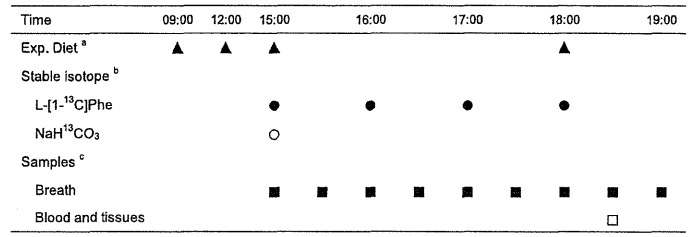

Fig. 1. The protocols employed for each IAAO study day. [a]The experimental diet was either a 4.3% or 17.2% casein diet. The diet was provided every 3 h (9:00–18:00). Each meal represented one-eighth of each rat's daily intake. [b]Isotope: Priming doses of L-[1-^{13}C]phenylalanine and $NaH^{13}CO_3$ were started with the third meal at 15:00, and the infusion of L-[1-^{13}C]phenylalanine was continued hourly until the end of the study. [c]Sample collection: Baseline breath sample was collected before the isotope protocol began. Nine breath samples were collected every 30 min after the initiation of the isotope protocol. Samples of blood, liver, and gastrocnemius muscle were collected at 18:30.

tures will be absorbed very rapidly, and protein utilization will show a higher efficiency, compared with slow proteins such as casein (*20*). Incidentally, a previous study by Moehn et al. (*21*) evaluated the metabolic availability of amino acids in peas, and they indicated the applicability of using IAAO for intact protein sources.

Measurements of the quality and quantity of the dietary protein employed can be used to facilitate adjustments to the diet to ensure that the metabolic demands for protein can be met sufficiently. Poor protein quality compromises the nutritional status and increases the protein requirement. In the 1991 FAO/WHO/UNU report (*22*), the protein digestibility corrected amino acid score (PDCAAS) value for casein is 1.00, compared with 0.25 for wheat gluten. Therefore, the protein requirement calculated for rats fed a wheat gluten diet is higher than that for rats fed a casein diet. In a clinical setting, the adequate quality and quantity of protein or amino acid for each disease might be estimated using the IAAO method.

The objective of the present study was to establish whether or not the IAAO method is viable for determining the metabolic demand for protein and to evaluate protein quality using protein itself, employing casein and wheat gluten as protein sources in experimental diets and using the IAAO method with L-[1-^{13}C]phenylalanine.

MATERIALS AND METHODS

Animals. This study was performed in accordance with the guidelines for animal experimentation at Kyoto Prefectural University, Japan. Male Wistar/ST rats (4 wk old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The rats were housed in individual mesh cages under controlled temperature (22±2°C) and lighting (lights on from 08:00 to 20:00) conditions. The rats were given free access to water and a 17.2% casein maintenance diet, and they were allowed to adapt to the laboratory environment for at least 1 wk before starting the experiment. After adaptation, 5- to 6-wk-old rats (initial BW=130.1±2.3 g) were used for the experiment. The amount of feed available and any feed not eaten were recorded for each rat for 3 d before the first study day, and the total daily intake for each rat, equivalent to the 24-h dietary intake, was calculated on the basis of the average intake during the previous 3 d.

Experiment 1. The objective of Experiment 1 was to examine the effect of L-[1-^{13}C]phenylalanine administration on breath $^{13}CO_2$ enrichment, and to evaluate whether the protein metabolism could be measured by the IAAO method in rats consuming different protein level diets. All of the eight rats were included in two IAAO studies, consuming both 4.3% and 17.2% casein diets (N×6.38) (*23*) with a time period of more than 2 d between the studies. The 17.2% casein maintenance diet employed for all of the studies was provided for at least 24 h. Then, the rats fasted overnight for 13 h from 20:00 on the day before the study day, but had free access to drinking water. The study protocol for all of the IAAO studies is depicted in Fig. 1. On the study day, the rats were weighed in the morning before feeding. Then, they received either 4.3% or 17.2% casein diets (Table 1). The study-day diet was provided in 4 isoenergetic, isonitrogenous diets, and each meal accounted for one-eighth of the rat's total daily intake. Specifically, the casein diet was consumed beginning at 09:00 and continued at each 3-h interval until 18:00 for a total of 4 meals. The rats were allowed free access to drinking water during the experiment period. The rats were fed the remaining half of the daily ration in the evening. The tracer protocol was started with the third meal at 15:00 to measure the phenylalanine kinetics with the use of L-[1-^{13}C]phenylalanine, and continued hourly until 18:00. The rats were placed in the chamber immediately after the oral administration of the ^{13}C substance. Breath samples were collected, and the $^{13}CO_2$ level in breath CO_2 was measured at 30-min intervals from 15:00 to 19:00. Baseline breath samples were collected before the isotope protocol began at 15:00. On a later day, the rats were dissected at 18:30; blood, liver and gastrocnemius muscle samples were collected for subsequent analysis of amino acid concentration in plasma and tissues.

Experiment 2. The protein intake for metabolic demands was measured using the IAAO method for rats fed the casein diets, and also for rats fed diets based on

Table 1. Composition of experimental diets.

Protein	Casein diet						Wheat gluten diet					
	4.3%	8.6%	12.9%	17.2%	21.5%	25.8%	7.2%	10.8%	14.4%	18.0%	21.6%	25.2%
	g/kg diet						g/kg diet					
Casein[1,2]	50	100	150	200	250	300	—	—	—	—	—	—
Wheat gluten[3,4]	—	—	—	—	—	—	100	150	200	250	300	350
Cornstarch[1]	557	523	490	457	423	390	527	498	470	440	411	383
Sucrose[1]	278	262	245	228	212	195	265	250	235	221	206	190
Rapeseed oil[5]	35	35	35	35	35	35	31	27	22	18	14	9
Soy bean oil[6]	15	15	15	15	15	15	12	10	8	6	4	3
Vitamins[1,7]	10	10	10	10	10	10	10	10	10	10	10	10
Minerals[1,8]	35	35	35	35	35	35	35	35	35	35	35	35
Cellulose[1]	20	20	20	20	20	20	20	20	20	20	20	20
L-Phenylalanine[9]	11	9	7	5	2	—	9	7	5	3	1	—
L-Tyrosine[10]	13	10	8	5	3	—	13	11	10	9	8	6
Energy (kJ/g)	15.4	15.4	15.5	15.5	15.5	15.6	15.5	15.5	15.5	15.5	15.6	15.6

[1] Oriental Yeast Co., Ltd., Japan.

[2] Protein, 86.2% (N×6.38). Amino acid (mg/100 g Casein): L-alanine, 2,700; L-arginine, 3,300; L-aspartic acid, 6,300; L-cysteine, 430; L-glutamic acid, 19,000; L-glycine, 1,600; L-histidine, 2,700; L-isoleucine, 4,900; L-leucine, 8,400; L-lysine, 7,100; L-methionine, 2,600; L-phenylalanine, 4,500; L-proline, 10,000; L-serine, 4,600; L-threonine, 3,700; L-tryptophan, 1,100; L-tyrosine, 5,000; L-valine, 6,000; total, 93,930.

[3] Weston Bioproducts Ltd., Queens land, Australia.

[4] Protein, 72.0% (N×5.70). Amino acid (mg/100 g wheat gluten): L-alanine, 2,100; L-arginine, 2,700; L-aspartic acid, 2,700; L-cysteine, 1,600; L-glutamic acid, 29,000; L-glycine, 2,700; L-histidine, 1,800; L-isoleucine, 3,000; L-leucine, 5,400; L-lysine, 1,400; L-methionine, 1,300; L-phenylalanine, 4,100; L-proline, 11,000; L-serine, 3,600; L-threonine, 2,000; L-tryptophan, 780; L-tyrosine, 2,500; L-valine, 3,300; total, 80,980.

[5] Nisshin Oillio Ltd., Japan.

[6] Wako Pure Chemical Industries, Ltd., Japan.

[7] AIN-76^{TM} vitamin mixture (per g mixture): vitamin A, 400 IU; vitamin D_3, 100 IU; vitamin E, 5 mg; vitamin K_3, 0.005 mg; vitamin B_1, 0.6 mg; vitamin B_2, 0.6 mg; vitamin B_6, 0.7 mg; vitamin B_{12}, 0.001 mg; D-biotin, 0.02 mg; folic acid, 0.2 mg; calcium pantothenate, 1.6 mg; nicotinic acid, 3 mg; choline chloride, 200 mg; sucrose, 0.968 g.

[8] AIN-76^{TM} mineral mixture (g/kg mixture): calcium phosphate dibasic, 500.0; sodium chloride, 74.0; potassium citrate, 220.0; potassium sulfate, 52.0; magnesium oxide, 24.0; manganese carbonate, 3.5; ferric citrate, 6.0; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.0066; chromium potassium sulfate, 0.55; sucrose, 118.03.

[9] L-Phenylalanine content was kept constant at 13,500 mg/kg diet in all diets, except the 25.2% wheat gluten diet (14,350 mg/kg diet).

[10] L-Tyrosine content was kept constant at 15,000 mg/kg diet in all diets.

wheat gluten instead of casein to determine whether it was important to consider the effects of the source of the protein in the diet. Sixteen rats were used, and even when they were measured for the wheat gluten diets, the 17.2% casein diet was provided as a maintenance diet for the 2 d before the study day for all of the IAAO studies. On the study day, eight rats received, in random order without repeats, one of six levels of the casein (4.3, 8.6, 12.9, 17.2, 21.5, 25.8%) diet (N×6.38) (*23*), and the other eight rats received one of six levels of the wheat gluten (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet (N×5.70) (*23*). The tracer protocol employed was the same as that employed in Experiment 1, and ^{13}C substance administration was performed for a total of four times at 15:00, 16:00, 17:00, and 18:00. However, breath samples were collected and the $^{13}CO_2$ level in the breath was measured only twice at 15:00 and 18:30. The experimental design was a completely randomized crossover design. Eight rats consumed the casein diet at all six levels, and the other eight rats consumed the wheat gluten diet at all six levels. Each IAAO study day was separated by 2 d, and the six IAAO studies were completed within 2 wk. Except for these points, all of the protocols were the same as those employed in Experiment 1.

Tracer administration protocol. L-[1-^{13}C]Phenylalanine (Cambridge Isotope Laboratories, Andover, MA) and $NaH^{13}CO_3$ (Cambridge Isotope Laboratories) were used as tracers. Labeled compounds were dissolved in saline and stored at 4°C. Isotopic solutions were prepared and administered in a volume of 2.5 mL/kg BW. Oral priming doses of 0.88 mg/kg BW $NaH^{13}CO_3$ and 7.92 mg/kg BW $NaHCO_3$ were given with the third meal at 15:00. An oral dosing protocol of 3.3 mg/kg BW L-[1-^{13}C]phenylalanine and 29.7 mg/kg BW phenylalanine was commenced simultaneously with the third meal, and administration of 6.0 mg/kg BW L-[1-^{13}C]phenylalanine and 54.0 mg/kg BW phenylalanine

was performed hourly until the end of the study.

Experimental diets. The composition and source of the powdery experimental casein and wheat gluten diets are shown in Table 1. Casein and wheat gluten provided the sole source of protein in the casein and wheat gluten diets, respectively. The compositions of the amino acids in the casein and wheat gluten are shown in the footnote to Table 1 (*23*). L-Phenylalanine and L-tyrosine were added to the diets to achieve an equal content of these amino acids in all diets. In the present study, L-phenylalanine (13.5 g/kg diet) and L-tyrosine (15.0 g/kg diet) were consumed in excess of these amino acid requirements for rodents (L-phenylalanine, 8.8 g/kg diet; L-tyrosine, 9.3 g/kg diet) (*24*), in order to minimize the net hydroxylation of phenylalanine to tyrosine. Each casein diet with varying protein content was kept at an identical energy level by varying the levels of sugar and starch. The oil levels in the wheat gluten diet were decreased because the energy level of wheat gluten is higher than those of casein. Thus, all of the diets had a similar energy level (15.4–15.6 kJ/g).

Breath sample collection and analysis. The instruments used for the collection of breath samples in the rats consisted of an acrylic chamber (10.6 L) fitted with a drinker, an aspiration pump (Columbus Instruments, Columbus, OH) and an air flow meter (Columbus Instruments). The chambers were continuously charged with fresh room air through the aspiration tube by a pump. The rats were moved outside the chamber for the administration of the ^{13}C substance, and thereafter moved back into the same chamber. Because the chambers filled with expired air were necessary in order to collect the breath samples, rats were placed in separate compartments for 30 min before the collection of the breath samples.

Breath samples of 200 mL volume drawn into a 200 mL syringe were injected into breath-sampling bags (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The $^{13}CO_2$ concentration in the expired air was measured by attaching the breath-sampling bags to the sampling joint of an infrared spectrometer (POCone; Otsuka Electronics Co., Ltd., Tokyo, Japan). Using the measurement system provided by POCone, the concentration of CO_2 in the aspirated air in the breath sampling bags was at least more than 0.5%. Therefore fresh room air was drawn through the system at comparatively low rates of approximately 0.4 L/min, and the CO_2 concentration within the chamber was stabilized at 0.8–1.2%. The $^{13}CO_2$ rate was measured as the $^{13}CO_2/^{12}CO_2$ ratio, and followed by a pulse of mixed gas composed of 5% CO_2, 12% O_2 and the rest of the mixture was N_2 for the control. Isotopic abundances were expressed relative to the international Vienna Pee Dee Belemnite standard (‰) as over the baseline ($\Delta - \Delta_0$) value, further normalized by each rat's weight.

Blood and tissue samples collection and analysis. Blood samples drawn from the inferior vena cava were collected in tubes with heparin, and plasma was separated from the blood samples by centrifugation at 1,500 $\times g$ for 5 min. The plasma was stored at −20°C until it was analyzed. The liver and gastrocnemius muscle were rapidly removed and snap-frozen in liquid nitrogen and stored at −80°C for analysis. Approximately 0.5 g of liver and muscle were homogenized in 4.5 mL of saline, centrifuged at 1,000 $\times g$ for 10 min.

A 100 μL plasma sample and the supernatant of liver and muscle obtained as described above were deproteinized with 300 mL ethanol and centrifuged at 1,500 $\times g$ for 10 min. A 200 mL sample of the supernatant fluid was cleared of contamination by using a strong cation exchanger (AG 50W-X8, Bio-Rad Laboratories, Hercules, CA), dried under a vacuum, derived to its 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivative using the Waters AccQ, Fluor Reagent Kit (Waters Corp., Milford, MA) and dried. Then the supernatant fluid was reconstituted in 200 μL of 0.1% formic acid. Phenylalanine and tyrosine concentrations were measured by an HPLC system. The individual amino acids were separated by an Inertsil ODS-3 column (250×4.6 mm, GL Sciences, Tokyo, Japan) with a binary LC gradient (0–60% aqueous acetonitrile containing 0.1% formic acid). The areas under the peaks were integrated using Peak Net 5.1c (Dionex Corp., Osaka, Japan). L-[1-^{13}C]Phenylanine and L-[1-^{13}C]tyrosine enrichment in the plasma and tissue samples was analyzed with a MS (LCQ Fleet, Thermo Scientific, Waltham, MA) coupled to the HPLC system. Selected ion chromatograms were obtained by monitoring ions *m/z* 336 and 337 for L-phenylalanine and L-[1-^{13}C]phenylalanine, *m/z* 352 and 353 for L-tyrosine and L-[1-^{13}C]tyrosine, respectively.

Statistical analysis. Data analysis was performed using Statcel2 software (Oms Publishing Inc., Tokyo, Japan). All results were presented as the mean±SE. Values of $p<0.05$ were considered statistically significant. Student's *t* test was used to analyze differences between two different groups, such as the protein intake. Statistical analysis for multiple comparisons was performed using one-way analysis of variance (ANOVA) with repeated measures followed by a Tukey-Kramer post hoc test.

The protein intake for metabolic demands was derived by applying a mixed-effect change-point model to breath $^{13}CO_2$ data (*25*), and the regression oxidation rate of the dietary protein contents. The first regression line showed a downward slope and the second line was horizontal with minimal or no slope. The breakpoint, the protein intake with a plateau in oxidation, was regarded as the protein intake for metabolic demand.

RESULTS

Experiment 1

The rats were given free access to a 17.2% casein diet as a maintenance diet for 3 d before the first study day, and the total daily intake for each rat was 16.5±0.5 g/d (calorie, 255.9±7.8 kJ/d; protein, 2.8±0.1 g/d). The body weights for the rats used for the 4.3% and 17.2% casein diet experiments were 144.1±5.7 g and 143.5±5.0 g, respectively.

Complete data sets of 9 breath samples were obtained

in only 7 of the rats fed the 17.2% casein diet. One rat did not consume its feed completely at 18:00, which affected the $^{13}CO_2$ values thereafter. Regardless of the protein intake and the 4.3% or 17.2% casein diets, breath $^{13}CO_2$ enrichment gradually increased after the initiation of the isotope protocol (Fig. 2). The plateau breath samples were collected during the isotopic steady state every 30 min during the period from 16:30 to 19:00 in rats fed the 17.2% casein diet, and from 17:30 to 19:00 in rats fed the 4.3% casein diet. This isotope protocol had been shown to achieve a satisfactory isotopic steady state 2.5 h after the start of L-[1-^{13}C]phenylalanine isotope administration. In addition, when the 4.3% casein diet was employed, the enrichment of breath $^{13}CO_2$ was greater than that achieved with the 17.2% casein diet, and during the period from 17:30 to 19:00, significant differences were shown between the 4.3% and 17.2% casein diets on breath $^{13}CO_2$ enrichment at 18:30 ($p<0.01$) and 19:00 ($p<0.01$).

The amino acid concentrations of plasma, liver and gastrocnemius muscle obtained at 18:30 on the IAAO study day are shown in Table 2. In both phenylalanine and tyrosine, ^{13}C-amino acid concentrations, ^{12}C-amino acid concentrations, and the total of these concentrations in the plasma and tissues of rats fed the 4.3% casein diet were similar to those of rats fed the 17.2% casein diet, and there were no significant differences.

Experiment 2

The rats were given free access to a 17.2% casein maintenance diet for 3 d before the first study day. The total daily intake for each rat used for the casein and wheat gluten diet experiments employing the IAAO method were 16.7±0.3 g/d (calorie, 258.9±4.3 kJ/d; protein, 2.9±0.1 g/d) and 17.3±0.5 g/d (calorie, 268.2±7.0 kJ/d; protein, 3.0±0.1 g/d), respectively. The body weights for the rats used for the 4.3, 8.6, 12.9, 17.2, 21.5, and 25.8% casein diet experiments were 149.4±5.7, 141.0±9.9, 155.2±10.8, 147.4±3.5, 158.0±2.4, and 184.7±3.1 g, respectively. The body weights for the rats used for the 7.2, 10.8, 14.4, 18.0, 21.6, and 25.2% wheat gluten diet experiments were 130.7±3.5, 148.8± 4.8, 157.1±5.4, 160.8±10.0, 134.4±2.8, and 142.4±3.0 g, respectively.

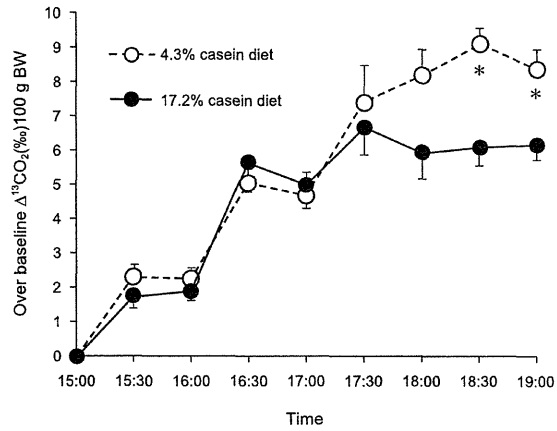

Fig. 2. The effect of L-[1-^{13}C]phenylalanine infusion on $^{13}CO_2$ enrichment of the breath. Values are mean±SE for the 4.3% ($n=8$ per mean) and 17.2% ($n=7$ per mean) casein diets. On the study day, the rats received either a 4.3% or 17.2% casein diet every 3 h from 09:00. Each meal represented one-eighth of the rat's total daily intake. The administrations of L-[1-^{13}C]phenylalanine were performed at 15:00, 16:00, 17:00, and 18:00. The establishment of a plateau in the breath samples on the basis of no significant differences among the timed samples was confirmed using repeated-measures ANOVA. The isotopic steady state was confirmed at 16:30–19:00 in rats fed the 17.2% casein diet, and at 17:30–19:00 in rats fed the 4.3% casein diet. *The asterisk marks shown significant differences ($p<0.01$) between the 4.3% and 17.2% casein diets at 18:30 and 19:00.

Table 2. The concentrations of phenylalanine and tyrosine in the plasma, liver and gastrocnemius muscle in rats fed 4.3% or 17.2% casein diets.

Diet	Phenylalanine			Tyrosine		
	^{13}C-Phe	^{12}C-Phe	Total	^{13}C-Tyr	^{12}C-Tyr	Total
			Plasma (nmol/mL)			
4.3% casein	13.2±2.9	47.2±4.3	60.4±7.0	7.5±2.0	113.0±29.4	120.6±30.7
17.2% casein	12.1±2.5	50.8±10.0	62.9±11.8	8.5±1.4	119.8±15.2	128.3±16.2
			Liver (nmol/g)			
4.3% casein	10.6±0.4	40.9±5.1	51.5±4.9	7.4±1.3	99.4±32.0	106.9±33.2
17.2% casein	10.4±2.1	43.1±10.5	53.6±12.3	8.8±2.8	92.5±7.5	101.4±9.2
			Gastrocnemius muscle (nmol/g)			
4.3% casein	13.0±1.7	46.6±4.5	59.6±5.7	8.4±0.8	91.9±8.7	100.3±8.5
17.2% casein	11.6±1.9	48.2±2.5	59.8±3.6	7.0±1.1	84.7±5.8	91.7±5.0

Values are shown as mean±SE for the 4.3% ($n=5$) and 17.2% ($n=5$) casein diets. Student's t test was performed to assess the effect of protein intake. No significant differences were demonstrated in the plasma and tissues phenylalanine or tyrosine concentrations between the 4.3% and 17.2% casein diets.

^{13}C-Phe, L-[1-^{13}C]phenylalanine; ^{12}C-Phe, L-[1–^{12}C]phenylalanine; ^{13}C-Tyr, L-[1-^{13}C]tyrosine; ^{12}C-Tyr, L-[1–^{12}C]tyrosine.

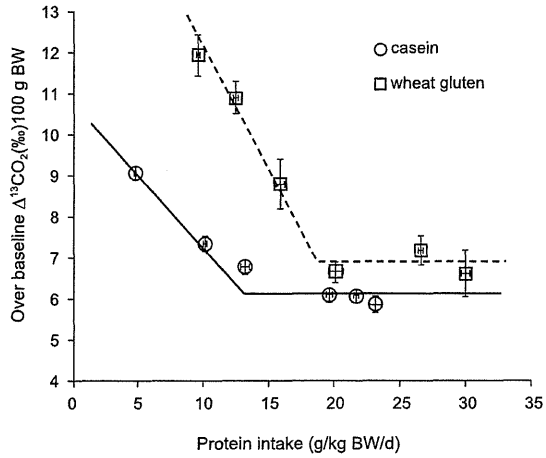

Fig. 3. The relationship between the intake of various proteins and the production of $^{13}CO_2$ from the oxidation of L-[1-^{13}C]phenylalanine when the rats were fed a casein diet (n=8 per mean) or a wheat gluten diet (n=8 per mean). Values are shown as mean±SE. The breakpoint estimates the mean protein intake for metabolic demands. The linear regression equation for the estimated protein intake for metabolic demands for the casein diet is as follows: $y=10.73-0.35x$ and $y=6.17$, for the wheat gluten diet is as follows: $y=18.87-0.66x$ and $y=6.92$, for the downward slope of the line and the level part of the line, respectively. The protein (%) included in the casein and wheat gluten diets was converted into protein intake (g) per day, and further normalized according to each rat's body weight. The mean protein intakes for metabolic demands for the casein and wheat gluten diets were estimated to be 13.1 g/kg BW/d and 18.1 g/kg BW/d, respectively.

Figure 3 shows the mean breakpoints illustrated in the breath $^{13}CO_2$ data, which were representative of the mean protein intake for metabolic demands. As the protein intake increased, breath $^{13}CO_2$ decreased steadily until the breakpoints were reached. There was no further decrease in breath $^{13}CO_2$ with the increase in protein intake. The protein (%) included in the casein and wheat gluten diets was converted into protein intake (g) per day, and further normalized according to each rat's body weight. Application of a mixed-effect change-point regression models to the breath $^{13}CO_2$ data resulted in the identification of a breakpoint at a dietary casein intake of 13.1 g/kg BW/d and a dietary wheat gluten intake of 18.1 g/kg BW/d. The enrichment of breath $^{13}CO_2$ was consistently higher in rats fed the wheat gluten diet, compared with the casein diet.

DISCUSSION

In the current IAAO study on rats, the protein intake for metabolic demands was estimated to be covered by a 13.1 g/kg BW/d for casein. This result was similar to the value recommended by the AIN-93G diet for laboratory rodents (a purified 20% casein (≥85% protein)), which was developed based on nitrogen balance studies. According to procedures recommended by the AIN, values were converted to dietary content by assuming a dietary intake of 15 g/rat/d for growing rats, and also for the rats fed 16.7±0.3 g/rat/d in the present study. This is the first study conducted that employed the IAAO method to determine the protein intake for metabolic demands using protein itself in rats, and the determined the protein intake for metabolic demands should be considered provisional.

Temperature, age, and physical activity influence the energy requirements of rats. It is difficult to estimate the energy requirement for growth due to variations in the composition of weight gain (*26–30*) and variations in the energetic efficiency of net protein and fat synthesis. However, it has been suggested that rats will generally consume enough food to meet their energy requirements (*31, 32*). The AIN-93 specifications indicate that a diet containing at least 15.0 kJ/g will meet the energy requirement for maintenance and growth if the rats are allowed free access to food and the diet is not deficient in other nutrients. In the present study, the rats accepted a 15.5 kJ/g diet containing 17.2% casein as a maintenance diet. Furthermore, the rats were given free access to this diet and the 24-h dietary intake was regarded as an individual rat's energy requirement.

Humayun et al. (*19*) reevaluated the protein requirement in young men employing the IAAO method, and the protein source of the experimental diet was consumed hourly in small meals consisting of a crystalline amino acid mixture. In the present study, casein and wheat gluten were employed as the protein source, and therefore, as the rats consumed the experimental diet at 3-h intervals, it can be considered that the mechanism of assimilation differed from that of the amino acid mixture. Amino acid mixtures will be absorbed very rapidly, and protein utilization will show a lower efficiency, compared with slow proteins such as casein (*20*).

The phenylalanine and L-[1-^{13}C]phenylalanine concentrations in the plasma, liver and gastrocnemius muscle were not affected by the amount of protein intake in the 4.3% or 17.2% casein diets, suggesting that the precursor pool for indicator oxidation did not change in size in response to the test protein intake. After phenylalanine is hydroxylated, conversion to tyrosine takes place, so the tyrosine concentration was also examined. In comparison with the ratio of L-[1-^{13}C]phenylalanine to the total phenylalanine concentration, only a trace of L-[1-^{13}C]tyrosine to the total tyrosine occurred in the plasma and tissues, regardless of the protein intake, suggesting that there was no tyrosine deficiency. In previous studies, the loss of the ^{13}C into the protein-bound tyrosine pool or tyrosine metabolites was minimized by providing a high-tyrosine diet before the study (*19*).

$^{13}CO_2$ breath tests are normally performed in the presence of a large background of naturally occurring isotope of approximately 1.1% ^{13}C (*33*). The ^{13}C rate of any unlabeled substrate ingested during a $^{13}CO_2$ breath test must be considered in order to eliminate artifacts that may reduce the sensitivity of the breath test and produce erroneous results (*33*). In our preliminary

examination, the stable rate of $^{13}CO_2$ production in breath was achieved between 5 h and 6 h and maintained until the end of the study. These results suggested that two meals received every 3 h were required to achieve constant $^{13}CO_2$ enrichment, and that the effect of the ^{13}C infusion could be evaluated correctly after the third meal at 15:00.

Experiment 1 demonstrated a similar pattern and a latter steady state ~2.5 h after the start of the stable isotope protocol (Fig. 2), so breath samples for the measurement of the protein metabolism were collected 210 min after the administration of the stable isotope began. Moreover, the protein intake level, the 4.3% or 17.2% casein diets, had a significant effect on breath $^{13}CO_2$ concentration at 18:30, showing that this protocol could detect differences in protein metabolism. These results reflected the supposition that if one indispensable amino acid (limiting) was deficient for protein synthesis, then all other indispensable amino acids (including the indicator amino acid, [^{13}C]phenylalanine) would be oxidized. Therefore, when the rats were fed a low protein diet, the 4.3% casein diet, most of amino acids were oxidized, and the $^{13}CO_2$ concentration in breath increased. By increasing the protein intake with the 17.2% casein diet, the intake of the limiting amino acid also increased, and the values produced by the IAAO method decreased, reflecting the increasing incorporation into protein.

The mean protein intakes for metabolic demands determined by the IAAO method were 13.1 g/kg BW/d for the casein and 18.1 g/kg BW/d for the wheat gluten. Therefore, the protein intakes for metabolic demands based on wheat gluten was higher than that based on casein. The differences between the casein and wheat gluten diets will be a function of the limiting amino acid in the respective protein source. This limiting amino acid will be dependent on both the amino acid profile and the digestibility of the protein. These results also conformed with our hypothesis, that the protein requirement will decrease with good quality (amino acid scoring pattern) protein intake, and increase with poor quality protein intake, validating the concept that the IAAO method could be employed to evaluate the quality of protein.

In regard to the measured phenylalanine oxidation, the enrichment of breath $^{13}CO_2$ differed between the rats fed the casein and wheat gluten diets. The enrichment of breath $^{13}CO_2$ was consistently higher in rats fed the wheat gluten diet, compared with rats fed the casein diet, even at the plateau line with a protein intake more than the metabolic demand for protein. According to intake of protein, specifically, the limiting amino acid, the indicator amino acid is partitioned between incorporation into proteins and oxidation. The quality of the protein also affected the $^{13}CO_2$ volume in the breath. Future extensions of this study to other protein sources will be necessary in order to confirm this relationship.

Hegsted (*34*) suggested the necessity of taking account of adaptation in their nitrogen balance methods, arguing that prior adaptation is required. The IAAO method can be conducted in short time periods because no period of adaptation to each intake is employed (*35*). Therefore, the IAAO method could be employed to evaluate the metabolic protein demand for all age groups (infants, children, adolescents, adults, and the elderly), as well as for post-operative patients or patients with injuries or infections that have specific metabolic conditions, such as a widely varying metabolic demand. In a clinical setting, the adequate quality and quantity of protein or amino acid for each specific condition could be estimated using the IAAO method.

The results of this study demonstrated that the IAAO method can be employed to evaluate not only the protein intake for metabolic demands, but the dietary protein quality in freely living rats. Further studies are necessary to assess the viability of the IAAO method in a clinical setting.

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REFERENCES

1) World Health Organization. 2007. Protein and amino acid requirements in human nutrition. Report of a joint WHO/FAO/UNU Expert Consultation (WHO Technical Report Series, No. 935), Geneva.
2) Forbes GB. 1973. Another source of error in the metabolic balance method. *Nutr Rev* **31**: 297–300.
3) Wallace WM. 1959. Nitrogen content of the body and its relation to retention and loss of nitrogen. *Fed Proc* **18**: 1125–1130.
4) Hegsted DM. 1976. Balance studies. *J Nutr* **106**: 307–311.
5) Hegsted DM. 1978. Assessment of nitrogen requirements. *Am J Clin Nutr* **31**: 1669–1677.
6) Young VR, Bier DM, Pellett PL. 1989. A theoretical basis for increasing current estimates of the amino acid requirements in adult man with experimental support. *Am J Clin Nutr* **50**: 80–92.
7) World Health Organization. 1985. Energy and protein requirements. Report of a Joint FAO/WHO/UNU Expert Consultation (WHO Technical Report Series, No. 724), Geneva.
8) Kim KI, McMillan I, Bayley HS. 1983. Determination of amino acid requirements of young pigs using an indicator amino acid. *Br J Nutr* **50**: 369–382.
9) Ball RO, Bayley HS. 1984. Tryptophan requirement of the 2.5-kg piglet determined by the oxidation of an indicator amino acid. *J Nutr* **114**: 1741–1746.
10) Lin FD, Smith TK, Bayley HS. 1986. Tryptophan requirement of growing swine as determined by the oxidation of an indicator amino acid. *J Anim Sci* **62**: 660–664.

11) Bertolo RF, Moehn S, Pencharz PB, Ball RO. 2005. Estimate of the variability of the lysine requirement of growing pigs using the indicator amino acid oxidation technique. *J Anim Sci* **83**: 2535–2542.
12) Zello GA, Pencharz PB, Ball RO. 1993. Dietary lysine requirement of young adult males determined by oxidation of L-[1-^{13}C]phenylalanine. *Am J Physiol* **264**: E677–E685.
13) Roberts SA, Thorpe JM, Ball RO, Pencharz PB. 2001. Tyrosine requirement of healthy men receiving a fixed phenylalanine intake determined by using indicator amino acid oxidation. *Am J Clin Nutr* **73**: 276–282.
14) Di Buono M, Wykes LJ, Ball RO, Pencharz PB. 2001. Total sulfur amino acid requirement in young men as determined by indicator amino acid oxidation with L-[1-^{13}C]phenylalanine. *Am J Clin Nutr* **74**: 756–760.
15) Turner JM, Humayun MA, Elango R, Rafii M, Langos V, Ball RO, Pencharz PB. 2006. Total sulfur amino acid requirement of healthy school-aged children as determined by indicator amino acid oxidation technique. *Am J Clin Nutr* **83**: 619–623.
16) Riazi R, Wykes LJ, Ball RO, Pencharz PB. 2003. The total branched-chain amino acid requirement in young healthy adult men determined by indicator amino acid oxidation by use of L-[1-^{13}C]phenylalanine. *J Nutr* **133**: 1383–1389.
17) Pillai RR, Elango R, Muthayya S, Ball RO, Kurpad AV, Pencharz PB. 2010. Lysine requirement of healthy, school-aged Indian children determined by the indicator amino acid oxidation technique. *J Nutr* **140**: 54–59.
18) Elango R, Humayun MA, Ball RO, Pencharz PB. 2009. Indicator amino acid oxidation is not affected by period of adaptation to a wide range of lysine intake in healthy young men. *J Nutr* **139**: 1082–1087.
19) Humayun MA, Elango R, Ball RO, Pencharz PB. 2007. Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. *Am J Clin Nutr* **86**: 995–1002.
20) Beaufrere B, Dangin M, Boirie Y. 2000. Fast and slow protein concept. *In*: Proteins, Peptides and Amino Acid in Enteral Nutrition (Furst P, Young V, eds) (Nestle Nutrition Workshop Series, Clinical & Performance Program Vol. 3), p 121–133. Karger, Basel.
21) Moehn S, Bertolo RF, Pencharz PB, Ball RO. 2005. Development of the indicator amino acid oxidation technique to determine the availability of amino acids from dietary protein in pigs. *J Nutr* **135**: 2866–2870.
22) Food and Agriculture Organization of the United Nations. 1991. Protein quality evaluation in human diets. Report of a Joint FAO/WHO/UNU Expert Consultation (FAO Food and Nutrition Paper No. 51), Rome.
23) Ministry of Education, Culture, Sports, Science and Technology. 2010. Standard tables of food composition in Japan, amino acid composition of foods 2010. Report of the Subdivision on Resources, The Council for Science and Technology. Tokyo.
24) Reeves PG, Nielsen FH, Fahey GC Jr. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr* **123**: 1939–1951.
25) Hayamizu K, Kato M, Hattori S. 2011. Determining amino acid requirements from repeated observations on indicator amino acid oxidation method by mixed-effect change-point regression models. *J Clin Biochem Nutr* **49**: 115–120.
26) Meyer JH. 1958. Interactions of dietary fiber and protein on food intake and body composition of growing rats. *Am J Physiol* **193**: 488–494.
27) Schemmel R, Mickelsen O, Motowi K. 1972. Conversion of dietary to body energy in rats as affected by strain, sex, and ration. *J Nutr* **102**: 1187–1197.
28) Hartsook EW, Hershberger TV, Nee JC. 1973. Effects of dietary protein content and ratio of fat to carbohydrate calories on energy metabolism and body composition of growing rats. *J Nutr* **103**: 167–178.
29) McCracken KJ. 1975. Effect of feeding pattern on the energy metabolism of rats given low-protein diets. *Br J Nutr* **33**: 277–289.
30) Deb S, Martin RJ, Hershberger TV. 1976. Maintenance requirement and energetic efficiency of lean and obese Zucker rats. *J Nutr* **106**: 191–197.
31) Berg RT, Bowland JP, Sibbald IR. 1956. Digestible energy in relation to food intake and nitrogen retention in the weaning rat. *J Nutr* **59**: 385–392.
32) Peterson AD, Baumgardt BR. 1971. Influence of level of energy demand on the ability of rats to compensate for diet dilution. *J Nutr* **101**: 1069–1074.
33) Schoeller DA, Klein PD, Watkins JB, Heim T, MacLean WC Jr. 1980. ^{13}C abundances of nutrients and the effect of variations in ^{13}C isotopic abundances of test meals formulated for $^{13}CO_2$ breath tests. *Am J Clin Nutr* **33**: 2375–2385.
34) Hegsted DM. 2000. From chick nutrition to nutrition policy. *Ann Rev Nutr* **20**: 1–19.
35) Brunton JA, Ball RO, Pencharz PB. 1998. Determination of amino acid requirements by indicator amino acid oxidation: applications in health and disease. *Curr Opin Clin Nutr Metab Care* **1**: 449–453.

Original Article

Association between 24 hour urinary α-tocopherol catabolite, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α-CEHC) and α-tocopherol intake in intervention and cross-sectional studies

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The objective is to determine the association between the 24 hour urinary α-tocopherol catabolite, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α-CEHC) and α-tocopherol intake in an intervention and a cross-sectional studies. In the 4-weeks intervention study, Japanese men (n = 10) consumed the test diet in week 1, and the test diet plus varying amounts of α-tocopherol in the three subsequent weeks: 21 μmol/d α-tocopherol in week 2, 63 μmol/d in week 3, and 125 μmol/d in week 4. A significant association between α-tocopherol intake and urinary α-CEHC was observed in this strictly controlled experiment ($r = 0.99$, $p<0.001$). In the cross-sectional study, all foods consumed over 4 consecutive days were recorded in 76 free-living young subjects (18-33 years). The association was weak, but a significant relationship was observed ($r = 0.29$, $p<0.05$) even in the cross-sectional study. In the cross-sectional study adults, mean estimated α-tocopherol intake calculated by urinary α-CEHC and the excretory ratio was 91% of their mean intake over the 4 days. The results show that urinary α-CEHC level reflected recent α-tocopherol intake in free-living young Japanese adults, and could be used as a measure of intake during the previous few days, both for group means and for individual rankings within a group.

Key Words: α-tocopherol, catabolism, CEHC, urine, biomarker

INTRODUCTION

Measurements of food intake are widely used for surveys of nutritional assessment. However, there are limitations in assessing only information from food surveys,[1] and methods that measure biological parameters can reveal new information. Urine, which is a noninvasive bio-sample, might overcome the limitations of nutritional assessment by food survey. For example, 24-hour urinary nitrogen level is established as a marker for protein intake,[2] urinary potassium level as a marker for potassium intake,[3] and urinary sugar level as a marker for sugar intake.[4,5] In previous studies, we investigated the relationship between water-soluble vitamin intake and their urinary excretion of these nutrients. We clarified that urinary water-soluble vitamin levels are strongly correlated with their intake.[6-9] These studies [6-9] have indicated that 24-h urinary excretion of water-soluble vitamins is a potential biomarker for recent vitamin intake in both intervention and cross-sectional studies.

Generally, fat-soluble vitamins are not excreted in urine. However, In 1995, Schultz *et al*,[10] reported that a catabolite of α-tocopherol, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α-CEHC), which is a metabolite with an intact chroman ring, is excreted in urine. Previously, α-CEHC was proposed as a potential excretion product of α-tocopherol in 1965 by Schmandke *et al*,[11] but had not been described again until Schultz's report. Schultz *et al*,[10] suggested that α-CEHC excretion indicates the saturated binding capacity of α-tocopherol in the plasma, and thus may be considered to be a marker of optimum α-tocopherol intake. This proposal was strengthened by Shuelke *et al*,[12] who found that α-CEHC was excreted into the urine of patients with α-tocopherol binding protein defects regardless of the plasma α-tocopherol concentration, whereas it was not excreted by healthy subjects until the plasma α-tocopherol concentration

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surpassed 40 μmol/L. These findings indicate that the urinary content of α-CEHC in the Japanese is almost below detection, because the average plasma concentration of α-tocopherol in the Japanese is around 20 μmol/L.[13] However, as Schultz *et al*,10 pointed out in their paper, their results were obtained with only seven participants, and therefore, further research is necessary to allow the results to be applied generally.

In 2003, Morinobu *et al*,[14] reported a straightforward and reliable method of determining α-CEHC, which was later modified by Stahl *et al*[15] and Lodge *et al*[16] Morinobu *et al*[14] also reported that α-CEHC was detected in healthy adult male Japanese volunteers (n = 14). Therefore, it is probable that α-CEHC is excreted into urine in Japanese individuals who take an ordinary amount of α-tocopherol.

The aim of the present study was to determine the possibility that α-CEHC can be used as a biomarker of α-tocopherol status in young Japanese adults. We examined the association between 24 h urinary α-CEHC levels and the intakes of α-tocopherol in strictly controlled-living and in free-living participants.

MATERIALS AND METHODS

Both studies were reviewed and approved by the ethics committee of The University of Shiga Prefecture. The purpose and protocol of this study was explained to all participants before joining the study, and written informed consent was obtained from each participant.

Subjects and experimental design

Intervention study group

We recruited students from a registered dietician department. All subjects (male Japanese college students, n = 10) were housed in the same facility and given the same diet. The experimental period was 4 weeks. They did not have regular use of medications or dietary supplements, or habitual alcohol or cigarette consumption. Age, body weight, height, and body mass index (mean ± SD) were 22.1 ± 2.3 years (18–25 years), 63.6 ± 5.2 kg, 174 ± 5 cm, and 21.0 ± 1.6 kg/m^2, respectively.

Cross-sectional study group

A total of 102 healthy, free-living Japanese females, aged 18-33 years, voluntarily participated in this study. The exclusion criteria were: presence of cold or influenza, and use of multivitamin supplements at least once during the previous month. In addition, we excluded participants whose 24-hour urine collection or dietary records were considered as incomplete, with a collection time outside the 22-26 h range, a urine volume of <250 ml, creatinine excretion in relation to body weight outside the 10.8-25.2 mg/kg range,[17,18] or extremely low or high energy intake (<2,090 or >16,700 kJ/d).[19] After these exclusions, 76 of the 102 female students were found to be eligible and were enrolled into the group.

Dietary records

Intervention study group

The diet given to the participants consisted of a breakfast of bread, margarine, ham, yoghurt, tomato, lettuce, and milk; a lunch of rice, toasted and seasoned laver (a type of seaweed), luncheon meat, boiled egg, raw cabbage, miso soup, and Japanese tea; and an evening meal of rice, soy sauce, seasonal Pacific saury (a type of fish), tofu (soybean curd), boiled spinach leaves, kiwi fruit and Japanese tea, with a midnight snack of cheese and jelly fruit mix. The daily intakes of energy and nutrients form the basal diet are shown in Table 1. Nutrients were calculated by using Standard Tables of Food Composition in Japan, (fifth revised and enlarged edition).[20] The subjects ate this diet on days 1-5 of each week over the experimental period of 4 weeks and were free to eat what they wanted on days 6 and 7. In the latter three weeks, the participants took α-tocopherol acetate in addition to the diet: 21 μmol/d in week 2, 63 μmol/d in week 3, and 125 μmol/d in week 4.

Table 1. Daily intake of energy and nutrients form the basal diet in intervention study

Energy and nutrients[1]	Amount
Energy (kJ)	11,100
Protein (g)	97.5
Fat (g)	86.7
Carbohydrates (g)	361
α-Tocopherol (mg)	8.7 (20.2 μmol)

[1]Nutrients were calculated from the Standard Tables of Food Composition in Japan.[20]

Cross-sectional study group

This group underwent a 4-day dietary assessment in which the participants were living freely in the university and consuming their normal diet. The 4-day assessment began on a Monday (day 1) and ended on Thursday (day 4). All food consumed during the 4-day period was recorded using a weighed food recording method.[21] A digital cooking scale capable of weighing in 1 g increments (Tanita Inc., Tokyo, Japan), a set of dietary record forms, a dietary record manual, and a disposable camera were distributed to the participants in advance. In the dietary record, the ingested food was described (eg "raw", "boiled", "cooked", "skin present", "a part of cooking ingredients", or "with or without seasoning"), and coded according to the Standard Tables of Food Composition in Japan (fifth revised and enlarged edition) as for the intervention group.[20] The participants took photographs with a disposable camera of the dishes before and after eating. Several experienced dietitians used the photographs to complete the data, and asked the participants to resolve any discrepancies or to obtain further information when needed. The food that remained after eating was measured on the digital scales and was deducted from the dietary record. Food, nutrient and energy intakes were calculated using SAS statistical software (version 6.12; SAS Institute, Cary, NC, USA), based on the Standard Tables of Food Composition in Japan.

Collection of 24 hour urine sample

For the intervention study group, the 24 hour urine samples were collected from the second urination on day 4 to the first urination after 06:30 hours (wake-up time) on day 5 in each week.

For the cross-sectional group, a single 24-hour urine sample was collected on day 4 to measure the α-

tocopherol metabolite, α-CEHC. In the morning, participants were asked to discard the first specimen and to record the time on the sheet. The following morning, participants were asked to collect a specimen at the same time as the discarded specimen from the previous morning and to record the time on the sheet.

After the urine samples were collected, the sample volumes were measured, and aliquots of the urine were stabilized to avoid destruction of α-CEHC. All treated urine samples were then stored at –20°C until analysis.

Chemicals

α-CEHC was purchased from Cayman Chemical Co., Ltd (Ann Arbor, Michigan, USA). β-Glucuronidase derived from *Escherichia coli* was obtained from Nacalai Tasque Co., Ltd (Kyoto, Japan). All other chemicals used were of the highest purity available from commercial sources.

Analysis of α-CEHC

The concentrations of α-CEHC in urine were measured by high performance liquid chromatography with electrochemical detection (HPLC-ECD), as described by Morinobu *et al.*[14] β-Glucuronidase (25,000 units) was dissolved in 2.5 ml of 0.1 mol/L sodium acetate-acetate buffer (pH 4.5) on ice and used to hydrolyze the conjugate immediately after preparation. Urine (1 ml) was placed into a tube with 1,000 U (100 μl) of β-glucuronidase, 100 μl of 57 mmol/L ascorbic acid, and 20 μl of 0.015% dibuthylhydroxytoluene (BHT). After which the mixture was incubated for 4 hours at 37°C to achieve hydrolysis, 50 μl of 6 mol/L HCl and 2 ml of diethylether were added to stop the reaction. After mixing by vortex and separating by centrifugation at 1,800×*g* for 10 min, 1 ml of the diethylether layer was collected and evaporated to dryness. The residue was dissolved in 200 μl of 0.015% BHT, and a 20 μl aliquot was injected into the HPLC-ECD.

Statistics

SPSS software (version 16 for Windows; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Values were presented as means ± SD. The daily measurements of urinary α-CEHC and the dietary α-tocopherol intakes were not normally distributed, therefore, the data were converted logarithmically. Pearson correlation coefficients were calculated to determine the association between urinary and dietary measurements. The value of the urinary excretory ratio (%) was calculated as follows: [α-CEHC excretion in the fourth day (μmol/d)/the average α-tocopherol intake during 4-days (μmol/d)]×100. An analysis of variance (ANOVA) random effects model was used to quantify inter- and intra-individual percentage coefficient of variance (% CV), which was used to estimate the variability in α-tocopherol intake.

RESULTS

Relationship between the intake of α-tocopherol and the urinary excretion of α-CEHC

Intervention study group

Figure 1 shows the relationship between the intake of α-tocopherol and the urinary excretion of α-CEHC. A strong significant association was observed ($r = 0.99$, $p = 0.0043$). The average urinary α-CEHC concentration at baseline (week 1) was 0.74 μmol/d, which increased to 0.94, 1.89, and 3.34 μmol/d after supplementation at the doses of 21, 63 and 125 μmol/d, respectively. The excretory ratio of α-CEHC was 3.7 ± 1.3, 2.3 ± 0.7, 2.3 ± 1.1, and 2.3 ± 0.6% for α-tocopherol intakes of 20, 41, 83 and 146 μmol/d, respectively.

Cross-sectional study group

The basic characteristics of the 76 young women are presented in Table 2. Each values were similar to those reported for young adults female aged 18–22 years.[19] In brief, the participants were considered as typical female university students in Japan, characterized by relatively low BMI (20.2 kg/m^2), and low intake of fat (28.4%). During the experimental period, all participants were living freely, and none of the participants were drinking or smoking. Average intake of α-tocopherol in participants was 13.7 ± 3.8 μmol/d, and daily intake of α-tocopherol

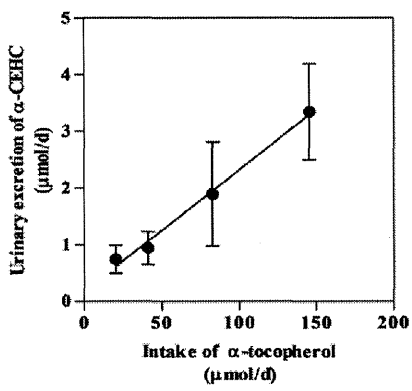

Figure 1. Relationship between urinary excretion of α-CEHC and the intake of α-tocopherol in strictly controlled participants (intervention study). Individual average intake of α-tocopherol is plotted on the *x*-axis, and the 24-h urinary excretion of α-CEHC, a catabolite of α-tocopherol, is plotted on the *y*-axis. In total, 10 healthy male Japanese college students aged 18-25 years were enrolled. Values are mean ± SD. A significant correlation ($r = 0.99$, $p = 0.0043$) was obtained. Regression line: $y = 0.0214 (\pm 0.0014) x + 0.180 (\pm 0.122)$.

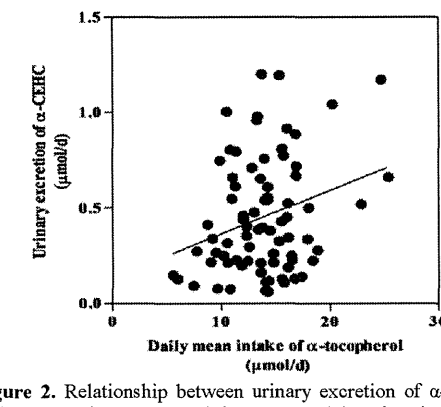

Figure 2. Relationship between urinary excretion of α-CEHC and intake of α-tocopherol in young adults' female (cross-sectional study). Measurements were taken of food intake on 4 consecutive days. The urine samples were collected at day 4. Individual average intake of α-tocopherol is plotted on the *x*-axis, and the urinary excretion of α-CEHC on day 4 is plotted on the *y*-axis. In total, 76 healthy, free-living, female college students aged 18–33 years were enrolled. A significant correlation ($r = 0.29$, $p = 0.0147$) was obtained. Regression line: $y = 0.0228 (\pm 0.0091) x + 0.137 (\pm 0.130)$.

Table 2. Characteristics and dietary intakes of female subjects

Variables	Young adult female (n = 76)
Anthropometric variable	
Age (years)	20.1 ± 2.3
Body height (cm)	158 ± 5
Body weight (kg)	50.6 ± 5.4
Body mass index (kg/m^2)	20.2 ± 1.8
Dietary mean intake at days 1-4	
Total energy (kJ/d)	6950 ± 1260
Protein (% of energy)	13.8 ± 2.2
Fat (% of energy)	28.4 ± 4.5
Carbohydrate (% of energy)	56.5 ± 4.9
α-Tocopherol intake (μmol/d)	13.7 ± 3.8
Inter-individual variations on the vitamin E intake (% CV)[1]	41.5
Intra-individual variations on the vitamin E intake (% CV)[1]	55.3
Urinary α-CEHC (μmol/d)	0.444 ± 0.292
Excretory ratio[2] of vitamin E (%)	3.58 ± 3.39

[1]% CV, percentage coefficient of variance.
[2]Calculated by the formula [α-CEHC excretion on day 4 (μmol/d) / the average α-tocopherol intake over the 4 days (μmol/d)] × 100.

Table 3. Mean dietary α-tocopherol intake and 24-hour urinary α-CEHC, excretory ratio, and estimated mean α-tocopherol intake in young adult females

	Young adult female (n = 76)
Mean α-tocopherol intake[1] (μmol/d)	13.7 ± 3.8
Excretory ratio[2] (%)	3.58 ± 3.39
Estimated mean α-tocopherol intake[3] (μmol/d)	12.4 ± 8.2
% ratio[4]	91

[1]Calculated by average α-tocopherol intake over the 4 days for each individual.
[2]Calculated by the formula [α-CEHC excretion on day 4 (μmol/d) / the average α-tocopherol intake over the 4 days (μmol/d)] × 100.
[3]The estimated α-tocopherol intake in young adult was calculated by the individual value of the 24-h urinary excretion of α-CEHC and the excretory ratio of 3.58 %, and the estimated mean α-tocopherol intake was calculated by the resulting estimated α-tocopherol intake.
[4]%Ratio between the mean and the estimated mean α-tocopherol intake.

was similar to adequate intake for Japan. [20] Urinary excretion of α-CEHC was twofold higher in elderly subjects than healthy peoples. The value of the excretory ratio of vitamin E in elderly subjects was 1.4-fold higher than in healthy individuals. Intra and inter-individual variations in α-tocopherol intakes, the values were both around 50% (Table 2).

Correlations between 24-hour urinary excretion of α-tocopherol metabolite, α-CEHC on day 4 and the average α-tocopherol intake on 4 consecutive days are shown in Figure 2. A significant association was observed in young adult females.

Estimated mean α-tocopherol intake calculated by urinary excretion of α-CEHC and the excretory ratio in the **cross-sectional study**

Mean dietary α-tocopherol intake and 24-hour urinary α-CEHC, excretory ratio, and estimated mean α-tocopherol intakes in young women are shown in Table 3. The excretory ratio was determined from the urinary excretion of α-CEHC and the average α-tocopherol intake over 4 days. The individual estimated α-tocopherol intake was calculated by average excretory ratio and the individual urinary α-CEHC value. The estimated mean α-tocopherol intake in young women was 91% of the real mean α-tocopherol intake over 4 days in young adult females.

DISCUSSION

Alpha-CEHC, a urinary metabolite of α-tocopherol, was described in 1995 by Schultz *et al*[10] Excretion of α-CEHC is considered to reflect saturation of α-tocopherol in the body, because it is not a metabolite of the α-tocopherol consumed for antioxidant defense. In other words, the detection of α-CEHC in urine generally indicates a better α-tocopherol nutritional status. Therefore, it is generally considered that the content of α-CEHC is below the limit of detection under an ordinary dietary habitant. Morinobu *et al*,[14] however, reported that α-CEHC was detected in healthy adult male Japanese volunteers. Therefore, it is probable that α-CEHC is excreted into urine in Japanese individuals who consume an ordinary amount of α-tocopherol. But, the data are based on only seven persons. Further research is necessary to allow the results to be applied generally.

In the present first study, the intervention study was performed to determine whether urinary α-CEHC excretion correlates with intake of α-tocopherol. We found a significant positive correlation between urinary α-CEHC and intake of α-tocopherol in healthy young male Japanese adults who consumed a strictly controlled diet with doses of α-tocopherol ranging from 20 to 145 μmol/d.

In the second experiment, to determine the usefulness of urinary α-CEHC as a biomarker for α-tocopherol nutritional status, a cross-sectional study was performed on free-living subjects. A weak but significant correlation was found between urinary α-CEHC and the mean α-tocopherol intake. These results indicate that α-CEHC levels in 24-h urine reflect dietary α-tocopherol intakes over the past few days, and suggest that α-tocopherol intake can be estimated from urinary α-CEHC values in free-living subjects.

This phenomenon might be only applicable for the Japanese and East-Asian populations. The subjects ate a lot of vegetable and were of relatively low BMI and had low intake of fat. The requirement of α-tocopherol is dependent on the intakes of polyunsaturated fatty acids. The average intakes of the polyunsaturated fatty acids were around 10 g/d. The optimum ratio of α-tocopherol (mg/d) to polyunsaturated fatty acids (g/d) is reported to be 0.60.[22] Its average ratio was about 0.60 in the present subjects. This is a reason why a significant association was observed even with low intake of α-tocopherol for the Japanese.

Metabolism of α-CEHC from α-tocopherol is somewhat different between Japanese, America and European populations. For example, the metabolic activity of α-tocopherol to α-CEHC might be higher in the Japanese than in the Americans and Europeans. The precise regulatory mechanism of post-absorption α-tocopherol elimination is not clear. The current knowledge is only of a pathway involving cytochrome P450-mediated ω-hydroxy lation of the α-tocopherol phytyl side chain, followed by stepwise removal of two or three carbon molecules such as acetyl-CoA or propionyl-CoA, ultimately yielding the α-CEHC that is excreted in urine.[23] Low fat intakes in the Japanese might bring about surplus ability to the β oxidation system, which results in increased activity of α-tocopherol to α-CEHC. Sesamin increases tissue α-tocopherol concentration by inhibiting the α-tocopherol oxidation pathway;[24] sesame oil, which contains sesamin, is often used in Japanese cooking, which might bring about the saving effect of α-tocopherol. Compared to Caucasian, Asian population had significantly lower plasma vitamin E levels in the same environment.[25] However, there are no data regarding its metabolism or immune functions among two populations. In Asian populations, they may be easily excreted into urine as α-CEHC compared to Caucasians. This is also a reason why the significant association was observed even with low intake of α-tocopherol for the Japanese.

The limiting factor of the transport capacity appears to be plasma lipid concentration. In general, subjects with a low total plasma lipid concentration did not accumulate as high concentration of α-tocopherol, as those with a higher total lipid content; and they started to excrete α-CEHC earlier[10]. If the urinary excretion of α-CEHC is related to the α-tocopherol content of plasma lipid, the range of thresholds of excretion is much wider and the correlation between plasma concentration and urinary excretion becomes more pronounced. In the present cross-sectional study, we did not measure plasma lipid concentration, and how plasma lipid affected urinary excretion of α-CEHC is unclear.

In terms of the completeness of the dietary assessment in the present study, there are several limitations in terms of using a weighed food record method. To reduce errors associated with self report, several dietitians reviewed the collated records along with the photographs. The selection of participants from a dietetics course also contributed to reduce reporting errors, as they had nutritional knowledge and were well trained. Another limitation exists in the present food composition table developed for Japan. In a dietary assessment of free-living people, potential errors caused by the quality of this table, such as defects in food composition, are inevitable. These limitations might cause the relatively low correlation in the free-living experiment population compared with that of the intervention study.

In conclusion, a significant relationship between the α-tocopherol intake and urinary excretion of α-CEHC was observed in young Japanese adults.

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AUTHOR DISCLOSURES

None of the authors had any financial or personal conflicts of interest associated with this manuscript.

REFERENCES

1. Livingstone MB, Black AE. Markers of the validity of reported energy intake. J Nutr. 2003;133:895S-20.
2. Bingham SA. Urine nitrogen as a biomarker for the validation of dietary protein intake. J Nutr 2003;133:921S-4.
3. Tasevska N, Runswick SA, Bingham SA. Urinary potassium is as reliable as urinary nitrogen for use as a recovery biomarker in dietary studies of free living individuals. J Nutr. 2006;136:1334-40.
4. Tasevska N, Runswick SA, Welch AA, McTaggart A, Bingham SA. Urinary sugars biomarkers relates better to extrinsic than to intrinsic sugars intake in a metabolic study with volunteers consuming their normal diet. Eur J Clin Nutr. 2009;63:653-9.
5. Bingham S, Luben R, Welch A, Tasevska N, Wareham N, Khaw KT. Epidemiologic assessment of sugars consumption using biomarkers: Comparisons of obese and nonobese individuals in the European prospective investigation of cancer Norfolk. Cancer Epidemiol Biomarkers Prev. 2007; 16:1651-4.
6. Shibata K, Fukuwatari T, Ohta M, Okamoto H, Watanabe T, Fukui T, et al. Values of water-soluble vitamins in blood and urine of Japanese young men and women consuming a semi-purified diet based on the Japanese Dietary Reference Intakes. J Nutr Sci Vitaminol. 2005;51:319-28.
7. Tsuji T, Fukuwatari T, Sasaki S, Shibata K. Twenty-four-hour urinary water-soluble vitamin levels correlate with their intakes in free-living Japanese school children. Public Health Nutr. 2010;25:1-7.
8. Tsuji T, Fukuwatari T, Sasaki S, Shibata K. Twenty-four-hour urinary water-soluble vitamins correlate with their vitamin intakes in free-living Japanese university students. Eur J Clin Nutr. 2010;64:800-7.
9. Tsuji T, Fukuwatari T, Sasaki S, Shibata K. Urinary excretion of vitamin B1, B2, B6, niacin, pantothenic acid, folate, and vitamin C correlates with dietary intakes of free-living elderly, female Japanese. Nutr Res. 2010;30:171-8.
10. Schultz M, Leist M, Petrzika M, Gassmann B, Brigelius-Flohé R. Novel urinary metabolite of alpha-tocopherol, 2, 5, 7, 8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman, as an indicator of an adequate α-tocopherol supply? Am J Clin Nutr. 1995;62:1527S-34.

11. Schmandke H. On the conversion of alpha-tocopherol and 2,5,7,8-tetramethyl-2-(beta-carboxyethyl)-6-acetoxychroman into tocopheronolactone in normal and tocopherol deficient organisms. (Zur Umwandlung von α-Tocopherol und 2.5.7.8-Tetramethyl-2-(β-carboxyäthyl)-6-acetoxychroman in Tocopheronolacton im Normal- und Tocopherolmangel-Organismus.) Int Z Vitaminforsch. 1965;35:346-52. (In German)
12. Schuelke M, Elsner A, Finckh B, Kohlschütter A, Hübner C, Brigelius-Flohé R. Urinary α-tocopherol metabolites in α-tocopherol transfer protein-deficient patients. J Lipid Res. 2000;4:1543-51.
13. Maruyama C, Imamura K, Oshima S, Suzukawa M, Egami A, Tonomoto M et al. Effects of tomato juice consumption on plasma and lipoprotein carotenoid concentrations and the susceptibility of low density lipoprotein to oxidative modification. J Nutr Sci Vitaminol. 2001;47:213-21.
14. Morinobu T, Yoshikawa S, Hamamura K, Tamai H. Measurement of α-tocopherol metabolites by high-performance liquid chromatography during high-dose administration of α-tocopherol. Eur J Clin Nutr. 2003;57:410-4.
15. Stahl W, Graf P, Brigelius-Flohé, Wechter W, Sies H. Quantification of the α- and γ tocopherol metabolites 2,5,7,8-tetramethyl- 2-(2'-carboxyethyl)-6-hydroxychroman and 2,7,8- trimethyl-2-(2'-carboxyethyl)-6-hydroxychroman in human serum. Anal Biochem. 1999;275:254-9.
16. Lodge JK, Traber MG, Elsner A, Brigelius-Flohe′ R. A rapid method for the extraction and determination of α-tocopherol metabolites in human urine. J Lipid Res. 2000;41: 148-54.
17. Stamler J, Elliott P, Dennis B, Dyer AR, Kesteloot H, Liu K, Ueshima H, Zhou BF. INTERMAP: background, aims, design, methods, and descriptive statistics (nondietary). J Human Hypertens. 2003;17:591-608.
18. Murakami K, Sasaki S, Takahashi Y, Uenishi K, Yamasaki M, Hayabuchi H et al. Misreporting of dietary energy, protein, potassium and sodium in relation to body mass index in young Japanese women. Eur J Clin Nutr. 2008;62:111-8.
19. Ministry of Health, Labour, and Welfare of Japan. Dietary Reference Intakes for Japanese, 2010, Tokyo; 2010. (In Japanese)
20. Ministry of Education, Culture, Sports, Science and Technology. Standard Tables of Food Composition in Japan, Fifth Revised and Enlarged Edition, Tokyo; 2007. (In Japanese)
21. Imai T, Sakai S, Mori K, Ando F, Niino N, Shimokata H. Nutritional assessment of 3-day dietary records in national institute for longevity science-longitudinal study of aging (NILS-LSA). J Epidemiol. 2000;10:70-6.
22. Valk EE, Hornstra G. Relationship between vitamin E requirement and polyunsaturated fatty acid intake in man: a review. Int Vitamin Nutr Res. 2000;70:31-42.
23. Sontag TJ, Parker RS. Cytochrome P450 ω-hydroxylase pathway of tocopherol catabolism. J Biol Chem. 2002;277: 25290-6.
24. Parker RS, Sontag TJ, Swanson JE. Cytochrome P4503A-dependent metabolism of tocopherols and inhibition by sesamin. Biochem Biophys Res Commun. 2000;277:531-4.
25. Benzie IF, Janus ED, Strain JJ. Plasma ascorbate and vitamin E levels in Hong Kong Chinese. Eur J Clin Nutr. 1998; 52:447-51.

Original Article

Association between 24 hour urinary α-tocopherol catabolite, 2,5,7,8-tetramethyl-2(2'-carboxyethyl)-6-hydroxychroman (α-CEHC) and α-tocopherol intake in intervention and cross-sectional studies

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24 小時尿液 α-生育醇代謝產物 2,5,7,8-tertranetgyl-2 (2'carboxyethyl)-6-hydroxychroman(α-CEHC)與 α-生育醇攝取之介入及橫斷研究之相關

本研究的目的為評估在介入及橫斷研究中，24 小時尿液的 α-生育醇代謝產物 2,5,7,8-tertranetgyl-2(2'carboxyethyl)-6-hydroxychroman (α-CEHC) 與 α-生育醇攝取量之相關性。在四週的介入性研究，日本男性 (n=10) 在第一週攝取測試飲食，後續三周攝取添加不同量 α-生育醇的測試飲食：第二週為 21 μmol/dα-生育醇、第三週為 63 μmol/dα-生育醇與第四週為 125 μmol/dα-生育醇。在這個嚴格控制的實驗中，觀察到 α-生育醇的攝取量與尿液中的 α-CEHC 有顯著的相關 (r=0.99, p<0.001)。在橫斷性研究，76 名年輕一般研究對象 (18-33 歲) 紀錄連續四天攝取的所有食物。在橫斷性研究這個相關性儘管不強，但是顯著的 (r=0.29, p<0.05)。在橫斷性研究中的成年人，以尿液 α-CEHC 計算其平均估計 α-生育醇攝取量，其排泄率為四日平均攝取量的 91%。無論組別平均值或是組別中個體的排序結果，都顯示尿液中的 α-CEHC 量可反映年輕日本一般成年人近期的 α-生育醇攝取量，且可以當作過去幾天的攝取量測量方法。

關鍵字：α-生育醇、代謝產物、CEHC、尿液、生物標記

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Vitamin Contents in Rat Milk and Effects of Dietary Vitamin Intakes of Dams on the Vitamin Contents in Their Milk

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Summary Studies of factors that affect milk vitamin contents are important. We investigated the vitamin contents in rat milk and the effects of dietary vitamin intakes of dams on the vitamin contents in their milk. A low-vitamin diet (0.2%) and a high-vitamin diet (4.0%) based on a diet containing 1% AIN-93-VX (normal diet) was given to female rats from pregnancy to lactation. Regarding the effects of the vitamin intakes, the concentrations of vitamins B_1, B_2, B_6, B_{12} and E were decreased with the low-vitamin diet, but were not increased with the high-vitamin diet. The concentrations of niacin, pantothenic acid and biotin were not decreased with the low-vitamin diet, but were increased with the high-vitamin mixture diet. The folate concentration remained constant regardless of the intake of folate. These findings clearly indicate that the levels of certain vitamins in milk are easily affected by the dietary vitamin intakes.

Key Words content, lactation, milk, rat, vitamin

It is generally believed that milk contains all of the nutrients for the proper development of infants. However, this is not the case, at least with regard to vitamins. Therefore, studies of factors that affect milk vitamin contents are important. Kirchgessner et al. (*1*) reported that the vitamin B_1 content in rat milk was lower in rats fed with a low-vitamin B_1 diet than in rats fed with a sufficient vitamin B_1 diet. Duerden and Bates (*2*) reported that the vitamin B_2 concentration in rat milk was extremely low when dams were fed a vitamin B_2-restricted diet compared with control dams. Regarding the vitamin B_6 content in rat milk, Kirksey and Susten (*3*) reported that the vitamin B_6 level was a more sensitive indicator than liver or muscle of chronically low intakes of vitamin B_6 by dams, while other investigators also reported that the level of vitamin B_6 in the milk of dams changed according to their intake of vitamin B_6 (*3–6*). Two groups reported that the concentration of vitamin B_{12} in milk was affected by the dietary intake of vitamin B_{12} (*7*, *8*). Meanwhile, O'Connor et al. (*9*) reported that the folate content of rat milk was increased according to increases in dietary folate. These reports clearly indicate that the levels of certain vitamins in rat milk are easily affected by the dietary vitamin intakes. For other vitamins, there is no available information.

In this study, we investigated nine kinds of vitamin contents in rat milk, the changes in the vitamin contents during lactation, and the effects of dietary vitamin intakes of the dams on the vitamin concentrations in their milk.

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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, a mineral mixture (AIN-93-M-MX) (*10*) and a vitamin mixture (AIN-93-VX containing chorine bitartrate) (*10*) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Thiamin hydrochloride (vitamin B_1, $C_{12}H_{17}ClN_4OS$-HCl=337.27), thiamin diphosphate chloride ($C_{12}H_{19}ClN_4O_7P_2S$=460.77), riboflavin (vitamin B_2, $C_{17}H_{20}N_4O_6$=376.37), cyanocobalamin (vitamin B_{12}, $C_{63}H_{88}CoN_{14}O_{14}P$=1,355.40), nicotinamide ($C_6H_6N_2O$ =122.13), calcium pantothenate (PaA-Ca, $C_{18}H_{32}N_2O_{10}$-Ca=476.54), folic acid ($C_{19}H_{19}N_7O_6$= 441.40), D(+)-biotin ($C_{10}H_{16}N_2O_3S$=244.31), (±)D-α-tocopheryl acetate ($C_{31}H_{52}O_3$=472.74), pyridoxal 5′-phosphate monohydrate ($C_8H_{10}NO_6P$-H_2O=265.169) and pyridoxal hydrochloride ($C_8H_9NO_3$-HCl=203.62) were purchased from Wako Pure Chemical Industries.

Nembutal (2.5 g/50 mL) was obtained from Dainippon Sumitomo Pharma (Osaka, Japan). Oxytocin (50 IU/mg) and lumiflavin ($C_{13}H_{12}N_4O_2$=256.3) were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan).

All other chemicals used were of the highest purity available from commercial sources.

Animals and diets. Male and female rats of the Wistar strain (8 wk old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). The rats were immediately placed in individual cages and fed a 20% casein diet containing 1% vitamin mixture (Table 1) for 1 wk to

Table 1. Compositions of the experimental diets.

	0.2% VX[1] (%)	1.0% VX (%)	4.0% VX (%)
Casein	20	20	20
L-Methionine	0.2	0.2	0.2
Gelatinized cornstarch	46.9	46.9	46.9
Sucrose	24.2	23.4	20.4
Corn oil	5	5	5
Mineral mixture (AIN-93-MX)	3.5	3.5	3.5
Vitamin mixture (AIN-93-VX)	0.2	1	4
(mg/100 g of diet)			
All-*trans*-retinyl palmitate (500,000 IU/g)	0.16	0.8	3.2
Cholecalciferol (400,000 IU/g)	0.05	0.25	1
All-rac-α-tocopheryl acetate (500 IU/g)	3	15	60
Phylloquinone	0.015	0.075	0.3
Thiamin-HCl	0.12	0.6	2.4
Riboflavin	0.12	0.6	2.4
Pyridoxine-HCl	0.14	0.7	2.8
Cyanocobalamin	0.0005	0.025	0.01
Nicotinic acid	0.6	3	12
Ca pantothenate	0.32	1.6	6.4
Folic acid	0.04	0.2	0.8
D-Biotin	0.004	0.02	0.08
Choline bitartrate	5	25	100
Sucrose	up to 200	up to 1,000	up to 4,000

[1] VX: vitamin mixture.

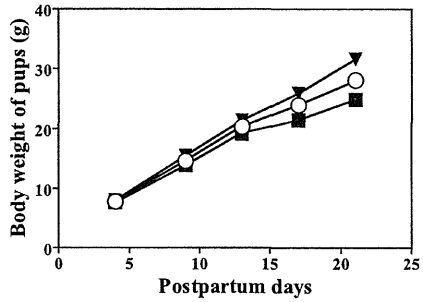

Fig. 1. Effect of feeding with the three levels of vitamin mixture diets to dams on the body weight gains of pups. ■, 0.2% vitamin mixture diet; ○, 1.0% vitamin mixture diet (normal diet); ▼, 4.0% vitamin mixture diet. Values are expressed as means±SE for 5–7 rats.

Table 2. The vitamin contents in rat milk.

Vitamins	Values	*n*
Vitamin B_1 (μg/mL)	0.204±0.082	16
Vitamin B_2 (μg/mL)	4.67±0.78	16
Vitamin B_6 (μg/mL)	1.49±0.23	13
Vitamin B_{12} (μg/mL)	0.032±0.008	16
Niacin (μg/mL)	7.02±2.28	16
Pantothenic acid (μg/mL)	15.2±6.6	16
Folate (μg/mL)	2.91±0.38	16
Biotin (μg/mL)	0.154±0.047	13
Vitamin E (μg/mL)	15.3±0.55	16

The values are means±SD for postpartum days 9, 13, 17, and 21.

allow them to acclimatize to their new circumstances. The female rats were then divided into three groups and fed one of three experimental diets containing 0.2, 1.0 or 4.0% vitamin mixture (Table 1) during mating, gestation and lactation. After the female rats delivered their pups, the pups that the mother rat brought up was adjusted to six. The day of postpartum was designated day 1. For collection of milk, the mother rat was separated from the litter at 09:00. At 15:00, the dam was anesthetized with 0.1 mL of nembutal (2.5 g/50 mL) and intraperitoneally injected with 2.5 IU of oxytocin. After confirming the effectiveness of the anesthesia, milk (about 2 mL) was collected using a special milking machine (Automatic Milker WAT-2001; Little Leonard Co. Ltd., Tokyo, Japan). The milk collection was carried out on days 4, 9, 13, 17 and 21. The collected milk was diluted by 1 : 2 by the addition of saline (0.85% NaCl), and the diluted milk was stored at −20°C until analysis.

The animal room was maintained at a temperature of about 22°C and a humidity of about 60% with a 12-h light (06:00–18:00)/12-h dark (18:00–06:00) cycle. The body weight of each pup was measured when it was separated from the mother. The care and treatment of the experimental animals conformed to The University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

Analyses. Vitamin B_1 (*11*), vitamin B_2 (*12*), vitamin B_6 (*13*), vitamin B_{12} (*14*), nicotinamide (*15*), pantothenic acid (*16*), folate (*17*), biotin (*18*) and vitamin E (*19*) in milk were measured as described in the cited references.

Statistical analysis. Each value was expressed as the mean±SE. The statistical significance of differences was determined by ANOVA and subsequent Tukey-Kramer multiple-comparison tests. Differences with values of $p<0.05$ were considered to be statistically significant. Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used for all analyses.

RESULTS

Effects of feeding the three vitamin diets to the dams on the body weight gains of pups

The low-vitamin (0.2%) and high-vitamin (4.0%) diets based on the AIN-93 diet (*10*) (1.0% vitamin diet; normal diet) were given to the female rats from pregnancy to lactation. Figure 1 shows the effects of feeding the three vitamin diets to the dams on the body weight gains of the pups. The weights of the pups were almost the same among the three groups.

Vitamin contents in milk of dams fed the normal diet

The reference values of rat's milk vitamin contents were assumed to be the average of the values on the days 9, 13, 17, and 21, because there were quite different values for vitamin B_1, vitamin B_2, and vitamin E on day 4. Table 2 shows the average vitamin contents in

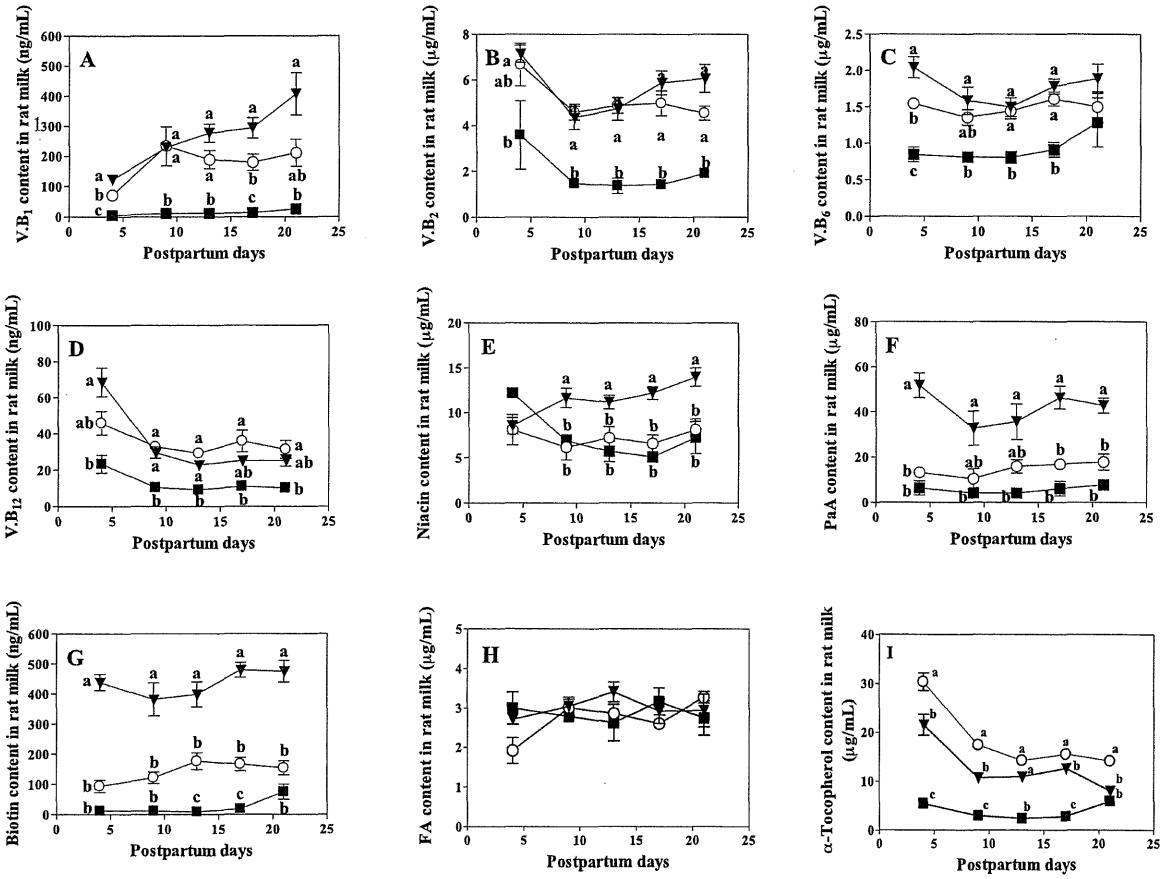

Fig. 2. Effect of feeding with the three levels of vitamin mixture diets to dams on the milk contents of vitamin B_1 (A), vitamin B_2 (B), vitamin B_6 (C), vitamin B_{12} (D), niacin (E), pantothenic acid (PaA) (F), biotin (G), folate (H), and vitamin E (I) contents in rat milk. ■, 0.2% vitamin mixture diet; ○, 1.0% vitamin mixture diet (normal diet); ▼, 4.0% vitamin mixture diet. Values are expressed as means±SE for 3–5 rat milks. Different letters on the symbols denote statistically significant differences (as determined by Tukey-Kramer multiple-comparison tests, $p<0.05$) in milk vitamin content among the groups of the three vitamin mixture diets in the same postpartum day.

rat milk collected on postpartum days 9, 13, 17 and 21 when the dams were fed the normal diet (1% vitamin mixture diet).

Changes in the vitamin contents during lactation of the dams fed the normal diet

Figure 2 shows the changes in the vitamin contents on specific postpartum days. The vitamin B_1 (Fig. 2A) content was lower on postpartum day 4 than on the other days. On the other hand, the contents of vitamins B_2 (Fig. 2B), B_{12} (Fig. 2D) and E (Fig. 2I) were higher on postpartum day 4 than on the other days. The contents of vitamin B_6 (Fig. 2C), niacin (Fig. 2E), biotin (Fig. 2G) and folate (Fig. 2H) remained relatively constant during lactation.

Effects of feeding the three vitamin diets to the dams on the vitamin contents in their milk

Figure 2 also shows the effects of the intake of the dietary vitamins on the vitamin contents in the rat milk during lactation. Although the concentrations of vitamins B_1 (Fig. 2A), B_2 (Fig. 2B), B_6 (Fig. 2C) and B_{12} (Fig. 2D) did not increase with the high-vitamin diet compared with the normal vitamin diet, these vitamin concentrations decreased with the low-vitamin diet.

The concentrations of niacin, pantothenic acid and biotin were not decreased by feeding the low-vitamin diet to the dams, although these vitamin concentrations were increased by feeding the high-vitamin mixture diet to the dams (Fig. 2E, 2F and 2G, respectively).

The folate concentration remained constant regardless of the amount of folate intake (Fig. 2H). The concentration of vitamin E was decreased with the low-vitamin diet, but was not increased with the high-vitamin diet compared with the normal vitamin diet (Fig. 2I).

DISCUSSION

The body weight gains of the pups were almost the same among the three groups regardless of whether the dams were fed diets containing low (0.2%), normal (1.0%) or high (4%) levels of the vitamin mixture. The present findings mean that the milk of lactating rats fed the low-vitamin diet (0.2%) was able to maintain normal growth of the pups. This finding is likely to have arisen because the AIN-vitamin mixture (*10*) contains

around five-fold higher vitamin contents than the required amounts.

The first purpose of the present study was to analyze the milk vitamin concentrations of rats fed the normal diet. As summarized in Table 2, we measured 9 kinds of vitamins, namely vitamin B_1, vitamin B_2, vitamin B_6, vitamin B_{12}, niacin, pantothenic acid, folate, biotin and vitamin E.

We did not measure the contents of vitamins A, D and K in rat milk. The reported values for vitamins A and D in rat milk are around 800 ng/mL (*20*) and 2 ng/mL (*21*), respectively. We could not find any data for the vitamin K content in rat milk.

The concentration of vitamin B_1 in the milk of dams fed the normal diet was around 200 ng/mL (Fig. 2A and Table 2). There is one previous report about the vitamin B_1 content in rat milk. Kirchgessner et al. (*1*) reported that the content increased according to the postpartum days, with values of 840, 1,600 and 2,500 ng/mL on days 2, 6 and 13, respectively, when the dams were fed a diet containing 0.67 mg/100 g diet (the same concentration in the 1% vitamin mixture diet as the normal diet). These values were about 10-fold higher than the present data for the 1% vitamin mixture diet. Kirchgessner et al. (*1*) used Sprague-Dawley rats as the experimental animals and fed a relatively high-fat diet (8.7% fat), while we used Wistar rats and fed a 5% fat diet. These differences may be reasons why the milk vitamin B_1 concentrations were so different.

The concentration of vitamin B_2 in the milk of dams fed the normal diet was around 5,000 ng/mL (Fig. 2B and Table 2). There is one previous report about the vitamin B_2 content in rat milk. Duerden and Bates (*2*) reported that the content was around 8,000 ng/mL when the dams were fed a diet containing 1.5 mg/100 g diet. The concentration of dietary vitamin B_2 was 2.5-fold higher than that in the present normal diet, and the content of vitamin B_2 in the milk was 1.6-fold higher than that in the present study. Duerden and Bates (*2*) also reported that the vitamin B_2 content in milk was significantly lower when the dams were fed a vitamin B_2-restricted diet. In the present study, the vitamin B_2 content in milk was lower in the dams fed the low-vitamin diet than in the dams fed the normal and high-vitamin diets.

The concentration of vitamin B_6 in the milk of dams fed the normal diet was around 1,500 ng/mL (Fig. 2C and Table 2). In previous reports, a constant value for the vitamin B_6 content in rat milk was not obtained. Thomas and Kirksey (*6*) reported that the vitamin B_6 content was 500 ng/mL when the dams were fed a diet containing 0.3 mg pyridoxine-HCl/100 g diet (this concentration is three-sevenths of that in our normal control diet). Felice and Kirksey (*5*) reported that the content was around 900 ng/mL when the dams were fed a diet containing 1.0 mg pyridoxine-HCl/100 g diet (this concentration is 1.4-fold higher that the concentration in our normal control diet). Debes and Kirksey (*4*) reported that content was around 500 ng/mL when the dams were fed a diet containing 2.0 mg pyridoxine-HCl/100 g diet (this concentration is 2.85-fold higher than the concentration in our normal control diet). In the present study, the vitamin B_6 content increased according to the change in diet from the low-vitamin (0.14 mg pyridoxine-HCl/100 g diet) to the normal diet (0.7 mg pyridoxine-HCl/100 g diet). However, the concentration did not increase when the dietary vitamin intake was increased from the normal diet to the high-vitamin diet (2.8 mg pyridoxine-HCl/100 g diet) (Fig. 2C). This finding is consistent with those in Kirksey and Susten (*3*) and Pang and Kirksey (*22*). They fed five levels of pyridoxine-HCl (0.12, 0.24, 0.48. 0.96 and 1.92 mg/100 g diet) to female rats, and found that the vitamin B_6 concentration in the milk reached a plateau of around 300 ng/mL with the 0.48 mg pyridoxine-HCl/100 g diet.

The concentration of vitamin B_{12} in the milk of dams fed the normal diet was around 30 ng/mL (Fig. 2D and Table 2). Regarding previous reports, the vitamin B_{12} content in rat milk was dramatically increased according to the intake of vitamin B_{12} of the dams. When the dams were changed from a diet containing 0.2 μg/100 g diet to a diet containing 20 μg/100 g diet, the concentration of vitamin B_{12} increased from about 7 ng/g milk curd to 120 ng/g milk curd (*22*). When the dams were fed a vitamin B_{12}-deficient diet, the content was around 5 ng/mL (*8*).

Regarding previous reports of the folate content in rat milk, values of 150 ng/mL milk (*9*) and 440 ng/mL milk (*23*) were observed when the dams were fed a diet containing 0.2 mg/100 g diet. In the present study, the folate concentration in the milk of dams fed the normal diet was around 3 μg/mL (Fig. 2H and Table 2). This value was about 10-fold higher than the previously reported values (*9*, *23*). The previously reported values were obtained using Sprague-Dawley rats as the experimental animals, while we used Wistar rats. This difference may be the reason why the folate concentrations in the milk were so different.

No data for the contents of niacin, pantothenic acid, biotin and vitamin E in rat milk have been reported. The concentration of niacin in the milk of dams fed the normal diet was around 7 μg/mL (Fig. 2E and Table 2). The concentration of pantothenic acid in the milk of dams fed the normal diet was around 15 μg/mL (Fig. 2F and Table 2). The concentration of biotin in the milk of dams fed the normal diet was around 150 ng/mL (Fig. 2G and Table 2). The concentration of vitamin E in the milk of dams fed the normal diet was around 15 μg/mL (Fig. 2I and Table 2).

The second purpose of the present study was to evaluate the changes in the vitamin contents during lactation. The content of vitamin B_1 was remarkably increased from day 9 (Fig. 2A). A similar phenomenon has already been reported by Kirchgessner et al. (*1*). On the other hand, the contents of vitamin B_2 (Fig. 2B) and vitamin E (Fig. 2I) remarkably decreased from day 9. These phenomena would be associated with the vitamin requirement in the pups and regulated through the expression of carrier proteins for the vitamins in the

mammary glands. The other vitamins remained at relatively constant concentrations during lactation. Regarding vitamin B_{12}, Williams and Spray (*8*) already reported a similar phenomenon to the present study. However, Felice and Kirksey (*5*) reported that the content of vitamin B_6 was significantly higher on day 21 than earlier in the lactation period. For vitamin B_2, niacin, pantothenic acid, biotin, folate and vitamin E, the changes in the vitamin contents during lactation are reported here for the first time.

The final purpose of the present study was to clarify the effects of dietary vitamin contents on the milk vitamin contents in rats. In previous reports, the milk contents of vitamins B_1 (*1*) and B_2 (*2*) were decreased when the dams were fed on corresponding vitamin-restricted diets, and the vitamin B_6 (*3*) and B_{12} (*7*) contents in the rat milk reflected the intakes of the respective vitamins. In the present study, the concentrations of vitamins B_1, B_2, B_6, B_{12} and E were decreased with the low-vitamin diet, but were not increased with the high-vitamin diet. The present findings for vitamin B_1 are similar to the findings of Kirchgessner et al. (*1*), who also found that the vitamin B_1 content was decreased by feeding a low-vitamin B_1 diet, but was not increased by feeding an excess vitamin B_1 diet. The concentrations of niacin, pantothenic acid and biotin were not decreased with the low-vitamin diet, but were increased with the high-vitamin diet. These results indicate that the concentrations of niacin, pantothenic acid and biotin in milk are not easily decreased, even with low intake, while the concentrations of vitamins B_1, B_2, B_6, B_{12} and E in milk are affected by their intakes. The folate concentration remained constant regardless of the folate intake. It is known that there is a well-developed epithelial folate transport system for the regulation of normal folate homeostasis (*24*, *25*). Therefore, the concentrations of the vitamins could also be well-regulated by transport systems in intestinal absorption and in secretion to the milk. However, the present findings suggest there is a specific regulation mechanism for each of the vitamins to maintain the milk vitamin contents. Regarding the vitamin E concentration in human milk, its concentration is associated with the total fat intake by mothers, while the vitamin E intake seems to have no effect (*26*).

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REFERENCES

1) Kirchgessner M, Trubswetter N, Stangl GI, Roth-Maier DA. 1997. Dietary thiamin supply during gestation effects thiamin status of lactating rats and their suckling offspring. *Int J Vitam Nut* **67**: 248–254.
2) Duerden JM, Bates CJ. 1985. Effect of riboflavin deficiency on reproductive performance and on biochemical indices of riboflavin status in the rat. *Br J Nutr* **53**: 97–105.
3) Kirksey A, Susten SS. 1978. Influence of different levels of dietary pyridoxine on milk composition in the rat. *J Nutr* **108**: 509–513.
4) Debes SA, Kirksey A. 1979. Influence of dietary pyridoxine on selected immune capacities of rat dams and pups. *J Nutr* **109**: 744–759.
5) Felice JH, Kirksey A. 1981. Effects of vitamin B-6 deficiency during lactation on the vitamin B-6 content of milk, liver and muscle of rats. *J Nutr* **111**: 610–617.
6) Thomas MR, Kirksey A. 1976. Influence of pyridoxine supplementation on vitamin B-6 levels in milk of rats deficient in the vitamin. *J Nutr* **106**: 509–514.
7) Daniel LJ, Gardiner M, Ottey LJ. 1953. Effect of vitamin B_{12} in the diet of the rat on the vitamin B_{12} contents of milk and livers of young. *J Nutr* **50**: 275–289.
8) Williams DL, Spray GH. 1971. Some observations on the vitamin B_{12} content of rat's milk. *Br J Nutr* **25**: 295–298.
9) O'Connor DL, Picciano MF, Tamura T, Shane B. 1990. Impaired milk folate secretion is not corrected by supplemental folate during iron deficiency in rats. *J Nutr* **120**: 499–506.
10) Reeves PG. 1997. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J Nutr* **127**: 838S–841S.
11) Fukuwatari T, Toriochi M, Ohta M, Sasaki R, Shibata K. 2004. Metabolic disturbance of tryptophan-nicotinamide conversion pathway by putative endocrine disruptors, bisphenol A and styrene monomer. *Shokuhin Eiseigaku Zasshi* **45**: 1–7.
12) Ohkawa H, Ohishi N, Yagi K. 1982. A simple method for micro-determination of flavin in human serum and whole blood by high-performance liquid chromatography. *Biochem Int* **4**: 187–194.
13) Rybak ME, Pfeiffer CM. 2004. Clinical analysis of vitamin B_6: determination of pyridoxal 5′-phosphate and 4-pyridoxic acid in human serum by reversed-phase high-performance liquid chromatography with chlorite post column derivatization. *Anal Biochem* **333**: 336–344.
14) Watanabe F, Abe K, Katsura H, Takenaka S, Mazumder ZH, Yamaji R, Ebara S, Fujita T, Tanimori S, Kirihata M, Nakano Y. 1998. Biological activity of hydroxo-vitamin B_{12} degradation product formed during microwave heating. *J Agric Food Chem* **46**: 5177–5180.
15) Shibata K, Kawada T, Iwai K. 1988. Simultaneous micro-determination of nicotinamide and its major metabolites, N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-3-pyridone-4-carboxamide, by high-performance liquid chromatography. *J Chromatogr* **424**: 23–28.
16) Skeggs HR, Wright LD. 1944. The use of *Lactobacillus arabinosus* in the microbiological determination of pantothenic acid. *J Biol Chem* **156**: 21–26.
17) Tamura T. 1990. Microbiological assay of folates. *In*: Folic Acid Metabolism in Health and Disease. Contem-

porary Issues in Clinical Nutrition (Picciano MF, Stolstad ELR, Gregory JF III, eds), Vol 13, p 121–137. Wiley-Liss, New York.

18) Fukui T, Iinuma K, Oizumi J, Izumi Y. 1994. Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. *J Nutr Sci Vitaminol* **40**: 491–498.

19) Yoshikawa S, Morinobu T, Hamamura K, Hirahara F, Iwamoto T, Tamai H. 2005. The effect of γ-tocopherol administration on α-tocopherol levels and metabolism in humans. *Eur J Clin Nutr* **59**: 900–905.

20) Green MH, Green JB, Akohoue SA, Kelley SK. 2001. Vitamin A intake affects the contribution of chylomicrons vs. retinol-binding protein to milk vitamin A in lactating rats. *J Nutr* **131**: 1279–1282.

21) Clements MR, Fraser DR. 1988. Vitamin D supply to the rat fetus and neonate. *J Clin Invest* **81**: 1768–1773.

22) Pang R, Kirksey A. 1974. Early postnatal changes in brain composition in pregnancy of rats fed different levels of dietary pyridoxine. *J Nutr* **104**: 111–117.

23) O'Connor DL, Picciano MF, Sherman AR, Burgert SL. 1987. Depressed folate incorporation into milk secondary to iron deficiency in the rat. *J Nutr* **117**: 1715–1720.

24) Balamurugan K, Said HM. 2003. Ontogenic regulation of folate transport across rat jejuna brush-border membrane. *Am J Physiol Gastrointest Liver Physiol* **285**: G1068–G1073.

25) Ashokkumar B, Mohmmed ZM, Vaziri ND, Said HM. 2007. Effect of folate oversupplementation on folate uptake by human intestinal and renal epithelial cells. *Am J Clin Nutr* **86**: 159–166.

26) Antonakou A, Chiou A, Andrikopoulos NK, Bakoula C, Matalas AL. 2011. Breast milk tocopherol content during the first six months in exclusively breastfeeding Greek women. *Eur J Nutr* **50**: 195–202.