Table 4. Urinary excretions and relative availability of water-soluble vitamins in Experiment 1

Vitamins	Data 1 (μmol/day)	Data 2 (μmol/day)	Data 3 (μmol/day)	Urinary excretion rate for vitamins in the test diet (%)	Urinary excretion rate for free vitamins (%)	Relative availability (%)
Vitamin B_1	0.698±0.372	3.437±1.556	2.628±1.198	21.4±11.4	44.7±20.4	58±23
Vitamin B_2	0.515±0.228	6.414±1.041	5.899±0.947	32.3±14.3	64.0±10.3	50±22
Vitamin B_6	2.33±0.52	10.70±1.05	8.37±1.11	39.3±8.7	48.2±6.4	84±24
Niacin	88.7±37.7	438.0±91.5	349.3±77.3	42.3±18.0	76.2±16.9	57±22
Pantothenic acid	17.6±2.6	68.6±9.0	51.0±8.7	40.1±5.9	59.2±10.1	70±16
Folate	0.019±0.004	0.159±0.047	0.140±0.045	5.2±1.1	10.8±3.5	52±20
Biotin	0.098±0.024	0.289±0.046	0.191±0.25	58.6±14.0	67.5±8.7	86±13
Vitamin C	475±56	879±57	408±40	79.9±9.5	79.7±7.8	101±16

Data 1: The values are urinary excretions of vitamins when only the diet is fed to the subjects. Data 2: The values are urinary excretions of vitamins when the diet and vitamin mixtures are fed to the subjects. Data 3: The values are calculated "Data 2" − "Data 1." Values are means±SD for 9 female subjects.

12の2回目の尿から翌日のDay 13の1回目までの尿を蓄尿し，これをDay 12の1日尿とした．

(4) 分析方法

尿中のチアミン[4]，リボフラビン[5]，ビタミンB_6代謝産物4-ピリドキシン酸（4-PIC）[6]はHPLC法により測定した．ニコチンアミド（Nam）[7]，N^1-メチルニコチンアミド（MNA）[8]，N^1-メチル-2-ピリドン-5-カルボキサミド（2-Py）[7]，N^1-メチル-4-ピリドン-3-カルボキサミド（4-Py）[7]はHPLC法により測定し，これらの合計を総ニコチンアミド代謝産物とした．パントテン酸[9]，葉酸[10]，ビオチン[11]は微生物学的定量法により測定した．ビタミンCは，アスコルビン酸，デヒドロアスコルビン酸，2,3-ジケトグロン酸の合計としてHPLC法により測定した[12]．

食事中のビタミンB_1，ビタミンB_2，ナイアシン，パントテン酸，葉酸，ビオチンは，結合型を遊離型に完全に消化したのち，上記の方法で測定した．ビタミンCは上記のHPLC法により測定した．ビタミンB_6は結合型を遊離型に完全に消化したのち，微生物学的定量法により測定した[13]．

(5) 相対利用率の計算方法

相対利用率の計算方法は前報に記載した[3]．簡略に記すと，規定食摂取時の水溶性ビタミン排泄量（データ1）を規定食中の水溶性ビタミン量で割り，規定食摂取時の水溶性ビタミン排泄率を求めた．遊離型水溶性ビタミン付加時の水溶性ビタミン排泄量（データ2）とデータ1から，遊離型水溶性ビタミン付加による増加分（データ3）を求めた．データ3を遊離型水溶性ビタミン量で割り，遊離型水溶性ビタミンの排泄率を求めた．規定食中の水溶性ビタミンの相対利用率は，規定食摂取時の水溶性ビタミン排泄率を遊離型水溶性ビタミンの排泄率で割って求めた．

3. 結　果

(1) 実験1

実験1では，成人女性9名を対象として，パンを主食とした食事に含まれる水溶性ビタミンの相対利用率を求めた．Table 1に示した栄養素組成の食事を女性に摂取させたときの水溶性ビタミンの相対利用率をTable 4に示した．実験1で用いた食事における各ビタミンの相対利用率は，ビタミンB_1では58±23%，ビタミンB_2では50±22%，ビタミンB_6では84±24%，ナイアシンでは57±22%，パントテン酸では70±16%，葉酸では52±20%，ビオチンでは86±13%，ビタミンCでは101±16%であった．

(2) 実験2

実験2では，成人女性5名を対象として実験1の再現性について確認するとともに，成人男性9名をも対象とすることにより相対利用率の男女間の比較を行った．Table 2に示した栄養素組成の食事を男性に，Table 3に示した食事を女性に摂取させたときの水溶性ビタミンの相対利用率をTable 5に示した．実験2で用いた食事における各ビタミンの相対利用率は，ビタミンB_1は男性で48±20%，女性で55±10%，ビタ

Table 5. Urinary excretions and relative availability of water-soluble vitamins in Experiment 2

Vitamins		Data 1 (μmol/day)	Data 2 (μmol/day)	Data 3 (μmol/day)	Urinary excretion rate for vitamins in the test diet (%)	Urinary excretion rate for free vitamins (%)	Relative availability (%)
Vitamin B_1	Male	0.554±0.207	2.654±0.480	2.100±0.422	13.9±5.2	29.5±5.9	48±20
	Female	0.731±0.101	3.474±0.569	2.743±0.535	20.9±2.9	38.5±7.5	55±10
	Total	0.628±0.188	2.995±0.650	2.368±0.558	16.8±5.5	33.2±7.8	51±17
Vitamin B_2	Male	0.605±0.347	4.036±1.612	3.431±1.313	19.5±11.2	37.4±14.3	51±17
	Female	0.394±0.117	4.563±1.770	4.170±1.674	18.5±5.5	45.4±18.2	43±9
	Total	0.517±0.288	4.256±1.622	3.739±1.450	19.1±8.9	40.8±15.8	47±14
Vitamin B_6	Male	3.48±0.24	14.53±1.14	11.05±1.07	58.2±4.0	63.6±6.2	92±10
	Female	2.84±0.54	13.48±1.11	10.64±0.76	53.9±10.3	61.2±4.4	88±16
	Total	3.21±0.50	14.09±1.21	10.88±0.94	56.4±7.2	62.6±5.4	90±12
Niacin	Male	64.4±12.8	261.9±19.0	197.5±11.1	26.9±5.3	43.1±2.4	62±11
	Female	47.9±7.9	243.1±47.8	195.2±47.1	24.1±4.0	42.6±10.3	59±18
	Total	57.5±13.6	254.1±33.5	196.5±29.6	25.7±4.8	42.9±6.4	61±14
Pantothenic acid	Male	20.2±2.3	65.7±3.5	45.5±2.2	38.0±4.4	52.5±2.6	72±9
	Female	13.9±2.4	63.9±7.5	50.0±5.6	35.8±6.2	57.6±6.4	62±8
	Total	17.6±4.0	65.0±5.3	47.4±4.4	37.1±5.1	54.6±5.1	68±10
Folate	Male	0.033±0.006	0.212±0.092	0.179±0.089	5.8±1.0	13.8±6.9	51±22
	Female	0.034±0.005	0.255±0.079	0.221±0.082	6.8±0.9	17.1±6.3	47±23
	Total	0.033±0.005	0.230±0.086	0.196±0.085	6.3±1.1	15.2±6.6	49±21
Biotin	Male	0.144±0.019	0.350±0.085	0.206±0.079	53.4±7.2	71.6±28.5	81±22
	Female	0.093±0.011	0.294±0.052	0.201±0.050	58.7±7.0	70.9±17.6	86±21
	Total	0.123±0.031	0.326±0.076	0.204±0.066	55.6±7.3	71.3±23.5	83±21
Vitamin C	Male	823±89	1,316±121	493±98	89.5±9.7	96.4±19.3	96±22
	Female	844±54	1,356±97	512±65	91.7±5.9	100.2±12.8	93±11
	Total	832±74	1,332±109	501±83	90.4±8.1	98.0±16.3	95±18

Data 1: The values are urinary excretions of vitamins when only the diet is fed to the subjects. Data 2: The values are urinary excretions of vitamins when the diet and vitamin mixtures are fed to the subjects. Data 3: The values are calculated "Data 2" − "Data 1." Values are means±SD for 7 for male subjects, 5 for female and 12 for total.

ミンB_2は男性で51±17%，女性で43±9%，ビタミンB_6は男性で92±10%，女性で88±16%，ナイアシンは男性で62±11%，女性で59±18%，パントテン酸は男性で72±9%，女性で62±8%，葉酸は男性で51±22%，女性で47±23%，ビオチンは男性で81±22%，女性で86±21%，ビタミンCは男性で96±22%，女性で93±11%であった．実験2の女性の相対利用率は，実験1で得られた値と近似したものであった．いずれの水溶性ビタミンにおいても，相対利用率に性差は認められなかった．男女計12名の相対利用率は，ビタミンB_1は51±17%，ビタミンB_2は47±14%，ビタミンB_6は90±12%，ナイアシンは61±14%，パントテン酸は68±10%，葉酸は49±22%，ビオチンは83±21%，ビタミンCは95±18%であった．

4. 結　論

本研究では，成人男性7名（19～25歳）および成人女性のべ14名（21～25歳）を対象とし，以下の結果を得た．パンを主食としたときの水溶性ビタミンの相対利用率は，概ね次のような値であった．ビタミン

B_1は55%，ビタミンB_2は50%，ビタミンB_6は85%，ナイアシンは60%，パントテン酸は70%，葉酸は50%，ビオチンは85%，ビタミンCは95%であった．なお，ビタミンB_{12}については，主要な排泄経路が尿ではなく腸管であることから[14]，本法では相対利用率を測定することはできないので，他の方法を考案しなければならない．

これらの値は，前報[3]のめしを主食としたときの相対利用率と近似した値であった．

なお，米国人を被験者とし，脂肪エネルギー含量が40%程度の食事ではビタミンB_6が75%，パントテン酸が50%程度と報告されている[15]．また，葉酸の生体利用率に関しては，50%程度と報告されている[16]．

本研究は，平成16年度～18年度厚生労働科学研究費補助金・循環器疾患等生活習慣病対策総合研究事業・日本人の食事摂取基準（栄養所要量）の策定に関する研究（主任研究者　柴田克己），および平成19年度厚生労働科学研究費補助金・循環器疾患等生活習慣病対策総合研究事業・日本人の食事摂取基準を改定するためのエビデンスの構築に関する研究―微量栄養素と多量栄養素摂取量のバランスの解明―（主任研究者　柴田克己）を受けて行ったものである．関係各位に謝意を表する．

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Comparison of the Nicotinamide Catabolism among Rat Strains

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We discovered markedly differing catabolism of nicotinamide among rat strains. We compared the catabolism of nicotinamide and also that of the other tryptophan-nicotinamide and water-soluble vitamins among the four strains, Wistar, Sprague-Dawley (SD), August-Copenhagen Irish (ACI) and Fischer 344. The major urinary catabolite of nicotinamide was N^1-methyl-4-pyridone-3-carboxamide in Wistar, SD and ACI, and N^1-methylnicotinamide in Fischer rats. This phenomenon was attributed to the enzyme activity involved in the reaction of N^1-methylnicotinamide to N^1-methyl-4-pyridone-3-carboxamide being much lower in Fischer than in the other three strains. With the water-soluble vitamins, this specific phenomenon was only observed in the catabolism of vitamin B_6; the urinary catabolite, 4-pyridoxic acid, was much lower too. It was found for the first time that the activities of oxidase were lower in Fischer than in the other strains. This study showed that Wistar, SD, ACI strains had similar water-soluble vitamin metabolism including nicotinamide catabolism.

Key words: nicotinamide; rat strain; urine; vitamin B_6; water-soluble vitamin

Nicotinamide (Nam) is a unique vitamin because mammals including humans can synthesize it from the essential amino acid, tryptophan. The Trp-Nam metabolic pathway (see Fig. 1) is very important, because over 400 enzymes need NAD or NADP as coenzymes, and elucidation of the mechanism regulating the pathway would provide useful information for maintaining the best health. For example, NAD and NADP are involved in numerous cellular reactions, and are a substrate for the reaction of poly(ADP-ribosyl)ation.[1] Poly(ADP-ribosyl)ation catalyzed by chromatin-associated poly(ADP-ribose)polymerase (PARP-1; EC 2.4.2.30) is related to the regulation of gene expression, cellular differentiation, apoptosis, DNA replication and repair.[2] The intermediates involved on the Trp-Nam pathway are excreted to the urine, and the amount of these substances reflects the changes in the activities of enzymes involved along the pathway. For example, the urinary excretion of N^1-methylnicotinamide (MNA), N^1-methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py), which are nicotinamide catabolites, can serve as surrogate biological markers of an adequate amino acid intake.[3] The urinary ratio of (2-Py + 4-Py)/MNA is high when rats eat an adequate amino acid diet, and the ratio is low when they eat an inadequate diet, because the activity of liver MNA oxidases decrease with an excess and lack of essential amino acids.[3]

Studies on Trp-Nam metabolism are often performed on humans, but when we cannot use humans as subjects, we almost exclusively use the Wistar strain of rats. We have believed that the findings obtained when using the Wistar strain of rats would be common to all strains of rat. We discovered in this study that the catabolism of nicotinamide markedly differed among rat strains. We therefore investigated whether the urinary excretion of the metabolites involved on the Trp-Nam pathway and the metabolism of water-soluble vitamins differed among strains or not. We measured the urinary excretion of the Trp-Nam metabolites and water-soluble vitamins by the four strains of rat, and compared the metabolism.

Materials and Methods

Chemicals. NAD^+ was purchased from Sigma Chemical Company (St. Louis, MO, USA). Vitamin-free milk casein, sucrose, and L-methionine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized cornstarch, the mineral mixture (AIN-93M)[4] and the vitamin mixture (AIN-93-VX containing choline bitartrate)[4] were obtained from Oriental Yeast Co. (Tokyo, Japan).

Thiamin hydrochloride (vitamin B_1, $C_{12}H_{17}ClN_4OS$–HCl = 337.27), riboflavin (vitamin B_2, $C_{17}H_{20}N_4O_6$ = 376.37), cyanocobalamin (vitamin B_{12}, $C_{63}H_{88}CoN_{14}O_{14}P$ = 1355.40), nicotinamide (Nam, $C_6H_6N_2O$ = 122.13), calcium pantothenate (PaA-Ca, $C_{18}H_{32}N_2O_{10}$–Ca = 476.54), folic acid (FA, $C_{19}H_{19}N_7O_6$ = 441.40), D(+)-biotin ($C_{10}H_{16}N_2O_3S$ = 244.31), L(+)-ascorbic acid (AsA, $C_6H_8O_6$ = 176.13), anthranilic acid (AnA, $C_7H_7O_2N$ = 137.14) and quinolinic acid (QA, $C_7H_5O_4N$ = 167.13) were purchased from Wako Pure Chemical Industries. 4-Pyridoxic acid (4-PIC, a vitamin B_6 catabolite, $C_8H_9NO_4$ = 183.16) was made by ICN Pharmaceuticals (Costa Mesa, CA, USA) and obtained through Wako Pure Chemical Industries. Xanthurenic acid (XA, $C_{10}H_7O_4N$ = 205.17), kynurenic acid (KA, $C_{10}H_9O_4N$ = 207.19), 3-hydroxyantranilic acid (3-HA, $C_7H_7O_3N$ = 154.14) and MNA chloride ($C_7H_9N_2O$–HCl = 159.61) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). 2-Py ($C_7H_8N_2O_2$ = 152.15) and 4-Py ($C_7H_8N_2O_2$ = 152.15) were synthesized by the methods of Pullman and Colowick[5] and Shibata *et al.*,[6] respectively. All other chemicals used were of the highest purity available from commercial sources.

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Abbreviations: Nam, nicotinamide; PARP-1, poly(ADP-ribose)polymerase; MNA, N^1-methylnicotinamide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide; PaA, pantothenate; FA, folic acid; AsA, L(+)-ascorbic acid; AnA, anthranilic acid; QA, quinolinic acid; 4-PIC, 4-pyridoxic acid; XA, xanthurenic acid; KA, kynurenic acid; 3-HA, 3-hydroxyantranilic acid; SD, Sprague-Dawley; ACI, August-Copenhagen Irish; F344, Fischer 344; HPLC, high-perfomance liquid chromatography

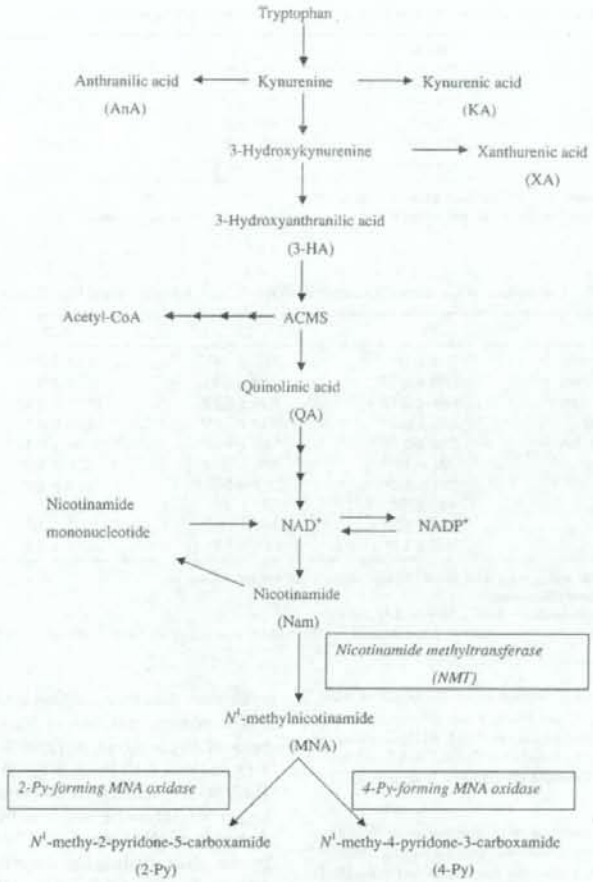

Fig. 1. Metabolic Pathway of Tryptophan to Nicotinamide.

Table 1. Composition of the Diet

	(g/100 g of diet)
Vitamin-free milk casein	20.0
L-Methionine	0.2
Gelatinized cornstarch	46.9
Sucrose	23.4
Corn oil	5.0
Mineral mixture (AIN-93M)	3.5
NiA-free vitamin mixture (AIN-93-VX containing chorine bitartrate)	1.0

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.

The animal room was maintained at a temperature of around 22 °C and at about 60% humidity with a 12-h light (06:00–18:00)/12-h dark (18:00–06:00) cycle. Body weight and food intake were measured daily at around 09:00 a.m., and the diet and water were renewed daily.

Five male rats each of the Wistar, Sprague-Dawley (SD), August-Copenhagen Irish (ACI) and Fischer 344 (F344) strains (8 weeks old each) were obtained from Clea Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; Clea Japan). They had free access to the diet (Table 1) for 11 d. Urine samples on the last day (09:00 a.m.–09:00 a.m.; 24-h urine) were collected in amber bottles with 1 ml of 1 M HCl and stored at −20 °C until needed for use. The rats were killed by decapitation after the last urine samples had been collected, a 10-μl sample of blood was taken from the carotid artery for measuring NAD and NADP, and the liver was removed for measuring the enzyme activities involved in the Nam catabolism.

Analysis. The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the high-performance liquid chromatographic (HPLC) method of Shibata *et al.*,[6] while the content of MNA in the urine was measured by the HPLC method of Shibata.[7] The contents of KA,[8] XA,[9] 3-HA,[9] AnA,[10] and QA[11] in the urine were measured by using the HPLC methods.

The concentrations of NAD ($NAD^+ + NADH$) and NADP ($NADP^+ + NADPH$) in the blood were respectively measured by the colorimetric method of Shibata and Murata,[12] and by the method of Shibata and Tanaka.[13]

Thiamin in the urine was measured by the HPLC post-labeled fluorescence method of Fukuwatari *et al.*[14]

The urinary concentration of riboflavin was analyzed according to the method of Ohkawa *et al.*[15] The urinary excretion of 4-PIC, which is a catabolite of vitamin B_6, was determined according to the method described by Gregory and Kirk.[16] The cyanocobalamin concentration in the urine was assayed by the microbiological method with *Lactobatillus delbueckii* subsp. *lactis* ATCC 7830.[17] The content of free PaA in the urine was directly measured by using *Lactobacillus plantarum* ATCC 8014.[18] The concentrations of urine folates were

Table 2. Comparison of the Body Weight Gain, Food Intake and Food Efficiency Ratio among Four Strains of Rats

	Wistar	SD	ACI	F344
Initial body weight (g)	210 ± 2^{a}	289 ± 2^{b}	173 ± 2^{c}	143 ± 1^{d}
Final body weight (g)	255 ± 3^{a}	356 ± 3^{b}	197 ± 2^{c}	184 ± 2^{d}
Body weight gain (g/10 d)	45 ± 1^{a}	67 ± 3^{b}	24 ± 1^{c}	41 ± 2^{a}
Food intake (g/10 d)	171 ± 2^{a}	223 ± 9^{b}	127 ± 4^{c}	133 ± 3^{c}
Food efficiency ratio*	26.6 ± 0.9^{a}	$29.8 \pm 0.4^{a,c}$	19.0 ± 0.4^{b}	31.0 ± 1.9^{c}

*Food efficiency ratio, body weight gain (g/10 d)/food intake (g/10 d) × 100

Each value is the mean ± SEM for five rats; values with different superscript letters in the same row are statistically different at $p < 0.05$, as determined by Tukey's multiple-comparison test.

Table 3. Comparison of the Urinary Excretion of Water-Soluble Vitamin among Four Strains of Rats

	Wistar	SD	ACI	F344
Food intake* (g/d)	17.4 ± 1^{a}	23.3 ± 1.5^{b}	13.2 ± 0.5^{c}	$15.4 \pm 0.3^{a,c}$
Vitamin B_1 (nmol/g of diet)	5.06 ± 0.77	3.69 ± 0.33	4.19 ± 0.49	4.59 ± 0.20
Vitamin B_2 (nmol/g of diet)	8.64 ± 0.19^{a}	7.40 ± 0.70^{a}	13.37 ± 0.83^{b}	6.88 ± 0.24^{a}
4-PIC** (nmol/g of diet)	9.42 ± 0.54^{a}	10.52 ± 1.05^{a}	8.02 ± 0.74^{a}	2.27 ± 0.39^{b}
Vitamin B_{12} (pmol/g of diet)	$2.26 \pm 0.15^{a,c}$	1.69 ± 0.10^{a}	3.96 ± 0.34^{b}	2.87 ± 0.13^{c}
SUM*** (nmol/g of diet)	$286 \pm 43^{a,b}$	402 ± 36^{a}	276 ± 16^{b}	$343 \pm 20^{a,b}$
PaA (nmol/g of diet)	43.4 ± 1.2^{a}	25.1 ± 2.6^{b}	50.5 ± 3.6^{a}	50.8 ± 4.0^{a}
FA (pmol/g of diet)	442 ± 50^{a}	295 ± 35^{b}	156 ± 14^{c}	87 ± 6^{c}
Biotin (pmol/g of diet)	$207 \pm 12^{a,b}$	$190 \pm 16^{a,b}$	223 ± 28^{b}	138 ± 17^{a}
AsA (nmol/g of diet)	38.8 ± 2.8^{a}	50.7 ± 8.5^{a}	45.4 ± 2.7^{a}	21.3 ± 1.1^{b}

*This value is for the food intake during urine collection (09:00 a.m. on day 9 to 09:00 a.m. on day 10).

**4-PIC, 4-pyridoxic acid, a vitamin B_6 catabolite

***SUM, nicotinamide and its metabolites = Nam + MNA + 2-Py + 4-Py

Each value is the mean ± SEM for five rats; values with different superscript letters in the same row are statistically different at $p < 0.05$, as determined by Tukey's multiple-comparison test.

determined by the microbioassay method, using *Lactobacillus casei* ATCC 7469.[19] The content of free biotin in the urine was directly measured by using *Lactobacillus plantarum* ATCC 8014 according to the agar plate assay developed by Fukui *et al.*[20] Total AsA in the urine was determined by the HPLC method according to Kishida *et al.*[21]

Statistical analysis. The significance of differences in the mean concentration between the four strains was tested by using one-way ANOVA followed by Tukey's multiple-comparison test. GraphPad Prism (version 4; obtained from GraphPad Software, San Diego, CA, USA) was used for all statistical analyses.

Results

Body weight and food intake

Table 2 shows the initial and final body weights, body weight gain, food intake and food efficiency ratio during experiments with each strain. The initial body weight differed among the strains, and up to the final day. Since the size differed among the four strains, the food intake, body weight gain and food efficiency ratio differed too. Therefore, in this study the urinary excretion was corrected to 1 g of food intake.

Urinary excretion of vitamins

The urinary excretion of vitamin B_1, vitamin B_2, 4-PIC, vitamin B_{12}, Nam and its metabolites (SUM), PaA, FA, biotin and AsA per g of diet, respectively, is shown in Table 3. The urinary excretion of vitamin B_1 did not differ among the strains. The urinary excretion of vitamin B_2 was significantly higher by ACI than by the other strains. The excretion of both 4-PIC and AsA was significantly lower by F344 than by the other strains. The excretion of PaA was significantly lower by SD than by the other strains. The urinary excretion of vitamin B_{12}, FA, biotin and SUM differed among some strains.

Urinary excretion of Nam and its metabolites

The urinary excretion of Nam, MNA, 2-Py and 4-Py per g of diet is shown in Table 4. The excretory ratios of 4-Py/2-Py and (2-Py + 4-Py)/MNA are also shown in Table 4. The urinary excretion of Nam was significantly higher by SD and F344 than by ACI and Wistar. The excretion of MNA was 6- to 17-fold higher by F344 than by the other strains. On the contrary, the excretion of 4-Py by F344 was 1.7–2.4% of that by the other strains. The excretion of 2-Py was significantly higher by ACI and SD than by F344 and Wistar.

The results for the Wistar strain showed 4-Py to be the most abundant metabolite, accounting for 83.7% of the urinary excretion of SUM, and was followed by MNA, 6.0%; 2-Py, 5.5%; and Nam, 4.8%. For the SD strain, 4-Py was the most abundant metabolite, accounting for 73.6% of SUM, and followed by MNA, 12.1%; Nam 7.8%; and 2-Py, 6.5%. For the ACI strain, 4-Py was the most abundant metabolite, accounting for 76.4%, and followed by 2-Py, 10%; MNA, 8.6%; and Nam, 5.0%. For the F344 strain, MNA was the most abundant metabolite, accounting for 85.1%, and followed by Nam, 8.0%; 2-Py, 5.4%; and 4-Py, 1.5%. Consequently, for F344, the excretory ratios of (2-Py + 4-Py)/MNA, which is an index of amino acid adequacy and possibly an index of liver MNA oxidase activity, and 4-Py/2-Py were significantly lower than for the other strains.

Urinary excretion of upper metabolites on the Trp-Nam pathway

The urinary excretion of AnA, KA, XA, 3-HA and QA per g of diet are shown in Table 4. The order of amount of AnA in urine of each strain was ACI, Wistar, F344, and SD, and a significant difference was observed between ACI and SD. The urinary excretion

Table 4. Comparison of the Urinary Excretion of the Metabolites on the Tryptophan-Nicotinamide Pathway among Four Strains of Rats

	Wistar	SD	ACI	F344
AnA (nmol/g of diet)	$3.99 \pm 0.10^{a,b}$	2.94 ± 0.22^{a}	4.63 ± 0.57^{b}	$3.49 \pm 0.13^{a,b}$
KA (nmol/g of diet)	78.9 ± 5.0^{a}	58.9 ± 2.4^{b}	34.7 ± 4.6^{c}	57.2 ± 2.7^{b}
XA (nmol/g of diet)	52.5 ± 1.7^{a}	59.3 ± 5.9^{a}	24.2 ± 3.4^{b}	35.3 ± 1.0^{b}
3-HA (nmol/g of diet)	3.98 ± 0.32^{a}	1.72 ± 0.35^{b}	5.05 ± 0.53^{a}	6.72 ± 0.39^{c}
QA (nmol/g of diet)	$28.8 \pm 2.1^{a,b}$	42.7 ± 8.0^{a}	15.3 ± 1.9^{b}	$27.2 \pm 5.2^{a,b}$
Nam (nmol/g of diet)	13.7 ± 1.7^{a}	31.5 ± 4.6^{b}	13.8 ± 0.7^{a}	27.5 ± 1.7^{b}
MNA (nmol/g of diet)	17.3 ± 2.0^{a}	48.5 ± 10.9^{a}	23.7 ± 2.0^{a}	292.2 ± 17.4^{b}
2-Py (nmol/g of diet)	15.6 ± 2.4^{a}	26.0 ± 1.5^{b}	27.7 ± 1.8^{b}	18.4 ± 1.3^{a}
4-Py (nmol/g of diet)	239 ± 37^{a}	296 ± 34^{a}	211 ± 12^{a}	5 ± 1^{b}
4-Py/2-Py	15.3 ± 0.4^{a}	11.3 ± 0.8^{b}	7.6 ± 0.1^{c}	0.3 ± 0.1^{d}
(2-Py + 4-Py)/MNA	14.58 ± 0.60^{a}	8.70 ± 2.30^{b}	$10.17 \pm 0.37^{a,b}$	0.08 ± 0.01^{c}
SUM* (μmol/d)	4.84 ± 0.42	9.29 ± 0.71	3.64 ± 0.14	5.30 ± 0.36
Trp intake** (μmol/d)	193 ± 11^{a}	258 ± 16^{b}	$147 \pm 6^{a,c}$	171 ± 3^{c}
Conversion ratio of Trp-Nam*** (%)	$2.58 \pm 0.38^{a,b}$	3.63 ± 0.32^{a}	2.49 ± 0.14^{b}	$3.10 \pm 1.77^{a,b}$

*SUM, Nam + MNA + 2-Py + 4-Py

**Trp intake (μmol/d), food intake (g/d) $\times 0.2 \times 0.875 \times 0.013 \times 1/204 \times 10^{6}$

***Conversion of Trp-Nam (%), SUM (mol/d)/Trp intake (mol/d) $\times$ 100

Each value is the mean ± SEM for five rats; values with different superscript letters in the same row are statistically different at $p < 0.05$, as determined by Tukey's multiple-comparison test.

Table 5. Comparison of the NAD and NADP Content in Whole Blood among Four Stains of Rats

	Wistar	SD	ACI	F344
$NAD^+ + NADH$ (nmol/ml of whole blood)	72.9 ± 2.6	70.0 ± 1.1	74.7 ± 2.2	73.5 ± 1.1
$NADP^+ + NADPH$ (nmol/ml of whole blood)	11.4 ± 0.5^{a}	11.7 ± 0.8^{a}	8.4 ± 0.7^{b}	11.4 ± 0.5^{a}

Each value is the mean ± SEM for five rats; values with different superscript letters in the same row are statistically different at $P < 0.05$, as determined by Tukey's multiple-comparison test.

of KA was significantly higher from Wistar than from the other strains, and significantly higher from SD and F344 than from ACI. The excretion of XA was significantly higher from SD and Wistar than from F344 and ACI. The 3-HA excretion was significantly higher from F344 than from the other strains, and higher from ACI and Wistar than from SD. The order of amount of excreted QA was SD, Wistar, F344, ACI, and a significant difference was observed between SD and ACI.

Conversion ratio of Trp to Nam

The conversion ratio of Trp to Nam is shown in Table 4. It was calculated from the Trp intake and urinary excretion of SUM. A significant difference was observed between the SD and ACI strains.

Concentration of NAD and NADP in whole blood

The concentrations of NAD ($NAD^+ + NADH$) and NADP ($NADP^+ + NADPH$) in whole blood are shown in Table 5. The concentration of NAD did not significantly differ among the strains. The concentration of NADP was significantly lower in ACI than in the other strains, but the total concentration of NAD and NADP did not significantly differ among the strains.

Enzyme activities involved in Nam catabolism

The enzyme activities of nicotinamide methyltransferase (NMT), 2-Py-forming MNA oxidase and 4-py-forming MNA oxidase in the liver are shown in Table 6. The enzyme activity of NMT was significantly higher in ACI than in the other strains. The activity of 2-Py-forming MNA oxidase was significantly higher in ACI than in the other strains, and in Wistar and SD than in F344. The activity of 4-Py-forming MNA oxidase in F344 was approximately 1/160 to 1/110 as high as in the other strains.

Discussion

We have previously studied the metabolism of the Trp-Nam pathway by using the Wistar strain of rats obtained from Clea Japan. Generally speaking, Nam is catabolized to 2-Py and 4-Py *via* MNA in rat liver, the most abundant catabolite being 4-Py which accounts for around 80% in the sum of Nam and its metabolites.[3,22] We discovered by accident that the catabolism of Nam extremely differed among rat species. We chose the four strains of Wistar, SD, ACI and F344 rats to represent normal rats, because these strains are commonly used for experimental purposes. We therefore investigated the metabolic characteristics of vitamins including Nam in the four strains of normal rats, Wistar, SD, F344 and ACI, obtained from Clea Japan.

We recognized that only the F344 rat had characteristic properties: the most abundant catabolite was MNA, accounting for 85%, and the amount of 4-Py was lower than that of 2-Py (middle part of Table 4). In fact, as shown in Table 6, the two MNA oxidase activities in F344 were extremely low compared with the case for

Table 6. Comparison of the Enzyme Activities in Nicotinamide Catabolism

	Wistar	SD	ACI	F344
Liver weight (g)	11.5 ± 0.2^a	16.1 ± 0.6^b	7.1 ± 0.1^c	7.8 ± 0.1^c
NMT* (nmol/h/g of liver)	77.5 ± 8.8^a	73.4 ± 5.4^a	125.2 ± 5.7^b	77.3 ± 4.3^a
2-Py-forming MNA oxidase (nmol/h/g of liver)	930 ± 95^a	753 ± 186^a	1421 ± 42^b	107 ± 55^c
4-Py-forming MNA oxidase (nmol/h/g of liver)	5047 ± 564^a	3327 ± 1004^a	4717 ± 327^a	31 ± 25^b

*NMT, nicotinamide methyltransferase

Each value is the mean ± SEM for five rats: values with different superscript letters in the same row are statistically different at $p < 0.05$, as determined by Tukey's multiple-comparison test.

the other three strains. Furthermore, the activity ratio of 4-Py-forming MNA oxidase/2-Py-forming MNA oxidase was also much lower in F344. This meant that the MNA oxidases in F344 were not only lower overall than in the other strains, but also that the 4-Py-forming MNA oxidase activity was lower than that of 2-Py-forming MNA oxidase. A similar phenomenon was observed with LEC rats[23] and the Wistar rats fed with an inadequate diet such as low in protein.[3,24] We had already clarified that the activity of 4-Py-forming MNA oxidase was affected by various nutrients.[25-27] So, we carried out a preliminary experiment on F344 rats fed with a high protein diet (40% casein) for 10 d to learn whether the activity of 4-Py-forming MNA oxidase increased or not. We had already shown that the activity increased when Wistar rats were fed with an appropriate diet such as 20% casein.[3] However, the result of the preliminary experiment was negative, and the activity of 4-Py-forming MNA oxidase in F344 rats did not increase even when fed with a 40% casein diet, and the urinary excretory ratio of (2-Py + 4-Py)/MNA did not change either. This result means that the catabolism of Nam in F344 would be rigidly controlled. In this connection, the urinary excretion of 4-PIC, a metabolite of vitamin B_6 pyridoxine and pyridoxal, was much lower in F344 than in the other three strains (Table 3). This result can be explained by the fact that the formation of 4-PIC is catalyzed by pyridoxal oxidase. The urinary excretion of AsA tended to be lower, which implies that L-gulonolactone oxidase would be lower in F344 than in the other strains. Several reports have supported the oxidases in F344 being lower. Duclos *et al.*[28] have reported that the cytochrome c oxidase activity in F344 was lower than that in Lewis rats, and Levy *et al.*[29] that the acyl-CoA oxidase activity in F344 was lower that than SD rats. These observations indicate that several oxidases in F344 were much lower than in the other strains of rats. This is characteristic of F344 rats.

Other metabolic characteristics in the water-soluble vitamins of F344 were not apparent, although trivial statistical differences were observed (Table 3). However, we do not disregard the phenomenon that the urinary excretion of folic acid was lower from F344 than from the other three strains.

Although the catabolic fate of Nam was extremely different between F344 and the other three strains, the sum of Nam and its metabolites was almost the same (Table 3). We therefore furthermore investigated the upper part of Nam metabolism, namely, the metabolites on the Trp-QA pathway. Such metabolites as AnA, KA, XA, 3-HA, and QA were marginally different, although this was trivial, because the maximum value for each did not differ 4-fold more than the minimum value. We therefore concluded that the metabolism of Trp-QA among the rats did not differ. In fact, the conversion ratio of Trp to Nam among the strains was no different (lower part of Table 4) and nor were the concentrations of NAD and NADP in the blood (Table 5).

Table 7. Comparison of the Urinary Excretory Pattern of Nam and Its Metabolites among Humans and the Four Strains

	Humans*	Wistar	SD	ACI	F344
Nam (%)	N.D.**	4.8	7.8	5.0	8.0
MNA (%)	35.2	6.0	12.1	8.6	85.1
2-Py (%)	56.3	5.5	6.5	10.0	5.4
4-Py (%)	8.5	83.7	73.6	76.4	1.5

*This data was taken from ref. 30.

**N.D., not detected

Each value is expressed as a percentage over the sum of Nam and its metabolites (Nam + MNA + 2-Py + 4-Py) in each animal.

Each value is the mean for eleven humans or five rats.

We compared the catabolism of Nam among humans and the four rat strains. Table 7 shows the urinary excretory pattern of Nam and its metabolites over the sum of them (Nam + MNA + 2-Py + 4-Py). In humans, Nam metabolized to MNA, and much of the MNA oxidized to 2-Py, the rate being approximately 65%.[30] In the Wistar, SD, and ACI strains, Nam was almost completely oxidized to 4-Py *via* MNA. In F344, Nam was catabolized to MNA, and a little of the MNA was catabolized into such pyridones as 2-Py and 4-Py, differing from the other strains. In the Wistar, SD, and ACI strains, MNA was efficiently oxidized into the pyridones like in humans, but the ratio of 2-Py/4-Py was the reverse. It was therefore found that the three strains apart from F344 would be suitable models for Trp-Nam metabolism instead of humans.

We further compared the metabolism of water-soluble vitamins among the Wistar, SD and ACI strains. Although trivial differences were apparent, nor essential distinction was observed (Tables 3–5). We concluded that the metabolism of water-soluble vitamins among the strains did not differ.

In summary, the Wistar strain, which our laboratory has usually used, had common metabolism and would reflect the result for the metabolism of the strains, expect for the Nam catabolism in F344 rats. The enzyme activities of various oxidases in F344 rats were extremely low compared with such other strains as Wistar, SD, and ACI. The reactions of MNA to 2-Py, MNA to 4-Py, and pyridoxal to 4-PIC were extremely weak in F344.

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Effects of UVA Irradiation on the Concentration of Folate in Human Blood

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Although it is well known that ultraviolet A (UVA) irradiation destroys folate, no definite conclusion for the biological degradation has yet been drawn. In the present study, we determined the effects of UVA exposure on the blood folate concentration *in vitro* and *in vivo*. UVA irradiation reduced the synthesized folate pteroylmonoglutamic acid (PGA) content in the blood, but not 5-methyltetrahydrofolate, a major folate form in the blood stream. Exposure to sunlight also decreased the plasma folate concentration in human subjects who took PGA prior to the exposure, but not in subjects who did not take PGA. These results suggest that UVA exposure destroyed PGA but not 5-methyltetrahydrofolate in human blood *in vivo*.

Key words: folate; ultraviolet A (UVA); pteroylmonoglutamic acid; 5-methyltetrahydrofolate; humans

Folate is a vitamin; therefore, folate deficiency, or impairment of the folate metabolism in humans, leads to several diseases such as megaloblastic anemia,[1] neural tube defects,[2] and increasing the risk of development of cardiovascular diseases.[3] It is well known that the requirement of folate increases in pregnant women.[4] Supplementation with folate during pregnancy is strongly recommended in many countries. Pteroylmonoglutamic acid (PGA) is a synthetic form of folate (Fig. 1) and is the oxidized and most stable form of the folates. Therefore, PGA is commonly used as a folate supplement. However, there is a deficit in masking vitamin B_{12} deficiency by PGA supplementation which is a more severe deficiency than folate deficiency.[5] Vorobey *et al.*[6] have reported that PGA in an aqueous solution was degraded by UVA. Furthermore, Der-Petrossian *et al.*[7] have reported that extracorporeal exposure of plasma to UVA during extracorporeal photophoresis led to photodegradation of folate. We have reported that the folate level in serum was higher in young Japanese women than in young men who had been given a vitamin mixture containing PGA.[8] We discussed that the phenomenon that a lower folate level in men would be a result of bathing in a lot of sunlight compared with women. On the other hand, the serum folate concentration of subjects exposed to UVA has been reported as unchanged.[9] No definite conclusion about the possibility of folate photodegradation *in vivo* has yet been drawn.

In vivo, folates exist mainly in the reduced form, for instance as 5-methyltetrahydrofolate (5-MTHF) (Fig. 1).[10] When PGA is taken, some appears in the blood stream in the form of PGA itself,[11] although a part of PGA is converted to 5-MTHF in the tissues of the small intestine. Staindal *et al.*[12] have reported that 5-MTHF absorbed less UVA when compared with PGA, and that 5-MTHF was not destroyed by UVA irradiation, but was by UVB and UVC. However, as UVB and UVC do not penetrate the atmosphere and reach the human skin, 5-MTHF in the blood is not destroyed by exposure to sunlight.

We have the hypothesis that PGA in blood would be destroyed, but not 5-MTHF, by exposure to sunlight. If humans do not take PGA, the blood folate concentration would not be decreased by exposure to sunlight, because the form of the blood folate is mainly 5-MTHF. On the contrary, if humans take PGA, the blood concentration would be decreased by exposure to sunlight, because the PGA taken appears as PGA itself in the blood stream. We conducted an experiment to prove this hypothesis, and as we were able to achieve some valid results.

Materials and Methods

Subjects. Healthy Japanese college students aged from 21 to 24 years old participated in the present experiments. They did not have regular use of medications or dietary supplements, or any habitual alcohol or cigarette consumption. This study was reviewed and approved by The Ethical Committee of the University of Shiga Prefecture.

Chemicals. PGA and 5-MTHF calcium salt were purchased from Wako Pure Chemical Industries (Osaka, Japan), and from Schircks Laboratories (Jona, Switzerland), respectively.

Experiment 1 (Change of PGA in an aqueous solution by UVA irradiation). An aqueous solution of 49 μM PGA was made, 200 μl of the solution was put into the wells of a microtiter plate (Sumilon multiwell plate, MS-8496F, 0.4 ml × 96 wells, flat bottom), and the plate was irradiated with UVA light (EBF-140L/J, Spectronics Corporation; the wavelength was 365 nm) for 0, 30, 60, 90, or 120 min at room temperature. The UVA dose in this study was 0, 800, 1600, 2400, and 3200 mJ/cm^2, respectively. The respective residual amount of PGA was measured by an HPLC method and microbiological assay recently described.

Experiment 2 (Changes of 5-MTHF in an aqueous solution by UVA or UVB irradiation). An aqueous solution of 20 μM 5-MTHF was made, 200 μl of the solution was put into wells of a microtiter plate

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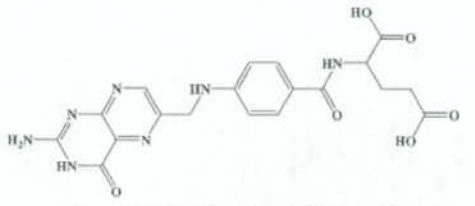
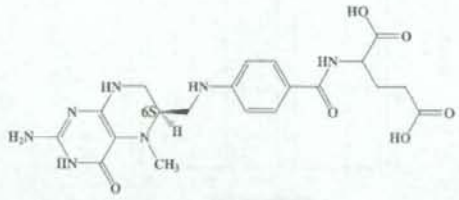
Abbreviations: PGA, pteroylmonoglutamic acid; 5-MTHF, 5-methyltetrahydrofolic acid; UVA, ultraviolet A; UVB, ultraviolet B; UVC, ultraviolet C



Folic acid = pteroylmonoglutamic acid = $C_{19}H_{19}N_7O_6$ = 441.4



5-Methyltetrahydrofolic acid = $C_{20}H_{23}CaN_7O_6$ =497.5

Fig. 1. Structures of Folic Acid and 5-Methyltetrafolic Acid.

(Sumilon multi-well plate, MS-8496F, 0.4 ml × 96 wells, flat bottom), and the plate was irradiated with UVA light (EBF-140L/J, Spectronics Corporation; the wavelength was 365 nm) or UVB light (EBF-260C/J, Spectronics Corporation; the wavelength was 312 nm) for 0, 30, 60, 90, or 120 min at room temperature. The UVA dose in this study was 0, 800, 1600, 2400, and 3200 mJ/cm^2, respectively, and the UVB dose was 0, 900, 1800, 2700, 3600 mJ/cm^2, respectively. The respective residual amount of 5-MTHF was measured by an HPLC method.

*Experiment 3 (Change of the folate concentration in blood by UVA irradiation (*in vitro *experiment)).* Blood was taken from the venous vain at 09:00 before breakfast from Japanese college students (4 males and 7 females), who ate freely, but had not taken any PGA supplements, by using a syringe coated with EDTA. A 3-ml amount of the blood in a plate (Sumilon dish, ϕ60 × 15 mm) was irradiated with UVA light (EBF-140L/J, Spectronics Corporation; the wavelength was 365 nm) for 120 min at room temperature. The UVA dose was 3200 mJ/cm^2. As a control, the dish was placed in the dark. The folate was measured by a microbioassay. To correct the vaporization under processing, the amount of protein in the blood was measured.

*Experiment 4 (Change of the folate concentration in blood, to which PGA or 5-MTHF has been added, by UVA irradiation (*in vitro *experiment)).* Blood was taken from the venous vein at 09:00 before breakfast from Japanese college students (7 males and 4 females), who ate freely, but had not taken any vitamin supplements, by using a syringe coated with EDTA. One milliliter of 110 μM PGA or 110 μM 5-MTHF was added to 6 ml of the blood taken and mixed well. Three milliliter of each sample was withdrawn from the PGA- or 5-MTHF-added blood, and the sample in a plate (Sumilon dish, ϕ60 × 15 mm) was irradiated with UVA light (EBF-140L/J, Spectronics Corporation; the wavelength was 365 nm) for 120 min at room temperature. The UVA dose was 3200 mJ/cm^2. As a control, the dish was placed in the dark. The folate was measured by a microbioassay. To correct the vaporization under processing, the amount of protein in the blood was measured.

Experiment 5 (Comparison of the folate concentrations in blood between male and female young adults who ate freely). The subjects were 23 male and 32 female students who ate freely. Blood was taken from the venous vein before lunch at around 12:00. The folate concentration was measured by a microbioassay.

*Experiment 6 (Change of the folate concentration in blood, withdrawn from the subjects who took no PGA supplements, by sunlight exposure (*in vivo *experiment)).* The subjects were 9 male (average (± SD) age, height, body weight, and BMI were 23.6 ± 2.7 years, 173.7 ± 4.6 cm, 69.2 ± 8.7 kg, and 22.9 ± 2.7 kg/m^2) and 14 female (average (± SD) age, height, body weight, and BMI were 21.8 ± 2.4 years, 160.0 ± 4.0 cm, 51.7 ± 4.4 kg, and 20.0 ± 1.3 kg/m^2) students who ate freely. Blood was taken from the venous vein before and after sunlight exposure at 11:00 and 13:00, respectively. The subjects with short trousers and the tank tops were exposed to sunlight from 11:00 to 13:00 in the summer. The dose of UVA was about 19,000 mJ/cm^2. The folate concentration was measured by a microbioassay.

*Experiment 7 (Change of the folate concentration in blood, withdrawn from the subjects who took PGA, by sunlight exposure (*in vivo *experiment)).* The subjects were 7 female students. Their

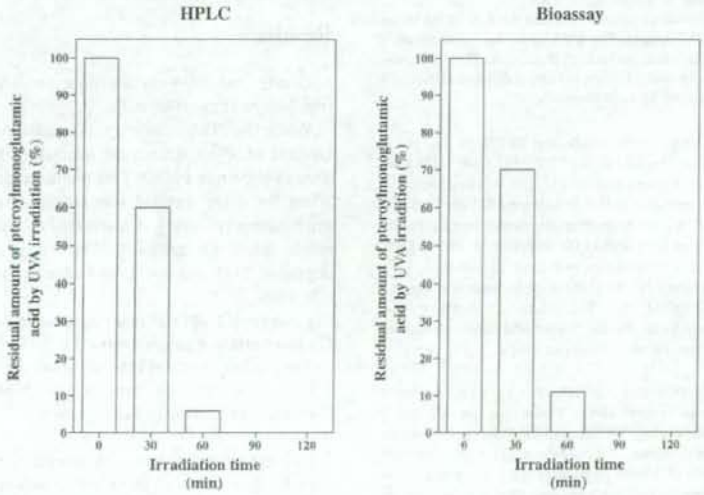


Fig. 2. Change of PGA in an Aqueous Solution by UVA Irradiation (Experiment 1).
A 200-μl amount of 49 μM PGA was irradiated with UV light for 0, 30, 60, 90, and 120 min. The respective residual amount of PGA was measured by an HPLC method and a microbiological assay. Each value is the mean of two separate experiments.

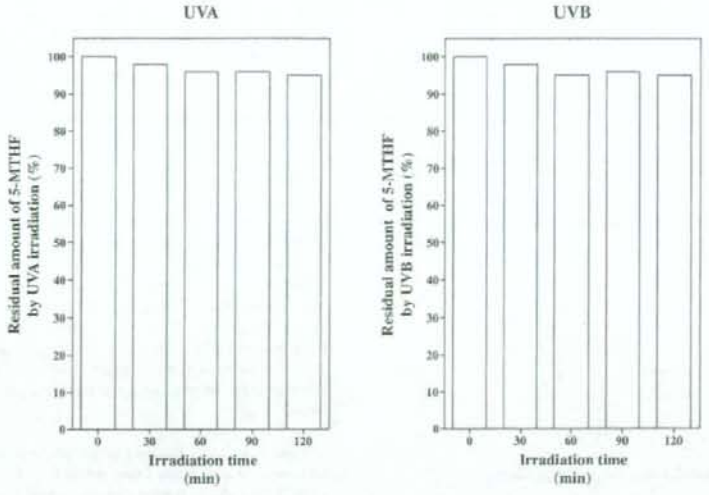


Fig. 3. Changes of 5-MTHF in an Aqueous Solution by UVA and UVB Irradiation (Experiment 2).
A 200-μl amount of 20 μM 5-MTHF was irradiated with UVA or UVB light for 0, 30, 60, 90, and 120 min at room temperature. The UVA dose in this study was 0, 800, 1600, 2400, and 3200 mJ/cm^2, respectively, and the UVB dose was 0, 900, 1800, 2700, and 3600 mJ/cm^2, respectively. The respective residual amount of 5-MTHF was measured by an HPLC method. Each values is the mean of two separate experiments.

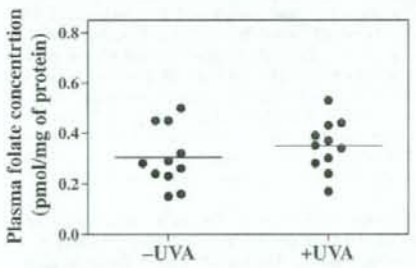


Fig. 4. Change of the Folate Concentration in Human Blood by UVA Exposure (Experiment 3, *in vitro* experiment).
Blood was taken from the venous vein, and the 3 ml of the blood was irradiated with UVA light. The UVA dose was 3,200 mJ/cm^2. As a control, blood in a dish was placed in the dark. The folate was measured by a microbioassay. Circles indicate individual values, and the horizontal line in the figure is the mean.

average (± SD) age, height, body weight, and BMI were 22.7 ± 1.8 years, 160.9 ± 5.5 cm, 49.9 ± 5.0 kg, and 19.2 ± 0.9 kg/m^2. They ate freely, however, the PGA preparation (0.25 mg) was administered to them at each meal for two days before and at breakfast on the blood collection day. Blood (3 ml) was taken from the venous vein before and after sunlight exposure at 11:00 and 13:00, respectively. The subjects put on short trousers and the tank tops to expose a lot of skin. They bathed in the sunlight from 11:00 to 13:00 in the summer. The dose of UVA was about 12,000 mJ/cm^2. The folate concentration was measured by a microbioassay. As the control, the same experiment was done on another day without sunlight exposure.

HPLC methods for PGA and 5-MTHF. The apparatus consisted of an LC-10AD pump, a SPD-10A UV detector, an SIL-10AD auto-injector, a column oven CTO-10A, and an Shiseido Superiorex ODS (φ4.6 × 250 mm) column. The mobile phase was a mixture of a degassed solution of 20 mM phosphoric acid containing 5 mM hexanesulfonate-acetonitrile (9:1, v/v) and was used at a flow rate of 1.0 ml/min. The column temperature was maintained at 40 °C, and the UV detector was set at 280 nm. The HPLC system was interfaced with a Shimadzu Chromatopac C-R8A instrument for data processing.

Microbioassay. Plasma was obtained from EDTA-treated blood by centrifuging at 3,000 × *g* for 5 min at 4 °C. The plasma obtained was directly used for a microbioassay using *Lactobacillus rhamnosus* ATCC 27773.[13]

Protein determination. Protein concentration was determined by a BioRad protein assay, with bovine serum albumin as the standard.

Statistical analysis. The computer program, GraphPad Prism version 4.03 (GraphPad Software, San Diego, USA) was used for data analysis. The D'agostino and Pearson omnibus normality test showed that the blood folate concentration in experiments 3, 4, 5 and 6 was normally distributed, and the Shapiro-Wilk normality test showed this in experiment 7. Statistical significance was assessed by two-tailed paired Student's *t* test in experiments 3, 4, 6 and 7, and by a two-tailed unpaired Student's *t* test in experiment 5.

Results

Change of PGA in an aqueous solution by UVA irradiation (Experiment 1)

When the PGA solution was irradiated with UVA, the amount of PGA decreased according to the exposure time as shown in Fig. 2. This phenomenon was observed when the assay method was changed from HPLC to a microbioassay, using *Lactobacillus rhamnosus* which needs folate for growing. This result means that the degraded PGA did not have folate activity.

Change of 5-MTHF in an aqueous solution by UVA or UVB irradiation (Experiment 2)

Even when the 5-MTHF solution was irradiated with UVA or UVB, the amount of 5-MTHF was not decreased as shown in Fig. 3.

Change of the folate concentration in human blood with UVA irradiation in vitro *(Experiment 3)*

The blood was withdrawn from college students, and the EDTA-treated blood was directly exposed to UVA for 120 min. However, the folate concentra-

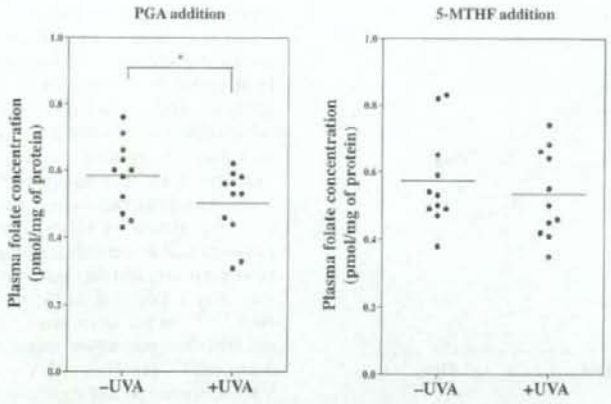


Fig. 5. Change of the Folate Concentration in Human Blood, to Which PGA or 5-MTHF Had Been Added, by UVA Irradiation (Experiment 4, *in vitro* experiment).

Blood was taken from the venous vein. One milliliter of 110 μM PGA or 110 μM 5-MTHF was added to 6 ml of the blood taken and mixed well. Three milliliter of the sample was withdrawn from the PGA- or 5-MTHF-added blood and irradiated with UVA light for 120 min. The UVA dose was 3,200 mJ/cm^2. As a control, the blood in a dish was placed in the dark. The folate was measured by a microbioassay. Circles indicate individual values, and the horizontal line in the figure is the mean. *Statistically significant at $p < 0.05$.

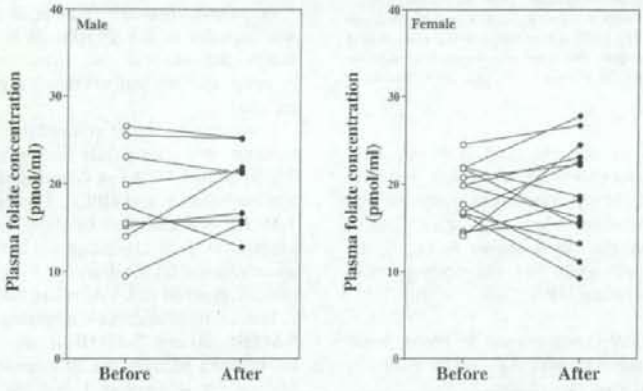


Fig. 6. Change of the Folate Concentration in Human Blood, Which Was Withdrawn from the Subjects Who Had Not Taken a PGA Supplement, by UVA Irradiation (Experiment 6, *in vivo* experiment).

The subjects were 9 male and 14 female students who ate freely. The blood was taken from the venous vein before and after sunlight exposure at 11:00 and 13:00, respectively. The folate concentration was measured by a microbioassay.

tion did not change with UVA irradiation as shown in Fig. 4.

Change of the folate concentration in human blood which PGA or 5-MTHF added to the blood with UVA irradiation in vitro *(Experiment 4)*

PGA or 5-MTHF was directly added to human blood, and the blood was exposed to UVA. As is shown in Fig. 5, the folate concentration in the PGA-added blood was significantly decreased with UVA irradiation, while that in the 5-MTHF-added blood did not decrease.

Comparison of the human blood folate concentrations between males and females who ate freely (Experiment 5)

The concentrations of blood folate in humans who ate freely, but had not taken folate as a supplement, were measured. We have previously reported that the concentration was higher in females than in males.[8] In the present experiment, the difference between males and females was not significant as shown in Table 1.

Table 1. Comparison of the Serum Folate Concentration between Male and Female Japanese Young Adults Who Ate Freely (Experiment 5)

	Mean	SD	Minimum	Maximum	Medium
Male (n = 23) Folates in serum (pmol/ml)	15.0	5.8	7.2	29.2	13.8
Female (n = 32) Folates in serum (pmol/ml)	17.7	5.9	9.5	31.5	15.7

The subjects were 23 male and 32 female students, who ate freely. Blood was taken from the venous vein before lunch at around 12:00. The folate concentration was measured by a microbioassay.

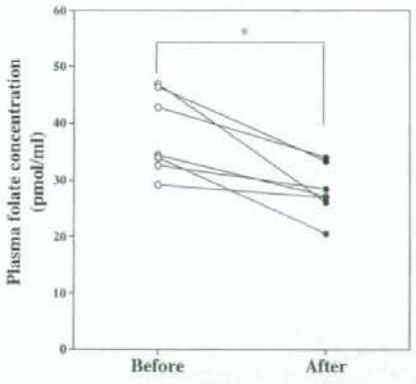


Fig. 7. Change of the Folate Concentration in Human Blood, Which Was Withdrawn from the Subjects Who Had Taken PGA, by Sunlight Exposure (Experiment 7, *in vivo* experiment).

The subjects were 7 female students who ate freely. The PGA preparation (0.25 mg) was administered to them at each meal for two days before and at breakfast on the blood collection day. The blood (3 ml) was taken from the venous vein before and after sunlight exposure at 11:00 and 13:00, respectively. The subjects wore shorts and a tank top to expose plenty of skins. They were exposed to the sunlight from 11:00 to 13:00 in the summer. The dose of UVA was about 12,000 mJ/cm^2. The folate concentration was measured by a microbioassay. As a control, the same experiment was done on another day without sunlight exposure. *Statistically significant at $p < 0.05$.

*Change of the folate concentration in blood, withdrawn from those subjects who had taken no PGA supplements, by sunlight exposure (*in vivo *experiment)*

The subjects who had not taken a folate supplement were exposed to sunlight. As is shown in Fig. 6, the concentration of blood folate did not decrease with sunlight exposure including UVA.

*Change of the folate concentration in blood, withdrawn from those subjects who had taken PGA, by sunlight exposure (*in vivo *experiment)*

The subjects who had been administered with PGA were exposed to sunlight. As is shown in Fig. 7, the concentration of blood folate significantly decreased with sunlight exposure (38.0 ± 7.2 pmol/ml *vs.* 28.1 ± 4.6 pmol/ml). On the contrary, the blood folate concentration in the subjects not exposed to sunlight was not altered (38.6 ± 3.3 pmol/ml *vs.* 38.2 ± 2.3 pmol/ml in 11:00 *vs.* 13:00). In other words, the blood folate concentration was decreased by 74% with sunlight exposure.

Discussion

Folate, a B-group vitamin, is the essential cofactor in the biosynthesis of a *de novo* purine base. So, the rate of folate catabolism progressively increases during pregnancy and the folate demand increases. For a example, Higgins *et al.*[14] have reported that the requirements of folate in the second trimester and in the third trimester were 430 and 540 μg/d, while that in nonpregnant women was 250 μg/d. Some investigators[15,16] have also advised women to consume a folate supplement for the duration of pregnancy and lactation. The main folate supplement that is available on the market is PGA, which is a synthetic form of folate, and is the oxidized and most stable form of folates. However, the demerit of PGA is that it is destroyed by UVA exposure[6] which partly penetrates into the blood stream. Therefore, there is the possibility of a reduction of the blood folate level with sunlight exposure. In fact, Der-Petrossian *et al.*[7] have reported that extracorporeal exposure of plasma to UVA during extracorporeal photophoresis led to the photodegradation of folate. Furthermore, it has been proposed that folate deficiency may result from intense solar exposure, and that sun-induced folate degradation may play a key role in the evolution of human skin color.[17,18] On the other hand, Gambichler *et al.*[9] have reported that the serum folate concentration was not decreased by exposure to UVA; their data suggest that UVA exposure do not significantly influence the serum folate level of healthy subjects and they concluded that the neural tube defects claimed to occur after per conceptual UVA exposure were probably not due to UVA-induced folate deficiency. Therefore, no definite conclusion about the biological significance of folate photodegradation *in vivo* can yet be drawn. We carried out the present experiment to elucidate this controversy.

Some investigators[6,7,19] have already shown that PGA was degraded by UVA exposure, however, these experiments just showed the detection of the degraded compounds. No quantitative experiment had been reported.

In experiment 1, we showed that PGA in an aqueous solution was completely destroyed by exposure to 3,200 mJ/cm^2 UVA, as determined by the methods of a microbioassay and HPLC. However, we showed that 5-MTHF was not destroyed by exposure to UVA in experiment 2. In experiments 3 and 4, the blood folate concentration did not decrease, even when the blood was directly exposed to UVA, while the folate concentration in blood with added PGA decreased and that with added 5-MTHF did not. 5-MTHF is the major form of blood folate when humans eat an ordinary food. From these findings, it is suggested that the blood folate level decreased when the blood contained PGA, while the blood folate level did not decrease when the blood did not contain PGA. We have already reported that the female blood folate level was higher than that of males when the subjects were fed on a semi-purified diet containing PGA.[8] However, as shown in Table 1 (experiment 5), the blood folate levels between males and females were no different when the subjects ate freely and had not taken PGA. From these data, we hypothesize that PGA in the blood is destroyed, but not 5-MTHF, by exposure of sunlight. If humans do not take PGA, the blood folate concentration would not be decreased by exposure to sunlight, because the form of blood folate is mainly 5-MTHF. On the contrary, if humans take PGA, the blood concentration would be decreased by exposure to sunlight, because the PGA taken appears in the blood stream as PGA itself. Thus, we carried out experiments 6 and 7. As was anticipated, when the subjects who had taken no PGA supplement were exposed to sunlight, the blood folate level did not decrease, while when the subjects who had taken PGA were exposed to sunlight, the folate level was significantly decreased (Fig. 8).

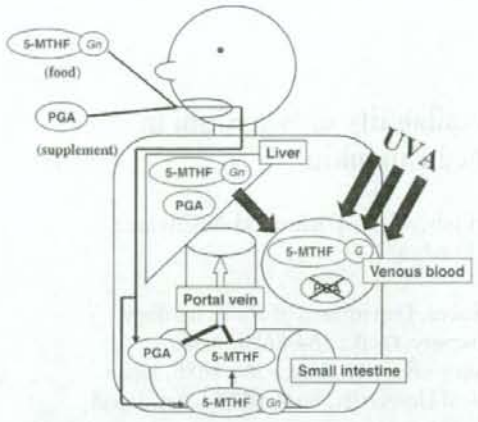


Fig. 8. Different Fates of PGA and 5-MTHF in the Blood Stream When Humans Are Exposed to Sunlight.

When humans take biological food concomitant with PGA as a folate supplement, 5-MTHG and PGA appear in the blood stream. Under such conditions, if humans are exposed to a sufficient amount of sunlight, only PGA is destroyed, so the blood folate level decreases with sunlight exposure. When humans take only biological food, only 5-MTHF-G is present in the blood, so even when humans are exposed to a sufficient amount of sunlight, the blood folate level does not decrease. 5-MTHF-Gn, 5-methyltetrahydrofolate polyglutamic acid.

In conclusion, only PGA, a synthetic form of folate in the blood stream was destroyed by UVA, but not 5-MTHF. We recommend that 5-MTHF is superior to PGA as a folate supplement.

Acknowledgment

This investigation is part of the results of "Studies on construction of evidence to the revised Japanese Dietary Reference Intakes-Elucidation of balance of dietary intake between micronutrient and macronutrient (principal investigator, Katsumi Shibata)," which was supported by The Ministry of Health, Labor and Welfare of Japan.

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Short Communication

Assessment of Nutritional Availability of Selenium in Selenium-enriched Pumpkin

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Abstract

The nutritional availability of selenium (Se) in Se-enriched pumpkin (Se-pumpkin) was assessed by comparing with selenite and Se-enriched *Kaiware* radish sprouts (Se-sprouts). Male weanling ddY mice were fed a *Torula* yeast-based Se-deficient diet. After feeding for 3 weeks, mice were divided into 7 groups and fed the basal diet or a diet supplemented with 0.05 or 0.25 μg/g of Se as either sodium selenite, Se-pumpkin or Se-sprouts for further one week. Supplementation of Se dose-dependently increased serum and liver Se concentrations and glutathione peroxidase (GPX) activities. In serum Se and GPX, the increases by Se supplementation did not significantly vary with Se source, but in the liver Se and GPX, the increases by selenite supplementation were significantly higher than those by supplementation with Se-pumpkin or Se-sprouts. A difference between the effect of Se-pumpkin and that of Se-sprouts was found in the elevation of liver Se concentration ; supplementation with Se-pumpkin caused significantly higher elevation of liver Se than that with Se-sprouts. When liver Se was used, the nutritional availabilities of Se from Se-pumpkin and that Se-sprouts were estimated to be 97% and 65% to selenite Se, respectively. However, when liver GPX was used for the estimation, the availability of Se from either Se-pumpkin or Se-sprouts was less than 50% to selenite Se.

Keywords : selenium, nutritional availability, glutathione peroxidase, selenium-enriched vegetables, selenium-enriched radish sprouts, selenium-enriched pumpkin

Introduction

Selenium (Se) is an essential trace element in human and animal nutrition, and plays several important roles in the form of selenoproteins that include the families of glutathione peroxidase (GPX), deiodinases or thioredoxine reductases [1]. Besides the nutritional roles, Se is thought to be associated with cancer prevention from the results of many epidemiological studies and animal experiments [2]. To prevent a low Se status, various Se-enriched foods were prepared and used to increase daily Se intake [3, 4]. In particular, several Se-enriched plant foods have been developed since their anti-tumor activities are expected to be higher than those of selenite, selenate or high Se yeast [4].

The utilization of dietary minerals including Se is the net result of several physiological and metabolic processes that converted a portion of ingested minerals to certain metabolically critical forms that are necessary for normal physiological function. In the view of mineral nutrition, it is necessary to show an extent of the biological utilization of dietary minerals for their critical or functional forms quantitatively. The quantitative description of biological utilization of dietary minerals has come to

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be called their "bioavailability" or "nutritional availability" [5]. There are three factors contributing to the nutritional availability ; physiological characteristics of host animals, dietary components ingested with minerals and chemical species of minerals. Since the composition of Se species in the Se-enriched plant foods is diverse [6], their nutritional availabilities are thought to vary with the kinds of plant species. In the present study, we attempted to compare the nutritional availability of Se in Se-enriched pumpkin (Se-pumpkin) to those in selenite and Se-enriched *Kaiware* radish sprouts (Se-sprouts) by using tissue Se deposition and GPX activity of mice given these Se sources.

Materials and Methods

Se-pumpkin was purchased from PhytoSelenium Research Laboratories (Kumamoto, Japan) [7], and Se-sprouts were prepared by hydroponics, which used 10 μg Se/ml of sodium selenite solution as described previously [8]. Both Se-enriched vegetables were freeze-dried and milled. Se contents of Se-pumpkin and Se-sprouts were 45 and 121 μg/g dry weight, respectively.

The protocol of the animal experiment was reviewed and approved by the Animal Ethics Committee of Kansai Medical University. Fifty-six male weanling ddY mice were fed a *Torula* yeast-based Se-deficient basal diet [9]. After feeding for 3 weeks, mice were divided into 7 groups and fed the basal diet or the basal diet supplemented with 0.05 or 0.25 μg/g of Se as either sodium selenite, Se-pumpkin or Se-sprouts for a further week. Serum and liver were then collected, and their Se contents and GPX activities were measured. Se was determined by inductively coupled plasma mass spectrometry [10] and GPX activities were assayed using *tert*-butyl hydroperoxide as the peroxide substrate [11].

In the present study, the nutritional availability of Se from Se-pumpkin or Se-sprouts was assessed using sodium selenite as reference Se. The concentration of Se and GPX activity in liver and serum were used as the responses to increasing amounts of dietary Se. As the responses (Y) to increasing amounts of dietary Se (X) can be described by the general equation $Y = m\ X + k$, the relative nutritional availability of Se from Se-enriched vegetables was estimated by a slope-ratio technique that compares the slope of dose-response plots to the slope observed for selenite Se [12].

Results

No significant difference was observed in the body weight among groups. At the end of the feeding period, the mean ± SD of body weight for all mice (n=56) was 33.2 ± 1.9 g.

Se concentration and GPX activities in the liver and serum are summarized in Tables 1. The Se concentration and GPX activities both increased gradually with an increase of the supplementary level of Se, regardless of its source or the tissue monitored. In the serum Se and GPX, the increases by Se supplementation did not vary with the Se source, but in the liver Se and GPX, the increases by selenite supplementation were higher than those supplemented with Se-pumpkin or Se-sprouts. In the elevation of liver Se concentration, supplementation with Se-

Table 1 Se concentration and GPX activities in serum and liver of rats fed experimental diets

Se supplemented to diet		Se concentration		GPX activity	
Source	Level (μg/g)	Serum (ng/ml)	Liver (ng/g)	Serum (unit/ml)	Liver (unit/g protein)
None	-	37 ± 5 ^{a}	54 ± 3 ^{a}	0.17 ± 0.01 ^{a}	17 ± 3 ^{a}
Selenite	0.05	162 ± 10 ^{b}	210 ± 20 ^{b}	0.36 ± 0.07 ^{ab}	75 ± 11 ^{a}
Se-pumpkin	0.05	113 ± 6 ^{b}	98 ± 3 ^{ab}	0.22 ± 0.03 ^{a}	24 ± 6 ^{a}
Se-sprouts	0.05	102 ± 4 ^{ab}	92 ± 3 ^{ab}	0.20 ± 0.03 ^{a}	16 ± 5 ^{a}
Selenite	0.25	449 ± 20 ^{c}	657 ± 43 ^{d}	0.81 ± 0.12 ^{c}	442 ± 35 ^{c}
Se-pumpkin	0.25	428 ± 24 ^{c}	598 ± 36 ^{d}	0.81 ± 0.13 ^{c}	212 ± 42 ^{b}
Se-sprouts	0.25	411 ± 23 ^{c}	463 ± 48 ^{c}	0.72 ± 0.13 ^{bc}	217 ± 42 ^{b}

Values are the means ± SEM (n=8). GPX units expressed as μmol NADPH oxidized per min. Means in the same column not sharing a common superscript differ significantly ($p < 0.05$) by analysis of variance followed by Tukey-Kramer multiple range test.

Table 2 Regression of supplementary Se level (X) with parameters of Se status (Y) and nutritional availability of Se from Se-pumpkin or Se-sprouts.

Parameters	Source of Se supplemented	Regression	Correlation coefficient (r)	Nutritional availability (%)
Serum Se	Selenite	Y = 1592 X + 56	0.973	-
	Se-pumpkin	Y = 1568 X + 36	0.975	98.5
	Se-sprouts	Y = 1362 X + 36	0.911	85.6
Serum GPX	Selenite	Y = 2.46 X + 0.20	0.767	-
	Se-pumpkin	Y = 2.67 X + 0.14	0.815	108.5
	Se-sprouts	Y = 2.06 X + 0.14	0.711	83.7
Liver Se	Selenite	Y = 2356 X + 71	0.954	-
	Se-pumpkin	Y = 2267 X + 23	0.967	96.2
	Se-sprouts	Y = 1529 X + 38	0.858	64.9
Liver GPX	Selenite	Y = 1735 X + 5	0.952	-
	Se-pumpkin	Y = 819 X + 3	0.804	47.2
	Se-sprouts	Y = 757 X + 2	0.746	43.6

Regression was fitted to the equation $Y = m\ X + k$, where Y represented the parameters in mice fed the basal diet or the diet supplemented with Se at X level (μg/g). Units of parameters are the same as in Table 1. Nutritional availability was estimated using the slope of the regression ; (slope of Se-pumpkin or Se-sprouts)/(slope of selenite) x 100.

pumpkin caused a higher elevation of liver Se than that with Se-sprouts.

The regression analyses of supplementary Se with the Se concentration or GPX activities are summarized in Table 2. As the increases of Se concentration and GPX activity in mouse serum or liver were significantly correlated with supplementary levels of each Se source, linear regression could be calculated in each combination. Accordingly, as also described in Table 2, the relative nutritional availability of Se from Se-pumpkin or Se-sprouts can be estimated by the slope ratio analysis, which uses sodium selenite as a reference. Based on the serum parameters, the availability of Se from either Se-pumpkin or Se-sprouts was more than 80% to selenite Se. However, based on the liver GPX, the availability of Se from either Se-pumpkin or Se-sprouts was less than 50% to selenite. When liver Se was used for analysis, the nutritional availability of Se from Se-pumpkin and that from Se-sprouts was estimated to be 97% and 65% to selenite Se, respectively.

Discussion

In the estimation of nutritional availability, each parameter of Se status gave difference values. Among the responses of parameters in mice supplemented with selenite, serum GPX gave the lowest correlation coefficient. Compared to liver GPX activity, serum GPX activity reaches a plateau level at lower dietary Se level [13]. Thus, this low correlation indicates a possibility that the response of serum GPX reached a plateau level in the tested range of Se supplemented. When comparison between liver GPX and Se concentration is made, GPX has been thought to be superior to Se concentration as an index for Se status because GPX is one of the functional forms of Se in tissues [5]. Accordingly, nutritional availability based on the liver GPX is the most reliable among the four parameters, and the availability of Se either from Se-pumpkin or Se-sprouts is less than 50% to selenite Se.

We have already identified the main Se species in Se-pumpkin and Se-sprouts as protein-bound selenomethionine (SeMet) [14] and *Se*-methylselenocysteine (MeSec) [8], respectively. Dietary Se must be metabolized to selenide before incorporation to selenoproteins [1]. The low nutritional availability of Se from Se-pumpkin or Se-sprouts estimated in the present study indicates that the formation of selenide either by demethylation of MeSec or transsulfuration of SeMet was not sufficient for the synthesis of selenoprotein including GPX.

When liver Se was used in the estimation of nutritional availability, Se from Se-pumpkin gave higher availability values than that from Se-sprouts. Since SeMet is a non-specific form of Se that is metabolized as a constituent of methionine pool, SeMet can be non-specifically incorporated into body proteins [15, 16]. Accordingly, this result

may indicate that SeMet in Se-pumpkin was nonspecifically incorporated into liver protein. The SeMet incorporated to liver protein can take part in the amino acid pool, be metabolized to selenide *via* the transsulfuration pathway gradually and then be incorporated into selenoproteins. Thus, with the long-term administration of Se-pumpkin, the nutritional availability of Se from Se-pumpkin may be higher than that obtained in the present study.

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原　著

誘導体化とガスクロマトグラフィー－質量分析によるセレン強化食品中の含セレンアミノ酸の同定－

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Identification of Selenoamino Acids in Selenium-enriched Foods by Derivatization and Gas Chromatography-Mass Spectrometry

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Summary

Selenoamino acids in selenium (Se)-enriched foods were identified by gas chromatography-mass spectrometry (GC-MS) after derivatization using a commercial amino acid analysis kit (EZ : faast™). After the derivatization, a compound eluted at the same retention time as derivatized *Se*-methylselenocysteine (MeSec) in GC was detected in Se-enriched *Kaiware* radish sprouts and Se-enriched garlic bulb. Mass spectrum of this derivatized compound was coincident with that of derivatized MeSec ; MeSec was identified in these Se-enriched foods. Similarly, selenomethionine was identified in Se-enriched yeast using EZ : faast™ and GC-MS. Analysis by high performance liquid chromatography-inductively coupled plasma mass spectrometry showed a presence of selenohomolanthionine (SeHL) in Se-enriched mung bean sprouts. However, SeHL could not be analyzed by GC-MS after the derivatization by EZ : faast™.

疫学調査や動物実験によって，必須微量元素のセレンには抗腫瘍作用のあることが明らかにされている[1]。しかし，セレン化合物は毒性も強いため，ヒトへの応用は進んでいない。セレンはイオウの同族元素であり，自然界には含硫アミノ酸のセレンアナログである含セレンアミノ酸が存在している。とくに，セレンを蓄積した植物には，*Se*-メチルセレノシステイン（MeSec）をはじめとする多様な含セレンアミノ酸が存在する[2,3]。われわれは，MeSecを豊富に含有するセレン強化カイワレダイコンスプラウトが亜セレン酸に比較してセレンとしての栄養有効性は低いが，抗腫瘍活性は高いことを示した[4,5]。このようにセレン強化植物には，機能性の高い含セレンアミノ酸が存在しており，高セレン環境下で野菜類を栽培する試みが世界各地で行われている[6]。

セレン化合物の同定には，誘導結合プラズマ質量分析（ICPMS）を検出に用いた高速液体クロマトグラフィー（HPLC）や液体クロマトグラフィー－質量分析（LC-MS）が頻用されている。前者ではセレン化合物を特異的に検出でき，後者では未知化合物の構造を推定できる。しかし，HPLC-ICPMSが有機溶媒，LC-MSが不揮発性の溶媒をそれぞれ嫌うことから，両者を共通のカラム－溶媒系で実施することは困難である。したがって，セレン化合物の同定においてHPLC-ICPMSとLC-MSは独立して用いられており，食品や生体中のセレン化合物の同定を効率よく進めることは現在でも難しい。

一方，ガスクロマトグラフィー－質量分析（GC-MS）は，未知化合物の同定技術として古くから確立している。含セレンアミノ酸のような非揮発性化合物の場合，GC-MSで分析するには誘導体化処理を行うことが必要である。近年，生体や食品中の遊離アミノ酸を固相抽出後，誘導体化処理するキット（アミノ酸誘導体化キット）が開発されており，アミノ酸類のGC-MS分析を簡便かつ短時間で実施することが可能となっている。今回，セレン強化食品中の含セレンアミノ酸をこのようなアミノ酸誘導体化キットとGC-MSを用いて同定することに成功したので報告する。

実験方法

1. 試料など

セレン強化カイワレダイコンスプラウトとセレン強化リョクトウスプラウトは，既報[2]に記載した方法に従って栽培したものを用いた。セレン強化ニンニク鱗茎は植物セレニウム研究所（熊本，阿蘇町）から購入した。セレン強化酵母乾燥粉末はBiospringer社（フランス）が生産した

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