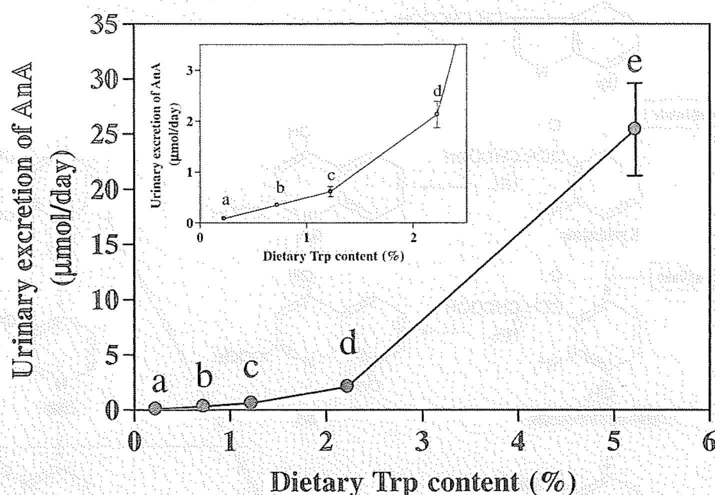

Fig. 3. Effect of Trp Intake on the Urinary Excretion of AnA in Rat.

The large graph shows the urinary excretion of AnA in the control group compared with the added 5% Trp supplemented group, and small graph shows that urinary excretion of AnA in control group to added 2% Trp group. A different letter on the curve means a significant difference at $p < 0.05$, as determined by the Tukey-Kramer multiple comparison test.

Table 2. Trp Intake and Metabolites Used for Indices

	Ctrl	+0.5%	+1%	+2%	+5%
Trp intake	213 ± 5	673 ± 25	1115 ± 43	2043 ± 32	4203 ± 256
(μmol/day)	(1)	(3.1)	(5.2)	(9.6)	(18.9)
AnA	82 ± 3	347 ± 20	614 ± 99	2126 ± 261	25420 ± 4207
(nmol/day)	(1)	(4.1)	(7.8)	(25)	(352)
KA	687 ± 57	1600 ± 80	2400 ± 160	3233 ± 118	6869 ± 364
(nmol/day)	(1)	(2.3)	(3.5)	(4.7)	(10)
XA	512 ± 39	1419 ± 142	2370 ± 157	3845 ± 321	7600 ± 400
(nmol/day)	(1)	(2.8)	(4.6)	(7.5)	(14.8)
3-HA	21 ± 3	71 ± 7	121 ± 22	148 ± 8	405 ± 7
(nmol/day)	(1)	(3.5)	(6.4)	(6.9)	(22)
Value of AnA/KA	0.12 ± 0.05	0.22 ± 0.07	0.26 ± 0.06	0.66 ± 0.10	3.7 ± 0.39
	(1)	(1.8)	(2.2)	(5.5)	(83.3)
Value of AnA/XA	0.16 ± 0.01	0.24 ± 0.08	0.26 ± 0.03	0.55 ± 0.04	3.3 ± 0.35
	(1)	(1.5)	(1.6)	(3.4)	(20.1)
Value of AnA/3-HA	3.9 ± 0.2	4.9 ± 0.3	5.1 ± 0.7	14.4 ± 1.0	62.8 ± 10.7
	(1)	(1.3)	(1.3)	(3.6)	(16.1)

Each value is expressed as the mean ± SEM of four rats, and in numbers parentheses are the relative to a control value of 1.

higher than the respective control group ratios. However, the excretion ratios were at the same level between the 0.5% and 1% Trp supplemented and the control groups.

Discussion

Trp has appeared on the market to treat sleep disorders,[14,15] and consequently, there is the risk of an excessive intake. It is therefore important to provide a useful index that will monitor an excessive tryptophan intake. Nutrient metabolite ratios change in urine in the case of an excessive nutrient intake. Our previous study has shown the effect of Nam when the nutrient intake exceeded the tolerable level. For example, when rats were fed with a diet containing different amounts of Nam, metabolic changes was observed; the urinary excretory ratio of (2-Py + 4-Py)/MNA was markedly reduced by a diet containing more than a tolerable intake of Nam.[16–18] We thought that an excessive Trp intake would also induce metabolic change which would be reflected in urinary excretion. Thus, we investigated the Trp-Nam metabolism in rats fed with a diet containing excessive Trp. Trp-Nam metabolites and metabolic changes might provide an index for the excessive intake of Trp.

In the present experiment, the urinary excretion of such Trp catabolites as KA, XA and 3-HA increased according to the intake of Trp (Fig. 2). Thus, the enzyme activity related with the conversion of Trp to 3-HA

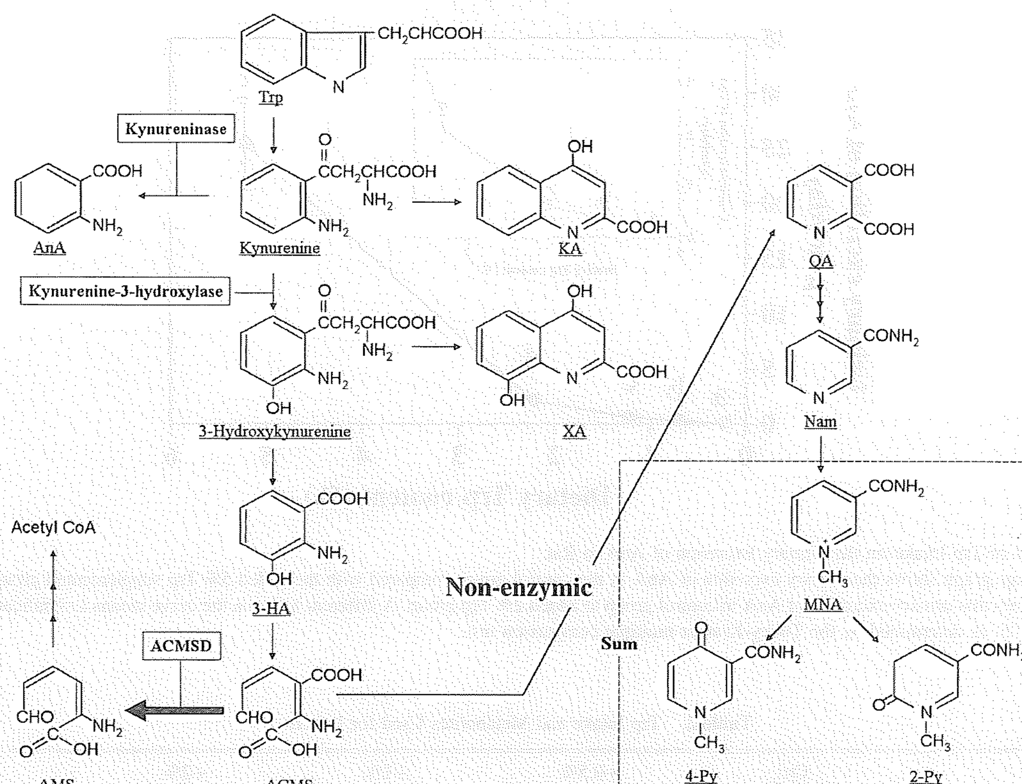

Fig. 4. Metabolism of Trp.
This whole metabolic pathway exists only in the liver.

might be sufficient to metabolize a diet containing up to 5% Trp.

QA is formed from ACMS and ACMS is formed from 3-HA. The reaction from 3-HA to ACMS is catalyzed by 3-hydroxyanthranilic acid oxygenase (3-HAO) whose enzyme activity is extremely high ($715 \pm 20\,\mu$mol/h/g of liver). In this study, we did not measure ACMS, although ACMS formation might increase because 3-HA was increased by an excessive Trp intake. The excretion of QA increased with increasing Trp intake up to 2%, but was almost the same form in the 2% and 5% Trp diet groups (Fig. 2D). This means that the activity of the enzyme, aminocarboxymuconate semialdehyde decarboxylase (ACMSD), was also increased which metabolizes α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) to α-aminomuconate-ε-semialdehyde (AMS) (the bold line in Fig. 4). The Trp–ACMS pathway branches at this metabolite; one branch is the reaction from ACMS to AMS catalyzed by ACMSD, and the other is the reaction from ACMS to QA by spontaneous autocyclization. The activity of ACMSD would be induced by Trp because it has been reported that ACMSD activity was altered by various nutritional factors and chemicals *in vivo*.[14,15,17,19]

The sum of the metabolites, Nam, MNA, 2-Py and 4-Py, was almost the same in the 1%, 2% and 5% Trp diet groups, which showed that the metabolism of QA to nicotinic acid mononucreotide was saturated in the 2% Trp diet group or the metabolite of ACMS to AMS was accelerated as already mentioned. Quinolinic acid phosphoribosyltransferase (QPRT) metabolized QA to NaMN. Rao *et al.*[18] have reported that QPRT was the limiting with the rate enzyme on the Trp–Nam pathway in rats.[20] Therefore, QPRT reached the limiting rate with the 2% Trp diet. We can explain these metabolic changes by enzymatic conversion.

Interestingly, the urinary excretion of AnA sharply increased above 2% Trp in the diet (Fig. 3), although KA, 3-HA and XA increased with increasing Trp intake. We cannot clearly explain why the excretion of AnA increased in this way. The Km for the value enzyme might be able to explain this result. The Km value for kynureninase, which catalyzes the reaction of kynurenine to AnA is 2.4×10^{-4} M. The Km value for kynurenine-3-hydroxylase, which catalyzes the reaction of kynurenine to 3-hydroxykynurenine, is 2.3×10^{-5} M, and the Km value for kynurenine amino transferase, which catalyzes the reaction kynurenine to KA, is 8.8×10^{-4} M (Fig. 4). Little AnA may be formed with intake of the in 1% Trp diet, but with intake of the 5% Trp diet, AnA formed might become extremely high (Fig. 4). Although the Km value is the same, the Kynurenine to KA reaction did not become extremely strong because this reaction may be complex. However, the synthesis of AnA increased by 25 and 308 times in the 2% and 5% Trp diet groups compared to the control

group although liver kynureninase, which is involved in the reaction of kynurenine to AnA did not change (data not shown). The reaction of kynurenine to 3-hydroxykynurenine is also involved in AnA formation, because a decrease in this reaction caused kynurenine to increase. Therefore, the increased urinary excretion of AnA was not attributable to any changes in the enzyme activities involved. We could not clearly explain why the excretion of AnA increased in the rats fed with the 2% and 5% Trp diets. But, Urinary excretion of AnA shows that extremely increase appeared lower level of adverse effect. We thought this might be able to be used as a bio marker.

Excess Trp intake is able to known by the daily amount excretion of AnA, but if we use metabolite of dependent on the Trp intake, it becomes easy. We calculated some ratios of AnA/some Trp metabolite. As Table 2 shows, the urinary excretory ratios of AnA/KA, AnA/XA and AnA/3-HA in the 2% and 5% Trp diet groups were higher than simple dose-dependence. When using the excretion ratio as a useful index to prevent excessive tryptophan intake, a spot test of urine can be used for the evaluation. Among the three ratios, the change in AnA/KA was the greatest. However this needs further examination because, in practice, we do not know when Trp metabolites appear in the urine.

In conclusion, we propose the urinary excretory ratio of AnA/KA, as an index to mark the excessive intake of Trp in rats. We want to study In the future whether it is possible to adapt this index.

Acknowledgment

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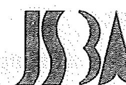

Communication

Molybdenum and Chromium Concentrations in Breast Milk from Japanese Women

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Molybdenum (Mo) and chromium (Cr) in 79 Japanese breast milk samples were measured by inductively coupled plasma-mass spectrometry. For Mo, 51 samples (64.6%) showed less than 5 ng/ml and only 12 samples (15.2%) showed more than 10 ng/ml. The range and median were <0.1 to 25.91 and 3.18 ng/ml respectively. For Cr, 38 samples (48.1%) showed less than 1 ng/ml, 20 samples (25.3%) showed 1 to 2 ng/ml, and only six samples (7.6%) showed more than 5 ng/ml. The range and median were <0.1 to 18.67 and 1.00 ng/ml respectively.

Key words: molybdenum; chromium; breast milk; dietary reference intake; inductively coupled plasma-mass spectrometry

Mo and Cr are essential trace elements in human nutrition, and deficiencies of them have been observed in patients with long-term total parenteral nutrition.[1,2] In Dietary Reference Intakes for Japanese in 2005 (DRI-J 2005), the recommended dietary allowances of Mo and Cr for adults were set at 20 to 25 µg/d and 25 to 40 µg/d respectively.[3]

Information on the secretion of trace elements in human milk is needed in order to estimate intake by breast-fed infants and, to establish the recommended intake for infants. In fact, adequate intake (AI) levels of several trace elements for infants (0 to 5 months) were set on the basis of the concentrations of those trace elements in breast milk of Japanese women in DRI-J 2005,[3] but, AI levels for Mo and Cr were not set in DRI-J 2005 because there was no available information on the concentration of these two trace elements in breast milk from Japanese women. In the present study, we measured Mo and Cr concentrations of breast milk samples from 79 Japanese women by inductively coupled plasma-mass spectrometry (ICPMS), and attempted to estimate AI levels for these two trace elements in Japanese infants.

The study was reviewed and approved by the Ethics Committee of the University of Shiga Prefecture, and it followed the Declaration of Helsinki. Seventy-nine healthy Japanese mothers who were breast-feeding exclusively and not taking vitamin or mineral supplements were recruited in several midwife clinics in Hokkaido, Chiba, Kanagawa, Kyoto, Hiroshima, and Nagasaki Prefectures in Japan from March 2005 to December 2006. The numbers of subjects recruited in the various prefectures were as follows: Hokkaido, 12; Chiba, 10; Kanagawa, 15; Kyoto, 30; Hiroshima, 2; and Nagasaki, 10. All the subjects had given birth to infants at term (gestational age 38 to 41 weeks). The mothers were 32.0 ± 4.1 years old (mean ± SD), with a range of 19 to 39 years. There were no health problems in their babies.

Breast milk was obtained from the subjects at an intermediate time during breast-feeding, placed in a nylon bag (Kaneson, Osaka, Japan) or a polypropylene centrifuge tube (Sumitomo Bakelite, Tokyo, Japan) and stored in a freezer at −20 °C until analysis. The postpartum day on which the sample was collected was 95.5 ± 46.8 d (mean ± SD) with a range of 5 to 191 d.

Two to 5 milliliters of breast milk was transferred to a ceramic melting pot ($32\phi \times 24$ mm), dried at 90 °C for 1 h in an electric oven, and then heated in an electric furnace (As One F-B1414M, Osaka, Japan) at 550 °C for 16 h. After dry incineration, the remaining ash was dissolved in 5 ml of 2% HNO_3. Mo and Cr in the sample solutions thus prepared were measured by ICPMS with

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Abbreviations: Mo, molybdenum; Cr, chromium; DRI-J 2005, Dietary Reference Intakes for Japanese in 2005; AI, adequate intake; ICPMS, inductively coupled plasma-mass spectrometry; Rh, rhodium; WHO, World Health Organization

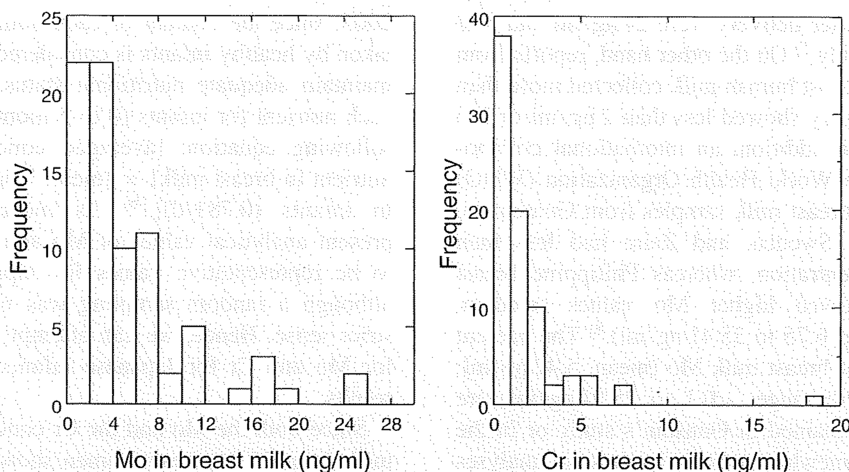

Fig. 1. Histograms of Mo and Cr Concentrations in Breast Milk from 79 Japanese Women.

direct nebulization. The ICPMS operating conditions were as follows: instrument, ICPM-8500 (Shimadzu, Kyoto, Japan); forward power, 1,200 W; coolant gas flow rate, 7.0 l/min; auxiliary gas flow rate, 1.5 l/min; nebulizer gas flow rate, 0.58 l/min; sampling depth, 5.0 mm; integration time, 2.0 s; number of runs, 20; mode of analysis, pulse; isotopes monitored, ^{52}Cr, ^{95}Mo, ^{97}Mo, and ^{98}Mo. A rhodium (Rh) isotope (^{103}Rh) was used as the internal standard. Since the three analytical values obtained from ion intensities at 95, 97, and 98 m/z were similar, the mean was used for Mo quantification. Mean values of triplicate analyses were used as Mo and Cr values for each subject. The detection limit was 0.1 ng/ml of breast milk for both elements.

Quadruplicate analyses of standard non-fat milk powder (SRM 1549, certified Cr content, 2.6 ± 0.7 ng/g; non-certified Mo content, 0.34 µg/g) showed values (mean ± SD) of 2.9 ± 0.6 ng/g as Cr content and 0.32 ± 0.04 µg/g as Mo content. On the other hand, quadruplicate analyses of pooled breast milk and a mixture of pooled breast milk with 1 ng/ml of standard Mo or Cr showed values (mean ± SD) of 5.22 ± 0.12 and 6.25 ± 0.10 ng/ml as Mo and 1.35 ± 0.11 and 2.26 ± 0.13 ng/ml as Cr respectively. In addition, quadruplicate analyses of pooled breast milk on another day showed 5.27 ± 0.08 ng/ml as Mo and 1.28 ± 0.09 ng/ml as Cr.

Among the 79 breast milk samples, only one sample had non-detectable Mo and 15 samples had non-detectable Cr. Figure 1 shows histograms of Mo and Cr concentrations in 79 breast milk samples. For Mo, 51 subjects (64.6%) showed less than 5 ng/ml, and only 12 subjects (15.2%) showed more than 10 ng/ml. This distribution of breast milk Mo is coincident with that observed in our preliminary study.[4] Similarly, for Cr, 38 subjects (48.1%) showed less than 1 ng/ml and 20 subjects (25.3%) showed values ranging from 1 to 2 ng/ml, while only six subjects (7.6%) showed more than 5 ng/ml. Except for samples with non-detectable

Table 1. Summary of Analyses of Molybdenum and Chromium Contents in Breast Milk from 79 Japanese Women

	Mo (ng/ml)	Cr (ng/ml)
Mean*	5.42	1.73
Standard deviation*	5.33	2.57
Minimum	<0.1	<0.1
Maximum	25.91	18.67
Geometric mean*	3.57	0.69
Median	3.18	1.00
25 percentile value	1.89	0.31
75 percentile value	7.16	2.32

*Non-detectable values were set to 0.05 ng/ml, which was half the detection limit.

Mo or Cr, skewness and kurtosis were calculated to be 0.210 ($z = -0.245$, NS) and -0.532 ($z = 6.853$, $p < 0.001$) for log Mo ($n = 78$) respectively, and -0.028 ($z = 0.094$, NS) and -0.101 ($z = 5.517$, $p < 0.001$) for log Cr ($n = 64$) respectively. These results indicate that both Mo and Cr show logarithmical normal distribution rather than normal distribution.

Table 1 summarizes the analytical results for Mo and Cr in 79 breast milk samples. In the calculation of these statistical values, we set all non-detectable values to 0.05 ng/ml, which was half the detection limit. The arithmetical means for Mo and Cr in the 79 samples were 5.42 and 1.73 ng/ml respectively. Since both elements showed logarithmical normal distribution, their geometric mean and median values were lower than their arithmetical mean values. The ranges of geometric mean ± geometric standard deviation were 1.33 to 9.57 ng/ml for Mo and 0.17 to 3.34 ng/ml for Cr. There was no significant association between Mo or Cr concentrations and days postpartum on which samples were collected. In addition, no regional variation was observed in Mo or Cr.

There have been several reports on Mo in breast milk. Gunshin *et al.* found that the mean and range of Mo concentration in breast milk from 24 Japanese women

from 19 to 384 d after delivery were 24 ng/ml and 5 to 63 ng/ml respectively.[5] On the other hand, reports from the US found that most human milk collected more than 1 month after delivery showed less than 2 ng/ml of Mo concentration.[6,7] In addition, an international collaborative study by the World Health Organization (WHO) showed that most breast milk samples from Guatemala, Hungary, Nigeria, Sweden, and Zaire had less than 5 ng/ml Mo concentration, whereas Philippine breast milk samples showed higher Mo values (median, 16.36 ng/ml; range, 6.75 to 35.41 ng/ml).[8] The present analytical values of breast milk Mo (mean, 5.42 ng/ml; median, 3.18 ng/ml; range, <0.1 to 25.91 ng/ml) are lower than those obtained in Gunshin's study or in the Philippines, but somewhat higher than those in analyses performed in many countries outside of Asia. Since rice and soybeans are rich in Mo,[9] the dietary Mo intake of Asian people who eat large amounts of rice and soybean products is expected to be higher than that of Western people. In fact, we confirmed that dietary Mo intake and serum Mo concentrations in Japanese is somewhat higher than in Americans or Europeans.[9,10] Accordingly, it is likely that the Mo concentration in Japanese breast milk is somewhat higher than in breast milk collected in the US or Europe. The present analytical values for breast milk Mo are reasonable and representative values for Japanese breast milk, although the cause of high Mo values in breast milk in Gunshin's study[5] and in the Philippines[8] is unclear.

There have also been several reports on Cr concentrations in breast milk. In Japanese subjects, values of 6.5 ng/ml and of a non-detectable level to 20.9 ng/ml were reported as the mean and range respectively for 24 Japanese subjects.[5] Another recent Japanese study of a large number of subjects ($n = 1{,}166$) reported 59 ± 47 ng/ml (mean $\pm$ SD) as the breast milk Cr concentration,[11] but, the values in the latter study are not reliable, since no accuracy evaluation of analytical values using standard reference materials was performed. Similarly, the reliability of the former study is also insufficient, since accuracy was evaluated using only orchard leaves (SRM 1571), which contained about 1,000-fold higher amounts of Cr than breast milk. On the other hand, several recent reports indicate that the amounts of Cr in breast milk from most American mothers is less than 1 ng/ml.[12,13] Accordingly, the Dietary Reference Intakes of the US has adopted a value of 0.25 ng/ml as the average Cr value in breast milk from American mothers.[14] The present analytical values (mean, 1.73 ng/ml; median, 1.00 ng/ml; range, <0.1 to 18.67 ng/ml) were somewhat higher than the US averaged values, but are coincident with breast milk Cr values observed in an international collaborative study performed by the WHO;[15] the present Cr values are therefore reasonable and representative values for Japanese breast milk.

The main purpose of this study was to estimate the AI values for Mo and Cr for Japanese infants. In DRI-J 2005, since the content of each nutrient in breast milk taken by healthy infants is considered to be sufficient to maintain adequate nutritional status, the AI value for each nutrient for infants (0 to 5 months) was set by the following equation: [averaged concentration of each nutrient in breast milk] $\times$ [mean volume of milk intake in infants (0.78 l/d)].[16] As mentioned above, the present analytical values of Mo and Cr are considered to be representative values for Japanese breast milk, although a random sampling was not performed in a strict sense. Hence, we can attempt to estimate the AI for Mo and Cr for Japanese infants using the present results.

Since both the Mo and the Cr concentration in breast milk from 79 Japanese women showed a logarithmical normal distribution, the geometric mean is suitable for their averaged values. However, when the data include values below detection limit, the geometric mean may vary with the way of treating them. In Table 1, we set all non-detectable values to 0.05 ng/ml, which was half the detection limit. This treatment is the most convenient and has been adopted in many studies, but the estimated geometric mean varies with the setting of the detection limit. Other approaches to estimating the geometric mean of data including non-detectable values are to use Cohen's maximum likelihood estimator method,[17] the normal plot method,[18] and the robust method.[19] Following Cohen's method, we calculated the geometric means of the data excluding non-detectable values and adjusted those geometric means to those of all the data using a detection limit value (0.10 ng/ml) and Cohen's λ.[20] According to Cohen's method, the geometric means for Mo and Cr in the 79 breast milk samples were estimated to be 3.52 and 0.71 ng/ml respectively. Following the normal plot method, we depicted two probability plots, as shown in Fig. 2, and calculated a regression equation of log Mo or log Cr *versus* normal scores. Based on X intercepts in the equation, the geometric means for Mo and Cr were estimated to be 3.66 and 0.82 ng/ml respectively. Following the robust method, we substituted normal scores of the non-detectable values for Y in the regression equation of Fig. 2 to estimate extrapolated values below the detection limit. After this extrapolation, the geometric means for Mo and Cr in the 79 samples were estimated to be 3.66 and 0.82 ng/ml respectively.

These geometric means estimated by Cohen's method, the normal plot method, and the robust method are different from those described in Table 1 (Mo, 3.57; Cr, 0.69 ng/ml). Thus, since the geometric mean of the data including non-detectable values varied with the treatment of non-detectable values, we used medians as averaged values for Mo and Cr in the 79 Japanese breast milk samples to estimate AI. When the median is used in the estimation, the AI values for Mo and Cr for Japanese infants (0 to 5 months) are 2.5 µg/d (3.18 µg/l $\times$ 0.78 l/d = 2.48 µg/d) and 0.8 µg/d (1.00 µg/l $\times$ 0.78 g/l = 0.78 µg/d) respectively.

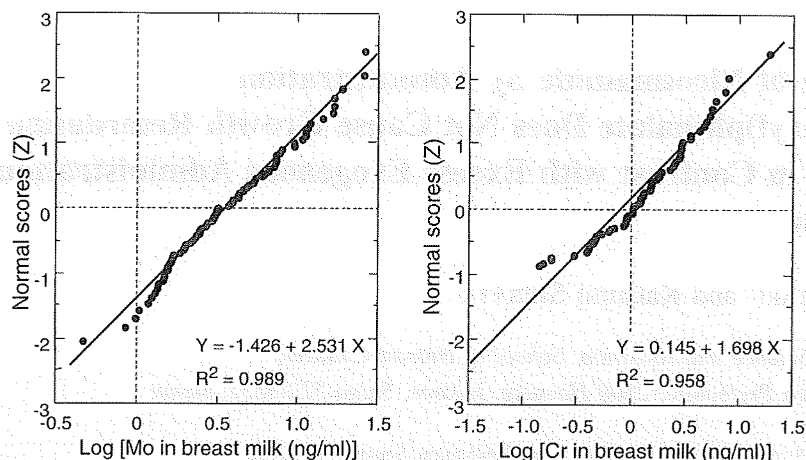

Fig. 2. Regression of Log Mo and Log Cr *versus* Normal Scores.

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Note

The Formation of Nicotinamide by Administration of Di(2-ethylhexyl)phthalate Does Not Cause Growth Retardation in Young Rats in Contrast with Excess Exogenous Administration of Nicotinamide

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We investigated the relationship between protein and tryptophan intake and the adverse-effect-level of di(2-ethylhexyl)phthalate (DEHP). Growth retardation of young rats due to DEHP was strengthened by increasing protein level. The addition of tryptophan to the diet caused extreme increases in the nicotinamide formation, but no growth retardation was observed.

Key words: nicotinamide; phthalic acid ester; tryptophan; toxicity; di(2-ethylhexyl)phthalate (DEHP)

DEHP is a ubiquitous environmental contaminant due to its extensive use as a plasticizer.[1] Since phthalate plasticizers are not chemically bound to polyvinylchloride plastics, they can leach, migrate, or evaporate into indoor air and atmosphere, foodstuffs, and other materials. These esters have been detected in animals and humans.[2–4] We have reported that the administration of phthalic acid esters such as dibutylphtahlate[5,6] and DEHP[7–12] disturbed the metabolism of *de novo* NAD biosynthesis. Concretely, the administration of phthalic acid esters increased the formation of QA (a key intermediate of the *de novo* NAD biosynthesis) and its metabolites.[7] For example, the conversion ratio of Trp to Nam was abnormally increased up to 30% by feeding of a diet containing 3% DEHP, while the conversion ratio of group on a non-DEHP diet was about 2%.[7] Handler *et al.*[13] and Shibata and Tanaka[14] have reported that the intake of an excess of Nam caused retarded growth in young rats. We have proposed that part of growth retardation in young rats due to phthalic acid esters is attributable to excess Nam formation. Objective of the present study was to test this hypothesis. Here we report that our hypothesis was clearly disconfirmed.

NAD^+ and $NADP^+$ were purchased from Sigma Chemical Company (St. Louis, MO). Vitamin-free milk casein, sucrose, L-methionine, Nam, and QA were purchased from Wako Pure Chemical Industries (Osaka, Japan). KA, XA, and MNA chloride were purchased from Tokyo Chemical Industry (Tokyo). 2-Py and 4-Py were synthesized by the methods of Pullman and Colowick[15] and Shibata *et al.*[16] respectively. Corn oil was purchased from Ajinomoto (Tokyo). Mineral and vitamin mixtures and gelatinized cornstarch were obtained from Oriental Yeast (Tokyo). All the other chemicals used were of the highest purity available from commercial sources.

The care and treatment of the experimental animals conformed to The University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. Male rats of the Wistar strain (3 weeks old with a body weight of about 40 g) were obtained from CLEA Japan (Tokyo). They were immediately placed in individual metabolic cages (CT-10; Clea Japan). In experiment 1, the rats were divided into five groups and fed *ad libitum* for 21 d on 40% casein diets[17] containing a niacin-free AIN-93 vitamin mixture with 0, 0.1, 0.5, 1.0, and 2.0% DEHP. In experiment 2, the rats were divided six groups and fed *ad libitum* for 21 d on niacin-free 20% casein diets,[7] with 0, 0.1, and 0.5% Trp with and without 0.1% DEHP. The room temperature was maintained at about 20 °C and about 60% humidity, and a 12-h light/12-h dark cycle was maintained. Body weight and food intake were measured daily at about 10:00 AM. In experiment 2, urine samples (24-h; 10:00 AM–10:00 AM) on the last day were collected in amber bottles containing 1 ml of 1 M HCl, stored at −20 °C until needed. The rats were killed by decapitation. To measure NAD (NAD^+ + NADH) and NADP ($NADP^+$ + NADPH), the

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Abbreviations: DEHP, di(2-ethylhexyl)phthalate; Trp, L-tryptophan; AnA, anthranilic acid; KA, kynurenic acid; XA, xanthurenic acid; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; Nam, nicotinamide; MNA, N^1-methylnicotinamide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide

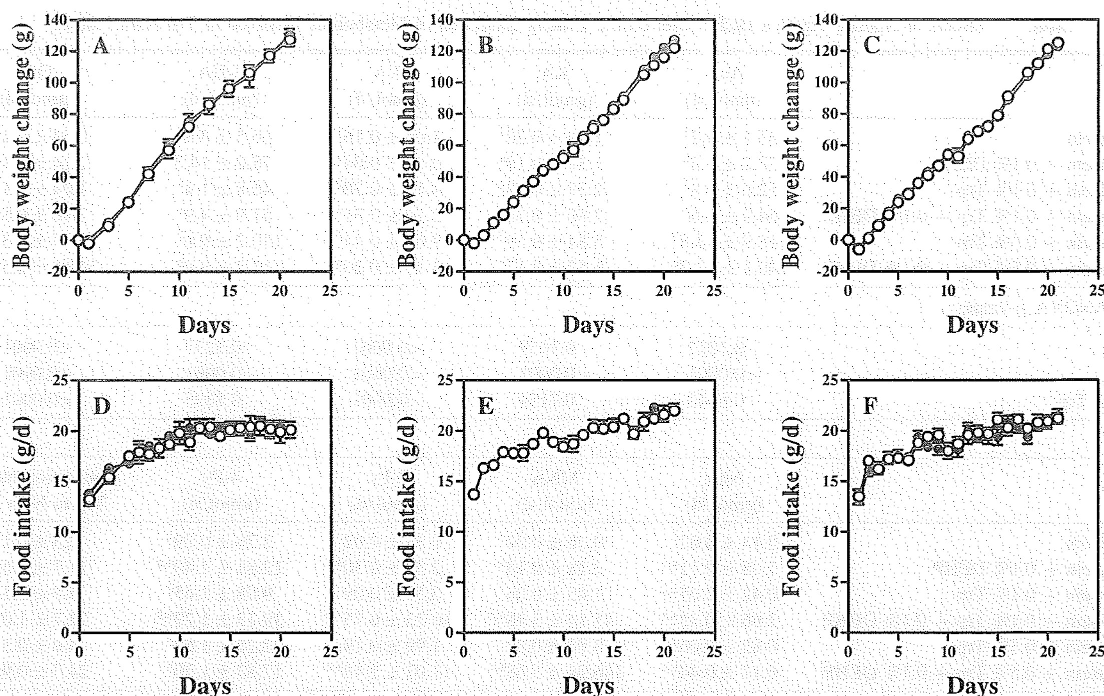

Fig. 1. Effect of Adding Free Trp to a 0.1% DEHP Diet on Body Weight and Food Intake.

Male rats of the Wistar strain (6 weeks old) were placed in individual metabolic cages. They were divided into six groups and then fed freely a 20% casein diet supplemented with 0% Trp (A and D), 0.1% Trp (B and E), or 0.5% Trp (C and F) with or without 0.1% DEHP for 21 d. ○, 0% DEHP diet; ●, 0.1% DEHP diet. Each point is the mean ± SEM (n = 5).

liver of each animal was removed, and a portion (approximately 0.2 g) was immediately treated as described in the literature.[18,19)]

The contents of NAD (NAD^+ + NADH) and NADP ($NADP^+$ + NADPH) were measured by the colorimetric methods of Shibata and Murata[18)] and Shibata and Tanaka[19)] respectively. The urinary contents of Nam and of the catabolic metabolites MNA, 2-Py, and 4-Py were measured. The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the HPLC method of Shibata *et al.*,[16)] while the content of MNA in the urine was measured by the HPLC method of Shibata.[20)] The contents of KA,[21)] XA,[22)] 3-HA,[22)] AnA,[23)] and QA[24)] in the urine were measured by the HPLC method.

We have reported that growth retardation of young rats due to DEHP appeared at a level of 1.0% on a 20% casein diet.[7)] Body weight gain on day 21 was 68.2 ± 2.5 g in the 40% casein diet group. It was 62.4 ± 2.7 g on a diet with 0.1% DEHP group, with no growth retardation, while growth retardation was observed at 0.5% on the 40% casein diet. Body weight gains at day 21 were 21.2 ± 3.9 g, 11.8 ± 3.5 g, and −9.4 ± 2.1 g on the diets with 0.5%, 1.0%, and 2.0% respectively.

The addition of free Trp to the DEHP diet did not affect body weight gain or food intake, as shown in Fig. 1. Liver weight was higher on each DEHP diet than on respective non-DEHP diet, as shown in Table 1. Liver weight was not affected by adding free Trp. NAD concentrations in the livers showed a tendency to increase with Trp intake. DEHP administration caused further increases in NAD concentration.

Table 1. Effect of Adding Free Trp to a DEHP Diet on Liver Weight and NAD Contents in Liver

	Liver weight (g/rat)	NAD (nmol/g of liver)
20% Casein	12.09 ± 0.36	830 ± 63[1]
20% Casein + 0.1% DEHP	17.61 ± 0.47*	1179 ± 86*,[a]
20% Casein + 0.1% Trp	12.31 ± 0.31	845 ± 51[1,2]
20% Casein + 0.1% Trp + 0.1% DEHP	17.54 ± 0.46*	1341 ± 95*,[a,b]
20% Casein + 0.5% Trp	12.61 ± 0.28	1066 ± 43[2]
20% Casein + 0.5% Trp + 0.1% DEHP	17.30 ± 0.71*	1458 ± 36*,[b]
2-way ANOVA p-values		
DEHP	<0.0001	<0.0001
Trp	0.9721	0.0024
DEHP × Trp	0.6560	0.5281

Each value is the mean ± SEM (n = 5); a different superscript letter means significant difference at $p < 0.05$, calculated by Bonferroni post test.
*; Significant difference between groups on the same Trp diets.
Numbers; Significant difference among groups on non-DEHP diets.
Letters; Significant difference among groups on 0.1% DEHP diets.

The metabolites of Trp to Nam were increased dose-dependent manner due to the addition of free Trp, as shown in Table 2. DEHP intake caused QA to increase extremely, as well as metabolites such as Nam, MNA, 2-Py, and 4-Py. Therefore, the conversion ratio of Trp to

Table 2. Effects of Adding Trp to a DEHP Diet on the Urinary Excretion of Metabolites Involved in Trp-Nam Pathway

	AnA (nmol/d)	KA (μmol/d)	XA (μmol/d)	3-HA (nmol/d)	QA (μmol/d)
20% Casein	33.1 ± 9.2^{1}	1.19 ± 0.20^{1}	1.05 ± 0.16^{1}	66.5 ± 3.8^{1}	0.54 ± 0.11
20% Casein + 0.1% DEHP	37.2 ± 5.2^{a}	1.37 ± 0.19^{a}	0.80 ± 0.04^{a}	78.0 ± 14.3^{a}	2.14 ± 0.19^{a}
20% Casein + 0.1% Trp	52.6 ± 5.8^{2}	2.39 ± 0.13^{2}	4.18 ± 0.30^{2}	46.8 ± 1.4^{1}	1.04 ± 0.11
20% Casein + 0.1% Trp + 0.1% DEHP	64.9 ± 1.6^{b}	2.80 ± 0.33^{b}	$2.10 \pm 0.31^{*,b}$	51.9 ± 4.9^{a}	7.90 ± 0.82^{a}
20% Casein + 0.5% Trp	118.9 ± 13.8^{3}	5.84 ± 0.59^{3}	9.67 ± 0.44^{3}	110.2 ± 9.6^{2}	2.74 ± 0.31
20% Casein + 0.5% Trp + 0.1% DEHP	140.1 ± 12.3^{c}	6.52 ± 0.17^{c}	$4.19 \pm 0.20^{*,c}$	114.2 ± 9.0^{b}	$54.66 \pm 8.51^{*,b}$
2-way ANOVA p-values					
DEHP	0.1022	0.1059	<0.0001	0.3237	<0.0001
Trp	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DEHP × Trp	0.6440	0.7154	<0.0001	0.8895	<0.0001

	Nam (μmol/d)	MNA (μmol/d)	2-Py (μmol/d)	4-Py (μmol/d)	Conversion ratio of Trp to Nam (%)
20% Casein	0.14 ± 0.02^{1}	0.36 ± 0.02	0.36 ± 0.02	3.76 ± 0.29^{1}	2.3 ± 0.1
20% Casein + 0.1% DEHP	$1.08 \pm 0.11^{*,a}$	2.28 ± 0.18^{a}	2.35 ± 0.30^{a}	$12.81 \pm 1.40^{*,a}$	$11.7 \pm 0.6^{*,a}$
20% Casein + 0.1% Trp	$0.42 \pm 0.05^{1,2}$	1.45 ± 0.06	0.57 ± 0.09	9.08 ± 1.45^{2}	3.7 ± 0.5
20% Casein + 0.1% Trp + 0.1% DEHP	$2.66 \pm 0.33^{*,b}$	$45.14 \pm 3.18^{*,b}$	$10.23 \pm 0.75^{*,b}$	$19.13 \pm 1.29^{*,b}$	$22.7 \pm 1.0^{*,b}$
20% Casein + 0.5% Trp	0.83 ± 0.06^{2}	3.92 ± 0.32	1.99 ± 0.19	22.28 ± 1.62^{3}	4.0 ± 0.3
20% Casein + 0.5% Trp + 0.1% DEHP	$6.17 \pm 0.44^{*,c}$	$146.94 \pm 5.68^{*,c}$	$17.07 \pm 1.48^{*,c}$	17.83 ± 1.70^{b}	$24.3 \pm 0.9^{*,b}$
2-way ANOVA p-values					
DEHP	<0.0001	<0.0001	<0.0001	0.0002	<0.0001
Trp	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
DEHP × Trp	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Each value is the mean ± SEM (n = 5); a different superscript letter means significant difference at $p < 0.05$, calculated by Bonferroni post test.
*; Significant difference between the groups on the same Trp diets.
Numbers; Significant difference among the groups on non-DEHP diets.
Letters; Significant difference among the groups on 0.1% DEHP diets.

Nam was higher in each of DEHP groups than in the respective non-DEHP groups.

Shibata and Iwai have reported in 1980 that phthalic acid, an analog of QA, is a strong competitive inhibitor of quinolinate phosphoribosyltransferase (QPRT).[25] Hence, we investigated to determine whether the administration of phthalic acid esters, widely used as plasticizers, would cause adverse effects on rats when they were fed a niacin-deficient diet. Shibata *et al.* found in 1982 that growth promoting activity due to appropriate phthalic acid esters occurred when rats were fed a niacin-free, Trp-limiting diet.[26] We were surprised at that phenomenon, and so we started to sort out the contradiction. We found and have reported that mono(ethylhexyl)phthalate (MEHP), a metabolite of DEHP, inhibits the enzyme activity of ACMSD, and increases the formation of QA, causing it to increase further metabolites such as Nam.[7–12] Phthalic acid does not accumulate in the liver, so the reaction of QPRT might not be inhibited.

Phthalic acid esters are known to have adverse effects on mammals.[4] We have reported that the addition of a large amount of DEHP caused growth restriction.[7] Under such conditions, the conversion ratio of Trp to Nam increased extremely up to 30%, which mean that Nam formation in the body was about 35 mg/kg body weight of rat.[7] Nam administration of 40 mg/kg of body weight of rat caused significant growth retardation.[14] Hence, we present the hypothesis that part of the growth retardation induced by phthalic acid esters is due to the production of excess Nam. In the present experiment, we investigated whether the administration of a high-protein diet that contained high Trp to rats could cause a strengthening of growth retardation by DEHP. Growth retardation was observed at a level of 0.5% DEHP in the 40% casein diets, while we have reported that in the 20% casein diets, the retardation appeared at a level of 1.0% DEHP.[7] We found that feeding the high protein diet strengthened growth retardation due to DEHP. We investigated to determine whether the strengthened growth retardation due to feeding the high protein diet is attributed to high Trp intake. The addition of Trp only to the 20% casein diet containing 0.1% DEHP did not cause apparent growth retardation. A concentration of 0.1% DEHP was chosen because we anticipated that growth retardation would be strengthened by the addition of Trp, but no retardation of the body weight gain or food intake in young rats was observed (Fig. 1), although the conversion ratio was over 20% in the group consuming a 0.1% free Trp added diet containing 0.1% DEHP (Table 2), which indicates 60 mg of Nam formation per kilogram of body weight. Furthermore,

in the group consuming a 0.5% free Trp added diet containing 0.1% DEHP, Nam formation in the body was about 150 mg per kilogram body weight. Nevertheless, the body weight gain and food intake were almost the same as in the group on the 20% casein diet (control). These results indicate that our hypothesis that part of DEHP-growth retardation is due to an excess of Nam, is not plausible. In addition, the present data indicate that the growth retardation level in young rats exogenously taken excess Nam is lower than in rats endogenously formed excess Nam. This phenomenon implies that the peak concentration of Nam is the most important point in the toxicity of Nam, that is, as to whether Nam causes growth retardation. Even if the endogenous formation of Nam in 1 d are almost the same as the administered amount of exogenous Nam in 1 d, the peak Nam concentration in the body should not be much lower in endogenous Nam than in exogenous Nam, because endogenous Nam is continuously metabolized, or synthesized and catabolized, while the exogenous Nam causes an abrupt increase in the concentration of Nam in the body.

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Age-Related Alterations of B-Group Vitamin Contents in Urine, Blood and Liver from Rats

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Summary To investigate how aging alters B-group vitamin metabolism, rats were fed with niacin-free 20% casein diet from 3 to 80 wk old, and the urinary excretions of the B group vitamins were periodically measured. The blood and liver B-group vitamin levels in 80-wk-old rats were also compared with those in 8-wk-old rats. The urinary excretion of thiamin, riboflavin, vitamin B_6 metabolite 4-pyridoxic acid, pantothenic acid, folic acid and biotin were not altered during 540 d. The urinary vitamin B_{12} increased by 8-fold at 29 wk old, and further increased at 80 wk old. Conversion of nicotinamide from tryptophan gradually decreased to 60% from 29 to 48 wk old. Plasma PLP, vitamin B_{12} and folate levels in 80-wk-old rats were lower than those in 8-wk-old rats, consistent with lower liver vitamin B_6 and folate levels in aged rats. Plasma and liver biotin levels in aged rats were higher than those in young rats. Other B-group vitamins such as vitamin B_1, vitamin B_2, niacin and pantothenic acid levels in blood and liver from aged rats were same as those from young rats. Alteration of vitamin B_6 metabolism in particular is similar to the observations in eldery humans reported previously. Our findings suggest that aged rats can be useful models to investigate aging-related B-group vitamin metabolism.

Key Words aging, vitamin B_2, vitamin B_6, folate, biomarker

The precise cascade of pathological age-related events is still not clearly understood, but enhanced production of free radicals and its deleterious effects on macro-biomolecules such as proteins and DNA (*1*, *2*) as well as polyunsaturated fatty acids (*3*) are well known during aging. Aging is a major risk factor for various chronic disorders including cancer. It is well known that taking in nutrients suitable for life stages is important to prevent the aging process and chronic disorders. Food, namely nutrient intake, is a key factor affecting aging and the incidence of many chronic disorders (*4–6*). Decreased food intake and a sedentary lifestyle reduce energy expenditure and may alter their metabolism in elderly adults, and alteration of metabolism may cause alteration of nutrient requirement. However, there is not sufficient information on how aging alters vitamin metabolism (*7*, *8*). To investigate age-related alterations of B-group vitamins, rats were kept for 540 d from 3 to 80 wk old, and the urinary excretions of the B group vitamins were periodically measured. The blood and liver B-group vitamin levels in 80-wk-old rats were also compared with those in 8-wk-old rats.

MATERIALS AND METHODS

Chemicals. Vitamin-free milk casein, sucrose, and L-methionine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized cornstarch, the mineral mixture (AIN-93M) (*9*), the niacin free-vitamin mixture (AIN-93-VX containing 25% choline bitartrate) (*9*) and nicotinamide adenine dinucleotide (NAD) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Thiamin hydrochloride, thiamin diphosphate (TDP) chloride, riboflavin, pyridoxine hydrochloride, pyridoxal 5′-phospahte (PLP), nicotinamide, calcium pantothenate, pteroylmonoglutamic acid (folic acid) and D(+)-biotin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Thiamin monophosphate (TMP) chloride dihydrate and lumiflavin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). 4-Pyridoxic acid (4-PIC) was made by ICN Pharmaceuticals (Costa Mesa, California, USA) and obtained through Wako Pure Chemical Industries. N^1-Methylnicotinamide (MNA) chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N^1-Methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized (*10*, *11*). All other chemicals used were of the highest purity available from commercial sources.

Animals and diets. The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

Male Wistar rats aged at 3 wk old were obtained from CLEA Japan, Inc. (Tokyo, Japan) and immediately placed in individual wire-bottomed cages (260×180×380 mm, wide×height×depth). Rats fed ad libitum a niacin free-20% casein diet (Table 1) for 35 or 540 d.

The room temperature was maintained at around 22°C and about 60% humidity, and a 12-h light

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Table 1. Composition of the diet.

	%
Milk casein (vitamin-free)	20.0
L-Methionine	0.2
Gelatinized cornstarch	46.8
Sucrose	23.5
Corn oil	5.0
Mineral mixture (AIN-93)	3.5
Nicotinic acid free-vitamin mixture (AIN-93-VX)	1.0

(06:00–18:00)/12-h dark (18:00–06:00) cycle was maintained. Body weight and food intake were measured every 2 or 3 d at around 09:00. Urine samples (24-h; 09:00–09:00) were collected in amber bottles containing 1 mL of 1 mol/L HCl at 14, 23, 32, 39, 51, 57, 63, 70, and 80 wk during the experiment, and were stored at −20˚C until needed. Five rats were killed at 8 wk old, and the other was at 80 wk old by decapitation at around 09:00. Blood was collected, and the liver was dissected to measure B-group vitamin.

Determination of vitamins and their metabolites in urine, blood and liver. For vitamin measurements, known concentration reconstituted samples were prepared as quality control (QC), and QC samples were analyzed for validation in all analysis. Stability of samples was also confirmed, and no degradation was observed for any vitamin measurement.

Vitamin B_1: Vitamin B_1 contents in blood and liver were determined as the sum of thiamin, TMP and TDP. Five percent trichloroacetic acid was added to whole blood, and supernatant of the mixture was used for measurement. Liver was homogenized in a 5% trichloroacetic acid, and supernatant of the homogenate was used for measurement. Urinary thiamin, blood vitamin B_1 and liver vitamin B_1 were determined by the HPLC-post labeled fluorescence method (*12*).

Vitamin B_2: Riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in blood and liver were converted to lumiflavin by photolysis, and lumflavin was determined as vitamin B_2 by the HPLC method (*13*). Urinary riboflavin was determined by the HPLC method (*13*).

Vitamin B_6: Vitamin B_6 vitamers including phosphate esters in the liver were converted to free vitamin B_6 vitamers such as pyridoxal and pyridoxine by autoclave under acidic condition, and measured as total vitamin B_6 by the microbioassay method using *Saccharomyces carlsbergenisis* strain 4228 ATCC 9080 (*14*). Plasma PLP was determined by the HPLC method (*15*). Urinary 4-PIC was determined by the HPLC method (*16*).

Vitamin B_{12}: Liver homogenate, plasma or urine were added to 0.2 mmol/L acetate buffer (pH 4.8), and the vitamin B_{12} was converted to cyanocobalamin by boiling with 0.0006% potassium cyanide at acidic pH (*17*). Cyanocobalamin was determined by the microbioassay method using *Lactobacillus leichmanii*, ATCC 7830 (*17*).

Niacin: NAD and NADP in the liver homogenate were converted to nicotinamide by autoclave, and nicotinamide was determined by the HPLC method (*11*). The contents of NAD (NAD^++NADH) in whole blood were measured by the colorimetric method (*18*). Urinary 2-Py, 4-Py and MNA, nicotinamide metabolites, were determined by the HPLC method (*11*).

Pantothenic acid: To digest bound pantothenic acid such as CoA and pantetheine in liver and blood to free form, liver homogenate or blood was incubated with 0.5 U alkaline phosphatase and 2% pigeon liver amidase in 250 μL of 20 mmol/L phosphate buffer (pH 7.0) at 37˚C for 2 h. Pantothenic acid in urine, liver and blood were determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014 (*19*).

Folic acid: Folate in liver was digested to pteroylmonoglutamic acid by conjugase and protease (*20*). Plasma and urinary folate, and pteroylmonoglutamic acid digested from liver were determined by the microbioassay method using *Lactobacillus casei* ATCC 2733 (*20*).

Biotin: Bound biotin in liver was converted to free form by autoclave under acidic conditions, and biotin in urine, plasma and liver were determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014 (*21*).

Statistical analysis. For the statistical evaluation, the significance of the differences in the mean values between young and aged rats was tested by using Student's, two-tailed *t*-test and non-parametric Mann-Whitney U test. The differences of $p<0.05$ were considered to be statistically significant. Instat software (version 2.00; obtained from GraphPad Software, Inc., San Diego, CA, USA) was used for all analyses.

RESULTS

Body weight and food intake

The body weight increased linearly until approximately 10 wk old, and gradually increased to 80 wk old (Fig. 1A). This growth curve was the same as the data obtained from CLEA Japan, Inc. (*22*). The daily food intake was increased until approximately 10 wk old, and then kept constant around 20 g/d (Fig. 1B).

Comparison of blood B-group vitamin concentrations between young and aged rats

Table 2 shows comparison of B-group vitamin concentrations in whole blood or plasma between 8-wk-old young and 80-wk-old aged rats. Blood vitamin B_1, blood vitamin B_2, blood NAD and blood pantothenic acid concentrations in aged rats were the same as those in young rats. Plasma PLP, plasma vitamin B_{12} and plasma folate concentrations in aged rats were significantly lower than those in young rats, while plasma biotin in aged rats was significantly higher than that in young animals.

Comparison of liver B-group vitamin levels between young and aged rats

Table 3 shows comparison of liver B-group vitamin levels between 8-wk-old young and 80-wk-old aged rats. Vitamin B_1, vitamin B_2, nicotinamide and pantothenic acid levels in aged rats were the same as those

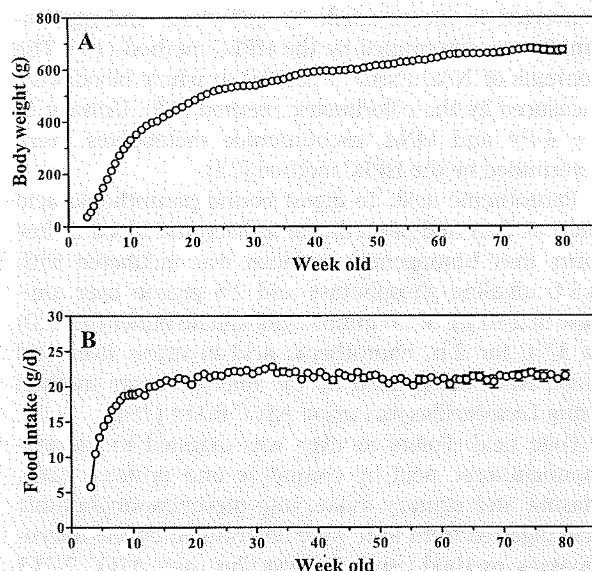

Fig. 1. Body weight (A) and daily food intake (B) in male rats of the Wistar strain fed with a 20% casein diet. Twenty male rats of the 3-wk-old Wistar strain were fed a niacin free-20% casein diet (Table 1) for 540 d. Values are expressed as means±SE for 20 rats.

Table 2. Comparison of the blood or plasma B-group vitamin levels between young and aged rats.

	Young rats[1] (8 wk old)	Aged rats[2] (80 wk old)
Blood vitamin B_1 (pmol/mL)	346±19	378±13
Blood vitamin B_2 (pmol/mL)	169±14	151±18
Plasma PLP (nmol/mL)	2.10±0.13	0.24±0.02*
Plasma vitamin B_{12} (pmol/mL)	7.18±0.09	1.13±0.06*
Blood NAD (NAD^++NADH) (nmol/mL)	79.4±3.8	78.4±2.9
Blood pantothenic acid (nmol/mL)	2.96±0.27	3.43±0.12
Plasma folate (pmol/mL)	277±11	52.4±2.9*
Plasma biotin (pmol/mL)	27.8±1.9	64.8±4.4*

[1]Values are expressed as mean±SE for 5 rats.
[2]Values are expressed as means±SE for 18–20 rats.
*Significant difference at $p<0.05$ between young and aged rats, as determined by non-parametric Mann-Whitney *U* test.

Table 3. Comparison of the liver B-group vitamin contents between young and aged rats.

	Young rats[1] (8 wk old)	Aged rats[2] (80 wk old)
Vitamin B_1 (nmol/g)	36.9±2.3	24.4±0.9
Vitamin B_1 (nmol/g)	74.3±3.8	64.8±2.2
Vitamin B_6 (nmol/g)	48.9±1.4	19.9±1.2*
Vitamin B_{12} (pmol/g)	91.5±9.7	not measured
Nicotinamide (nmol/g)	932±28	1,160±70
Pantothenic acid (nmol/g)	551±37	455±13
Folate (nmol/g)	26.3±1.3	13.6±0.6*
Biotin (nmol/g)	1.26±0.10	1.86±0.13*

[1]Values are expressed as mean±SE for 5 rats.
[2]Values are expressed as means±SE for 18–20 rats.
*Significant difference at $p<0.05$ between young and aged rats, as determined by non-parametric Mann-Whitney *U* test.

in young rats. Vitamin B_6 and folate levels in aged rats were significantly lower than those in young rats, while biotin in aged rats was significantly higher than that in young rats.

Age-associated alteration of urinary excretion of B-group vitamins

Urinary excretion of thiamin, riboflavin, vitamin B_6 metabolite 4-PIC, pantothenic acid and folic acid were not altered from 14 to 80 wk old (Fig. 2A, B, C, E and F). The urinary recovery rate of thiamin, riboflavin, 4-PIC, pantothenic acid and folic acid were approximately 20, 30, 40, 60 and 10%, respectively. The urinary excretion of cyanocobalamin increased from 23±2 to 178±9 pmol/d between 23 and 32 wk old, and then increased from 217±20 to 518±40 pmol/d between 70 and 80 wk old (Fig. 2D). The urinary recovery rate of cyanocobalamin was approximately 10% at 23 wk old, 40% at 51 wk old and 130% at 80 wk old. The urinary excretion of biotin increased from 1.9±0.1 to 4.9±0.2 nmol/d between 23 and 32 wk old, and kept constant after 32 wk old (Fig. 2G). The urinary recovery rate of biotin was approximately 10% at 23 wk old and 30% at older than 32 wk old.

Age-associated alteration of the conversion ratio of tryptophan-nicotinamide

Since nicotinamide and its coenzyme forms were sufficiently supplied only from tryptophan via the tryptophan-nicotinamide conversion pathway in rats fed with a niacin-free 20% casein diet (*23*), and tryptophan-nicotinamide metabolism can be assessed in the rats fed with a niacin-free 20% casein diet. The urinary excretion of nicotinamide metabolites gradually decreased to 60% from 32 to 51 wk old (Fig. 3A). The conversion ratio of tryptophan to nicotinamide also gradually decreased from 1.5 to 0.8% (Fig. 3B).

DISCUSSION

To investigate the alterations of B-group vitamin metabolism during aging, 3 wk old rats were kept for 540 d until 80 wk old. Urinary excretion of B-group vitamins was periodically measured from 14 to 80 wk old, and blood and liver B-group vitamin levels in aged rats were compared with those in young rats. As for vitamin B_6 and folic acid, the blood and liver contents in aged rats were much lower than those in young rats, while aging did not affect their urinary excretion. Plasma vitamin B_{12} in aged rats was also lower than that in young rats. Plasma and liver biotin levels in aged rats were higher than those in young rats, and urinary biotin also increased in aged rats. As for vitamin B_1, vitamin B_2 and pantothenic acid, aging did not affect their blood and liver contents or urinary excretion. These findings show that aging affects the metabolism

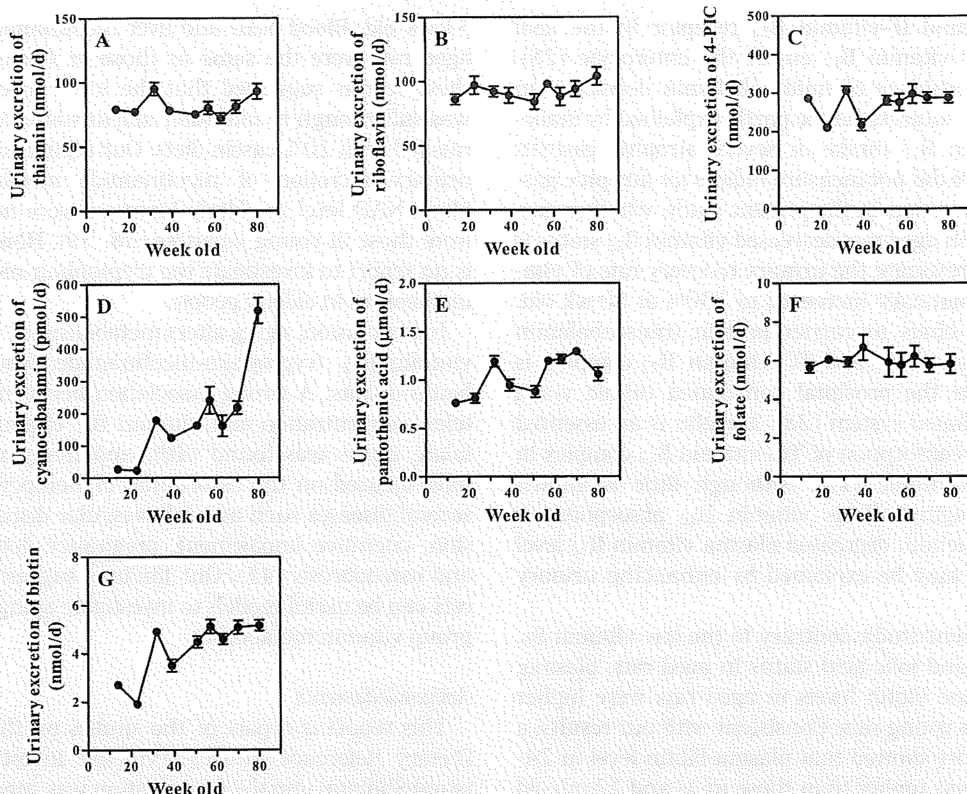

Fig. 2. Age-associated alteration of the urinary excretion of thiamin (A), riboflavin (B), 4-pyridoxic acid (4-PIC) (C), cyanocobalamin (D), pantothenic acid (E), folate (F) and biotin (G). Values are expressed as means±SE for 20 rats.

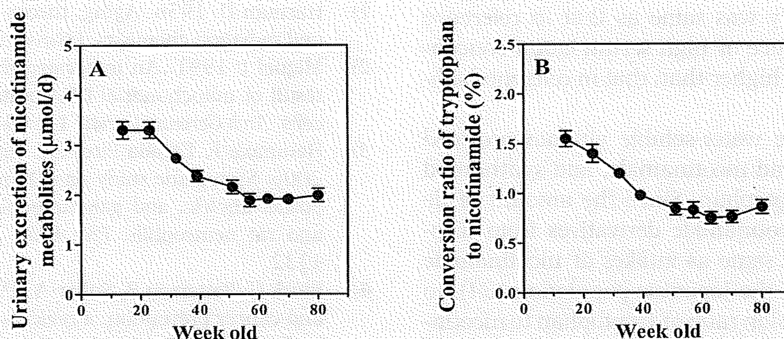

Fig. 3. Age-associated alteration of the urinary excretion of nicotinamide metabolites. Values are expressed as means±SE for 20 rats.

of some B-group vitamins, such as vitamin B_6, vitamin B_{12}, folic acid and biotin.

Plasma PLP concentration in elderly adults aged 65–75 y was lower than that in young adults aged 25–35 y, while dietary vitamin B_6 intake and urinary 4-PIC were the same among these groups (*24*), consistent with the present results for age-related alteration of vitamin B_6 metabolism obtained from the animal experiment. The vitamin B_6 requirements of the elderly over 60 y were determined as 1.9–2.0 mg/d from their plasma PLP and urinary 4-PIC (*25*), and these values are higher than those reported for younger adults (*26*). An oral vitamin B_6 loading study shows that vitamin B_6 absorption and ability to synthesize PLP are not different between elderly and young adult (*24*). Since urinary 4-PIC did not change in elderly adults (*24*) or aged rats, one of the possibilities to explain age-related PLP decrease may the regulation of 4-PIC production in the liver. Further study will elucidate the mechanism of age-related vitamin B_6 metabolism.

The framingham cohort study showed that prevalence of low serum cobalamin concentration in elderly people was 2.3 times higher than that in control young subjects (*27*). Vitamin B_{12} is different from other B-group vitamin because of its mechanism of absorption. Free vitamin B_{12} binds with haptocorrin and gastric intrinsic factor (IF) in the stomach and the duodenum, respectively, and this IF-vitamin B_{12} complex attaches

to the intestinal IF-vitamin B_{12} receptor in the ileal mucosa, and vitamin B_{12} enters the enterocyte (*28*). The high prevalence of mild cobalamin deficiency in healthy older subjects can be partly explained by inadequate vitamin B_{12} intake or severe atrophic gastritis (*29*). Since we did not measure indices for atrophic gastritis or IF excretion in the present study, whether gastric function in aged rats decreased vitamin B_{12} status is unclear. Interestingly, the urinary recovery rate of vitamin B_{12} dramatically increased to 130% at 80 wk old. Vitamin B_{12} binds to carrier protein transcobalamin (TC) in serum (*30*), and TC–vitamin B_{12} complex is reabsorbed in the proximal convoluted tubule via a receptor-mediated system (*28*). Megalin is an essential receptor for reabsorption of TC–vitamin B_{12} complex in the proximal tubule (*31*). Although little is known about how aging affects vitamin B_{12} absorption or megalin's function, decreased plasma vitamin B_{12} level in aged rats may be explained by enhancing urinary loss.

In the present study, contrary to the low vitamin B_6, vitamin B_{12} and folic acid status in aged rats, plasma, liver and urine biotin levels in aged rats were higher than those in young rats. Consistent with our results, a previous report showed that plasma biotin level in 24-mo old rats was higher than those in 3- and 12 mo old rats (*32*). This age-related alteration of biotin status is due to an increase of biotin transport in the intestine (*32*). An epidemiological study for elderly Japanese people showed that the mean serum biotin level in elderly people aged over 65 y was same as that in reference people, while prevalence of high serum biotin concentration in elderly was higher than that in reference subjects (*33*).

Niacin is a unique water-soluble vitamin provided from nicotinic acid and nicotinamide, and synthesized from amino acid tryptophan. Since the amount of de novo synthesized nicotinamide derivatives from tryptophan is almost the same as intakes of nicotinamide and nicotinic acid in the Japanese population (*34*), to determine the conversion ratio of tryptophan to nicotinamide is important to calculate niacin equivalent. The rats were given a niacin-free diet in the present experiment, because to assess the age-related alteration of tryptophan-nicotinamide metabolism was difficult in rats fed with a niacin-containing diet. Weaning rats need 1 mg (8 μmol) of niacin in 100 g of diet or 85 mg (420 μmol) of tryptophan in 100 g of niacin-free diet for maximum growth (*35*, *36*). Since the 20% casein diet used in the present study contains 230 mg (1.1 mmol) of tryptophan in 100 g of diet, and young rats convert 2% of tryptophan into nicotinamide in molar basis (*37*), the 20% casein diet contains enough tryptophan to supply nicotinamide for maximum growth in rats. Therefore our findings in the rats fed with the niacin-free 20% casein diet are considered to be applied to rats fed with niacin-containing diet. In the present study, the conversion ratio of tryptophan to nicotinamide was 1.6% at 14 wk old, gradually decreased to 0.8% until 51 wk old, and then kept this level at over 51 wk old. Blood NAD and liver nicotinamide levels in aged rats were the same as those in young rats, and these results suggested that the low conversion ratio was still enough to maintain niacin status for the aged rats fed with 20% casein diets. Our reports showed that urinary excretion of nicotinamide metabolites and blood NAD level in elderly Japanese were not different from those in young Japanese (*38*, *39*). However, there is no report to investigate the tryptophan-nicotinamide metabolism in elderly people.

In conclusion, aging alters metabolism of vitamin B_6, vitamin B_{12}, tryptophan-nicotinamide, folic acid and biotin in rats. A strong association between homocysteine concentration and vitamin B_6, vitamin B_{12} and folate status was found (*40*), and many researchers have focused on the association of homocysteine and several diseases such as cardiovascular disease, depression, cognitive impairment, pregnancy complications and osteoporosis (*41*). Our findings suggest that aged rats can be useful models to investigate aging-related B-group vitamin metabolism.

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資　料

水溶性ビタミン混合剤投与中止1週間後の尿中水溶性ビタミン排泄量

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福渡　努，柴田　克己

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Urinary Excretion of Water-Soluble Vitamins One Week After Stopping Administration of the Water-soluble Vitamin Mixture

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The number of people taking large amounts of vitamin supplements is increasing in Japan. Taking large amounts of water-soluble vitamins is not considered risky because excess water-soluble vitamins are quickly excreted in the urine. In the present study, we investigated the clearance of water-soluble vitamins in Japanese. In the first week, six young female subjects ate the diet. In the second week, they ate the same diet with 6 times the recommended water-soluble vitamin dose based on the Dietary Reference Intake (DRI) for Japanese. In the third week, they ate the same diet without the vitamin mixture. The 24-h urine samples were collected every week. All water-soluble vitamins except vitamin B_{12} in 24-h urine samples were measured. The urinary excretion of nicotinamide metabolites, biotin, and ascorbic acid in the third week was same as that in the first week. Urinary thiamin in the third week was 4.4 times higher than in the first week, riboflavin was 3.4 times, vitamin B_6 metabolite 4-pyridoxic acid 2.0 times, pantothenic acid 2.4 times, and folic acid 3.1 times.

Key words: vitamin mixture, human, urine, excess, water-soluble vitamins

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緒　言

ある種の疾病がビタミン欠乏に起因することが20世紀初頭から半世紀をかけて明らかにされてきた[1]．20世紀の後半から現在にかけては，ビタミン欠乏症を予防するにはどの程度のビタミン摂取が必要であるかが明らかにされつつある[2]．このような中で，20世紀末には，ビタミンCに欠乏症である壊血病を予防する生理機能だけではなく，新たに抗酸化作用が見いだされ，この抗酸化作用を通じて疾病予防が期待できることが明らかにされたことにより[3)-6)]，あらためてビタミンの機能が注目されてきた．

このことがきっかけとなり，必要量を超えた各種ビタミンを摂取するヒトが増えてきた．水溶性ビタミンといっても，化学構造上の共通性はないため，異化代謝経路もさまざまであると推察される．また，大量に摂取した後のクリアランスの速度も異なることが予想されるが，未だ明確なデータはみあたらない．また，疫学調査において，ビタミン剤の摂取が無いときのビタミンの尿中排泄量を調べるうえで，ビタミン剤の補給を中止する期間を知る

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必要がある．本研究では，ヒトにおける各水溶性ビタミンのクリアランスを比較した．

実験方法

被験者

被験者は，あらかじめ実験内容の説明を受け，書類にて，実験への参加を希望した女子学生 6 名で，喫煙，飲酒の習慣がなく，朝食など規則正しい食習慣をもつ者である．被験者の身体的特徴を Table 1 に示した．本研究は，滋賀県立大学倫理審査委員会において承認を受け，ヘルシンキ宣言の精神に則って行われた．

食　事

2 種類の規定食を摂取させた．その栄養素成分は Table 2 と Table 3 に示した．ビタミン B_{12} を除く 8 種類の水溶性ビタミンは実測値である．他は五訂増補日本食品標準成分表を用いて計算した [7]．

実験計画

概略を Fig. 1 に示した．規定食摂取時の第 1 週の Day 4 の尿を採取・測定し，対照値とし，Data 1 とした．Day 5 ～ Day 7 は自由食としたが，日本人の食事摂取基準 (2005 年版) に示された量の約 6 倍量の水溶性ビタミン混合（ビタミン B_{12} を除く）を投与した．第 2 週の Day 1 ～ Day 4 は規定食に加えて日本人の食事摂取基準 (2005 年版) に示された量の約 6 倍量の水溶性ビタミン混合 (Table 4) を投与した．第 2 週の Day 4 の尿を採取・測定し，Data 2 とした．Day 5 ～ Day 7 は自由食を摂取させた．但し，ビタミン混合の付加はしなかった．第 3 週は，クリアランスを知るために，第 1 週と同じ規定食を摂取させ，Day 4 の尿を採取・測定し，Data 3 とした．なお，付加量を食事摂取基準に示されたビタミン量を各々約 6 倍量とした理由は，明確に尿中排泄量の増大が認められ，かつ 1 週間適度の間投与し続けても過剰摂取による健康障害が現れないと思われた量と考えたからである．

蓄尿中の尿は氷中に保存し，24 時間尿の採取が終わると，直ちに容量を測定した．ビタミン B_1，ビタミン B_2，ビタミン B_6 異化代謝産物である 4-ピリドキシン酸，ニコチンアミド異化代謝産物である N^1-メチルニコチンアミド (MNA)，N^1-メチル-2-ピリドン-5-カルボキサミド (2-Py)，N^1-メチル-4-ピリドン-3-カルボキサミド (4-Py) の測定には，尿 9 mL に 1 mol/L HCl を 1 mL 添加し，-20℃で保存したサンプルを用いた．ビタミン C の測定には，尿 5 mL に 10％メタリン酸 5 mL を加え，-20℃で保存したサンプルを用いた．葉酸の測定には，尿 8.1 mL に 1 mol/L L-アスコルビン酸溶液 0.9 mL を添加し，-20℃で保存したサンプルを用いた．パントテン酸とビオチンの測定には，無処理尿を -20℃で保存したサンプルを用いた．

分析方法

ビタミン B_1

定量の標準品として使用したチアミン塩酸塩は和光純薬工業株式会社 (大阪) から購入した．尿中のビタミン B_1 の定量方法は文献 8 に示した HPLC 法で行った．値はチアミンとして示した．

ビタミン B_2

定量の標準品として使用したリボフラビンは和光純薬工業株式会社 (大阪) から購入した．尿中のビタミン B_2 の定量方法は，文献 9 に記載された HPLC 法に従って測定した．

ビタミン B_6 異化代謝産物 4-ピリドキシン酸

定量の標準品として使用した 4-ピリドキシン酸は Sigma Chemical Company (米国) から購入した．尿中の 4-ピリドキシン酸の定量方法は文献 10 に記載された HPLC 法を用いて測定した．

ニコチンアミド異化代謝産物 MNA，2-Py，4-Py

MNA 定量の標準品として使用した MNA 塩酸塩は東京化成工業株式会社 (東京) から購入した．尿中の MNA の定量は，MNA を強アルカリ性下でアセトフェノンと縮合させることにより蛍光物質に変換した後測定する HPLC 法を用いた [11]．

2-Py 定量の標準品として使用した 2-Py は Pullman と Colowick[12] の方法にしたがって合成した．4-Py 定量の標準品として使用した 4-Py は Shibata ら [13] の方法にしたがって合成した．尿中の 2-Py および 4-Py は弱アルカリ性下で，

Table 1. Characteristics of the Subjects

Subjects	Age (Yr)	Height (cm)	Body weight (kg)	BMI
Female 1	21	161.0	50.0	19.3
Female 2	21	161.0	52.5	20.3
Female 3	21	162.0	46.0	17.5
Female 4	22	154.0	48.0	20.2
Female 5	21	160.5	53.0	20.6
Female 6	21	165.0	52.5	19.3
Mean	21.2	160.6	50.3	19.5
SD	0.4	3.6	2.9	1.1