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25% choline bitartrate)[23] were obtained from Oriental Yeast Company, Limited.

Thiamin hydrochloride ($C_{12}H_{17}ClN_4OS$-HCl; molecular weight 337·27), riboflavin ($C_{17}H_{20}N_4O_6$; 376·37), pyridoxine hydrochloride ($C_8H_{11}NO_3$-HCl; 205·63), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$; 1355·40), nicotinamide ($C_6H_6N_2O$; 122·13), calcium pantothenate ($C_{18}H_{32}N_2O_{10}$-Ca; 476·54), folic acid ($C_{19}H_{19}N_7O_6$; 441·40) and D(+)-biotin ($C_{10}H_{16}N_2O_3S$; 244·31) were purchased from Wako Pure Chemical Industries. 4-Pyridoxic acid ($C_8H_9NO_4$ = 183·16) was made by ICN Pharmaceuticals and obtained through Wako Pure Chemical Industries.

N^1-Methylnicotinamide chloride ($C_7H_9N_2O$-HCl; 159·61) was purchased from Tokyo Kasei Kogyo. N^1-Methyl-2-pyridone-5-carboxamide (2-Py, $C_7H_8N_2O_2$ 152·15) and N^1-methyl-4-pyridone-3-carboxamide (4-Py, $C_7H_8N_2O_2$ 152·15) were synthesised by the methods of Pullman & Colowick[24] and Shibata *et al.*[25], respectively. All other chemicals used were of highest purity available from commercial sources.

Animals and treatment

The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. The animals were maintained under controlled temperature (22°C), 60% humidity and light conditions (12 h light–12 h dark cycle).

Effects of ethanol feeding on the B-group vitamin contents of liver, blood and urine in rats fed a diet containing a sufficient-vitamin mixture (Expt 1)

Male Wistar rats (3 weeks old) obtained from CLEA Japan were fed freely with a conventional purified diet, consisting of 20% vitamin-free milk casein, 0·2% L-methionine, 46·9% gelatinised maize starch, 23·4% sucrose, 5% maize oil, 3·5% AIN-93-G mineral mixture[14] and 1% AIN-93 vitamin mixture[14] containing chorine bitartrate, but without nicotinic acid, to acclimatise for 7 d. Nicotinic acid had not been added to this diet because it is supplied enough from tryptophan in casein[26], and a dietary fibre-free diet was used because it is a tradition not to use dietary fibre in our laboratory which is not essential for normal growth[27].

The rats were divided into two groups (n 5 each). Group 1 was fed with a diet containing the 1% vitamin mixture (a sufficient-vitamin diet) and allowed to drink water for 28 d. Group 2 was fed with a diet containing the 1% vitamin mixture (a sufficient-vitamin diet) and forced to drink a 15% ethanol solution instead of water for 28 d. The 24 h urine samples were collected in amber bottles containing 1 ml of 1 M-HCl at 09.00–09.00 hours of the last day and were stored at −25°C until required. The rats were killed at about 09.00 hours; blood was collected and tissues were taken to measure the weights and the contents of B-group vitamins in the liver, blood and urine. Liver samples were preserved at −25°C until required.

Effects of ethanol feeding on the B-group vitamin contents of liver, blood and urine in rats fed a diet containing a low-vitamin mixture (Expt 2)

A preliminary experiment revealed that the body-weight gain of young rats was the same when fed a diet containing the 1% AIN-93 vitamin mixture and the 0·3% AIN-93 vitamin mixture, whereas the body-weight gain was lower in rats fed a diet containing the 0·2% AIN-93 vitamin mixture than in those fed a diet containing the 1 or 0·3% diets. Thus, we determined tentatively whether the diet containing the 0·3% AIN-93 vitamin mixture could supply a minimum amount of vitamins for the growing rats.

Male Wistar rats (3 weeks old) obtained from CLEA Japan were fed freely with the conventional purified diet (mentioned above) to acclimatise for 7 d. The rats were then divided into two groups (n 5 each). Group 1 was fed a diet containing the 0·3% vitamin mixture and allowed to drink water for 28 d. Group 2 was fed a diet containing the 0·3% vitamin mixture and forced to drink a 15% ethanol solution instead of water for 28 d. The 24 h urine samples and tissues were collected. Levels of alanine aminotransferase, aspartate aminotransferase and γ-glutamyltranspeptidase were measured at Mitsubishi Chemical Medience (Tokyo, Japan).

Measurement of B-group vitamins in urine and blood

Preparation and measurement of the extracts of the B-group vitamins from the urine and blood are described as follows[28].

Vitamin B_1

Frozen liver samples, about 0·5 g, were thawed, minced, and then added to ten volumes of 5% ice-cold TCA and homogenised with a Digital Homogenizer Hom (Iuchi). The acidified homogenate was centrifuged at 10 000 ***g*** for 10 min at 4°C, and the supernatant was retained and used for the measurement of vitamin B_1[29].

Vitamin B_2

Frozen liver samples, about 0·5 g, were thawed, minced, and then added to ten volumes of 50 mM-KH_2PO_4–K_2HPO_4 buffer (pH 7·0) and homogenised with a Teflon/glass homogeniser (Nikko Hansen). To 0·1 ml of the homogenate, 0·44 ml of water and 0·26 ml of 0·5 M-H_2SO_4 were added and then kept at 80°C for 15 min. After cooling, 0·2 ml of 10% TCA were added and centrifuged at 10 000 ***g*** for 3 min at 4°C. From the supernatant obtained, 0·2 ml was withdrawn and added to 0·2 ml of 1 M-NaOH. The alkalised mixture was irradiated with a fluorescent lamp for 30 min and then 0·02 ml of glacial acetic acid were added to the mixture. The neutralised mixture was passed though a 0·45 μm microfilter and the filtrate was directly injected into the HPLC system for measuring lumiflavin[30].

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Vitamin B_6

Frozen liver samples, about 0·5 g, were thawed, minced, and then added to 90 ml of 55 mM-HCl and homogenised with a Waring blender. The homogenate was autoclaved at 121°C for 3 h. After cooling, the mixture was adjusted to pH 5·0 with 1 M-NaOH and then made up to 100 ml with water. The solution was filtered with qualitative filter no. 2 (ADVANTEC MFS, Inc.). The filtrate was used for measuring vitamin B_6 as described previously[31].

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 % potassium cyanide (KCN). The suspension was homogenised with a Teflon/glass homogeniser. The homogenate was then put into a boiling water-bath for 5 min. After cooling, 0·15 ml of 10 % metaphosphoric acid were added and made up to 10 ml with water. The solution was filtered with qualitative filter no. 2 (ADVANTEC MFS, Inc.). The filtrate was used for measuring vitamin B_{12} as described previously[32].

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 homogenised with a Teflon/glass homogeniser. The homogenate (1 ml) was withdrawn and added to 4 ml of water, and then autoclaved at 121°C for 10 min. After cooling, the mixture was centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was retained and the precipitated materials were extracted again with 5 ml of water, and the supernatant was retained. Both the retained supernatants were combined, and the extract was used for measuring nicotinamide as described previously[25].

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 at 37°C overnight to convert free pantothenic acid from the bound type of pantothenate compounds. The reaction was stopped by putting it 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 and the precipitated materials were extracted again with 2 ml of water, and the supernatant was retained. Both the retained supernatants were combined, and the extract was used for measuring pantothenic acid as described previously[33].

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 with a Teflon/glass homogeniser. The homogenate was autoclaved at 121°C for 5 min. After cooling, 2·5 ml of pronase (5 mg/ml; Pronase MS; Kaken Pharmaceutical Company, Limited) were added and then incubated at 37°C for 3 h. The reaction was stopped by putting it 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) were added and incubated at 37°C overnight. The reaction was stopped by putting it into 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, and the precipitated materials were extracted again with 3 ml of water, and the supernatant was retained. Both the retained supernatants were combined, and the extract was used for measuring folate as described previously[34]. The conjugase solution was made as follows: 60 ml of 50 mM-KH_2PO_4–K_2HPO_4 buffer (pH 7·0) were added to 20 g 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 of the kidney acetone powder. The dialysed conjugase solution was used.

Biotin

Frozen liver samples, about 0·5 g, were thawed, minced, and then added to two volumes of 2·25 M-H_2SO_4 and then homogenised with a Waring blender. The suspension was hydrolysed by autoclaving for 1 h at 121°C. After cooling, the suspension was centrifuged at 10 000 ***g*** for 10 min at 4°C, and the supernatant was used for measuring biotin[35].

Analyses

The measurements of the B-group vitamins except for vitamin B_6 were described previously[19]. The urinary excretion of 4-pyridoxic acid, a catabolite of vitamin B_6, was measured according to the method of Gregory & Kirk[36].

Statistical analysis

Mean values between the treatment groups were compared using the Mann–Whitney U two-tailed t test. $P<0·05$ was considered to be statistically significant. All statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software).

Results

Effects of ethanol feeding on the B-group vitamin contents of liver, blood and urine in rats fed a diet containing a sufficient-vitamin mixture (Expt 1)

There were no differences in body-weight gain and liver weights between the groups. No differences in the levels of vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, nicotinamide, pantothenic acid, folate and biotin were observed in the liver

and blood. Although the 24 h urinary excretion of some of the vitamins was slightly lower in the ethanol-treated group than in the control, the differences were not significant (data not shown). Thus, ethanol consumption did not affect the B-group vitamin contents in the liver, blood and urine when the rats were fed a diet containing sufficient amounts of the vitamins.

Effects of ethanol feeding on the B-group vitamin contents of liver, blood and urine in rats fed a diet containing a low-vitamin mixture (Expt 2)

As shown in Table 1, body-weight gain, food intake and liver weights were lower in the ethanol-fed group than in the controls. The overall food intake was lower in the ethanol-fed group than in the controls, but energy intake was almost the same because of ethanol intake.

The effects of ethanol consumption on the activities of alanine aminotransferase, aspartate aminotransferase and γ-glutamyltranspeptidase in plasma are shown in Table 2. No significant effects of ethanol consumption were observed for these indices of liver function.

The effects of ethanol consumption on the B-group vitamin contents of the liver are shown in Table 3. The contents of the vitamins in liver are measured as storage amounts of the vitamins, thus are expressed as mol/liver. The contents of vitamin B_1, vitamin B_2 and pantothenic acid were lower in the ethanol-fed group than in the controls, whereas the contents of vitamin B_6, vitamin B_{12}, nicotinamide, folate and biotin were not significantly different.

The effects of ethanol consumption on the B-group vitamin contents of the blood are shown in Table 4. The contents of vitamin B_1, vitamin B_2, vitamin B_6 and pantothenic acid were lower in the ethanol-fed group than in the controls, whereas the contents of vitamin B_{12}, nicotinamide, folate and biotin were not significantly different.

The effects of ethanol consumption on the 24 h urinary excretion of the B-group vitamins are shown in Table 5. The excretion of vitamin B_1, vitamin B_2, 4-pyridoxic acid (a catabolite of vitamin B_6), vitamin B_{12}, pantothenic acid, folate and biotin was lower in the ethanol-fed group than in the controls, whereas the contents of nicotinamide (sum of the contents of nicotinamide and its catabolites such as N^1-methylnicotinamide, 2-Py and 4-Py) were not significantly different.

Food intake was different in the two groups, so that urinary excretion ratios of the vitamins were calculated. As shown in Table 5, the excretion ratios of all vitamins except for vitamin B_{12} were lower in the ethanol-fed group.

Table 1. Effects of ethanol consumption on rat body-weight gain, food intake, ethanol intake, water intake, energy intake, food efficiency ratio and liver weight (Expt 2)

(Mean values with their standard errors for five rats per group)

	Control		15% Ethanol	
	Mean	SEM	Mean	SEM
Initial body weight (g)	36	1	36	1
Final body weight (g)	204	7	164*	8
Body-weight gain (g/28 d)	168	7	128*	3
Food intake (g/28 d)	363	14	258*	6
Ethanol intake† (g/28 d)	–		45	3
Water intake (ml/28 d)	396	26	–	
Energy intake‡ (kcal/28 d)	1488	58	1396	56
Energy intake‡ (kJ/28 d)	6230	242	5845	234
Food efficiency ratio§	0·46	0·01	0·50	0·00
Energy efficiency ratio‖	0·113	0·020	0·092	0·006
Liver weight (g)	9·70	0·55	8·47	0·36

* Mean values were significantly different from those of the control group ($P<0·05$; Mann–Whitney U two-tailed t test).
† The value is expressed in g of pure ethanol and not as the volume of 15% ethanol.
‡ Energy of 1 g ethanol was calculated as 29·3 kJ (7 kcal)/g.
§ (Body-weight gain/food intake) × 100.
‖ (Body-weight gain/energy intake) × 100.

Table 2. Effects of ethanol consumption on the activities of alanine aminotransferase, aspartate aminotransferase and γ-glutamyltranspeptidase in plasma

(Mean values with their standard errors for five rats per group)

	Control		15% Ethanol	
	Mean	SEM	Mean	SEM
Alanine aminotransferase (IU/l)	22·4	1·9	24·8	2·0
Aspartate aminotransferase (IU/l)	157	11	136	10
γ-Glutamyltranspeptidase (IU/l)	3·2	0·9	3·2	0·9

Discussion

An ordinary diet for rats generally contains sufficient amounts of nutrients including vitamins[23]. Under well-nourished conditions, rats are generally little affected by factors such as ethanol consumption. In fact, the present study proves that ethanol consumption did not affect the body-weight gain or the vitamin contents in the liver and blood when rats were fed a diet containing sufficient amounts of vitamins. On the other hand, when rats were fed a diet low in vitamins, body-weight gain was lower in the ethanol-fed group than in the control group and some vitamin contents of the liver and blood, and urinary excretion were decreased. These results show that chronic ethanol consumption affects

Table 3. Effect of ethanol consumption on liver B-group vitamin contents (Expt 2)

(Mean values with their standard errors for five rats per group)

	Control		15% Ethanol	
	Mean	SEM	Mean	SEM
Vitamin B_1 (nmol/liver)	127	6	100*	4
Vitamin B_2 (nmol/liver)	686	62	422*	16
Vitamin B_6 (nmol/liver)	229	16	281	23
Vitamin B_{12} (nmol/liver)	0·39	0·03	0·38	0·02
Niacin (μmol/liver)	18·2	1·8	16·6	1·3
Pantothenic acid (μmol/liver)	3·16	0·19	2·42*	0·18
Folate (nmol/liver)	70·0	9·7	73·6	9·3
Biotin (nmol/liver)	9·31	1·10	9·65	0·46

* Mean values were significantly different from those of the control group ($P<0·05$; Mann–Whitney U two-tailed t test).

Table 4. Effect of ethanol consumption on blood B-group vitamin contents (Expt 2)

(Mean values with their standard errors for five rats per group)

	Control		15 % Ethanol	
	Mean	SEM	Mean	SEM
Vitamin B_1 (pmol/ml)	159	4	139*	6
Vitamin B_2 (pmol/ml)	177	5	142*	4
Vitamin B_6 (nmol/ml)	0·49	0·04	0·34*	0·02
Vitamin B_{12} (pmol/ml)	1·55	0·03	1·41	0·01
Niacin (nmol/ml)	127	6	117	2
Pantothenic acid (nmol/ml)	1·13	0·04	0·89*	0·04
Folate (pmol/ml)	149	4	138	10
Biotin (pmol/ml)	30·4	3·4	25·9	1·0

* Mean values were significantly different from those of the control group ($P<0·05$; Mann–Whitney U two-tailed t test).

absorption, distribution and excretion of vitamins, as reported previously[1–19]. The present findings are not consistent with the *in vitro* perfusion of rat liver with ethanol, which caused the release of all B-vitamins except biotin from the liver stores[23]. This phenomenon was not observed in the present whole-body experiment, because the vitamin contents of the blood were not increased by ethanol consumption. In the present *in vivo* experiment, any vitamins released from the liver were quickly absorbed by non-hepatic tissues. In humans, the typical dietary vitamin intakes are generally around the minimum requirements. Thus, the nutritional status of rats fed a diet low in vitamins was similar to that of humans. Ethanol consumption was 45 g over 28 d, so that daily average ethanol consumption was about 1·6 g/d, which corresponds to an energy intake of 46·9 kJ (11·2 kcal)/d. The energy intake in the ethanol-fed group, including ethanol energy, was 5845 kJ (1396 kcal) over 28 d (about 209 kJ (50 kcal)/d). Thus, ethanol accounted for 20 % of dietary energy. Under these conditions, liver functions in rats were not injured. If humans were to consume 10 467 kJ (2500 kcal)/d, the equivalent ethanol consumption would be about 70 g/d, which corresponds to 1 litre of typical beer.

Vitamin depletion, common in malnourished alcoholic patients[10], can occur despite vitamin supplementation. Vitamin malabsorption[37], exacerbated by malnutrition, contributes to this depletion[38]. Also, in alcoholic patients, the impaired ability of the liver to bind and store vitamins might contribute to this depletion. This may probably be due to the hepatotoxicity of ethanol, which impairs not only the vitamin-binding capacity but also the vitamin storage of the liver. In the present study, a diet containing 20 % casein supplemented with methionine was used, which is an excellent protein source from a nutritional standpoint. This suggest the reasons why ethanol consumption did not cause any severe damage, such as an extremely low food intake and body-weight gain and roughness of fur for the rats, even when they were fed a low-vitamin diet.

Sorrell *et al.*[21] reported that the *in vitro* perfusion of rat liver with ethanol caused the release of all vitamins from the liver stores, especially thiamin. It is generally considered that this phenomenon causes increased urinary excretion of vitamins, but in the present *in vivo* experiments, ethanol consumption did not cause increased urinary excretion, but rather decreased it. This discrepancy between the expected and the actual findings may be attributed to the difference between the *in vitro* and *in vivo* experiments. Moreover, there are differences in short-term and long-term adjustment mechanisms for ethanol toxicity. The protein nutritional status was high in the present study because the diet used 20 % casein supplemented with methionine. Protein plays a pivotal role in vitamin absorption and storage in hepatocytes. Protein malnutrition causes malabsorption, reduced storage and impaired utilisation of vitamins. Thus, an adequate intake of vitamins, and also protein, is essential for preventing ethanol toxicity.

In the present study on the low-vitamin diet, vitamin B_1, vitamin B_2 and pantothenic acid contents in the liver and blood were lower in the ethanol-fed group than in the controls, even when rats were fed a high-protein diet. Furthermore, the total urinary excretion and excretion ratios of all three vitamins were also lower in the ethanol-fed group. Thus, ethanol consumption reduced the intestinal absorption of these vitamins, as reported by Subramanya *et al.*[12], Hamid *et al.*[13,14,16,17] and Wani & Kaur[19]. Vitamins such as

Table 5. Effect of ethanol consumption on urinary B-group vitamin excretion (upper row) and urinary excretion ratio (lower row) for each of the vitamins (Expt 2)†

(Mean values with their standard errors for five rats per group)

	Control		15 % Ethanol	
	Mean	SEM	Mean	SEM
Vitamin B_1				
nmol/d	3·5	0·1	1·8*	0·1
%	3·4	0·2	2·7*	0·2
Vitamin B_2				
nmol/d	3·6	0·3	0·15*	0·04
%	3·8	0·2	0·24*	0·05
4-PIC‡				
nmol/d	29·4	1·9	7·3*	0·5
%	15·6	0·5	4·5*	0·3
Vitamin B_{12}				
pmol/d	9·1	0·4	6·7*	0·2
%	8·9	0·3	9·1	0·2
Niacin§				
μmol/d	2·00	0·16	1·82	0·24
%	−‖		−‖	
Pantothenic acid				
nmol/d	24·3	2·4	6·3*	0·3
%	6·5	0·5	2·4*	0·2
Folate				
nmol/d	1·85	0·19	0·77*	0·11
%	7·3	0·7	4·4*	0·6
Biotin				
nmol/d	0·21	0·02	0·09*	0·01
%	5·0	0·4	3·0*	0·25

4-PIC, 4-pyridoxic acid.

* Mean values were significantly different from those of the control group ($P<0·05$; Mann–Whitney U two-tailed t test).

† Percentage urinary excretion ratio was calculated using the following equation: (24 h urinary excretion (mol/d)/intake of the vitamin during urine collection (mol/d)) × 100.

‡ A catabolite of vitamin B_6.

§ Niacin content was calculated as the sum of the nicotinamide content and its catabolites such as N^1-methylnicotinamide, N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide.

‖ Urinary excretion ratio was not calculated as niacin was derived from tryptophan.

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vitamin B_1, vitamin B_2 and pantothenic acid might be directly and/or indirectly involved in the metabolism of ethanol, indicating that the vitamin catabolites increased and were excreted into the urine. Of these three vitamins, only the catabolic fate of vitamin B_1 is relatively well known. It has been reported that the excretion of vitamin B_1 metabolites usually exceeds by far the excretion of intact vitamin B_1 using radioactive tracer experiments[39]. The major metabolites of vitamin B_1 in rat urine are 2-methyl-4-amino-5-pyridinecarboxylic acid[40], 4-methylthiazole-5-acetic acid[41] and thiamine acetic acid[42]. Pearson[39] reported that the sum of the metabolites accounted for about 50% of the total urinary excretion of vitamin B_1 and its catabolites from radioactive tracer experiments. Although we cannot measure the catabolites of vitamin B_1, these metabolites might increase in the urine of the ethanol-fed rats. It is likely that a similar phenomenon would apply for the fates of vitamin B_2 and pantothenic acid.

The content of vitamin B_6 in the blood was lower in the ethanol-fed group, but the content of vitamin B_6 in the liver was slightly higher in the ethanol-fed group than in the control. The urinary excretion of vitamin B_6, determined from its catabolite 4-pyridoxic acid, was much lower in the ethanol-fed group than in the control. Probably ethanol consumption resulted in an increased storage of vitamin B_6 in the liver.

Other B-group vitamin contents in the liver and blood, such as vitamin B_{12}, nicotinamide, folate and biotin, were not affected by ethanol consumption. The lack of any effect of ethanol consumption on the niacin content in this experiment was probably because nicotinamide was synthesised from tryptophan, which was present in the diet as casein and was supplied adequately[43]. For rats, NAD precursors such as nicotinic acid and nicotinamide are not essential. In fact, the urinary excretion of nicotinamide did not differ between the two groups. Concerning the effect of ethanol consumption on biotin, Sorrell *et al.*[21] reported that the *in vitro* perfusion of rat liver with ethanol did not cause the release of biotin, but caused the release of vitamin B_{12} first. In the present experiment, a similar phenomenon was observed for biotin, but not for vitamin B_{12}. Frank *et al.*[44] reported that the first vitamin released into the circulation during hepatic insult by ethanol is vitamin B_{12}. This disparity between the reported and the present findings might also arise from the difference in protein nutritional status.

There are many reports concerning how ethanol consumption affects folate absorption and metabolism[13–18,45–53]. Some studies have reported that ethanol consumption increased the urinary excretion of folates[46,47,50–53] and caused decreased serum folate levels. Romanoff *et al.*[53] reported that acute ethanol exposure inhibits the apical transport of 5-methyltetrahydrofolate in cultured human proximal tubule cells, and in subchronic ethanol studies, increasing concentrations of ethanol resulted in an up-regulation of folate transporters. Furthermore, Romanoff *et al.*[53] reported that both the folate receptor and reduced folate carrier transporter proteins were up-regulated in rats receiving an ethanol diet. On the contrary, Hamid *et al.*[13,14,16,17] and Wani & Kaur[19] reported that ethanol reduced the intestinal uptake of folate by altering the binding and transport kinetics of the folate transport system and also the expression of folate transporters in the intestine. In addition, Hamid & Kaur[15] reported that ethanol consumption reduces folate re-uptake in the renal absorption system by the decreased expression of transporters. The present data for folate are not consistent with previous reports[13–18,45–53]; the contents of folate in the liver and blood were not affected by ethanol consumption, and the urinary excretion of folate and the excretion ratio were decreased markedly. A study[52] reported that urinary folate excretion increased in ethanol-fed rats consuming folate-containing diets, but not in rats fed folate-deficient diets. In the present study, the urinary excretion of folate did not increase, but decreased. This was because the diet was low in folate. In the present study, the urinary excretion of folate was lower in the ethanol-fed group than in the non-ethanol group, suggesting that ethanol consumption and the feeding of a low-folate diet up-regulated the folate receptor and reduced folate carrier transporter proteins. This up-regulation was probably a compensatory response to counteract the effects of ethanol in inhibiting the reabsorption of folate. Therefore, the effects of ethanol would depend on the dose and duration of treatment.

In summary, these results show that ethanol consumption affects the absorption, distribution and excretion of each of the vitamins in rats fed a diet containing a low-vitamin mixture. On the other hand, when rats were fed a 20% casein diet containing a sufficient amount of vitamins, ethanol consumption did not affect any factors that we measured.

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Correlation between Mineral Intake and Urinary Excretion in Free-Living Japanese Young Women

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ABSTRACT

To clarify whether the urinary excretion of calcium, magnesium, phosphorus, iron, zinc, copper, manganese, selenium and molybdenum can be used as an index of their intake, the association between urinary excretion and intake in free-living individuals was examined. A total of 102 healthy free-living female university dietetics students aged 18 - 33 years voluntarily participated in this study, of which 76 students were eligible for this assessment. All food consumed for four consecutive days was recorded accurately by a weighed food record method. A 24-h urine sample was collected on the fourth day, and the urinary levels of sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, copper, manganese, selenium and molybdenum were measured. Significant correlation between urinary excretion and intake was observed in sodium ($r = 0.596$, $p < 0.001$), potassium ($r = 0.583$, $p < 0.001$), calcium ($r = 0.402$, $p < 0.001$), magnesium ($r = 0.365$, $p < 0.01$), phosphorus ($r = 0.509$, $p < 0.001$), selenium ($r = 0.349$, $p < 0.01$) and molybdenum ($r = 0.265$, $p < 0.01$). On the other hand, urinary excretion was very low and completely independent of the intake in iron, zinc, copper and manganese. These results indicate that urinary calcium, magnesium, phosphorus, selenium and molybdenum can be used as an index of their intake, similarly to sodium and potassium.

Keywords: Mineral Intake; Trace Elements; Urinary Excretion; Assessment; Japanese Young Women

1. Introduction

To assess the nutritional status of healthy free-living humans, the weighed food record method has been used widely to record the dietary intake and to calculate nutrient intake [1]. Although this method can provide relatively precise information regarding dietary intake compared with other dietary assessment [2], substantial effort is required for respondents to complete the dietary records and to weigh all food consumed. This often leads to errors in the records, which reveals the limitation of a weighed food record method in terms of accuracy [3]. Alternatively, other methods using quantitative biological information, such as urinary excretion, or concentrations of nutrient or their metabolites in blood, as biomarkers to assess dietary intake or nutritional status have been well studied in recent years.

Many preceding studies have investigated urinary excretion as a biomarker for assessing dietary intake. For example, 24-h urinary nitrogen is established as a marker for protein intake [4], urinary sugars for sugar intake [5,6], and urinary thiamine for thiamine intake [7]. As regards minerals, urinary potassium is established as a marker for potassium intake [8] and urinary iodine for iodine intake [9] as well as urinary sodium for sodium intake [10,11].

In the present study, we measured sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, copper, manganese, selenium and molybdenum in 24-h urine and examined the association between urinary mineral excretion and their intake in free-living individuals. In addition, we examined whether the urinary excretion of calcium, magnesium, phosphorus, iron, zinc, copper, manganese, selenium and molybdenum can be used as an index of their intake, similarly to sodium and potassium.

2. Subjects and Methods

2.1. Subjects

This study was reviewed and approved by the Ethics Committee of The University of Shiga Prefecture. A total of 102 healthy free-living female university dietetics students aged 18 - 33 years voluntarily participated in this

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study. The purpose and protocol of this study was explained to all participants before joining the study, and written informed consent was obtained from each participant, and from parents of participants aged < 20 years. We excluded participants diagnosed with cold or influenza, and those who had taken mineral supplements at least once during the previous month. In addition, we excluded participants whose 24-h urine collection or dietary records were considered as incomplete, with a collection time outside the 22 - 26 h range, urine volume < 250 mL, creatinine excretion in relation to body weight outside the 10.8 - 25.2 mg/kg range [12], or extremely low or high energy intake (<500 or >4000 kcal/d). After screening, 76 participants were found to be eligible. Anthropometric profiles of the 76 participants are shown and compared with those of general Japanese young women in **Table 1**. No difference was observed between subjects and general women.

2.2. Dietary Records

This was a 4-day dietary assessment in which the participants were living freely at college and consuming their normal diet. The first day (Monday) of the experimental period was defined as Day 1, etc. To measure dietary intake during the 4-day period precisely, we used a weighed food record method, which is the highest quality in Japan at this time [13,14]. A digital cooking scale (1 g unit; Tanita Inc., Tokyo, Japan), a set of dietary record forms, a dietary record manual, and a disposable camera were distributed to the participants in advance. Upon entry of the dietary record, the status of food at oral intake was identified as "raw", "cooked", "the presence of skin", "cooking ingredient", or "with or without seasoning", and coded according to the Fifth Revised and Enlarged Edition of the Standard Tables of Food Composition in Japan [15]. The participants took photographs with a disposable camera of the dish before and after eating. Several experienced dietitians used the photographs to complete the data, and asked the participants to resolve any discrepancies or to obtain further information when needed. The food that remained after eating was measured by a digital scale and was deduced from the dietary record. Food, nutrient and energy intake was calculated using the Standard Tables of Food Composition in Japan. For mineral intake, sodium, potassium, calcium, phosphorus, iron, zinc, copper and manganese were assessed. Because selenium and molybdenum are not designated in the Standard Table of Food Composition in Japan, intake of these microminerals was calculated using averaged values of the contents for every food groups described in the literature [16,17].

Table 1. Comparison of anthropometric profiles between subjects and general Japanese young women.

	Subjects (n = 76)	NHNSJ-2008[1] (n = 284)
Age	20.1 ± 2.3	20 - 29
Height (cm)	158.3 ± 5.0	158.3 ± 5.4
Weight (kg)	50.8 ± 5.2	51.9 ± 9.5
Body mass index (kg/m^2)	20.2 ± 1.7	20.7 ± 3.6

Values are the means ±SD. [1]Values for general Japanese young women aged 20 to 29 years described in the National Health and Nutrition Survey of Japan in 2008.

2.3. 24-h Urine Sampling

A single 24-h urine sample was collected on Day 4 to measure urinary mineral excretion. In the morning, participants were asked to discard the first specimen and to record the time on the sheet. The next morning, participants were asked to collect the last specimen at the same time as when the specimen had been discarded the previous morning, and to record the time on the sheet. After the urine sample had been collected, the volume of the sample was measured. The urine samples were stored at –20˚C until analysis.

2.4. Measurement of Urinary Minerals

Urine samples were diluted with 9 or more volumes of 0.1 M HNO_3 and filtrated through a 0.45-μm-membrane filter. Filtrate thus obtained was used for the measurement of minerals. Sodium, potassium, calcium and magnesium were determined by atomic absorption spectrometer (AA-6300; Shimadzu, Kyoto, Japan). Phosphorus, iron, zinc and copper were determined by inductively coupled plasma-atomic emission spectrometer (ULTIMA2; Horiba Ltd., Kyoto, Japan). Manganese, selenium and molybdenum were determined by inductively coupled plasma-mass spectrometer (ICPM-8500; Shimadzu) using rhodium (for manganese and molybdenum) and tellurium (for selenium) as internal standards. In these urinalyses, recovery of each mineral adding urine was 97% to 101%.

2.5. Statistical Analysis

For each subject, means of daily nutrient and energy intake were calculated from the consecutive 4-day dietary records. The mean values of the subjects were calculated based on the resulting individual mean values. Pearson correlation coefficients were calculated to determine the association between urinary and dietary measurements of minerals. These statistical tests were performed using a personal computer (eMac; Apple Computer, Cupertino, CA, USA) with the operating system Mac OS 9.2 and statistical program package StatView-J version 5.0 (Abacus Concept, Berkeley, CA).

3. Results and Discussion

In **Table 2**, the daily energy and nutrient intake of the 76

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Table 2. Daily intake of energy, major nutrients and minerals of subjects at experimental period.

	Subjects[1] (n = 76)	NHNSJ-2008[2] (n = 418)
Energy (kcal)	1658 ± 302	1669 ± 475
Protein (g)	57.3 ± 11.9	61.0 ± 21.4
Lipid (g)	52.8 ± 15.5	53.7 ± 22.6
Carbohydrate (g)	232.8 ± 39.8	227.3 ± 66.6
Minerals		
Sodium (mg)	2923 ± 834	3617 ± 1415 [3]
Potassium (mg)	1873 ± 472	1886 ± 710
Calcium (mg)	503 ± 142	406 ± 205 [3]
Magnesium (mg)	194 ± 53	201 ± 70
Phosphorus (mg)	852 ± 193	844 ± 292
Iron (mg)	6.7 ± 1.9	6.7 ± 2.7
Zinc (mg)	6.9 ± 1.5	7.2 ± 2.6
Copper (mg)	0.90 ± 0.21	0.98 ± 0.34
Manganese (mg)	2.8 ± 0.8	-
Selenium (μg)	189 ± 67	-
Molybdenum (μg)	272 ± 77	-

Values are the means ±SD. [1]Daily intake was assessed from the consecutive 4-day dietary records. [2]Values for general Japanese young women aged 18 to 29 years described in the National Health and Nutrition Survey of Japan in 2008. [3]Significant difference was observed between subjects and general Japanese young women at $p < 0.001$ by Student's t-test.

eligible participants is presented and compared with those of general Japanese young women described in the National Health and Nutritional Survey of Japan (NHNSJ) [18]. Similarity was observed between the subjects and general Japanese in the intake of energy and macronutrients. Among minerals, no difference was observed in potassium, magnesium, phosphorus, iron, zinc and copper intake. In addition, manganese and molybdenum intake in the participants was close to the reported values for general Japanese [19,20]. On the other hand, lower sodium intake and higher calcium intake were observed in the subjects than in general young women. In Japan, because excess intake of sodium and low intake of calcium have been major nutritional problems, dietetics students have received education so that sodium intake is reduced and calcium intake is increased; therefore, it is thought that the subjects made efforts to reduce their sodium intake and increase their calcium intake intentionally. Selenium intake in the participants was quite a bit higher than the reported value for general Japanese [16,21]. This indicates that overestimation arose in selenium intake roughly calculated using averaged values of the contents for every food group because no difference was observed between the subjects and general Japanese adolescents in the intake of energy and many nutrients.

Table 3 shows 24-h urinary excretion and the apparent urinary excretion rate of minerals. As regards manganese, since almost all samples showed less than the detection limit (<10 μg/L), it is excluded from the table.

Table 3. Daily urinary mineral excretion in subjects.

	Excretion amounts	Apparent excretion rate
	(mg/d)	(%)
Sodium	2616 ± 1010	90.7 ± 30.8
Potassium	1456 ± 498	79.5 ± 23.0
Calcium	100.5 ± 36.4	20.9 ± 8.2
Magnesium	39.9 ± 16.4	22.4 ± 15.4
Phosphorus	660 ± 223	79.1 ± 23.8
	(μg/d)	
Iron	220 ± 138	3.6 ± 2.5
Zinc	374 ± 125	6.3 ± 2.8
Copper	52.5 ± 37.1	6.3 ± 5.1
Selenium	84.8 ± 26.6	49.7 ± 21.3
Molybdenum	211 ± 93	82.2 ± 44.3

Values are the means ±SD. Apparent excretion rate was calculated as follows: (daily urinary excretion amounts)/(daily intake) × 100.

A high rate of urinary excretion (>70%) was observed for sodium and potassium, which intake has been assessed using urine. In addition, phosphorus and molybdenum also showed a high excretion rate, parallel to sodium and potassium. Because most phosphorus and molybdenum ingested from food are absorbed in the intestine and their main excretion route is urine [20,22], this high excretion rate is valid. Although dietary selenium is also mostly absorbed and its main excretion route is urine [23], the excretion rate was 50%, which was lower than several reported values [24]. This was surely caused by an overestimation of selenium intake; if the excretion rate were 70%, selenium intake would be estimated to be about 120 μg/d, which is almost coincident with the reported value for general Japanese [16,21].

The apparent urinary excretion rate of calcium and magnesium was about 20%, which was coincident with the reported value [22,25]. On the other hand, urinary excretion of iron, zinc and copper was very low, which reflects that urine is not the main excretion route of these minerals [26-28].

Figure 1 shows the correlation between daily intake and 24-h urinary excretion of sodium, potassium, calcium, magnesium and phosphorus. Significant correlation was observed with all of these five minerals. In particular, a strong correlation ($r > 0.5$) was observed for sodium, potassium and phosphorus; therefore, in these three minerals, intake could be estimated from the amount of urinary excretion for every individual with high accuracy. Urinary sodium and potassium are already used as important indices of their intake for individuals [10,11]. In addition, urinary phosphorus could also be used as an index of its intake.

Also, in the case of calcium and magnesium, a significant correlation between urinary excretion and intake was observed. The intestinal absorption rate of calcium and magnesium is 30% to 50% and the main excretion

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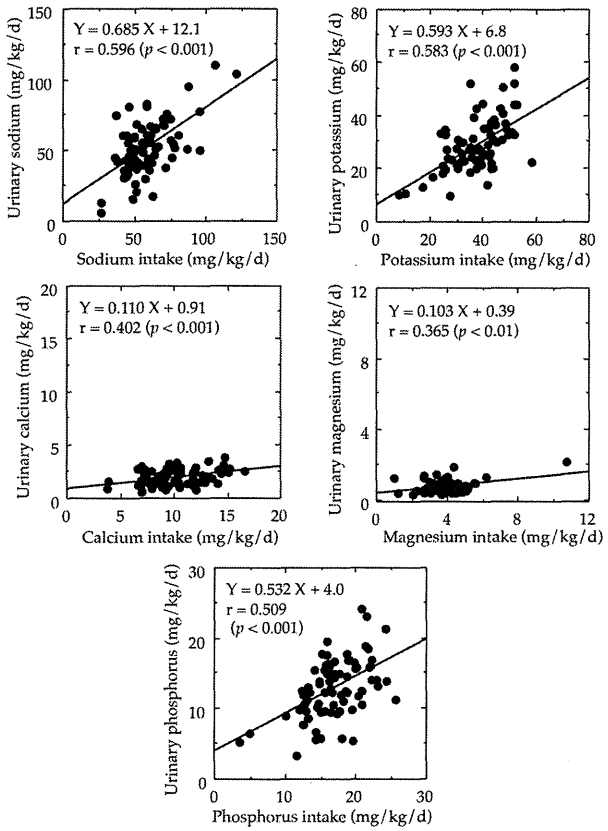

Figure 1. Correlation between daily intake and urinary excretion of sodium, potassium, calcium, magnesium and phosphorus in subjects.

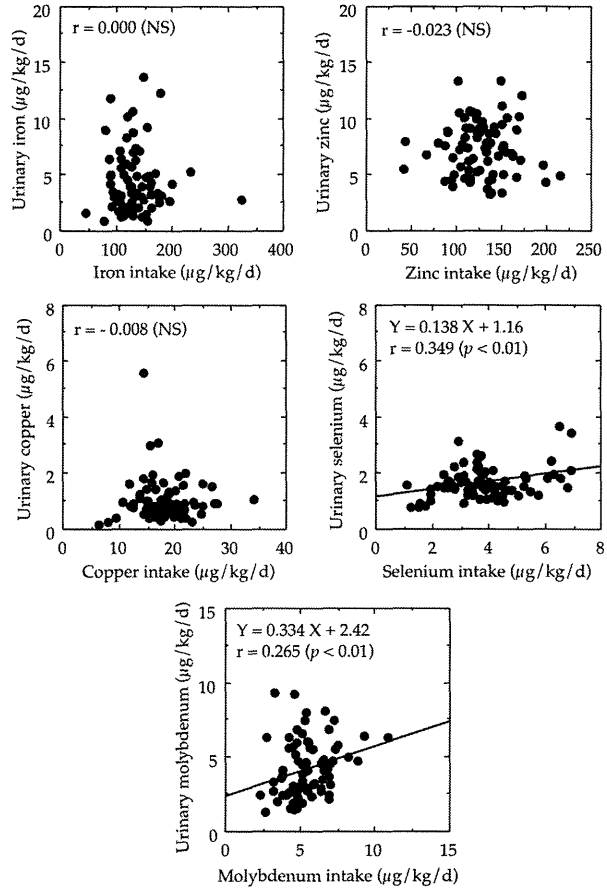

Figure 2. Correlation between intake and urinary excretion of iron, zinc, copper, selenium and molybdenum of subjects.

route is urine [22,25]; therefore, urinary excretion of these minerals reflects absorption amounts. Since intestinal absorption of these minerals changes with various factors [29], it may be difficult to estimate the intake of these minerals from the urinary excretion for every individual. Nevertheless, it will be possible to estimate the intake from urinary excretion at least in a group.

Figure 2 shows correlation between intake and urinary excretion in iron, zinc, copper, selenium and molybdenum. In iron, zinc and copper, the scale is changed between the X- and Y-axis since their excretion rate to urine is very low. In these three minerals, urinary excretion was almost completely independent of the intake. Accordingly, intake of these minerals cannot be estimated from urinary excretion. In addition, because urinary manganese excretion was very low, similarly to iron, zinc and copper, it may be difficult to use urinary manganese as an index of manganese intake. Probably, it is the reason that their urinary excretion is constantly low regardless of the intake, since they are bound to protein in blood. In the case of selenium and molybdenum, a significant correlation was observed; however, in spite of having said that a large part of ingested selenium and molybdenum was excreted into urine, similarly to potassium, sodium and phosphorus [20,23], the correlation coefficients were smaller than those of calcium and magnesium. Probably, these weak correlations were due to rough intake estimation using averaged values of the contents for every food group; therefore, it is considered that a greater correlation coefficient was obtained when intake was estimated using the content of every food, as for other minerals.

In the present study, it was confirmed that excretion amounts in 24-h urine were good indices of daily intake of phosphorus, calcium, magnesium, selenium and molybdenum similarly to sodium and potassium. In minerals, estimation of the intake using 24-h urine is possible when the main excretion route is urine. To estimate the intake of these minerals from the urinary excretion, the precise regression between intake and urinary excretion needs to be established by a balance test in the future.

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Different variations of tissue B-group vitamin concentrations in short- and long-term starved rats

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Abstract

Prolonged starvation changes energy metabolism; therefore, the metabolic response to starvation is divided into three phases according to changes in glucose, lipid and protein utilisation. B-group vitamins are involved in energy metabolism via metabolism of carbohydrates, fatty acids and amino acids. To determine how changes in energy metabolism alter B-group vitamin concentrations during starvation, we measured the concentration of eight kinds of B-group vitamins daily in rat blood, urine and in nine tissues including cerebrum, heart, lung, stomach, kidney, liver, spleen, testis and skeletal muscle during 8 d of starvation. Vitamin B_1, vitamin B_6, pantothenic acid, folate and biotin concentrations in the blood reduced after 6 or 8 d of starvation, and other vitamins did not change. Urinary excretion was decreased during starvation for all B-group vitamins except pantothenic acid and biotin. Less variation in B-group vitamin concentrations was found in the cerebrum and spleen. Concentrations of vitamin B_1, vitamin B_6, nicotinamide and pantothenic acid increased in the liver. The skeletal muscle and stomach showed reduced concentrations of five vitamins including vitamin B_1, vitamin B_2, vitamin B_6, pantothenic acid and folate. Concentrations of two or three vitamins decreased in the kidney, testis and heart, and these changes showed different patterns in each tissue and for each vitamin. The concentration of pantothenic acid rapidly decreased in the heart, stomach, kidney and testis, whereas concentrations of nicotinamide were stable in all tissues except the liver. Different variations in B-group vitamin concentrations in the tissues of starved rats were found. The present findings will lead to a suitable supplementation of vitamins for the prevention of the re-feeding syndrome.

Key words: **Starvation: Fasting: Energy metabolism**

Starvation produces a series of metabolic changes that lead to a reduction in body weight, alterations in body composition and metabolic gene expression[1,2]. In mammals and birds, three distinct levels of energy depletion have been established[3–10]. The first phase (phase 1) is a rapid period of adaptation marked by an increase in mobilisation of fat stores and a lowering in protein utilisation. During the second phase (phase 2), which is a long period of thrift, most of the energy expenditure is derived from fats, and then fat stores are progressively exhausted, while body proteins are efficiently spared. The third phase (phase 3) is characterised by an increase in protein utilisation. In humans, the negative energy balance resulting from starvation can arise due to disease, eating or psychological disorders, or hunger strikes. Starvation and consequent re-feeding syndrome can lead to electrolyte disorders, especially hypophosphataemia, along with neurological, pulmonary, cardiac, neuromuscular and haematological complications[11]. To avoid the re-feeding syndrome, an additional load of vitamins has been suggested to correct the vitamin deficiencies[11]. However, little is known about B-group vitamin status during starvation.

Several B-group vitamins take part in energy metabolism. For instance, vitamin B_2 functions as FAD and FMN in redox reactions including the electron transport chain and fatty acid oxidation. Nicotinamide is involved in more than 200 reactions, including the metabolism of carbohydrates, amino acids and fatty acids, and also in the electron transport chain. Vitamin B_1 catalyses carbohydrate metabolism including decarboxylation of α-ketoacids and *trans*-ketolation as a cofactor thiamin diphosphate; vitamin B_6 functions as pyridoxal 5′-phosphate in amino acid metabolism including aminotransferases,

Abbreviations: 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-5-carboxamide; 3-HBA, 3-hydroxybutyric acid; MNA, N^1-methylnicotinamide.

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decarboxylases, racemases and dehydratases as pyridoxal 5′-phosphate; and pantothenic acid is involved in fatty acid metabolism such as oxidation and synthesis. For these reasons, in the 'Dietary Reference Intakes for Japanese, 2010', dietary requirements for vitamin B_1, vitamin B_2 and niacin are expressed per 4186 kJ (1000 kcal), and the requirement for vitamin B_6 is expressed in terms of protein intake[12].

As mentioned earlier, prolonged starvation sifts the energy source from glucose to fats and then to protein, and B-group vitamins are involved in the metabolism of carbohydrates, fatty acids and amino acids. Thus, in the present study, we investigated how changes in energy metabolism altered B-group vitamin utilisation during starvation. We comprehensively determined eight kinds of B-group vitamin concentrations in rat blood, urine and tissues including the brain, heart, lung, stomach, kidney, liver, spleen, testis and skeletal muscle during 8 d of starvation.

Materials and methods

Diets

The composition of the purified diet is shown in Table 1. Vitamin-free milk casein, L-methionine and sucrose were purchased from Wako Pure Chemical Industries Limited (Osaka, Japan). Maize oil was purchased from Nisshin OilliO Group, Limited (Tokyo, Japan). Gelatinised maize starch, the mineral mixture (AIN-93G) and the vitamin mixture (AIN-93VX) were obtained from Oriental Yeast Company, Limited (Tokyo, Japan).

Animals

Male rats of the Wistar strain, weighing 225–235 g, were obtained from CLEA Japan, Inc. (Tokyo, Japan). The rats were individually housed in a temperature-controlled room (22 ± 2°C and 50–60% humidity) with a 12 h light–12 h dark cycle and were allowed to acclimate to the environment for 7 d before starting the experiment. Body mass, food consumption and water intake were recorded daily (±0·1 g). We also collected 24 h urine samples every day.

Experimental procedures

A total of twenty-five rats were randomly divided into five groups. After 1 week of acclimatisation, five rats were killed by decapitation as a control group (CONT, n 5). The other rats were deprived from food for 1 d (S1, n 5), 2 d (S2, n 5), 6 d (S6, n 5) or until they had been in phase 3 for 2 d; that is, they were starved for a total duration of 6–9 d (P3, n 5). The starving phase was determined by calculating the specific daily rate of body mass loss dM/Mdt (g/kg per d) for each animal (dM represents the loss of body mass during $dt = t_1$-t_0 and M is the body mass of the rat at t_0[10,14]. Blood was taken from the tail vein at 09.00 hours every day, and 3-hydroxybutyric acid (3-HBA) concentration in the blood was measured with a 3-HBA Kit (Abbott Japan Company, Limited, Tokyo, Japan) to confirm the metabolic state of each animal because blood 3-HBA reflects fatty acid oxidation.

Table 1. Composition of the diet

	(g/100 g)
Vitamin-free milk casein	20·0
L-Met	0·2
Gelatinised maize starch	46·9
Sucrose	23·4
Maize oil	5·0
Mineral mixture (AIN-93-G)	3·5
Vitamin mixture (AIN-93VX)*	1·0

* The composition of the vitamin mixture is described by Reeves *et al.*[13].

After the animals were killed, blood samples were collected into EDTA-2K tubes from the carotid artery and were centrifuged at 1700 ***g*** for 10 min at 4°C. Plasma glucose, TAG, urea N, aspartate aminotransferase and alanine aminotransferase were measured with FUJI DRI-CHEM (FUJIFILM Company, Tokyo, Japan).

The cerebrum, heart, lungs, stomach, kidneys, liver, spleen, testes and leg muscles were dissected and weighed (±0·001 g). The stomach was cleared of its contents. All tissue samples were immediately homogenised in ultra-pure water at 1:10 (w/v) using a Teflon glass homogeniser and stored at −20°C until needed. The present study was conducted according to the guidelines for the care and use of laboratory animals, and was approved by the Ethics Committee of the University of Shiga Prefecture (Shiga, Japan).

Analytical methods

Vitamin B_1. Thiamin in urine was measured directly. The vitamin B_1 content in the blood and tissue was determined as the sum of thiamin, thiamin monophosphate and thiamin diphosphate and was expressed as total thiamin. TCA (5%) was added to whole blood and tissue homogenates, and the blood and homogenates were centrifuged for 5 min at 20 000 ***g***, and the supernatant of the mixture was used for measurement. Vitamin B_1 levels in the urine, blood and tissue were determined by the HPLC post-labelled fluorescence method[15].

Vitamin B_2. Riboflavin in urine was measured directly by HPLC[16]. Riboflavin, FMN and FAD in blood and tissue were converted to lumiflavin by photolysis. Briefly, the supernatant from a TCA-treated blood or tissue sample was added to an equal volume of 1 M-NaOH. The alkalised mixture was irradiated with a fluorescent lamp for 30 min, and acetic acid was added to the mixture. The neutralised mixture was filtered with a 0·45 μm microfilter and the filtrate was directly injected into the HPLC system for the measurement of lumiflavin[17]. The measured lumiflavin was expressed as total vitamin B_2.

Vitamin B_6. 4-Pyridoxic acid, a catabolite of vitamin B_6, in urine was measured directly by HPLC[18]. Serum pyridoxal and pyridoxal 5′-phosphate were determined by the HPLC method[19]. Vitamin B_6 vitamers, including phosphate esters in the tissue, were converted to free vitamin B_6 vitamers such as pyridoxal and pyridoxamine using an autoclave under acidic conditions. Briefly, the homogenate was added

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to 0·06 M-HCl at 1:8 (v/v) and autoclaved at 121°C for 3 h, and the mixture was adjusted to pH 5·0 using 1 M-NaOH. These were measured as total vitamin B_6 by the microbioassay method using *Saccharomyces carlsbergensis* strain 4228 ATCC 9080[20].

Vitamin B_{12}. Urine, plasma and tissue homogenates were added to a 0·2 mM-acetate buffer (pH 4·8) with 0·0006 % potassium cyanide. These were put into a boiling water bath for 5 min to be converted to cyanocobalamin, and then 10 % metaphosphoric acid was added to be neutralised. Cyanocobalamin was determined by the microbioassay method using *Lactobacillus leichmannii* ATCC 7830[21].

Niacin. Nicotinamide[22] and its catabolites, N^1-methylnicotinamide (MNA)[23], N^1-methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py)[22], in urine were measured directly by HPLC. For measuring the total nicotinamide content in blood and tissues, the whole blood and tissue homogenates were autoclaved at 121°C for 20 min to convert the coenzymes to nicotinamide. The resulting nicotinamide was then determined by the HPLC method[22,24].

Pantothenic acid. Pantothenic acid in urine was determined by HPLC[25]. To digest the bound pantothenic acid including coenzyme A and phosphopantetheine in tissue and plasma to free form, the homogenate or blood was incubated at 37°C for 24 h. Pantothenic acid in the plasma and tissue was determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014[26].

Folate. Folate in urine and plasma was directly determined by the microbioassay method using *Lactobacillus casei* ATCC 27 773[27]. Folate in tissues was digested to monoglutamate forms by treatment with protease and conjugase. Briefly, 1 M-KH_2PO_4–K_2HPO_4 buffer (pH 6·1) was added to the tissue homogenate at 1:9 (v/v), and the homogenate was autoclaved at 121°C for 5 min. Proteinase MS (Kaken Pharmaceutical Company, Limited, Tokyo, Japan) was added to the homogenate at a final concentration of 2·5 mg/ml and then incubated at 37°C for 3 h. The reaction mixture was added to the conjugase solution (extract from porcine kidney acetone powder, Sigma, Porcine, Type II) at 30:1 (v/v) and incubated at 37°C for 12 h. After centrifugation at 10 000 ***g*** for 10 min, the supernatant was used for determination by the microbioassay.

Biotin. Bound biotin in tissues was converted to the free form using autoclave under acidic conditions. Briefly, 1·5 M-H_2SO_4 was added to the homogenate at 1:1 (v/v), and the homogenate was autoclaved for 1 h at 121°C. The suspension was centrifuged at 10 000 ***g*** for 10 min at 4°C, and the supernatant was used to measure biotin. Biotin in urine and plasma was measured directly. The biotin content in urine, plasma and tissue was determined by the microbioassay method using *L. plantarum* ATCC 8014[28].

Statistical analysis

Values are expressed as means with their standard errors. P3 rats (starved for 6–9 d) were expressed at 8 d on the graph for convenience. To test the significance of the differences in mean values among all groups, one-way ANOVA with Tukey's *post hoc* test was employed. Repeated ANOVA with Bonferroni's *post hoc* test was used to analyse urinary excretion of B-group vitamins in P3 rats, and individual data points were compared with their data at day 0. All differences at $P<0·05$ were considered to be statistically significant. Prism software (version 5; obtained from GraphPad Software, Inc., San Diego, CA, USA) was used for all analyses.

Results

Changes in body mass during starvation

Changes in body mass during starvation are shown in Table 2. Starvation for the first 24 h produced a weight loss of 7 %. From the second day to the last day of starvation, the rats lost 5 % weight for each 24 h (data not shown). The specific daily rate of body mass loss (dM/Mdt) *v.* time in starved rats is presented in Fig. 1. The pattern of dM/dMt showed a

Table 2. Body mass and organ mass in the control and starved rats
(Mean values with their standard errors, *n* 5)

	CONT*		S1		S2		S6		P3†	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Initial body mass (g)	252·9	3·3	253·1	2·8	246·4	6·2	252·2	3·1	249·0	3·2
Final body mass (g)	252·9^{a}	3·3	235·1^{a,b}	2·3	219·3^{b}	5·6	182·7^{c}	2·5	166·6^{c}	5·4
Organ mass (g, wet wt)										
Cerebrum	1·29	0·02	1·30	0·01	1·28	0·01	1·27	0·02	1·23	0·03
Heart	0·84^{a}	0·04	0·87^{a}	0·03	0·81^{a}	0·04	0·66^{b}	0·02	0·58^{b}	0·02
Lungs	1·28	0·08	1·19	0·10	1·09	0·06	1·09	0·13	0·95	0·06
Stomach	1·16	0·02	1·15	0·04	1·14	0·04	1·20	0·05	1·19	0·08
Kidneys	1·94^{a}	0·05	1·89^{a,b}	0·6	1·69^{b,c}	0·03	1·53^{c,d}	0·02	1·43^{d}	0·04
Spleen	0·75^{a}	0·04	0·67^{a}	0·03	0·50^{a}	0·05	0·30^{b}	0·01	0·25^{c}	0·04
Testes	2·75^{a,b}	0·07	2·66^{b,c}	0·05	2·66^{b,c}	0·04	2·47^{b,c}	0·03	2·45^{c}	0·06
Liver	11·18^{a}	0·23	7·25^{b}	0·22	6·07^{b}	0·19	4·69^{c}	0·12	3·83^{c}	0·42

CONT, non-starved control rats; S1, 1-day starved rats; S2, 2-day starved rats; S6, 6-day starved rats; P3, starved to phase 3 rats.
^{a,b,c}Mean values within a row with unlike superscript letters were significantly different determined by one-way ANOVA with Tukey's multiple comparison tests ($P<0·05$).
* Since the control rats were killed at the beginning of the experiment, the initial body weight was same as the final body weight.
† Phase 3 is determined by the rapid increase in dM/dMt (refer to Fig. 1).

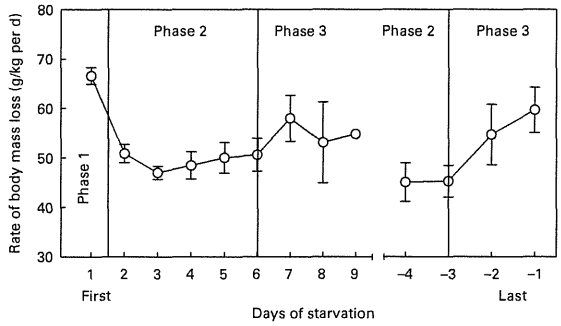

Fig. 1. Rate of body mass loss (d*M*/d*t*) in starved rats. Values are means with their standard errors (*n* 1–20 per d in the left; *n* 5 per d in the right). d*M*/d*t* (d*M* represents the loss of body mass during $dt = t_1 - t_0$ and *M* is the body mass of rat at t_0) was calculated for each animal. Abscissa: left, days counted from the beginning of starvation in all rats; right, counted to the end of starvation in the P3 group.

sharp decrease during the first hours of starvation and a steady rate at days 2–6 of starvation. Since obvious rapid increase was not observed after day 7 of starvation, we showed that the last part of starvation was counted to the end of starvation in the P3 group. The d*M*/d*Mt* in the P3 group clearly showed a rapid increase from 3 d before the end of starvation. These patterns were exactly consistent with previous reports[10,14]. Therefore, we defined the phase at first decrease as phase 1, that of steady rate as phase 2 and the third part of the curve as phase 3 according to previous reports[10,14]. The S6 group showed the steady rate of body mass loss and the low blood 3-HBA concentration, and these were characteristics of both phases 2 and 3. These results showed that the S1 group was representative of phase 1, the S2 was of phase 2, the S6 group was in the marginal range between phases 2 and 3, and the P3 group was in phase 3.

Changes in mass of individual organs during starvation

Table 2 shows the changes in the mass of individual organs during starvation. The cerebrum, lung and stomach mass was not affected by starvation. The liver weight was gradually reduced by starvation, and that in the P3 group was 30% of the control group. From 2 d of starvation, kidney mass decreased. Heart and spleen mass decreased from 6 d. Testes mass decreased in P3 rats. Prolonged starvation reduced the spleen and liver weight the most.

Blood/plasma parameters

Table 3 shows the blood parameters. Blood 3-HBA increased more in S1 and S2 rats than in control rats. In contrast, the urea concentration in plasma was significantly higher in S6 and P3 rats, whereas there was a non-significant increase in the S1 and S2 rats. Plasma glucose level was 60% significantly lower in the S1, S2 and S6 rats than in the control rats. Interestingly, plasma glucose returned to the basal level in the P3 rats. Plasma TAG was dramatically decreased after 1 d of starvation and then continued to decrease gradually throughout the remainder of the starvation period. Plasma aspartate aminotransferase was not affected by starvation. Plasma alanine aminotransferase began to increase after 6 d of starvation.

Effect of starvation on vitamin status

Table 4 shows B-group vitamin content in tissue, blood and urine in the control rats. We determined the B-group vitamin contents in nine tissues including the cerebrum, heart, lung, stomach, kidney, spleen, testis, skeletal muscle and liver, and five tissues were selected as representative variations in Fig. 2.

Cerebrum (Fig. 2(A)) and spleen. With the exception of biotin, all vitamin concentrations were unchanged by starvation. Biotin concentration was initially elevated to 150% in the S1 rats, and then returned to basal level. B-group vitamin concentrations in the spleen showed a similar pattern that starvation did not affect their concentrations except for vitamin B_2. Vitamin B_2 concentration in the testis was elevated to 130% after 6 d of starvation.

Heart (Fig. 2(B)). Vitamin B_1 and folate concentrations significantly decreased to approximately 60% after 6 d of starvation. Pantothenic acid concentration was significantly lower in the S2 and S6 rats than in the control rats. Biotin and vitamin B_6 concentrations significantly increased to 160 and 250% in the S1 and S6 rats, respectively. The other B-group vitamin concentrations were unchanged.

Table 3. Blood parameters in the control and starved rats
(Mean values with their standard errors, *n* 5)

	CONT		S1		S2		S6		P3	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
3-HBA (mmol/l)*	0·1[a]	0·0	1·8[a]	0·1	2·5[b]	0·2	0·7[a]	0·1	0·3[a]	0·1
Glucose (mmol/l)	6·36[a]	0·28	3·86[b]	0·32	3·84[b]	0·29	3·71[b]	0·37	6·44[a]	0·60
TAG (mmol/l)	3·01[a]	0·45	0·82[b]	0·08	0·81[b]	0·07	0·54[b]	0·10	0·46[b]	0·05
Urea (mmol/l)	7·45[a]	0·27	6·21[a]	0·42	6·21[a]	0·30	8·23[b]	2·36	13·08[b]	0·68
AST (U/l)	263	21	245	10	242	21	240	10	252	8
ALT (U/l)	39·8[a]	3·8	29·4[a]	1·9	31·8[a]	1·8	65·5[b]	10·0	73·3[b]	5·9

CONT, non-starved control rats; S1, 1-day starved rats; S2, 2-day starved rats; S6, 6-day starved rats; P3, starved to phase 3 rats; 3-HBA, 3-hydroxybutyrate; AST aspartate aminotransferase; ALT, alanine aminotransferase.
[a,b] Mean values within a row with unlike superscript letters were significantly different determined by one-way ANOVA with Tukey's multiple comparison tests ($P<0·05$).
* 3-HBA was measured in whole blood, and the others in serum.

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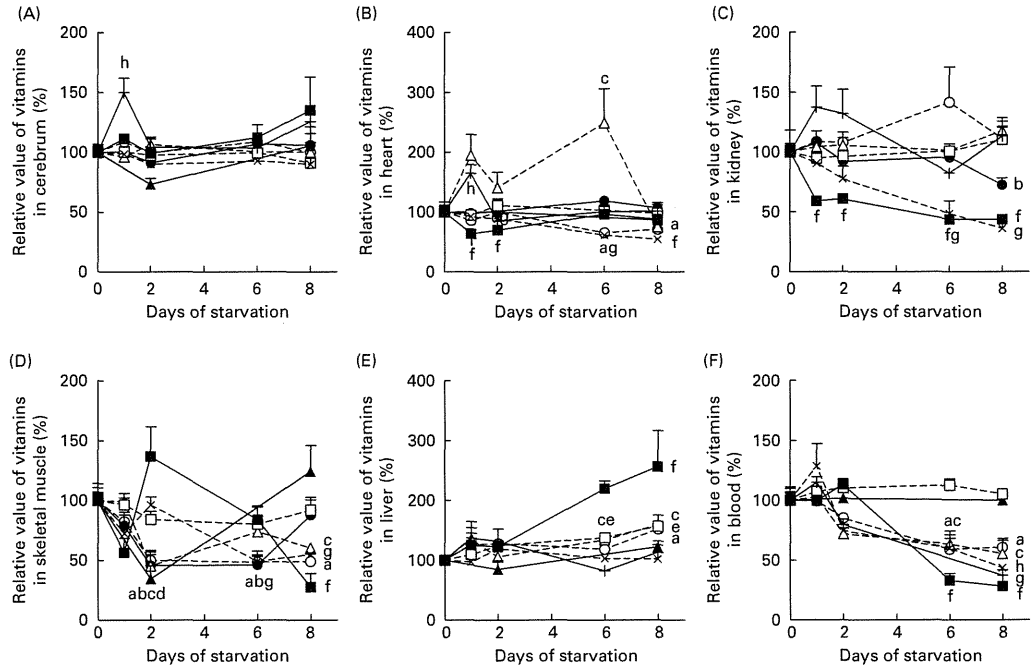

Fig. 2. Relative value of B-group vitamin concentrations in (A) cerebrum, (B) stomach, (C) kidney, (D) skeletal muscle, (E) liver and (F) blood of rats during starvation. * Sum of serum pyridoxal and pyridoxal-5′-phosphate is expressed as serum vitamin B_6. Values are reported as means with their standard errors, *n* 5 per d. Values of control rats are expressed as 100%. P3 is expressed at 8 d of starvation. Means with unlike letters were significantly different from day 0 in [a]vitamin B_1 (○), [b]vitamin B_2 (●), [c]vitamin B_6* (Δ), [d]vitamin B_{12} (▲), [e]nicotinamide (□), [f]pantothenic acid (■), [g]folate (×) and [h]biotin (+; $P<0.05$).

Kidney (Fig. 2(C)) and testis. Pantothenic acid concentration dramatically decreased to 50% of control during starvation. Folate concentration significantly decreased to 40% in the S6 and P3 rats. Vitamin B_2 concentration significantly decreased to 70% in the P3 rats. The reduction in pantothenic acid concentration from day 1 of starvation was also observed in the stomach and testis, and their maximal reduction was 60%. In the testis, vitamin B_2 concentration also significantly reduced to 50% during starvation, and other vitamin concentrations were not changed.

Skeletal muscle (Fig. 2(D)) and stomach. Concentrations of vitamin B_1, vitamin B_2 and vitamin B_6 significantly decreased to 50% from 2 d of starvation, but only vitamin B_2 concentration returned to control levels in the P3 rats. Vitamin B_{12} concentration significantly decreased to 40% only in the S2 rats. Pantothenic acid concentration significantly decreased in the P3 rats. Folate concentration significantly decreased to 50% in the S6 and P3 rats. Pantothenic acid concentration was same as control until 6 d of starvation, and then dramatically decreased to 30% in the P3 rats. Similar pattern was observed in the stomach that starvation reduced several B-group vitamin concentrations. In brief, pantothenic acid and biotin concentrations reduced to 30 and 60% from day 1 of starvation, respectively. Vitamin B_6 concentration significantly decreased to 50% from day 2, and vitamin B_2 did to 40% from day 6 of starvation.

Liver (Fig. 2(E)). Vitamin B_6 concentration was significantly higher in the S1, S6 and P3 rats, and the relative value in the P3 rats was 160% of the control animals. Nicotinamide concentration increased in the S2, S6 and P3 rats. Vitamin B_1 and pantothenic acid concentrations were higher in the P3 rats, and their values were 150 and 250%, respectively. Other B-group vitamins concentrations were unchanged.

Blood (Fig. 2(F)). Whole blood vitamin B_1, serum vitamin B_6 and plasma pantothenic acid concentrations decreased in the S6 and P3 rats. Plasma folate and biotin concentrations decreased in the P3 rats. The relative values of vitamin B_1 and vitamin B_6 in the P3 rats were 60% of control, those of folate and biotin were 50%, and those of pantothenic acid were 30%.

Urinary contents of B-group vitamins (Fig. 3). Vitamin B_1 excretion acutely decreased to 10% after 1 d of starvation. Urinary excretion of riboflavin, pyridoxal metabolite 4-pyridoxic acid and vitamin B_{12} gradually decreased during 4 d of starvation. Subsequently these values were stable, at approximately 20, 20 and 50% of each control value. Urinary folate was initially unchanged in the S1 rats and then decreased to 40% of the baseline value. Urinary pantothenic acid was increased to 170% in 3rd and 4th days of starvation, and then returned to the control level. Although biotin excretion increased to 460% during the first 3 d of starvation, it subsequently returned to the basal level.

Urinary contents of nicotinamide and its catabolites (Fig. 4). Nicotinamide excretion increased after 1 d of starvation and then returned to the basal level. 2-Py and 4-Py decreased after an initial increase on day 1. In contrast, MNA excretion

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Table 4. Contents of B-group vitamins in each tissue, blood and urine of control rats
(Mean values with their standard errors, n 4–5)

	Vitamin B_1 (nmol/g tissue)		Vitamin B_2 (nmol/g tissue)		Vitamin B_6 (nmol/g tissue)		Vitamin B_{12} (pmol/g tissue)		Nicotinamide (nmol/g tissue)		Pantothenic acid (nmol/g tissue)		Folate (nmol/g tissue)		Biotin (nmol/g tissue)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Cerebrum	9·1	0·4	6·6	0·1	18·3	0·4	35	2	292	12	61	3	0·52	0·02	0·19	0·01
Heart	18·9	1·5	34·4	1·6	14·5	1·6	202	20	578	46	166	9	0·75	0·03	0·23	0·04
Lung	5·7	0·5	8·4	0·6	3·6	0·3	49	4	375	11	59	8	0·78	0·03	0·13	0·02
Stomach	11·7	1·7	11·5	0·3	3·4	0·6	77	12	221	22	51	5	1·14	0·02	0·27	0·01
Kidney	16·8	3·1	57·2	2·7	18·8	1·1	269	19	920	27	250	18	6·47	0·35	1·40	0·12
Spleen	7·0	0·6	6·4	0·4	1·4	0·2	74	8	645	26	33	4	1·17	0·04	0·05	0·01
Testis	25·2	6·4	9·6	0·8	13·9	0·4	61	7	186	7	101	8	0·07	0·00	0·08	0·01
Skeletal muscle	4·3	0·2	7·5	0·5	35·5	1·8	38	5	400	24	22	2	0·30	0·01	0·03	0·00
Liver	32·2	1·8	68·0	3·2	17·1	0·6	144	10	862	34	242	10	14·14	0·69	1·84	0·11
	(pmol/ml WB)		(pmol/ml WB)		(pmol/ml serum)		(pmol/ml plasma)		(nmol/ml WB)		(nmol/ml plasma)		(pmol/ml plasma)		(pmol/ml plasma)	
Blood	285	15	208	12	1·46	0·16	4·97	0·13	121	5	7·02	0·39	43·2	3·6	54·6	3·5
	(nmol/d)		(nmol/d)		(nmol/d)		(pmol/d)		(μmol/d)		(nmol/d)		(nmol/d)		(pmol/d)	
Urine	96	4	65	12	345	4	38	2	3·93	0·17	567	68	6·20	0·68	2·96	0·25

WB, whole blood.

increased during the starvation period. Urinary excretion of the sum of nicotinamide and its catabolites increased 1·4-fold after 1 d of starvation and then decreased by less than half of the food sufficient state.

Discussion

The effects of metabolic changes, which are designated as the changes in the main energy sources such as glucose, lipids and protein, during starvation on the tissue and urine vitamin concentrations are currently poorly understood[11]. Elucidation of the effects will lead to a suitable supplementation for preventing the refeeding syndrome. Therefore, we investigated the effects of short- and long-term starvation on the vitamin concentrations in organs, muscle, blood and urine in rats.

Vitamin concentrations in organs and muscle showed different patterns for each vitamin. For noticeable characteristics, biotin concentration, which means the value in terms of g tissue, was increased in most organs of the S1 rats. A part of the reason is a reduced organ mass at S1. It was unclear why the biotin concentrations in organs remained at the same level regardless of organ mass during starvation. Vitamin B_1 is the vitamin that has the most rapid turnover[30], but the levels in the kidney were maintained. This may point to the necessity of vitamin B_1 in kidneys of starving rats. In terms of the metabolic state, vitamin B_1 was expected to decrease in the early days of starvation, because glucid is the main energy source in this period[3]. However, vitamin B_1 concentrations in tissues and blood were stable in the S1 rats. This is due to the sharp decrease in liver weight and in the urinary excretion of vitamin B_1. Along with the shifts in the main energy source from glucid to fat, vitamin demands appear to change. Next to vitamin B_1, pantothenic acid requirement may be the highest because it is involved in the metabolism of fatty acids[31], and also, biotin requirement may be higher because the gluconeogenesis is more active at the deficient state of glucose[32]. However, the present results were contrary to our expectations. Pantothenic acid concentrations in the heart, stomach, kidneys and testes were decreased in the S1 rats, and a similar phenomenon was observed in biotin concentrations. The urinary excretion of pantothenic acid and biotin was significantly increased by starvation. A similar phenomenon was already reported by Fukuwatari *et al.*[30]. Shibata and co-workers reported[33–38] that the urinary excretion of water-soluble vitamins reflects recent intake of the vitamins over the last few days, and in addition, the decreased urinary excretion of vitamins means the elevated demand for vitamins, whereas the increased urinary excretion of vitamins means the reduced demand for vitamins when their intake of vitamins is almost the same[33]. The increased urinary excretion suggests that the requirement of pantothenic acid and biotin was reduced by starvation, that is, lower concentrations of pantothenic acid and biotin in the body might prefer to live for a long time during starvation. A possible inferable reason for the increase in urinary pantothenic acid and biotin might be a mechanism to prevent the stored fat in the body from overspending or to decrease the amount of acetyl-coenzyme A,

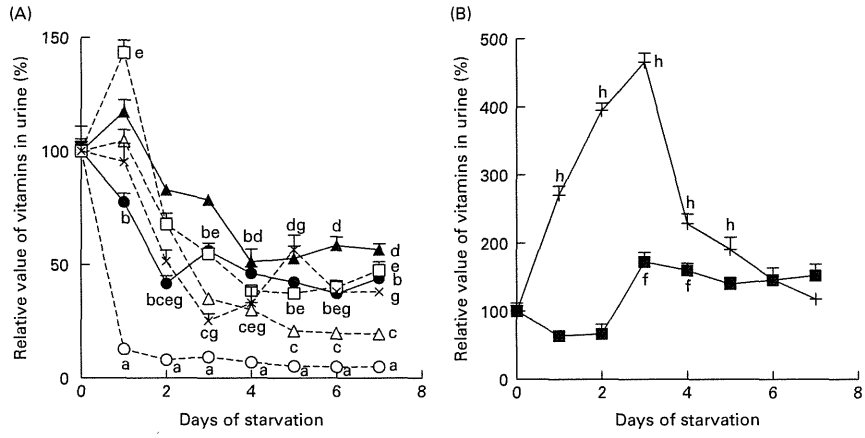

Fig. 3. Relative value of urinary B-group vitamin contents in P3 rats during starvation. Those of vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, nicotinamide and folate are shown in (A), and pantothenic acid and biotin in (B). Thiamin is expressed as vitamin B_1, riboflavin as vitamin B_2, 4-pyridoxic acid as vitamin B_6, and sum of nicotinamide and its catabolites as nicotinamide. Values are reported as means with their standard errors, n 5 per d. Values of control rats are expressed as 100 %. P3 is expressed at 8 d of starvation. Means with unlike letters were significantly different from day 0 in [a]vitamin B_1 (○), [b]vitamin B_2 (●), [c]vitamin B_6* (Δ), [d]vitamin B_{12} (▲), [e]nicotinamide (□), [f]pantothenic acid (■), [g]folate (×) and [h]biotin (+; $P<0{\cdot}05$).

which modifies several functional proteins such as histone[39] and some enzymes[40], and in addition, to decrease holoenzymes of carboxylases[41,42]. Acetylation generally activated some enzymes in fatty acid oxidation[40]. The physiologically active form of biotin is covalently attached at the active site of a class of important metabolic enzymes in gluconeogenesis, lipogenesis and amino acid metabolism[41,42]. Accordingly, decreased acetylation and biotin-dependent enzymes lead to reduced fatty acid oxidation and to save fat in the body.

Vitamin B_6 concentrations, expected to be the last vitamin decreased in tissues by starvation[3], decreased in the stomach, skeletal muscle and serum of the S2 rats. Vitamin B_6 in the

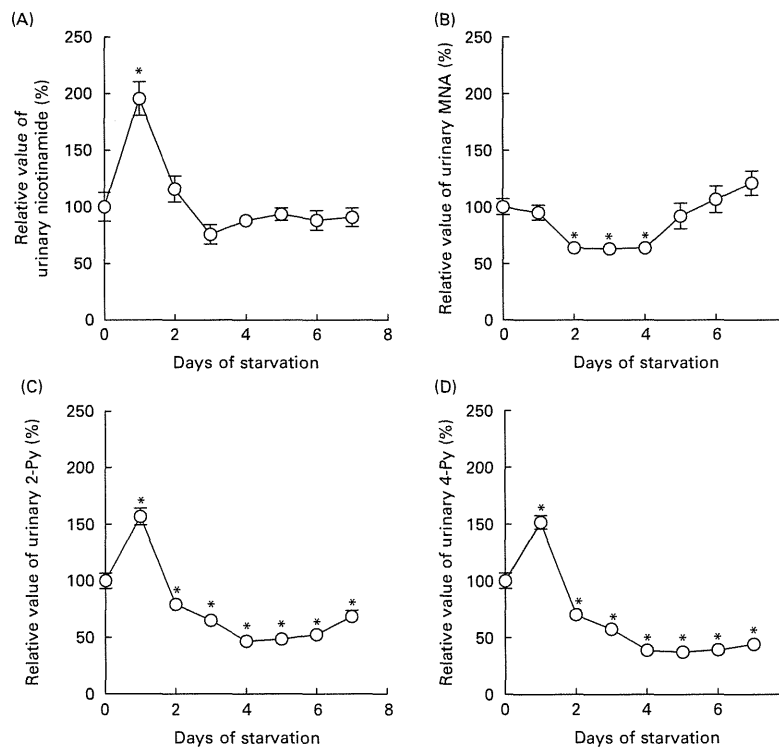

Fig. 4. Relative value of urinary nicotinamide (a) and its catabolites MNA (b), 2-Py (c) and 4-Py (d) contents in P3 rats during starvation. Values are reported as means with their standard errors, n 5 per d. * Mean values were significantly different from day 0 determined by one-way ANOVA with Tukey's multiple comparison tests ($P<0{\cdot}05$). MNA, N^1-methylnicotinamide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide.

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