Table 3. Composition of the female diet.

	(g/d)	Remarks
Wheat flour (Soft flour), first grade	315	Protein: 315 g×0.08=25.2 g. Tryptophan content was 1.1%, so 277 mg of tryptophan were supplied. Carbohydrate: 315 g×0.759=239 g Lipid: 315 g×0.017=5.4 g
Gluten	37.3	The gluten contained 81.6% protein, so the net protein amount was 29.8 g. Tryptophan content was 1.1%, so 328 mg of tryptophan was supplied. Total protein=55 g Total tryptophan=605 mg
Cornstarch	33	
Sucrose	20	Total carbohydrate=292 g
Fats		Total fat=40 g
Soybean oil	4.8	
Rapeseed oil	16.8	
Coconut oil	7.1	
Lard	5.9	
Dietary fiber		Insoluble dietary fiber used, Ramie powder, was obtained from Tosco Co., Ltd. (Tokyo, Japan).
Soluble	0	
Insoluble	10.1	
Mineral mixtures	12.0	The composition is shown below.
Total amount	462.0	Total energy=ca. 1,800 kcal=7,524 kJ

For breakfast and supper, 139 g of the above powder mixture was added to 91 mL of water, and mixed well, and was baked for 9 min at 250°C. The weight of the baked meal was ca. 175 g. The meal and 0.3 g of the vitamin mixture (composition shown below) were given to the subjects. For lunch, 185 g of the above mixture was added to 122 mL of water, and mixed well, and was baked for 10 min at 250°C. The weight of the baked meal was ca. 233 g. The meal and 0.4 g of the vitamin mixture (composition shown below) were given to the subjects.

Composition of the mineral mixture: 1,100 mg of $CaHPO_4$-$2H_2O$, 900 mg of $CaCO_3$, 2,200 mg of KH_2PO_4, 3,500 mg of $KHCO_3$, 2,083 mg of $MgCl_2$-$2H_2O$, 60 mg of $FeSO_4$-$5H_2O$, 12.2 mg of $MnSO_4$-$5H_2O$, 19 mg of $ZnCl_2$, 6.3 mg of $CuSO_4$-$5H_2O$, 0.2 mg of KI, and 2,120 mg of NaCl.

Composition of the vitamin mixture: 3.6 mg (1,800 IU) of retinal acetate reagent (500,000 IU/g), 2.5 μg of cholecalciferol, 4.47 mg of *dl*-α-tocopherol (5 mg were supplied from oils), 24 μg of phylloquinone (31 μg was supplied from oils), 0.9 mg of thiamin-HCl, 1.0 mg of riboflavin, 1.5 mg of pyridoxine-HCl, 2.8 mg of nicotinamide (10.0 mg of nicotinamide was supplied from 605 mg of tryptophan in protein), 2.4 μg of cyanocobalamin, 5.5 mg of calcium panthotenate, 200 μg of pteroylmonoglutamic acid, 30 μg of D(+)-biotin, 100 mg of ascorbic acid, made up to 1 g with sucrose.

HPLC-lumiflavin method of Ohkawa et al. (5) modified slightly. Urinary concentration of riboflavin was analyzed according to the method of Ohkawa et al. (6).

Vitamin B_{12} (cyanocobalamin): Serum vitamin B_{12} concentration was determined by using a fully automated chemiluminescence analyzer (ACS 180; Beckman Coulter, Inc.) according to the manufacturer's instructions. The serum, prepared in the usual way, was directly applied to the analyzer.

Vitamin B_{12} concentration in urine was assayed by the microbiological method with *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 (*7*).

Niacin: The total nicotinamide content in whole blood was measured by the method of Shibata et al. (*8*).

The quantities of Nam, 2-Py and 4-Py in urine were measured simultaneously by the HPLC method of Shibata et al. (*3*). The content of MNA was measured by the method of Shibata (*9*).

Pantothenic acid: The heat-extracted supernatant of whole blood was treated with a mixture of 2 enzymes (phosphatase and amidase) for decomposing bound pantothenic acid such as CoA, phosphopantetheine, and pantetheine to form free pantothenic acid (*10*). The enzyme-treated materials were directly used for measuring total pantothenic acid by using *Lactobacillus plantarum* ATCC 8014 (*11*). Total pantothenic acid means the sum of free pantothenic acid, and that derived from pantetheine, phosphopantethine, CoA, and acyl CoA including acetyl CoA.

The content of free pantothenic acid in urine was directly measured by using *Lactobacillus plantarum* ATCC 8014 (*11*).

Folates: Serum concentrations of folates were determined by an automated method based on the competitive protein-binding assay using the ACS at the Clinical Laboratory of Mitsubishi BCL in Kobe. Herbert (*12*) suggested that there was no significant loss of serum folate activity after storage at −20°C for up to 106 d. We also found that the solution of folic acid was stable for at least 3 wk in the freezer at −20°C. Therefore, ascorbic acid was not added prior to storage of serum and urine samples in this study.

The concentrations of urinary folates were determined by an automated method based on the competitive protein-binding assay using the ACS as mentioned above.

Biotin: Biotin levels in the serum were microbiologically quantified with *Lactobacillus plantarum* ATCC 8014 according to the agar plate assay developed by Fukui et al. (*13*).

The content of free biotin in urine was directly measured by using *Lactobacillus plantarum* ATCC 8014.

Vitamin C: Total ascorbic acid (vitamin C) was determined by an HPLC-UV method according to Fujiwara et al. (*14*).

Vitamin C in urine was determined by the 2,4-dinitrophenylhydrazine (DNPH) method according to Shigeoka et al. (*15*).

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

RESULTS AND DISCUSSION

Vitamin B_1

The content of total vitamin B_1 (free thiamin+thiamin monophosphate: TMP+thiamin diphosphate: TDP+thiamin triphosphate: TTP) in whole blood is shown in Table 4. Vitamin B_1 concentrations in males were 104 ± 17 pmol/mL (range 81–122) and in females 90 ± 23 pmol/mL (range 38–122) on the final day of the study. No significant difference between males and females was observed. However, a wide distribution range in blood vitamin B_1 concentrations of females was found; in particular, very low vitamin B_1 concentrations (38 pmol/mL) could be found. On the first day of the study, blood vitamin B_1 concentrations in males were 82 ± 10 pmol/mL (range 74–106) and in females 88 ± 19 pmol/mL (range 48–112).

The reference value of erythrocyte vitamin B_1 (70–90 pmol/mL) was proposed by the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes (USA and Canada) (*16*). However, the reference value in whole blood has not yet been proposed. In Japan, the content of vitamin B_1 in whole blood is frequently used. Kimura et al. (*17*) reported that the total vitamin B_1 concentration of farm village inhabitants was 109 ± 10 pmol/mL and that of fishing village inhabitants was 119 ± 19 pmol/mL in a survey of rural areas in 1982. And after these subjects were supplied with vitamin B_1 (1.5 mg/d) in daily foods for 1 wk, the total vitamin B_1 concentration of farm village inhabitants was 137 ± 7 pmol/mL and that of fishing village inhabitants was 157 ± 16 pmol/mL. The total vitamin B_1 concentration in whole blood of young men (29.4 ± 4.5 y old) was 140 ± 36 pmol/mL ($n=10$) and that of young women (23.5 ± 3.1 y old), 117 ± 21 pmol/mL ($n=10$), which was reported by Itokawa et al. (*18*). Takeda et al. (*19*) reported that the total vitamin B_1 concentration in whole blood of Japanese adult males and females was 119 ± 33 pmol/mL ($n=524$) and 104 ± 27 pmol/mL ($n=345$), respectively. In the survey study of other countries by Kimura et al. (*20*), the total vitamin B_1 concentration in the whole blood of Chinese adult males and females living in urban areas was 164 ± 34 pmol/mL ($n=100$) and 159 ± 37 pmol/mL ($n=100$). In the survey for vitamin B_1 status of inhabitants in northeast rural Thailand by Kimura et al. (*21*), the total vitamin B_1 concentration in whole blood of

Table 4. Blood values of water-soluble vitamins for Japanese college students who consumed on a semi-purified diet based on the Japanese dietary reference intakes.

	Mean	SD	Minimum	Maximum	Median
Males ($n=10$)					
Total thiamin in whole blood (pmol/mL)	104	17	81	122	106
Total riboflavin in whole blood (pmol/mL)	216	25	175	259	211
Cyanocobalamin in serum (pmol/mL)	0.34	0.05	0.26	0.43	0.34
Total Nam in whole blood (nmol/mL)	59.1	5.0	52.8	69.9	60.0
Total pantothenic acid in whole blood (nmol/mL)	2.45	0.37	1.86	3.27	2.36
Folates in serum (pmol/mL)	15.6	4.6	9.9	23.1	15.0
Biotin in serum (pmol/mL)	8.3	0.5	7.8	9.0	8.2
Ascorbic acid in plasma (nmol/mL)	62	10	47	80	60
Females ($n=10$)					
Total thiamin in whole blood (pmol/mL)	90	23	38	122	91
Total riboflavin in whole blood (pmol/mL)	234	18	205	259	238
Cyanocobalamin in serum (pmol/mL)	0.67*	0.20	0.41	0.92	0.67
Total Nam in whole blood (nmol/mL)	61.9	6.0	55.4	75.4	61.0
Total pantothenic acid in whole blood (nmol/mL)	2.48	0.30	2.08	2.83	2.51
Folates in serum (pmol/mL)	30.2*	8.6	21.0	51.7	29.0
Biotin in serum (pmol/mL)	8.4	0.3	7.8	9.0	8.6
Ascorbic acid in plasma (nmol/mL)	67	14	47	100	61

* Significant difference from male value at $p<0.05$, calculated by Student's *t* test.

Table 5. Urinary values of water-soluble vitamins for Japanese college students who consumed a semi-purified diet based on the Japanese dietary reference intakes.

	Mean	SD	Minimum	Maximum	Median
Males (n=10)					
Thiamin (nmol/d)	665	114	467	821	669
Riboflavin (nmol/d)	562	325	155	1,208	492
Cyanocobalamin (pmol/d)	93	31	69	170	85
Sum (MNA+2-Py;4-Py) (μmol/d)	84	26	54	128	74
Pantothenic acid (μmol/d)	9.3	2.3	6.2	12.2	10.2
Folates (nmol/d)	19.4	2.8	14.5	23.9	19.6
Biotin (pmol/d)	83	18	59	112	78
Ascorbic acid (μmol/d)	148	51	87	231	134
Females (n=10)					
Thiamin (nmol/d)	495*	212	286	988	458
Riboflavin (nmol/d)	580	146	366	849	569
Cyanocobalamin (pmol/d)	145*	49	94	252	112
Sum (MNA+2-Py:4-Py) (μmol/d)	83	19	53	111	81
Pantothenic acid (μmol/d)	16.9*	1.3	14.8	18.6	17.3
Folates (nmol/d)	22.7*	2.7	20.4	29.0	22.3
Biotin (pmol/d)	83	23	54	120	78
Ascorbic acid (μmol/d)	140	51	89	257	131

* Significant difference from male value at $p<0.05$, calculated by Student's t test.

males and females working on farms was 74±37 pmol/mL (n=59) and 76±46 pmol/mL (n=47), and the total vitamin B_1 concentration in the whole blood of males and females working in factories was 96±34 pmol/mL (n=279) and 105±27 pmol/mL (n=21), respectively.

The urinary excretion of free vitamin B_1 in males and females is shown in Table 5. A significant difference between sexes was observed. The reference value of urinary excretion of vitamin B_1 (133–333 nmol/d) has been proposed by the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes (USA and Canada) (*16*). All of the present values were over this reference value. So, the RDA of vitamin B_1 in the 6th revision was evaluated as good in adult men and women.

Murata et al. (*22*) reported that vitamin B_1 levels of 24 h-urine samples collected from 13 women in summer and winter were 545±402 nmol/d and 522±445 nmol/d, respectively. Itokawa et al. (*23*) reported that the urinary excretion of vitamin B_1 in adult men (21–24 y old) was 472±339 nmol/d (n=21). Therefore, the present values were almost the same as the previously reported ones (*22, 23*).

Based on the results of a thiamin deficiency study, Nishio et al. (*24*) concluded that the urinary excretion of vitamin B_1 would not be a useful index of thiamin depletion because the urinary excretion decreased so quickly before the clinical deficiency was revealed. But the use of the urinary values helps to assess the vitamin B_1 nutritional status, because the urinary excretion reflects the immediate vitamin B_1 intake.

Vitamin B_2

The total vitamin B_2 content expressed in whole blood is shown in Table 4. The values were almost the same between the 2 groups. Hiraoka (*25*) reported that the mean value and the reference interval were 225 pmol/mL and 154–311 pmol/mL, respectively, for the blood riboflavin levels of healthy female students aged 21–22 y whose intake of vitamin B_2 was above the RDA. The value obtained in the present study coincides with their data. There is a paper stating that over 180 pmol/mL of vitamin B_2 in whole blood indicates a well-nourished individual (*16*). In the present experiment, only one person was below the value. Therefore, the RDA of vitamin B_2 in the 6th revision would be evaluated as good.

The vitamin B_2 content in urine is shown in Table 5. The values were almost the same between the male and female students. Roughead and McCormick (*26*) reported that the amount of urinary riboflavin determined by the HPLC procedure exceeded 319 nmol/d under conditions of riboflavin intake of ca. 1.5 mg/d in healthy residents of rural Georgia, USA. Two of our subjects were below the value of 319 nmol/d. Urinary riboflavin separated from other flavin derivatives including catabolites by HPLC is considered to be useful for estimation of the vitamin B_2 requirement. The average in our study was ca. 550 nmol/d and the range was 155 to 1,208 nmol/d.

Vitamin B_{12}

The concentration of vitamin B_{12} in serum reflects both vitamin B_{12} intake and storage. The lower limit (cut-off value) of the serum vitamin B_{12} concentration is considered to be approximately 0.12–0.18 pmol/mL for adults (*16*). The serum concentrations of vitamin B_{12} found in the present study are shown in Table 4. These values are over the cut-off value, and so the RDA in the 6th revision was evaluated as good. But, the diet

used for the experiment contained a small amount of vitamin B_{12}. So, the accurate amount of vitamin B_{12} consumed was 4.2 μg/d. The mean values for males and females were 0.34 pmol/mL and 0.67 pmol/mL, respectively, and this difference was significant. Those values at day −1 were 0.34±0.07 pmol/mL and 0.48±0.14 pmol/mL, respectively. Therefore, daily intake of 2.4 μg of cyanocobalamin for 7 d maintained the serum vitamin B_{12} level in the male subjects, and increased it in the female subjects. Fernandes-Costa et al. (*27*) reported mean serum vitamin B_{12} values for young men (n=77) and women (n=82) to be 0.477 and 0.604 pmol/mL, respectively. Similar results have been also obtained by other workers (*28, 29*). At the present time, we cannot explain why there is such a difference in the serum vitamin B_{12} concentration between men and women and do not know whether there is any gender-based difference in vitamin B_{12} requirements.

Amounts of urinary vitamin B_{12} are shown in Table 5. The mean value in men and women was 93 pmol/d and 145 pmol/d, respectively, and this difference was significant.

Mollin and Ross (*30*) reported that the daily excretion of vitamin B_{12} in urine ranged from 81.2 to 199.3 pmol/d in 6 normal subjects, but decreased significantly [to less than 36.9 pmol] in 7 patients with pernicious anemia.

Adams (*31*) demonstrated that the mean value of urinary vitamin B_{12} was 121 pmol/d in non-vitamin B_{12}-deficient subjects and found a linear correlation between the serum vitamin B_{12} level and daily loss of vitamin B_{12} in the urine. From the present data, the urinary excretion of vitamin B_{12} in Japanese would be ca. 100 pmol/d in men and 150 pmol/d in women.

The values for serum and urinary vitamin B_{12} between men and women were significantly different, as stated above. However, the RDA of vitamin B_{12} in the 6th revision is the same for men and women. This problem should be resolved in the future.

Niacin (total Nam)

The content of total Nam in whole blood is shown in Table 4. There was no difference in the value between sexes. The present mean value for men and women was ca. 60 μmol/mL of whole blood. This value is about the same as was reported previously (*32*), in a study in which the subjects were female college students (n=19), who consumed self-selected foods. In a report published in 1950, Nose (*33*) reported that the content of total niacin in whole blood was 62.7±5.3 nmol/mL (n=10). In 1953, Katsura and Sakakida (*34*) reported a mean value of 61±5 nmol/mL for 10 men (24–29 y old) and 56±6 nmol/mL for 10 women (19–50 y old). Therefore, the RDA of niacin equivalent intake in the 6th revision was evaluated as good.

In humans, nicotinamide itself is minimally excreted into urine, but its catabolic metabolites, MNA, 2-Py, and 4-Py are excreted. The sum of the urinary excretion of MNA+2-Py+4-Py for the male and female students is shown in Table 5. Shibata and Matsuo (*35*) reported a value of ca. 100 μmol/d, for female college students who consumed self-selected foods. Okamoto et al. (*36*) reported that when female college students consumed a diet containing 25 mg niacin equivalent/d, the urinary excretion of the sum was ca. 80 μmol/d. The present and previous data (*35, 36*) indicate that the urinary excretion of the sum of MNA+2-Py+4-Py changes according to the niacin equivalent intake. In fact, Shibata and Matsuo (*35*) and Moyer et al. (*37*) revealed a high correlation coefficient between the sum and the niacin equivalent intake. Therefore, determination of the sum of MNA, 2-Py, and 4-Py would provide an excellent index for a niacin nutritional assessment. We would like to propose that adult humans (18–69 y olds) including men and women should ingest sufficient niacin equivalent to maintain this urinary sum at a level over 50 μmol/d.

Pantothenic acid

The content of total pantothenic acid in whole blood is shown in Table 4. No difference in value was observed between sexes. The present mean value was ca. 2.5 nmol/mL of whole blood. Wittwer et al. (*38*) reported that a normal value for pantothenic acid in the whole blood ranged from 1.57 to 2.66 nmol/mL, and Song et al. (*39*) reported the concentration in adult women to be 2.40±0.05 (n=47) nmol/mL. They also reported that the correlation between total pantothenic acid in blood and the intake of pantothenic acid was weak. As to the paper by Fry et al. (*40*), the total pantothenic acid did not decrease even when the subjects were fed a diet without pantothenic acid for 9 wk. Therefore, the total pantothenic acid in blood does not reflect the intake of pantothenic acid and should not be used for assessing the nutritional status of pantothenic acid.

The urinary content of pantothenic acid in the males and the females is shown in Table 5. The value was about 2 times higher in the females than in the males, and this difference was significant. There is clear evidence that the urinary excretion of pantothenic acid is a reliable index for assessing the nutritional status of pantothenic acid. For example, Fox and Linkswiler (*41*) reported that the correlation coefficient between dietary pantothenic acid intake and the urinary excretion of pantothenic acid was 0.805. Previously reported values (*40, 41*) for urinary pantothenic acid were ca. 10–20 μmol/d in adults. So, we conclude the RDA of pantothenic acid in the 6th revision is good, and propose that the urinary excretion of pantothenic acid is a suitable index for the nutritional assessment of pantothenic acid. A value of 10 μmol/d would be a suitable intake of pantothenic acid for maintaining health in males, whereas 15 μmol/d would be appropriate for females.

Folates

The serum content of folates is presented in Table 4. The serum concentrations of folates were 15.6±4.6 pmol/mL for males and 30.2±8.6 pmol/mL for females. The large sex difference was observed with the females showing a significantly higher level. Those values at

day −1 were 19.6±6.2 pmol/mL and 25.4±5.8 pmol/mL, respectively. Therefore, daily intake of 200 μg of pteroylmonoglutamic acid for 7 d maintained the serum level of folates in the male subjects, and increased it in the female subjects. No subjects with extremely low amounts of serum folates (6.8 pmol/mL) were found in either group.

Milne et al. (*42*) reported that the serum content of folates was 18.9±6.1 pmol/mL in 40 male volunteers, aged 19 to 54 y, without underlying disease in the USA. The mean plasma concentration of folates was 23.3±6.3 pmol/mL (n=39) and 23.3±7.5 pmol/mL (n=41) in 80 free-living men and women aged 50–87 y in the USA (*43*). In 6 healthy Caucasian males (aged 22–31 y) the serum value was 18 pmol/mL (*44*). It was 17.1±1.3 pmol/mL in 30 apparently healthy subjects (12 males and 18 females from 37 to 69 y of age) in Turkey (*45*). The Framingham Heart Study of 885 elderly people found the plasma folates concentrations to be greater for women (*46*): The level was 12.7 pmol/mL for men, which was significantly different from the 16.3 pmol/mL for women. Thus, a significant sexual difference was observed, which is in accordance with the present findings. On the other hand, Yukawa et al. (*47*) reported that in a folate-deficient group of the neurological patients, serum folates levels were significantly lower in females (5.0±2.0 pmol/mL, n=11) than in males (6.6±2.0 pmol/mL, n=25). A normal value for serum folates has not yet been proposed, but from these findings, the baseline values of folates in serum (or plasma) ranged from approximately 15 to 25 pmol/mL. There was also no difference of race among Japanese, Asians and Caucasians. From these findings, we conclude the RDA of folic acid in the 6th revision value of 200 μg/d is good.

The urinary content of folates is shown in Table 5. The urinary concentrations of folate were 19.4±2.8 nmol/d in the males and 22.7±2.7 nmol/d in the females. A significant difference in the values of the two groups was observed. The urinary excretion of folates ranged from 15 to 30 nmol/d in the present study, which is closely in accord with the previously reported value (11.3–90.7 nmol/d) (*48*). This would be a normal value for Japanese subjects.

Biotin

The amount of biotin supplemented in the semi-purified diet in this study was 30 μg/d, which followed the Japanese DRIs. As biotin is widely distributed in many foodstuffs, biotin is also contained in natural sources such as wheat gluten and raw cornstarch compounded as ingredients of the semi-purified diet. Therefore, the daily biotin intake was 142.7 μg for young men and 118.2 μg for young women.

Table 4 presents the serum biotin concentrations in both groups. The serum concentrations of biotin were 8.3±0.5 pmol/mL in men and 8.4±0.3 pmol/mL in women.

In a large Japanese population of 190 healthy adults (18 to 66 y, mean 34.4 y), Fukui et al. (*13*) found the mean total biotin level in their serum to be 10.9±2.2 pmol/mL. From these findings, normal values for total biotin in serum were from 7.8 to 12.2 pmol/mL. Watanabe et al. (*49*) also demonstrated that the serum biotin level in 685 elderly people was 10.2±7.2 pmol/mL, and that in 2,004 reference people, 9.4±1.4 pmol/mL, and the mean and SD of biotin levels did not differ between men and women. The normal value for serum biotin in reference people was determined to range from 6.5 to 12.1 pmol/mL. So, the present data are within this range. However, the ingested biotin was over 30 μg/d because the ingredients, raw cornstarch and gluten, contained a great deal of biotin. The evaluation of the present RDA (30 μg/d) of biotin could not be precisely made in the present experiment.

The biotin content in the urine is shown in Table 5. The urinary concentrations of biotin were 83.0±18.3 pmol/d in men and 83.2±22.5 pmol/d in women. No sex difference was observed. These results are in accord with the findings of previous studies in the USA and UK: Sweetman et al. (*50*) reported that 600 adults, aged 20–50 y, excreted biotin at the rate of 24.6–204.7 pmol/d. Similar findings were also obtained by Jung et al. (*51*) and Bitsch et al. (*52*), who demonstrated 187.1±44.6 pmol/d in 40 adults (45.7±10.9 y) and 40.9 pmol/d in 28 adults, respectively. As the ingestion of biotin was over 30 μg/d, the evaluation of the present RDA of biotin should be made carefully.

Vitamin C

The plasma level of vitamin C is shown in Table 4. No difference in the values of vitamin C was observed between sexes. The mean value of plasma vitamin C was ca. 64.5 nmol/mL. Levine et al. (*53*) reported a mean value of vitamin C in plasma of 62.0±10.2 nmol/mL for 15 women. Kobata et al. (*54*) reported that when 7 female subjects (average age of 25 y) were given a diet containing 111.0±33.0 mg/d of vitamin C, the plasma level of vitamin C remained constant through out the experimental period, at 56.8±10.8 nmol/mL. It was also reported that the mean value of vitamin C in plasma was 56.0±4.0 nmol/mL for 7 men (20–26 y old), who were administered 50 mg of the vitamin twice daily in the fasting state, at least 2-h before breakfast and dinner (*53, 54*). Levine et al. (*55*) demonstrated that the plasma saturation of vitamin C occurred between 200 and 400 mg daily doses, whereas a 100 mg daily dose resulted in saturation of vitamin C in neurophils, monocytes, and lymphocytes. Furthermore, they indicated that a 100 mg daily dose would be a suitable intake of vitamin C for maintaining the steady-state plasma concentration at a value corresponding to the RDA of vitamin C (*56*). Recently Ihara et al. (*57*) performed an experiment with 176 young Japanese women (19–26 y old) and reported that a vitamin C intake of ca. 99-mg daily is sufficient to produce a serum vitamin C concentration above the lower reference limit of 40 nmol/mL (7 mg/L). The data reported here clearly agree with the RDA of vitamin C for the Japanese population i.e., 100 mg daily (*19*). Healthy subjects require 100 mg daily to keep the plasma con-

centration at more than 40 nmol/mL.

The sum of urinary excretion of vitamin C in men and women is shown in Table 5. No sex difference was observed, and the mean value was ca. 144 μmol/d. Levine et al. (*53, 55*) reported the effect of 30–1,250 mg doses daily on the urinary excretion of vitamin C. In their report (*53, 55*), no vitamin C was excreted in the urine of men or women until a 100-mg dose has been given orally or intravenously. With intravenous administration, the entire dose was excreted at the 500–1,250 mg doses. At an oral dose of 500 mg daily and higher, vitamin C excretion increased linearly. It was also reported that bioavailability or gastrointestinal absorption declined at intravenously delivered doses above 200 mg or at oral doses above 500 mg (*53, 55*). Our data presented here and the data reported previously indicate that a 100-mg daily dose would be a suitable intake of vitamin C to maintain the steady-state plasma concentration without affecting the urinary excretion.

CONCLUSION

We measured the values of water-soluble vitamins except for vitamin B_6 in the blood and urine of Japanese young men and women who consumed a diet based on the DRIs in the 6th revision of Recommended Dietary Allowance in the Japanese population (*1*). The RDAs of water-soluble vitamins except for vitamin B_{12} and biotin were evaluated. All of the values of water-soluble vitamins indicated these RDAs to be good. So, we propose that the present values except for vitamin B_{12} and biotin might be used as normal values for Japanese adult men and women. The requirement of vitamin B_{12} and biotin were so low that we could not precisely control them in the present diet.

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Effects of Excess Pantothenic Acid Administration on the Other Water-Soluble Vitamin Metabolisms in Rats

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Summary To acquire the data concerning the tolerable upper intake level which prevents health problems from an excessive intake of pantothenic acid, an animal experiment was done. Rats of the Wistar strain (male, 3 wk old) were fed on a diet which contains 0%, 0.0016% (control group), 1%, or 3% calcium pantothenate for 29 d. The amount of weight increase, the food intake, and the organ weights were measured, as well as the pantothenic acid contents in urine, the liver and blood. Moreover, to learn the influence of excessive pantothenic acid on other water-soluble vitamin metabolism, thiamin, riboflavin, a vitamin B_6 catabolite, the niacin catabolites, and ascorbic acid in urine were measured. As for the 3% addition group, enlargement of the testis, diarrhea, and hair damage were observed, and the amount of weight increase and the food intake were less than those of the control group. However, abnormality was not seen in the 1% addition group. The amount of pantothenic acid in urine, the liver, and blood showed a high correlation with intake level of pantothenic acid. It was only for 4-pyridoxic acid, a vitamin B_6 catabolite, in urine that a remarkable difference was observed against the control group. Moreover, the (2-Py+4-Py)/MNA excretion ratio for these metabolites of the nicotinamide also indicated a low value in the 3% pantothenic acid group. As for the calcium pantothenate, it was found that the 3% level in the diet was the lowest-observed-adverse-effect-level (LOAEL) and the 1% level was the no-observed-adverse-effect-level (NOAEL).

Key Words pantothenic acid, excess administration, rat, urine, vitamin

Pantothenic acid (PaA), a vitamin, is essential for humans and animals for growth and normal physiological functions. It is an integral part of the acylation carriers, CoA and acyl carrier proteins, which are involved in more than 100 different metabolic pathways including energy metabolism of carbohydrates, proteins and lipids, and the synthesis of lipids, neurotransmitters, and steroid hormones (*1, 2*).

It was reported that PaA deficiency induced in experimental animals when fed on a diet without PaA led to growth retardation with reduced food intake (*3, 4*) and functional impairments in all systems (*5–13*). PaA deficiency has also been induced in humans by use of a metabolic antagonist, ω-methyl PaA along with a PaA-deficient diet (*14*): Signs and symptoms reported include depression, personality changes, cardiac instability, frequent infection, fatigue, abdominal pains, sleep disturbances and neurological disorders including numbness, paresthesia (abnormal sensation such as "burning feet" syndrome), muscle weakness and cramps. Naturally occurring PaA deficiency in humans is very rare and has been observed only in cases of severe malnutrition: World War II prisoners in the Philippines, Burma, and Japan experienced numbness and painful burning and tingling in their feet ("burning feet" syndrome), which was relieved specifically by PaA administration (*15*). The cause of this syndrome must originate from stresses because PaA involves the formation of adrenocortical hormones (*16*). Therefore, the specific PaA deficiency in humans would be "burning feet" syndrome. There is an interesting hypothesis that the formation of ketone bodies under fasting conditions induces a deficiency of cellular PaA (*17*). Supplementation of this vitamin would facilitate complete catabolism of fatty acids and thus the formation of ketone bodies could be circumvented. Oral contraceptives (birth control pills) containing estrogen and progestin may increase the requirement for PaA (*18*). Recently, a report that PaA protects cells and organs against peroxidative damage by increasing the content of cell glutathione was published (*19*).

The reports (*17–19*) that pantothenic acid prevents stresses, peroxidation reactions and the formation of the ketone bodies predict a possibility that the intake of pantothenic acid will increase more and more in the future.

PaA is not known to be toxic in humans. The only adverse effect noted was diarrhea resulting from very high intakes of 10 to 20 g/d of calcium D-pantothenate (*18*). However, there is one case report of life-threatening eosinophilic pleuropericardial effusion in an elderly woman who took a combination of 10 mg/d of biotin

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and 300 mg/d of PaA for 2 mo (*20*). In rats, there are two reports on the effect of excess PaA: one details changes in hepatocellular lipid production because of an excess of PaA (*21*) and the other is a report on adrenocortical alterations induced by deficiency and excess of PaA (*22*). In the present paper, we report the effects of administering excess PaA to rats to acquire data concerning the tolerable upper intake level to prevent health problems especially the effects on the other water-soluble vitamin metabolisms.

MATERIALS AND METHODS

Chemicals. Vitamin-free milk casein, sucrose, and L-methionine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized cornstarch, the mineral mixture (AIN-93M) (*23*) and the vitamin mixture (AIN-93-VX containing 25% choline bitartrate) (*23*) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Thiamin hydrochloride ($C_{12}H_{17}ClN_4OS$-HCl=337.27), riboflavin ($C_{17}H_{20}N_4O_6$=376.37), nicotinamide ($C_6H_6N_2O$=122.13), calcium pantothenate (PaA-Ca, $C_{18}H_{32}N_2O_{10}$-Ca=476.54), and L(+)-ascorbic acid ($C_6H_8O_6$=176.13) were purchased from Wako Pure Chemical Industries, Ltd. 4-Pyridoxic acid (4-PIC, $C_8H_9NO_4$=183.16) was made by ICN Pharmaceuticals (Costa Mesa, California, USA) and obtained through Wako. N^1-Methylnicotinamide (MNA) chloride ($C_7H_9N_2O$-HCl=159.61) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N^1-Methyl-2-pyridone-5-carboxamide (2-Py, $C_7H_8N_2O_2$=152.15) and N^1-methyl-4-pyridone-3-carboxamide (4-Py, $C_7H_8N_2O_2$=152.15) were synthesized by the methods of Pullman and Colowick (*24*) and Shibata et al. (*25*), respectively. All other chemicals used were of the highest purity available from commercial sources.

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

Male rats of the Wistar strain (3 wk old with a body weight of around 40 g) were obtained from CLEA Japan, Inc. (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; CLEA Japan, Inc.). They were then divided into four groups and fed ad libitum for 29 d, one group with a PaA-free, 20% casein diet, and the others with the same diet+0.0016% PaA-Ca (used as the control group),+1.0% PaA-Ca, or+3.0% PaA-Ca (Table 1).

The room temperature was maintained at around 22°C and about 60% humidity, and a 12-h light (06:00–18:00)/12-h dark (18:00–06:00) cycle was maintained. Body weight and food intake were measured every 2 d at around 10:00. Urine samples (24-h; 10:00–10:00) were collected in amber bottles containing 1 mL of 1 mol/L HCl on the last day of the experiment, and were stored at −25°C until needed. The rats were killed by decapitation at around 10:00 on the last day (day 29) after the collection of the urine sample had been completed, and the various tissues were removed and measured. The liver of each animal was removed, and a portion (approximately 0.2 g) was immediately treated as described in the literature to measure PaA (*26*) and CoA (*27*).

Analyses.

Vitamin B_1 (thiamin): The determination of vitamin B_1 in urine was measured by the HPLC-post labeled fluorescence method of Kimura et al. (*28*).

Vitamin B_2 (riboflavin): Urinary concentration of riboflavin was analyzed according to the method of Ohkawa et al. (*29*).

4-PIC: Urinary excretion of 4-PIC, which is a catabolite of vitamin B_6, was determined according to the method described by Gregory and Kirk (*30*).

Niacin: The quantities of Nam, 2-Py and 4-Py in urine were measured simultaneously by the HPLC method of Shibata et al. (*25*). The content of MNA was measured by the method of Shibata (*31*).

Pantothenic acid: The content of free pantothenic acid in urine was directly measured by using *Lactobacillus plantarum* ATCC 8014 (*26*).

Ascorbic acid: The contents of the reduced and oxidized ascorbic acids and 2,3-diketoglutaric acid were measured by the method of Kishida et al. (*32*).

CoA: The content of CoA in liver was measured by the methods of Alled and Guy (*33*).

Statistical analysis. For the statistical evaluation, the significance of the differences in the mean concentrations among groups was treated with ANOVA and when the analysis of ANOVA was significant, the Tukey-Kramer multiple comparisons test was performed. Dif-

Table 1. The composition of the diets.

	0% PaA-Ca diet	0.0016% PaA-Ca diet	1% PaA-Ca diet	3% PaA-Ca diet
Vitamin-free milk casein	20	20	20	20
L-Methionine	0.2	0.2	0.2	0.2
Gelatinized-cornstarch	45.9	45.9	45.4	44.4
Sucrose	22.9	22.9	22.4	21.4
Corn oil	5	5	5	5
Mineral mixture (AIN-93M)	5	5	5	5
Vitamin mixture (PaA-Ca free) (AIN-93-VX containing 25% choline bitartrate)	1	1	1	1
PaA-Ca	0	0.0016	1	3

ferences of $p<0.05$ were considered to be statistically significant. Instat software (version 2.00; obtained from GraphPad Software, Inc., San Diego, CA, USA) was used for all analyses.

RESULTS

Effects of excessive PaA administration on the body weight gain and food intake in rats

The diet containing 0.0016% PaA-Ca was considered as the control. The body weight gain and food intake were almost the same between the control and the 0% PaA-Ca groups in the first 5–7 d; however, these significantly decreased in the 0% group compared with the control after that day as shown in Fig. 1. The result means that some days are needed for appearance of PaA-deficiency. On the other hand, the body weight gain and food intake of the rats fed on the 3% PaA-Ca diet were significantly lower than those in the control group, especially in the initial 5 d of the experiment with the food intake (Fig. 1). The food intakes in the 3% group became similar in amount with those of the control group from around day 20 of the experiment. The body weight gains in the control and the 3% PaA groups were almost the same from day 7. This finding complies with the possibility that the rats acquire a detoxification process of PaA by exposure to an excess amount of PaA. No adverse effects were observed in the rats fed on the 1% PaA-Ca diet.

Effects of excessive PaA administration on the tissue weights of rats

Table 2 shows the tissue weights of the rats fed on the diets supplemented with various amounts of PaA-Ca. The values were expressed as g/100 g of body weight of rat. The weights of heart, liver, and kidneys revealed no significant difference among the four groups. In the present experiment, the group fed on the 0.0016% PaA-Ca diet is the control group. The brain and testis weights were significantly higher in the PaA-deficient group than in the other PaA-containing groups. In the comparison between the control and 1% PaA-Ca groups, all of the measured tissue weights were almost the same. But the weights of lung and spleen were significantly higher in the 3% group than in the control group.

Effects of excessive PaA administration on the PaA contents in urine and liver and the CoA content in liver

The urinary excretion of PaA increased with the intake of PaA as shown in Fig. 2A. The PaA content in the liver also increased with the intake of PaA as shown in Fig. 2B; however, the degree of the increase was not so dramatic compared with the result of the urine samples, while the CoA content in the liver did not increase with the intake of PaA as shown in Fig. 2C. The saturation of CoA in the liver of rats was attained by feeding the diet containing 0.0016% PaA-Ca, namely by feeding the control diet. The excessive administration to the rats did not yield any significant increase in CoA level in the liver although the PaA level in the liver increased with the excessive administration of PaA.

Effects of excessive PaA administration on the other B-group vitamins concerned with energy metabolism

The conenzymes such as TDP (thiamin diphosphate), FAD (flavin adenine dinucleotide), PLP (pyridoxal phos-

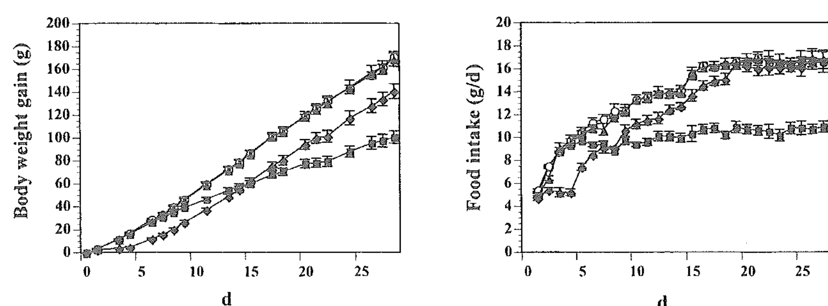

Fig. 1. Effects of PaA-Ca administration on the body weight gain (A) and food intake (B) of the weaning rats. Male rats of the Wistar strain (3 wk old) were obtained and immediately placed in individual metabolic cages (CT-10; Clea Japan). They were fed ad libitum (Table 1) for 29 d. ■, 0% PaA-Ca diet; ○, 0.0016% PaA-Ca diet (control group); ▲, 1% PaA-diet; ◆, 3% PaA-Ca diet. Values are means±SE for five rats.

Table 2. Effect of PaA-Ca administration on the tissue weights in rats.

	Body weight (g)	Brain (g/100 g BW)	Heart (g/100 g BW)	Lung (g/100 g BW)	Liver (g/100 g BW)	Spleen (g/100 g BW)	Kidney (g/100 g BW)	Testis (g/100 g BW)
0% PaA-Ca	139 ± 7.7^{a}	1.14 ± 0.06^{a}	0.38 ± 0.01	0.51 ± 0.01^{a}	4.96 ± 0.33	0.27 ± 0.02^{a}	0.96 ± 0.03	1.30 ± 0.08^{a}
0.0016% PaA-Ca*	211 ± 4.5^{b}	0.74 ± 0.07^{b}	0.37 ± 0.00	0.51 ± 0.01^{a}	4.67 ± 0.06	$0.32\pm0.01^{a,c}$	0.85 ± 0.00	1.03 ± 0.01^{b}
1% PaA-Ca	217 ± 6.7^{b}	0.78 ± 0.07^{b}	0.37 ± 0.01	$0.56\pm0.01^{a,c}$	4.73 ± 0.15	$0.35\pm0.03^{a,c}$	0.89 ± 0.02	1.09 ± 0.02^{b}
3% PaA-Ca	185 ± 5.4^{c}	0.87 ± 0.05^{b}	0.39 ± 0.01	$0.61\pm0.03^{b,c}$	5.19 ± 0.14	$0.39\pm0.03^{b,c}$	0.96 ± 0.02	0.99 ± 0.04^{b}

*Control group.
Values are means±SE for five rats. Different superscript letters indicate significant differences at $p<0.05$ in the Tukey-Kramer multiple comparisons test.

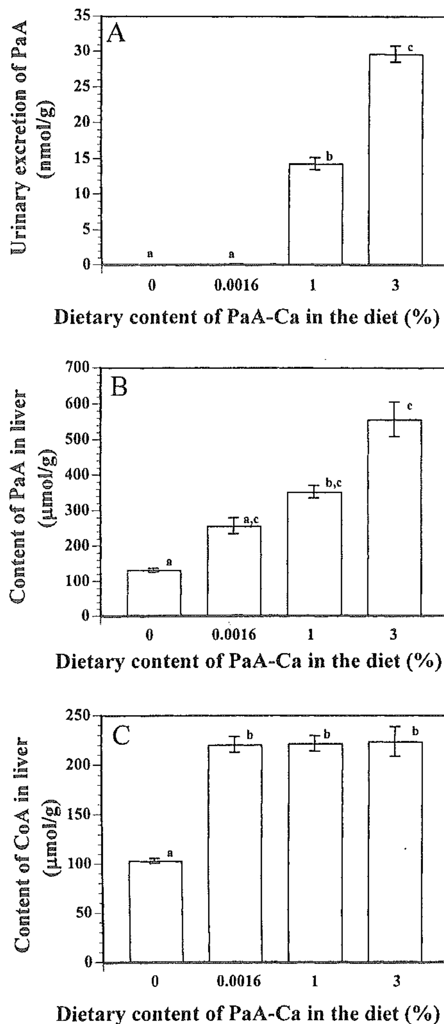

Fig. 2. Effects of PaA-Ca administration on the urinary excretion of PaA (A), the content of PaA in liver (B), and the content of CoA in liver (C). A: Twenty-four hour urine samples were collected on the last day of the experiment. The PaA content in urine samples were measured. B, C: The rats were killed after urine samples had been collected and the livers removed. The livers were treated as described in "Materials and Methods," and total PaA and CoA contents in the liver measured. Values are means±SE for five rats; different superscript letters indicate significant differences at $p<0.05$ in the Tukey-Kramer multiple comparisons test.

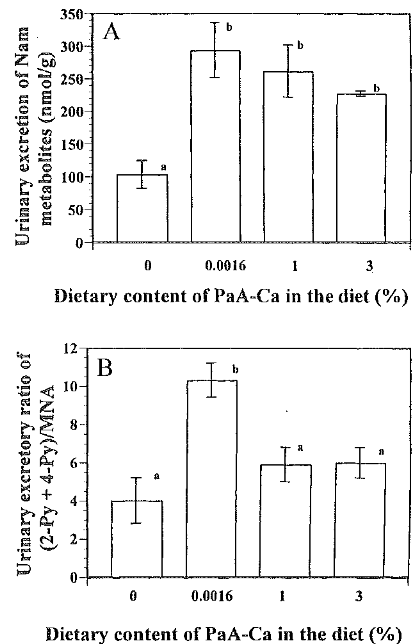

Fig. 3. Effects of PaA-Ca administration on the urinary excretion of sum of Nam and its metabolites such as MNA, 2-Py, and 4-Py (A) and the excretory ratio of the (2-Py+4-Py)/MNA (B). Twenty-four hour urine samples were collected on the last day of the experiment. A: The contents of Nam, MNA, 2-Py, and 4-Py were measured. B: The excretory ratio of the (2-Py+4-Py)/MNA was calculated. Nam, nicotinamide; MNA, N^1-methylnictoiamide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide. Values are means±SE for five rats; different superscript letters indicate significant differences at $p<0.05$ in the Tukey-Kramer multiple comparisons test.

phate) and NAD (nicotinamide adenine dinucleotide) were concerned in the metabolism of glucides, amino acids, and fatty acids as well as CoA. Thus, the excessive PaA administration had effects on the metabolism of vitamin B_1, vitamin B_2, vitamin B_6, and niacin (vitamin B_3).

Niacin and PaA are especially concerned with energy metabolism. The effects of excessive administration of PaA on the metabolism of the de novo nicotinamide synthetic pathway (tryptophan-quinolinic acid pathway) were investigated. As results, the respective urinary excretion of kynurenic acid, anthranilic acid, xanthurenic acid, 3-hydroxyanthranilic acid, and quinolinic acid was not changed by the administration of excessive PaA (data not shown). The excessive PaA administration did not affect the total urinary excretion of nicotinamide, MNA, 2-Py, and 4-Py (Fig. 3A), though it did affect the urinary excretory ratio of (2-Py+4-Py)/MNA (Fig. 3B), in comparison with the control group. The decreased ratio means that the excessive PaA induced some adverse effects in the rats, because the low value of the ratio of (2-Py+4-Py)/MNA indicates retrogression of the metabolism of nicotinamide.

Furthermore, the present data (Fig. 3) show that the deficiency of PaA affects the nicotinamide metabolism. The decreased total urinary excretion in the 0% PaA group compared with the control means that PaA deficiency increases the demand for niacin requirement.

Figure 4 shows the effects of PaA administration on the urinary excretion of vitamin B_1, vitamin B_2, and 4-PIC (a catabolite of vitamin B_6). The urinary excretion of vitamin B_1 and 4-PIC decreased according to the increase in the intake of PaA, while that of vitamin B_2 did not.

Effects of excessive PaA administration on the urinary excretion of ascorbic acid

In rats, ascorbic acid can be made from glucose, so ascorbic acid is not a vitamin. Therefore, many reports

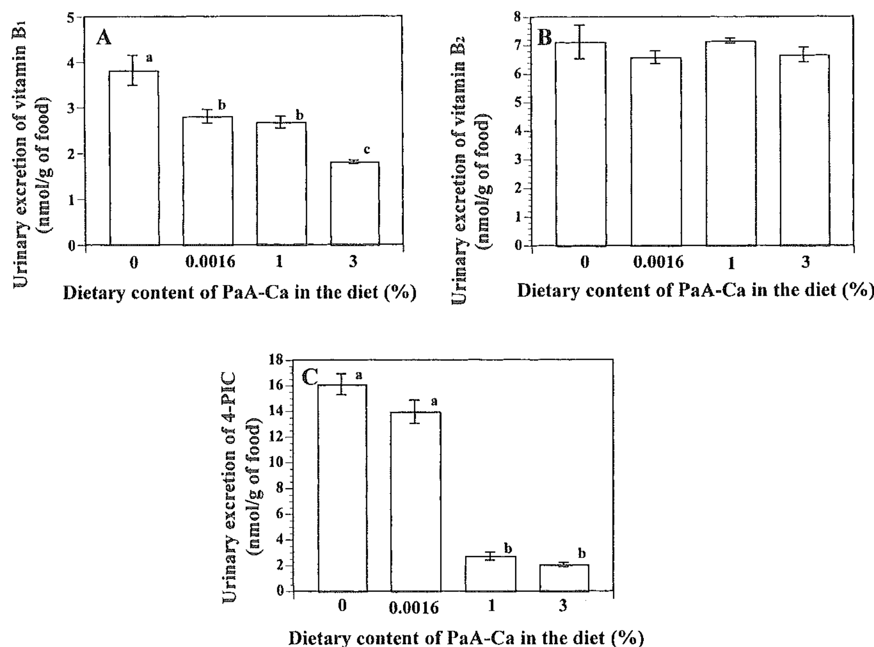

Fig. 4. Effects of PaA-Ca administration on the urinary excretion of vitamin B_1 (A), vitamin B_2 (B), and vitamin B_6 metabolites 4-PIC (C). Twenty-four hour urine samples were collected on the last day of the experiment. The contents of vitamin B_1 (A), vitamin B_2 (B), and 4-PIC (C) were measured. Values are means±SE for five rats; different superscript letters indicate significant differences at $p<0.05$ in the Tukey-Kramer multiple comparisons test.

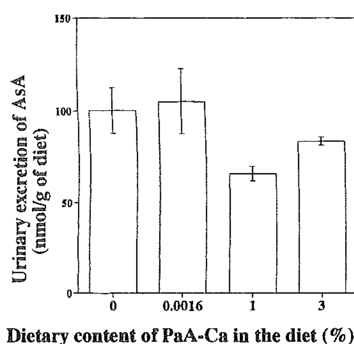

Fig. 5. Effects of PaA-Ca administration on the urinary excretion of ascorbic acid. Twenty-four hour urine samples were collected on the last day of the experiment. The content of ascorbic acid was measured. Values are means±SE for five rats.

have been published that the production of ascorbic acid is changeable by many factors. In the present experiment, the urinary excretion of ascorbic acid was not different among the four groups as shown in Fig. 5.

DISCUSSION

PaA is an integral part of CoA, which is an essential coenzyme in a variety of reactions that sustain life. CoA is required for biochemical reactions that generate energy from food (fats, carbohydrates, and proteins). The synthesis of essential fats, cholesterol, and steroid hormones requires CoA, as does the synthesis of the neurotransmitter, acetylcholine, and the hormone, melatonin (*2*). Metabolism of a number of drugs and toxins by the liver requires CoA (*34*). Most acetylated proteins in the body have been modified by the addition of an acetate group that was donated by CoA. Protein acetylation affects the 3-dimensional structure of proteins, potentially altering their function, the activity of peptide hormones, and appears to play a role in cell division and DNA replication. Protein acetylation also affects gene expression by facilitating the transcription of mRNA. A number of proteins are also modified by the attachment of long-chain fatty acids donated by CoA. These modifications are known as protein acylation, and appear to play a central role in cell signaling (*1, 2*).

As mentioned in the introduction, supplementation of PaA would facilitate complete catabolism of fatty acids (*17*) and oral contraceptives (birth control pills) containing estrogen and progestin may increase the requirement for PaA (*18*). Furthermore, PaA protects cells and organs against peroxidative damage by increasing the content of cellular glutathione (*19*). These findings induce use of excessive PaA for prevention against stresses, peroxidation, the formation of ketone bodies and so on.

The present experiments in rats clearly indicated that excessive intake of PaA had an adverse effect on body weight gain (Fig. 1A), food intake (Fig. 1B), the excretory ratio of (2-Py+4-Py)/MNA (Fig. 3B), the urinary excretion of vitamin B_1 (Fig. 4A), and the urinary excretion of 4-PIC (Fig. 4C) as well as PaA deficiency. The level causing the no-observed-adverse-effect level (NOAEL) in the rats was 1% in the diet and that causing the lowest-observed-adverse-effect-level (LOAEL) was 3% in the diet. The rats in the 1% PaA group consumed around16 g/d of their diet during day 20 to day 29 and the mean body weight during the days was about

160 g. So, the PaA intake was calculated as 3,000 mg/kg body weight a day. The rats in the 3% PaA group consumed around 16 g/d during day 20 to day 29 and the mean body weight during the days was about 180 g. So, the PaA intake was calculated as 1,000 mg/kg body weight a day, which is a proposed acceptable daily intake of PaA-Ca for rats. Although the data were not shown in the present experiments, we also done an experiment in which rats were fed on a 5% PaA diet. But, four of the five rats died with sever diarrhea within 2 d of starting the diet. The surviving rat, although maintaining the same diet, was restored to vigor after 1 wk and gained as much weight as the control. This phenomenon looked like the results in the 3% PaA group (Fig. 1A).

The excessive intake of PaA affected the metabolism of other B-group vitamins. The decreased excretory ratio of (2-Py+4-Py)/MNA, but no effect on the sum of the nicotinamide catabolites, MNA+2-Py+4-Py, means that excessive PaA inhibits the activity of MNA oxidase, which catalyzes the reactions of MNA → 2-Py and 4-Py (*35*), although the conversion pathway of tryptophan to nicotinamide was not affected. The excess intake of PaA also decreased the urinary excretion of 4-PIC, a catabolite of pyridoxal. The reaction of pyridoxal → 4-PIC is catalyzed by a similar enzyme, aldehyde oxidase (*35*). So, the excess intake of PaA might influence the oxidases. The urinary excretion of vitamin B_1 decreased in the 3% PaA group compared with that in the control and 1% groups. The decreased excretion indicated that the rats fed on the 3% PaA diet required a higher amount of vitamin B_1 compared with those fed on the control and 1% diets. The mechanism is not clear.

It is known that the ascorbic acid formation increases when the rats are exposed to xenobiotics to metabolize and excrete into urine (*36*). A large amount of PaA administration to the rats did not induce the formation of ascorbic acid (Fig. 5). So, excess amount of PaA is not recognized as xenobiotic in the rats.

In conclusion, the NOAEL of PaA, an essential part of CoA that sustains life was 1% in the dietary level and the LOAEL was 3% in the dietary level. In a daily intake per kg of body weight of rat, the NOAEL was around 1,000 mg, while the LOAEL was around 3,000 mg. If a safety factor would be 100 (species difference 10, individual difference 10) (*37*), the tolerable upper intake level is around 10 mg/kg body weight a day. We would propose 10 mg of PaA-Ca/kg body weight a day as a tentative tolerable upper intake level.

Acknowledgments

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総合論文：「高齢者とＢ群ビタミン」

寿命とニコチンアミド*

滋賀県立大学人間文化学部生活文化学科食生活専攻**

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Senior Citizen and B-group Vitamins: Longevity and Nicotinamide

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The research on the relation between the reactions of acetylation and deacetylation of histone and suppression and activation of the transcription has been recently preceded. In 1999, it was reported that yeast which had one extra copy of *Sir2* was found having 1.4 times longevity, while, the mutant to lack *Sir2* being shortened to 1/2 compared with the wild types. In addition, in 2002, it was clarified that Sir2 (= NAD^+-dependent histone deacetylase) was inhibited by nicotinamide that was one of the products. In 2003, as for the extension of the longevity that induced by the energy restriction, it was reported that it happened through increasing the appearance of *PNC1* (a gene that codes nicotinamidase), and however, the effect disappeared due to the lack of *Sir2*. Well, the mammal has the *sirtuin* (*SIRT*) family, and it is known that *SIRT1* is homolog that is the nearest to the yeast *Sir2*. In 2004, it was reported that the appearance of *SIRT1* had increased with the brain, fat tissues, the kidney, and the liver, when the rat was kept for 12 months under the energy restriction of 60%. From these reports, I have thought that it becomes a key that extends longevity that the relation between the Sir2 protein (NAD^+-dependence histone deacetylase) and nicotinamidase controls aging. The nicotinamide metabolism in the nuclei was presumed in relation to Sir2 and nicotinamidase. Moreover, it was reported that the activities of rat liver nicotinamidase, and nicotinamide methyltransferase which is another nicotinamide metabolizing enzyme were increased by the energy restriction. In mammals, the catabolism of nicotinamide being increased by the dietary restriction (limitation of amount of level that the weight of the maturity rat is maintained constantly) becomes clear.

Keywords: longevity, nicotinamide, NAD, metabolism, energy restriction

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1. 緒　　論

「腹八分に医者いらず」ということわざがある．摂取エネルギー制限により老化が抑制され，寿命が延びることは，ラットやマウスで繰り返し証明されているし，酵母，線虫，クモなどでも認められている[1]．したがって，摂取エネルギーの制限が老化を抑制し，寿命を延ばすことは動物にとって共通的に認められる現象のようである．では，どれだけエネルギー摂取を制限しなくてはいけないかというと，動物実験では自由摂取群の 1/2 ～ 2/3 に制限する必

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要がある．酵母では通常の2％グルコース培地で培養したものに対して0.5％培地で培養するとその寿命が1.25倍にもなる[2]．かなりきびしいエネルギー制限のように思えるが，人で考えれば，20歳代の体重を生涯維持することと同じ程度のエネルギー制限である．最近，エネルギー制限により得られる寿命の延長にニコチンアミドの代謝が重要な役割を果たしていることがわかってきた．

2. なぜニコチンアミドの代謝が寿命と関わっていると考えられるようになったのか？

2-1. クロマチン構造におけるヒストンのアセチル化と脱アセチル化の重要性

この研究がなぜ行われるようになったのか，そのいきさつを紹介する．

「転写が活発に行われている遺伝子領域では，ヒストンのアセチル化が亢進している」という現象は古くから知られていた．しかしながら，これは単なる副産物的な結果にすぎないとされ，ヒストンのアセチル化と転写活性の調節との関係に興味を持つ研究者は少なかった．ところが，1996年に転写のコアクチベーターとして同定されていた分子であるCBP/p300にヒストンアセチルトランスフェラーゼ(HAT)活性が報告され[3]，また同年，ヒストン脱アセチル化酵素(HDAC)として精製されていたタンパク質が出芽酵母の転写抑制因子RPD3のホモログであることが明らかにされた[4]ことなどから，「ヒストンのアセチル化・脱アセチル化反応」と「転写の活性化・抑制」という二つの現象がつながってきた．さらに，1997年，DNA結合性の転写因子p53がCBP/p300によってアセチル化されるという報告[5]がきっかけとなり，タンパク質のアセチル化・脱アセチル化の問題は，転写修飾の中心的命題となってきた．

2-2. Silent information regulator 2はNAD+依存性脱アセチル化酵素活性をもつ

そのような中で，2000年，Sir2(Silent information regulator 2：酵母において転写のサイレンシングに関与していることが1995年に報告されていたタンパク質)に[6]，NAD+依存性のヒストン脱アセチル化酵素活性があることが報告された[7]．この酵素は，NAD+とアセチル化されたヒストン(この状態では活性クロマチン構造をとっている)を基質として，脱アセチル化されたヒストン，ニコチンアミドおよび*O*-アセチルADPリボースを産生する(NAD+ + アセチル化ヒストン → ニコチンアミド + *O*-アセチルADPリボース + 脱アセチル化ヒストン)．ヒストンが脱アセチル化されるとサイレンシング，すなわち不活性クロマチン構造となる．その結果，老化が抑制され，寿命がのびるという仮説である．

たとえば，*Sir2*を1コピー余分に持つ酵母は野生型の酵母(21～23回分裂)よりも1.4倍の寿命を持つのに対し，*Sir2*を欠く変異体の寿命は野生型の1/2にまで短縮する[8]．つまり，Sir2遺伝子の産物であるSir2タンパク質は，酵母の老化・寿命の制御に必須であることが明らかとなった．このSir2の機能は酵母におけるエネルギー制限による寿命延長効果にも必須であることが明らかにされている[9]．すなわち，酵母をグルコース制限培地(0.5％グルコース)で培養すると非制限培地(2％グルコース)と比較して寿命が1.25倍程度延長されるが，*Sir2*を欠損させた変異株では延長されない[10]．さらに，Sir2のホモログであるsir-2.1の高発現が線虫の*Caenorhabditis elegans*の寿命を延ばすこと[11]，ヒトにおいてSir2に最も近いホモログであるSIRT1がp53の脱アセチル化を介してアポトーシスを阻害することが報告された[12][13]．これらの報告から，Sir2とそのホモログは生物界に広く保存された寿命に関わる因子であることを示唆している．従って，ヒトにおいてもエネルギー制限による老化の抑制と寿命の延長の可能性がでてきた．

ここで，Sir2が核内に存在することおよびSir2がNAD+の存在下でアセチル化ヒストンを脱アセチル化することを思い出していただきたい．NAD+はNADHに還元されるのではなく，ニコチンアミドと*O*-アセチルADPリボースに分解されてしまう．このSir2の脱アセチル化反応は生成物のニコチンアミドにより強く阻害される[14]．以上の結果から，野生型酵母においてエネルギー制限下でSir2活性が上昇する機構として次の三つの可能性が考えられる．①Sir2タンパク質レベルが上昇する．②Sir2による脱アセチル化反応の基質NAD+の供給が高まり，Sir2活性が上昇する．③脱アセチル化反応によって阻害物質であるニコチンアミドが迅速に代謝され，Sir2活性が上昇する．このうち①の可能性については，エネルギー制限下でもSir2レベルが変化しないことが判明している[15]ので除外される．そのような背景からハーバード大学医学部のSinclairらの研究グループは，出芽酵母を用いて，Sir2とNAD+代謝との関連を研究し始め，以下に示すことを明らかにしている[2][14][15]．なお，出芽酵母においては，3種類(接合型遺伝子，テロメア，rDNA)のサイレンシング遺伝子座(転写抑制遺伝子座)が同定されている．

2-3. NAD+生合成との関連性[15]

理解を助けるために，図1に出芽酵母のNAD+代謝の概要を示した．NAD+代謝と寿命とエネルギー制限との関係については，以下のことが明らかにされた．

①Nicotinate phosphoribosyltransferaseをコードする*NPT1*を高発現させた酵母では寿命が通常の酵母よりも1.6倍も延びた．しかしながら，NAD+濃度とNAD+/NADH比は変化していなかった．

②NAD+生合成に関わる酵素をコードする遺伝子であ

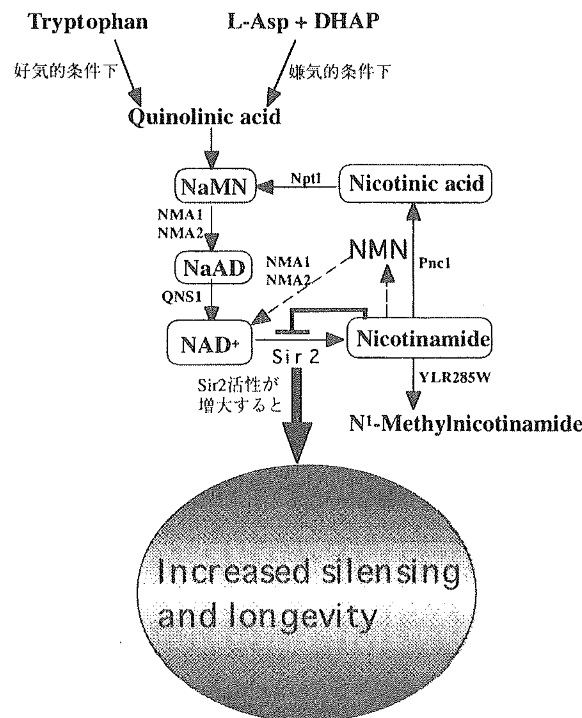

図 1．NAD⁺ 代謝の概要(出芽酵母)．
Sir2(NAD⁺ 依存性脱アセチル化酵素の一つ)は NAD⁺ 代謝からみれば，NAD⁺ 分解酵素の一つである．*Sir2* の欠損は酵母の NAD⁺ 含量に影響を及ぼさないことから，NAD⁺ 分解酵素としての役割は重要ではない．しかし，Sir2 活性が増大すると，活性型クロマチンを不活性型クロマチンに変換し，転写が抑制(サイレンシング)され，寿命が延びる．なお，点線の矢印で示したニコチンアミド→NMN（ニコチンアミドモノヌクレオチド)→NAD⁺ の経路はほ乳類では主要な NAD⁺ 生合成経路であるが，出芽酵母で存在するか否かは不明である．DHAP=Dihydroxyacetonephosphate.

る *PNC1* (Nicotinamidase)，*NMA1* (Nicotinic acid mononucleotide and nicotinamide mononucleotide adenylyltransferase)，あるいは *NMA2* (Nicotinic acid mononucleotide and nicotinamide mononucleotide adenylyltransferase)を導入すると，rDNA (rRNA 遺伝子)の安定化が増し，すなわち rRNA の合成量の低下が起こった．また，テロメア領域のサイレンシング(転写抑制)が高まった．しかし，*QNS1* (NAD⁺ synthetase)の導入はサイレンシングに影響を与えなかった．

③ *Sir2* 欠損酵母菌における NAD⁺ 量は通常の酵母と変動がなかった(このことは，Sir2 は NAD⁺ の主要な分解酵素ではないことを意味している．)．

④エネルギー制限によって，Nicotinate phosphoribosyltransferase 量は増大せず，局在も変動しなかった．これらの結果から，彼らは，Sir2 活性は *salvage* NAD⁺ 生合成経路(ニコチンアミド→ニコチン酸→ニコチン酸モノヌクレオチド→ニコチン酸アデニンジヌクレオチド→ニコチンアミドアデニンジヌクレオチド(NAD⁺))から流入する NAD⁺ レベル，あるいは NAD⁺ に由来する物質によって調節されている可能性を示唆した．つまり，NAD⁺ そのものの量は寿命とは関係ないらしい．

2-4．ニコチンアミドは Sir2 の阻害剤 [14)]

さらに，重要なことが見いだされた．

① Sir2 の反応産物の 1 つであるニコチンアミドは酵母のサイレンシングを強く阻害した．

②ニコチンアミドは Sir2 タンパク質の生成を抑制していなかった．

③ニコチンアミドは野生型酵母の rDNA の組み換え頻度を高めたが，元々 rDNA の組み換え頻度の高い *Sir2* 欠損酵母では影響を及ぼすことはなかった．

④野性型の酵母を 5 mM ニコチンアミド含有培地で培養すると寿命が非含有培地の寿命の約半分にまで低下した．この低下した寿命は *Sir2* 欠損酵母と同じであった．

⑤ *Sir2* 欠損酵母をニコチンアミド過剰含有培地で培養しても寿命の低下は認められなかった．

⑥ニコチンアミドは *in vitro* で Sir2 と SIRT1(ヒトの Sir2 ホモログ)の非拮抗阻害剤であることが証明された．IC_{50} は 50 μM 程度であった．

つまり，核内におけるニコチンアミドのすみやかな除去が寿命と関わりがあることがわかってきた．

2-5. Nicotinamidase の重要性[2)]

つまり，Nicotinamidase がヒストンの脱アセチル化反応に重要な役割を果たしている可能性がでてきた．そこで，この点に関する研究の進展をまとめると，

①野生型の酵母を 0.5 % グルコース培地で培養すると寿命が延びるが，この延長は Nicotinamidase をコードする遺伝子 *PNC1* を欠損させると消失した．

②野生型の酵母を 37℃で生育させると(熱ショック処理)，通常の培養温度の 30℃と比べて寿命が延びるが，この延長も Nicotinamidase をコードする遺伝子 *PNC1* を欠損させると消失した．

③野生株に 5 倍量の *PNC1* を導入した酵母の寿命は，約 2 倍も延長した．

④ Pnc1 レベルは培地中のグルコース濃度が低下するに従って増大した．

⑤ *cdc25-10* アレル(エネルギー制限の模倣を引き起こす)の導入は Pnc1 レベルの上昇を引き起こした．

⑥アミノ酸制限，塩ストレス，熱ストレス，ソルビトール添加はすべて，Pnc1 レベルの上昇を引き起こした．

⑦実際に Nicotinamidase 活性も Pnc1 タンパク質レベルに応じて認められた．

⑧ニコチン酸の添加は rDNA のサイレンシングを増大させなかった．

⑨ *de novo* NAD^+ 生合成経路を欠損させた酵母(*bna6* Δ；quinolinate phosphoribosyltransferase を欠く酵母)をさらに *pnc1* Δにしても通常の培地で生育した．このことは Pnc1 すなわち Nicotinamidase は，NAD^+ 生合成において決定的な役割を果たしていないことを意味している．

⑩ *npt1* Δ (nicotinate phosphoribosyltransferase の遺伝子欠損株) はサイレンシング欠陥株であるが，*PNC1* (nicotinamidase の遺伝子) を導入すると若干欠陥が回復する．ちなみに，*npt1* Δ (nicotinate phosphoribosyltransferase の遺伝子欠損株) の細胞内 NAD^+ レベルは野生株の 1/2 程度である．そこで，培地に *de novo* 生合成経路の中間体であるキノリン酸を添加して NAD^+ レベルを正常にすると，*npt1* Δ, *3×PNC1* 株の rDNA のサイレンシング(すなわち rRNA の転写抑制)を野生株のレベルまで回復させた．

⑪ヒトの *NNMT* (nicotinamide methyltransferase をコードする遺伝子)を酵母に導入すると，rDNA のサイレンシングが上昇した．また，酵母の *NNMT* 遺伝子と推定される YLR285W の導入もサイレンシングを上昇させた．

⑫ YLR285W の導入は酵母の寿命を延ばしたが，グルコース飢餓培地で培養しても，さらに寿命は延びなかった．しかしながら YLR285W 欠損株は野生株と同じようにグルコース飢餓により寿命が延びることから，YLR285W は *PNC1* と異なり，本当の寿命調節因子ではない．

ここまでのことを，想像も含めて図示すると，図 2 のような代謝図がかける．

3. ほ乳動物における Sir2 ファミリー

では，酵母のような細胞レベルの寿命と，人などの高等動物の個体としての寿命とを同一に論じることには多少の疑問を感じていたが，ほ乳動物のエネルギー制限と Sir2 ファミリーに関する論文[16)-24)]がでた．

ほ乳動物には Sir2 のホモログとして sirtuin (SIRT) ファミリーが存在し，SIRT1 が酵母 Sir2 に最も近いホモログである．となると，ヒトにおいても SIRT1 によって寿命を延長することが可能であるのか，そして，NAD^+ やニコチンアミド代謝を調節することによって SIRT1 の活性を制御できるのか大変興味深い．

3-1. ラットでのエネルギー制限と SIRT1 の発現量との関係

Cohen ら[16)]は，自由摂取群の 60 % に制限した食餌で離乳直後から 12 ヶ月間飼育したラットを用い，各組織における SIRT1 の発現量を調べたところ，脳，脂肪組織，腎臓，肝臓など多くの組織で自由摂取群に比べて発現量が増加していたことを報告している．したがって，酵母と同様にほ乳動物においてもエネルギー制限によって SIRT1 が活性化することを示している．15 時間絶食したマウスの肝臓，骨格筋においても SIRT1 発現量が増加することから[17)]，SIRT1 の標的となる転写因子として p53[12)13)]，フォークヘッド転写因子[18)-21)]，NF-κB[22)]が報告されている．これらの転写因子はいずれも細胞周期，アポトーシスに関与する．SIRT1 発現量の増加には p53，フォークヘッド転写因子の Foxo3a を必要とすることから[17)]，ネットワークを形成した複雑な制御機構かもしれないが，SIRT1 はこれらの転写因子を制御あるいは相互作用を介して細胞を死から生存の方向へ切り替える役割を持つようである．

3-2. SIRT1 は脂肪の蓄積を抑制し糖新生を活発にする

出芽酵母では，培地のグルコース濃度を通常の 2 % から 0.5 % に下げると，その寿命が 1.25 倍にのびることを[2)]，またほ乳動物でも無制限に食べさせたものよりも，成熟動物の体重を維持できるだけの摂取量に制限したものの方が，SIRT1 (Sir2 のホモログ) の発現量が高くなること[16)17)]を紹介した．Sir2 も SIRT1 は NAD^+-依存性脱アセチル化酵素である．では，脱アセチル化されるタンパク質には，どのようなものがあるのか．上述のように，アセチル化されたヒストンが最も有名である．アセチル化されたヒストンからアセチル基が取れると，ヒストンは DNA と強固に結合して不活性クロマチン状態となる．この脱アセチル化が NAD^+ 依存であることがきわめて重要である．一方，アセチル化はアセチル CoA が関与する．つまり，SIRT1 は細胞のエネルギー代謝に関与する情報をアセチル

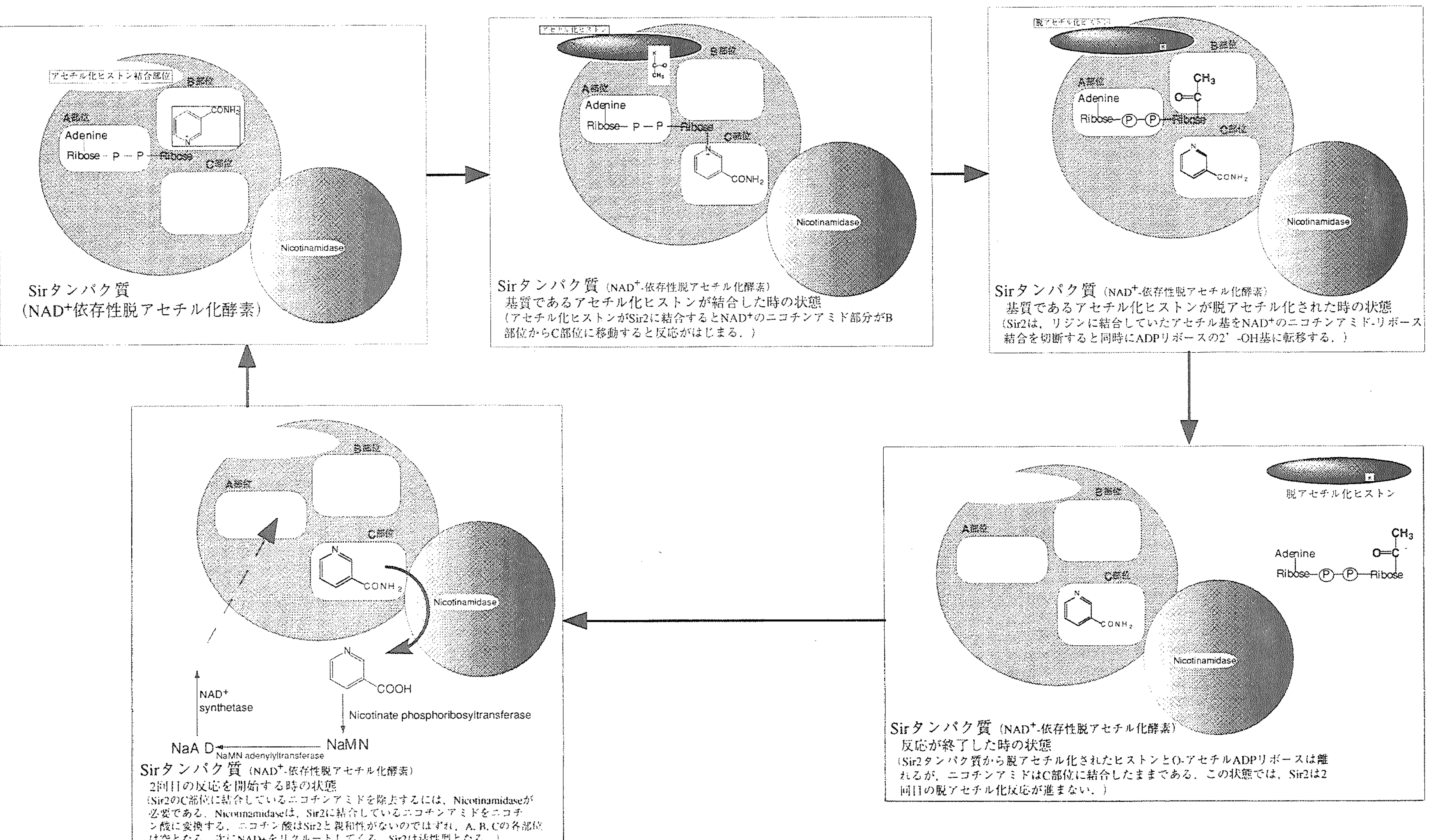

図2. Sir2, ニコチンアミダーゼ, NAD^+ およびニコチンアミドの関係図.

CoA と NAD+ を介して受け取り，遺伝子のサイレンシングにはたらいていると考えることができる．

では，このNAD+-依存性脱アセチル化酵素の基質特異性はどの程度厳密なのか．実は，あまり厳密ではない．FOXO4も基質となり[23]，脱アセチル化されることで転写活性が増大し，脂肪細胞においては，成熟脂肪細胞への分化が抑制される．繊維芽細胞を筋芽細胞に形質転換する因子として発見された転写因子であるMyoDもSIRT1によって脱アセチル化され，筋芽細胞への分化が促進される[24]．一方，転写調節因子であるp53というタンパク質はアセチル化を受けて活性を有する状態となるが，p53の脱アセチル化も触媒する[13]．つまり，SIRT1はp53活性を抑制することで，アポトーシスを抑制する．また，リガンド依存型核内受容体転写因子群のPPARγによる転写活性化は脱アセチル化されることで抑制され，脂肪組織における脂肪の蓄積が抑制される[25]．さらに，PPARγの転写共役因子として同定されたPGC1（PPARγ coactivator 1）もSIRT1によって脱アセチル化される．脱アセチル化されたPGC1は糖新生に関わる遺伝子を誘導し，グルコース量を高める[26]．

これらの報告から，SIRT1の活性増大は，脂肪が蓄積しないような代謝変動をもたらし，その一方で，糖新生，グルコース代謝を盛んにするような代謝変動をもたらすことで寿命を延長するらしい．しかしながら，なぜ，このような代謝変動によって寿命がのびるのか．精力的な研究が続いているので，近いうちに明らかにされるであろう．

3-3. ニコチンアミド代謝とSIRT1との関係

ニコチンアミド代謝，NAD代謝とSIRT1との関係については，ワーラー変性遅延マウスという，神経損傷後の軸索変性（ワーラー変性）を起こしにくいマウスの解析から見つかった．

このマウスでは，ユビキチン鎖の伸長に関与するUfd2a（ubiquitin fusion degradation protein 2a）とNAD+合成酵素であるニコチンアミドモノヌクレオチドアデニリルトランスフェラーゼ（Nmnat1：ニコチンアミドモノヌクレオチド（ニコチン酸モノヌクレオチド）+ ATP → NAD+（NaAD+）+ PPi．本酵素は核内に局在する）とのキメラタンパク質が過剰発現している．Arakiらは[27]初代後根神経節細胞を用いてNmnat1の過剰発現が軸索変性を遅延することを明らかにした．軸索変性遅延はNAD+添加によっても認められ，この作用はSIRT1 siRNAによって抑制された．すなわち，ワーラー変性遅延マウスに見られた神経保護作用は，Nmnat1によって核内NAD+レベルが上昇し，その結果，SIRT1が活性化されたことに起因するものであると思われた．

Revolloら[28]は，マウス繊維芽細胞を用い，ニコチンアミドをニコチンアミドモノヌクレオチドに変換するニコチンアミドホスホリボシルトランスフェラーゼ（Nampt：本酵素は生理的濃度のNAD+によりフィードバック阻害を受けることにより，細胞内のNAD+濃度を一定に維持する機能を有する）の過剰発現によって細胞内NAD+レベルとSIRT1発現が上昇したが，Nmnatの過剰発現およびニコチンアミド添加のいずれにもこの効果が認められなかったことを明らかにした．ニコチンアミド代謝がSIRT1活性にどのような影響をあたえるのか明らかではないが，少なくともほ乳動物においてもNAD+代謝を調節することによってSIRT1の活性調節が可能であるようだ．

3-4. エネルギー制限による他の効果

エネルギー制限の効果には寿命延長のみならず，腫瘍形成，神経変性，自己免疫疾患，糖尿病など様々な疾病を防ぐことが知られている[29]．上述したように，Arakiらの報告[27]はSIRT1による神経保護作用を示している．また，SIRT1の過剰発現により脂肪細胞形成が抑制され，脂肪細胞の脂肪分解が促進されることは，インスリン抵抗性，2型糖尿病，アテローム性動脈硬化など肥満に関連した疾病の治療・予防に重要な知見を与えるものである[30]．このように，今後は，様々な疾病をSIRT1がどのように防ぐことができるのかという研究が広く行われるようになることが予想される．さらに，NAD+代謝，ニコチンアミド代謝によってSIRT1を制御できるようになることを期待したい．

4. ラットにおけるエネルギー制限がニコチンアミドの異化代謝におよぼす影響[31]

このようなことを背景にして，今まで私どもが明らかに

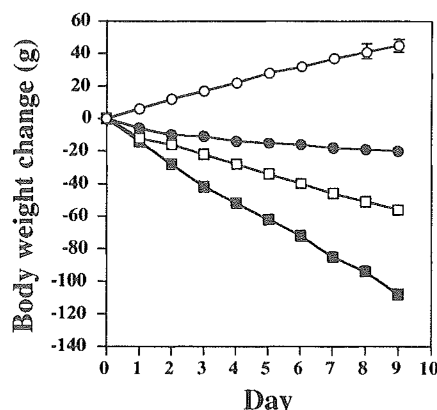

図3. エネルギー制限下で飼育したラットの体重変化．
13週齢の雄Wistar系ラットを，通常食で10日間飼育した．飼料の摂取量を図中に示したように制限した．Control群は，自由に摂取させた群，2/3群は自由摂取群の飼料摂取量の2/3量を摂取させた．1/2群は自由摂取群の飼料摂取量の1/2量を摂取させた．0群は飼料を与えなかった飢餓群である．数値は平均値 ± SEM（n=5）で示した．○，CONTROL群；● 2/3群；□，1/2群：■，飢餓群．