-free diet on the concentrations of B-group vitamins in blood and plasma

Nicotinamide was not detected in plasma, even in control rats. Hence, we measured the concentration of nicotinamide in whole blood. Its concentration did not change even when the vitamin-free diet was fed to rats for 5 d. This is because nicotinamide is biosynthesised from tryptophan. With respect to blood, only the concentrations of vitamin B_1, pyridoxal phosphate (a coenzyme of vitamin B_6), and biotin decreased to 15 % ($P < 0{\cdot}0001$), 7 % ($P < 0{\cdot}0001$) and 2 % ($P < 0{\cdot}0001$) on day 5, respectively, compared with the values at the beginning of the experiment, whereas the reduction in the concentrations of vitamin B_2 and folate was not so steep (Fig. 3). The plasma concentrations of vitamin B_{12} and pantothenic acid were not affected.

Effects of feeding vitamin-free diet on liver concentrations of B-group vitamins

The liver contents of all of the B-group vitamins did not decrease even if rats were fed the vitamin-free diet for 5 d (Fig. 4). The liver samples were not perfused, so a proportion of the levels of vitamins might originate from those found in the blood. However, the concentrations of respective B-group vitamins were much lower in blood samples than in the liver samples (Figs. 3 and 4). Therefore, the blood vitamin levels most probably did not have a significant impact on the vitamin levels observed in the liver samples.

Discussion

We have reported previously that the urinary excretion of B-group vitamins reflects recent dietary intakes of these vitamins[14–21], and proposed reference values of the urinary levels of B-group vitamins for evaluating nutritional status. This method has been applied to evaluate human nutritional status[21,41]. However, the question remains as to whether the urinary excretion of B-group vitamins decreases immediately before animals and human subjects become B-group vitamin insufficient or when they are fed a diet low in vitamins.

In the present study, we investigated the changes in the urinary excretion of B-group vitamins in rats when they were fed a vitamin-free diet. Feeding the vitamin-free diet to growing rats for 5 d did not affect the food intake, body weight and vitamin concentrations in the liver. These findings indicate that this treatment did not induce B-group vitamin insufficiency.

We measured the plasma B-group vitamins in rats during the feeding of the vitamin-free diet. The concentrations of only three vitamins of the eight B-group vitamins (vitamin B_1, pyridoxal phosphate and biotin) were decreased by feeding the vitamin-free diet, but the speed of the decrease was slower and the magnitude was lower than urinary excretion levels. For other B-group vitamins, the concentrations of vitamin B_2 and folate were slightly reduced, whereas those of vitamin B_{12}, nicotinamide and pantothenic acid were not decreased. These findings suggested that when rats were fed the vitamin-free diet, the concentrations of vitamins are maintained in the body by restriction of the elimination of B-group vitamins into urine. Therefore, the blood samples were not suitable to use for evaluating the nutritional status of B-group vitamins.

As was anticipated, the urinary excretion of B-group vitamins such as vitamin B_1, vitamin B_2, vitamin B_6, pantothenic acid, folate and biotin was steeply decreased by feeding the vitamin-free diet. The decrease was rapid and dramatic (Fig. 2). These findings support our proposal that the urinary excretion of B-group vitamins is a better indicator of B-group vitamin nutritional status than blood concentrations.

In the present study, because sufficient niacin is biosynthesised from tryptophan, the total excretion of nicotinamide and its catabolites was not decreased by feeding the vitamin-free diet. When the rats were fed a tryptophan-limited and niacin-free diet, the urinary excretion of niacin decreased significantly[42]. Therefore, the urinary excretion of nicotinamide and its metabolites can be used as an index of niacin nutritional status.

The urinary excretion of vitamin B_{12} was not affected by feeding the vitamin-free diet. This can be explained by the very slow turnover rate of vitamin B_{12}, and the fact that its elimination route is via the bile[40] and not via the kidney. In addition, a large amount of vitamin B_{12} is stored in the liver and kidneys[43].

In conclusion, in animals and human subjects consuming diets low in vitamins, the first potential mechanism to prevent insufficiency of B-group vitamins, except for vitamin B_{12}, is to reduce the urinary elimination of B-group vitamins via the kidney[44]. Therefore, the measurement of the urinary B-group vitamins, except for vitamin B_{12}, is a suitable method for individual nutritional status assessment. The present data complement our previous proposal that the urinary excretion of B-group vitamins reflects the nutritional status of these vitamins[14–21,41].

Acknowledgements

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