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# Comparison of Metabolic Fates of Nicotinamide, $NAD^+$ and NADH Administered Orally and Intraperitoneally; Characterization of Oral NADH

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***Summary*** Since NADH has been implicated in medication for some symptoms and as a possible supplement for health, we characterized the metabolic fate of NADH orally given to mice by comparing with those of nicotinamide (Nam), $NAD^+$ and NADH intraperitoneally or orally administered. Mice were individually housed in metabolic cages, and divided into two sets of four groups. Within each set, one group was intraperitoneally or orally administered saline and the other three groups received intraperitoneal or oral administration of a pharmacological dose of Nam, $NAD^+$ or NADH (5 $\mu$mol/mouse). Twenty-four hour urine samples for the day before and days 1 to 4 after administration were collected and analyzed for Nam and its metabolites. When mice were administered saline alone, urinary excretion of Nam and its metabolites, such as nicotinamide *N*-oxide (Nam *N*-oxide), $N^1$-methylnicotinamide (MNA), $N^1$-methyl-2-pyridone-5-carboxamide (2-Py), and $N^1$-methyl-4-pyridone-3-carboxamide (4-Py), was unchanged from day 0 to day 4. Intraperitoneal injection of Nam, $NAD^+$ and NADH produced significant increases in urinary excretion of Nam and its metabolites. Similar results were obtained when Nam and $NAD^+$ were given orally. On the other hand, oral administration of NADH did not bring about an increase in urinary excretion of Nam and its metabolites, suggesting that NADH in digestive organs has been decomposed to a compound(s) that cannot yield Nam. In fact, incubation of NADH at acidic pH to mimic the stomach resulted in rapid conversion of NADH to an unknown compound. Better understanding of the fate of oral NADH is needed for its therapeutic and supplemental use.

***Key Words*** NADH, $NAD^+$, oral administration, intraperitoneal administration, mouse

Reduced nicotinamide adenine dinucleotide (NADH) and its oxidized form ($NAD^+$) in cells are synthesized mainly from dietary nicotinamide (Nam) (Fig. 1). Nam is actively absorbed into intestinal cells and distributed into various tissues, where it is used for biosynthesis of pyridine nucleotide coenzymes (*1*, *2*). These coenzymes are also synthesized partly from tryptophan and nicotinic acid. Excess Nam is converted into nicotinamide *N*-oxide (Nam *N*-oxide), $N^1$-methylnicotinamide (MNA), $N^1$-methyl-2-pyridone-5-carboxamide (2-Py), and $N^1$-methyl-4-pyridone-3-carboxamide (4-Py) and these catabolites are excreted into urine in mice (*3*). It has been shown that orally taken $NAD^+$ also supplies Nam, since $NAD^+$ is metabolized to Nam in the small intestinal tract (*1*).

In contrast, the metabolic fate of oral NADH is unclear. It appears that instability of NADH in an acidic condition (gastric juice) (*4*) has made it difficult to pursue its fate. Nevertheless, NADH has been used as a novel medication for Parkinson's disease (PD) patients (*5–7*). Although support for this trial includes findings that NADH stimulates dopamine production through activation of tyrosine hydroxylase (*8*), which is the rate-limiting step of dopamine biosynthesis (*9*), and the intravenous or oral NADH administration improves PD rating scale, it is still controversial for several reasons whether NADH is recommendable as a therapeutic agent of PD (*5–7*). Furthermore, it has been reported that NADH appears to act against jet lag (*10*) and malaise (*11*, *12*).

Such a high incidence of NADH ingestion has prompted us to explore in vitro and in vivo changes of NADH. In the present study, we show that exposure of NADH to an acidic condition yields unknown products and that the metabolic fate of NADH orally given to mice markedly differs from that of intraperitoneal NADH. Metabolism of Nam, $NAD^+$ and NADH administered either orally or intraperitoneally has also been compared by measuring urinary Nam and its metabolites.

## MATERIALS AND METHODS

*Chemicals.* Vitamin-free milk casein, sucrose, L-methionine and Nam were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). $N^1$-Methylnicotinamide (MNA) chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). $NAD^+$ and NADH were

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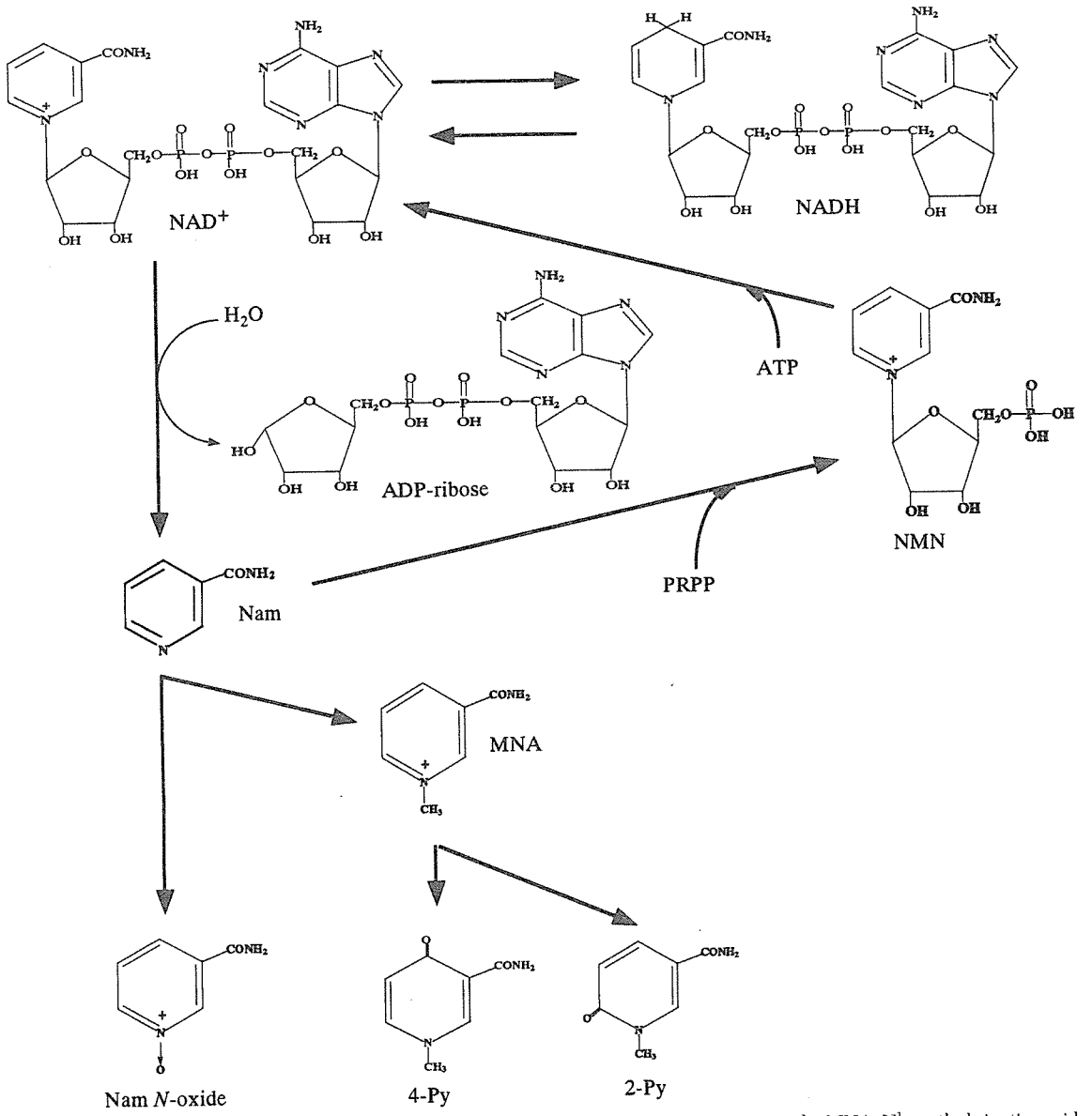


Fig. 1. Proposed metabolic fate of the intraperitoneally injected NADH. Nam, nicotinamide; MNA, $N^1$-methylnicotinamide; Nam *N*-oxide, nicotinamide *N*-oxide; 2-Py, $N^1$-methyl-2-pyridone-5-carboxamide; 4-Py, $N^1$-methyl-4-pyridone-3-carboxamide; NMN, nicotinamide mononucleotide; PRPP, 5-phosphoribosyl 1-pyrophosphate.

purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Nam *N*-oxide was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). $N^1$-Methyl-2-pyridone-5-carboxamide (2-Py) and $N^1$-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick (*13*) and Shibata et al. (*14*), respectively. Corn oil was purchased from Ajinomoto (Tokyo, Japan). The mineral and vitamin mixtures and the gelatinized cornstarch were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals used were of the highest purity available from commercial sources.

*Stability of NAD+ and NADH in acidic solution.* $NAD^+$ and NADH were each dissolved in 0.1 mol/L HCl at 0.1 mg/mL and kept at 25°C. Each solution was directly injected to an HPLC system. NADH was dissolved in $H_2O$ at 0.1 mg/mL and immediately injected into the HPLC. NADH was dissolved in 0.1 mol/L HCl at 0.1 mg/mL at 25°C, and then injected into the HPLC at 1 min after the dissolution, 40 min after the dissolution, and 3 h after the dissolution. The chromatographic conditions were constant: column, Chemcosorb 7-ODS-L (4.6, i.d., ×250 mm); mobile phase, 10 mmol/L $KH_2PO_4$ (pH 3.0 adjusted by $H_3PO_4$) : acetonitrile=96 : 4; column temperature, 30°C; detection, UV (260 nm); flow-rate, 1.0 mL/min; sample volume, 20 μL.

*Animals and diets.* This experimental design was approved by the Animal Experiment Committee of The University of Shiga Prefecture and the mice were handled according to Guidelines for Care and Use of Labora-

tory Animals.

Experiment 1: Male mice of the ICR strain (11 wk old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were individually housed in metabolic cages (CM-10S; CLEA Japan, Inc.) and fed a complete 20% casein diet (Table 1) and allowed free access to water throughout the experimental period. Body weight and food intake were measured daily at 09:00. The environmental conditions were constant: 12-h light/dark cycle, room temperature of 22±2°C, humidity of about 60%.

Table 1. Composition of the diets.

| | Control diet (NiA-free, 20% casein diet) |
|---|---|
| | (g/kg of diet) |
| Milk casein (Vitamin-free) | 200 |
| L-Methionine | 2 |
| Gelatinized-cornstarch | 459 |
| Sucrose | 229 |
| Corn oil | 50 |
| Mineral mixture[1] | 50 |
| Vitamin mixture (NiA-free)[2] | 10 |

[1] Provided the following (g/kg of diet): $CaCo_3$, 14.645; $CaHPO_4 \cdot 2H_2O$, 0.215; $KH_2PO_4$, 17.155; NaCl, 12.53; $MgSO_4 \cdot 7H_2O$, 4.99; $Fe(C_6H_5O_7) \cdot 6H_2O$, 0.31115; $CuSO_4 \cdot 5H_2O$, 0.078; $MnSO_4 \cdot H_2O$, 0.0605; $ZnCl_2$, 0.01; KI, 0.00025; and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.00125. Obtained from Oriental Yeast Co., Ltd., Tokyo, Japan.

[2] Provided the following (mg/kg of diet, except as indicated): retinyl acetate, 5,000 IU; cholecalciferol, 1,000 IU; tocopherol acetate, 50; menadione, 52; thiamine-HCl, 12; riboflavin, 40; pyridoxine-HCl, 8; cyanocobalamin, 0.005; ascorbic acid, 300; D-biotin, 0.2; folate, 2; calcium pantothenate, 50; *para*-aminobenzoic acid, 50; nicotinic acid, 60; inositol, 60; choline chloride, 2,000; and made up to 10 g with cellulose powder. Obtained from Oriental Yeast Co., Ltd.

After 1 wk, they were divided into four groups of five each. One group was intraperitoneally injected with a sterile physiological saline solution (0.1 mL), while the other three groups were intraperitoneally injected with an appropriate dose of Nam, $NAD^+$ or NADH (5 μmol/mouse) dissolved in sterile saline (0.1 mL) at 09:00. In the preliminary experiment of feeding a NiA-free 20% casein diet, the sum of Nam and its metabolites in the 24 h urine was about 1 μmol per mouse. For this reason, we decided dosage at 5 μmol as the amount of the lowest addition at which the increase of the excretion to the urine would be obviously confirmed. Twenty-four hour (09:00–09:00) urine samples from the day before (day 0) and days 1 to 4 after the injection were collected into bottles containing 1 mL of 1 mol/L HCl and stored at −25°C until analysis for Nam and its metabolites.

Experiment 2: The methods were the same as in Experiment 1 except for the route of administration. After 1 wk, they were divided into four groups of five. One group was orally administered a sterile physiological saline solution (0.1 mL), while to the other three groups was orally administered Nam, $NAD^+$ or NADH (5 μmol/mouse) dissolved in sterile saline (0.1 mL) at 09:00.

*Analyses.* The quantities of Nam, 2-Py and 4-Py in the urine were measured simultaneously by the HPLC method of Shibata et al. (*14*). The urinary content of

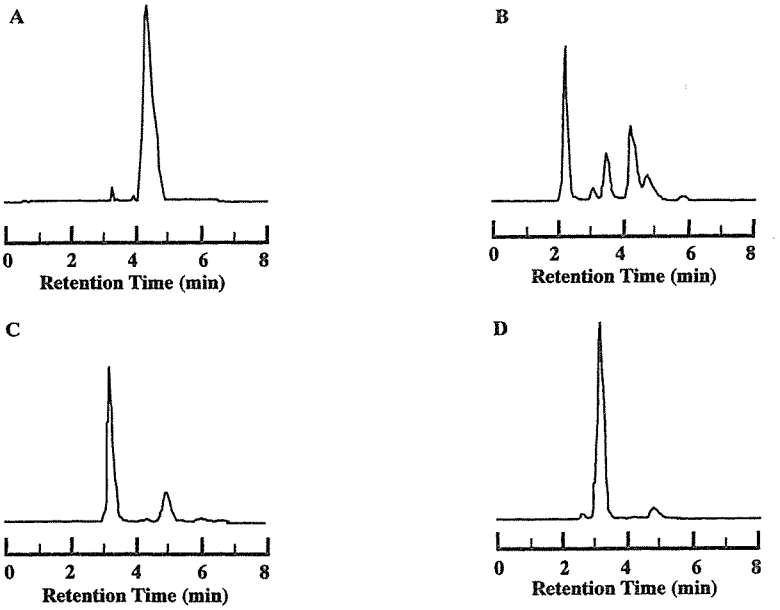


Fig. 2. Time-dependent changes in the HPLC chromatogram of NADH dissolved in 0.1 mol/L HCl. A: NADH was dissolved in $H_2O$ at 0.1 mg/mL and immediately injected into the HPLC. B–D: NADH was dissolved in 0.1 M HCl at 0.1 mg/mL at 25°C, and then injected into the HPLC at (B) 1 min after the dissolution, (C) 40 min after the dissolution, and (D) 3 h after the dissolution.

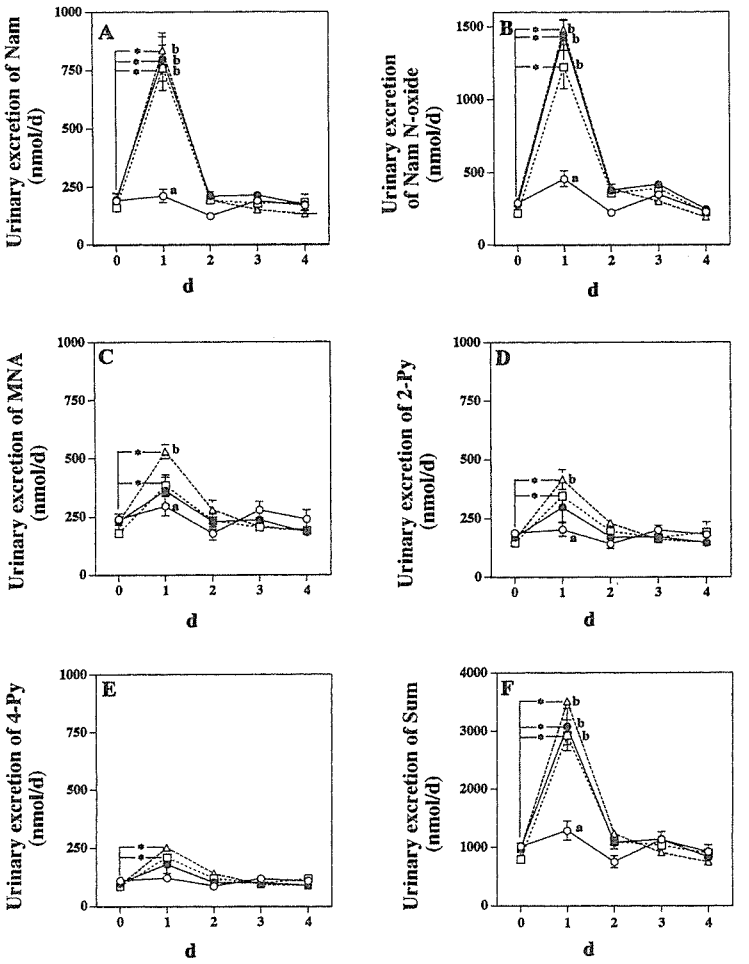


Fig. 3. Effects of intraperitoneal injection of Nam, $NAD^+$ or NADH on the urinary excretion of nicotinamide and its metabolites in mice. On day 1 at 09:00, 5 μmol/mouse of Nam (●), $NAD^+$ (□) or NADH (△) dissolved in 0.1 mL of sterile saline was intraperitoneally injected into mice. As a control (○), 0.1 mL of sterile saline was injected. Twenty-four hour (09:00–09:00) urine samples were collected for 1 d before the administration (day 0) and days 1 to 4 after the injection. Sum=Nam+Nam *N*-oxide+MNA+2-Py+4-Py. Each point is the mean±SE for 5 mice. Values with different superscript letters in the same figure are statistically significantly different at $p<0.05$ vs. the control group on the same day. *Significant at $p<0.05$ compared with the value of the respective day 0.

MNA or Nam *N*-oxide was measured by HPLC as previously described (*15*, *16*).

*Statistics.* All data are presented as means±SE, $n=5$. Statistical analysis was carried out by two-way ANOVA followed by Dunnett's multiple comparison test; the mice injected or administered with a sterile physiological saline solution were defined as the control groups (Stat View 5.0, SAS Institute Inc.; Cary, NC, USA).

## RESULTS

*Changes of NADH and $NAD^+$ in acidic solution*

It is widely accepted that NADH is unstable under acidic conditions but it is stable under alkaline conditions, while $NAD^+$ shows the opposite properties (*4*). We first examined the breakdown of NADH dissolved in 0.1 mol/L HCl (Fig. 2). Figure 2A is a HPLC chromatogram of NADH dissolved in water; a single peak of NADH was observed. Figure 2B, C, and D are HPLC chromatograms of the acidified NADH solution after incubation at 25°C for 1 min, 40 min and 3 h, respectively. Significant breakdown of NADH was seen after exposure for only 1 min (Fig. 2B) and incubation for 40 min resulted in a major breakdown product eluted at around 3 min (Fig. 2C). After incubation for 3 h, most NADH had been converted to this major product whose structure remains yet unknown (Fig. 2D). This product does not correspond to Nam, because Nam is eluted at around 6 min under the HPLC conditions used. As expected, $NAD^+$ in acidic solution was unchanged after 3 h incubation at 25°C (data not shown).

*Body weight and food intake*

The daily changes in body weight and food intake among all the groups in Experiments 1 and 2 were almost constant (data not shown). Therefore, there was

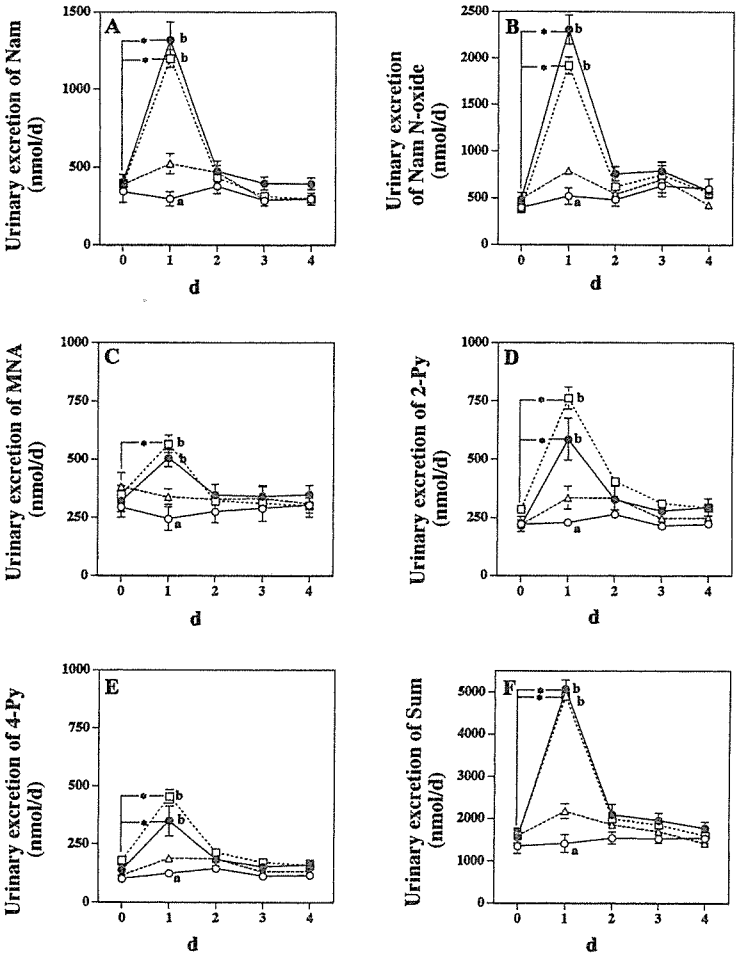


Fig. 4. Effects of oral administration of Nam, $NAD^+$ or NADH on the urinary excretion of nicotinamide and its metabolites in mice. On day 1 at 09:00, 5 μmol/mouse of Nam (●), $NAD^+$ (□) or NADH (△) dissolved in 0.1 mL of sterile saline was orally administered to mice. As a control (○), 0.1 mL of sterile saline was orally administered. The others are the same as in the legend for Fig. 3.

no influence on body weight or food intake by the route of administering NADH.

*Urinary excretion of Nam and its metabolites, Nam N-oxide, MNA, 2-Py and 4-Py*

*Experiment 1.* Mice received intraperitoneal injection of saline, Nam, $NAD^+$ or NADH, and 24-h urine samples were collected to investigate their metabolic fates. The results are shown in Fig. 3. Day 0 means the urine sample of the day before administration and day 1 indicates the day of administration. When saline only was administered to mice, daily excretions into the urine of Nam and its metabolites, such as Nam *N*-oxide, MNA, 2-Py, and 4-Py, were almost constant from day 0 to day 4 (Fig. 3A–E). Injection of Nam, $NAD^+$ and NADH produced significant increases in urinary excretions of Nam (Fig. 3A), Nam *N*-oxide (Fig. 3B), MNA (Fig. 3C), 2-Py (Fig. 3D), and 4-Py (Fig. 3E). The increment was seen in the 24-h urine samples after injection, but their metabolite excreta into the urine samples from day 2 to 4 were similar to those in saline-injected mice. Likewise, the sums of Nam, Nam *N*-oxide, MNA, 2-Py and 4-Py excreted into the 24-h urine samples just after the injection of Nam, $NAD^+$ or NADH were significantly higher than those in saline-injected mice (Fig. 3F). It is noted that NADH injected intraperitoneally is nearly equivalent to that of Nam or $NAD^+$ with respect to increases in urinary excretion of Nam and its metabolites. These results suggest that the intraperitoneally injected NADH is efficiently converted to $NAD^+$, which is then deglycosidated into Nam and ADP-ribose in cells. Then Nam would take its normal metabolic pathway including urinary excretion.

*Experiment 2.* Saline, Nam, $NAD^+$ or NADH was orally administered to mice, and 24-h urine samples were collected to investigate their metabolic fates. The results are shown in Fig. 4. The administration of Nam and $NAD^+$ produced significant increases in Nam (Fig. 4A), Nam *N*-oxide (Fig. 4B), MNA (Fig. 4C), 2-Py (Fig. 4D) and 4-Py (Fig. 4E). The sums of Nam, Nam *N*-oxide, MNA, 2-Py and 4-Py excreted into urine were significantly increased (Fig. 4F) by administration of Nam or $NAD^+$ as compared with that of saline. These increases

were seen in the urine samples collected on day 1 but thereafter the urinary levels of metabolites returned to those of controls, which are similar to when Nam or $NAD^+$ was given intraperitoneally. In contrast, oral administration of NADH did not produce any increases in Nam or its metabolites.

## DISCUSSION

This study was undertaken to investigate metabolic fate of NADH, because this compound has been tested as a pharmacological agent to ameliorate some symptoms including Alzheimer's disease (*17*), chronic fatigue syndrome (*11*, *12*), and jet lag (*10*). Primarily, in the patient with PD to which NADH was intravenously administered, a beneficial clinical effect was observed (*18*); therefore, it was investigated whether orally given NADH has a similar effect (*19*, *20*). Afterwards, the safety of the stabilized orally absorbable form of NADH tablet was tested in the rat (*21*) and the dog (*22*). Rainer et al. (*23*) reported that they found no evidence for any cognitive effect by oral NADH in dementia. NADH might also be used as a dietary supplement, but it is important to understand the fate of orally given NADH.

NADH is unstable in acidic conditions, while $NAD^+$ is stable (*4*). Gross and Henderson (*1*) revealed that $NAD^+$ is efficiently digested in the small intestinal tract, producing Nam that is transported into the blood and distributed to various tissues. Nam in the circulation appears to take two metabolic pathways depending on the cellular conditions; Nam is reused for biosynthesis of $NAD^+$ and NADH or it gives rise to some downstream metabolites whose physiological functions are not known. Excess Nam and its metabolites are also excreted into the urine. In agreement with this notion, $NAD^+$ given orally increased urinary excretion of Nam (Fig. 4) and its metabolites in a manner similar to that found when $NAD^+$ was intraperitoneally administered (Fig. 3). $NAD^+$-induced elevation of the urinary excretion was also similar to that caused by oral (Fig. 4) or intraperitoneal (Fig. 3) administration of Nam.

NADH appears to be almost equivalent to Nam and $NAD^+$ when it was given intraperitoneally (Fig. 3) but the fate of orally administrated NADH (Fig. 4) is entirely different; oral administration of NADH showed little effect on the urinary excretion of Nam and its metabolites (Fig. 4). These results present three possibilities that orally administered NADH a) may not be oxidized to $NAD^+$, b) may not be absorbed by the mouse gastrointestinal system, or c) may have been converted to a compound(s) before absorption that cannot yield Nam. Incubation of NADH under acidic conditions similar to gastric juice has made the third possibility very likely; treatment only for 1 min caused degradation of NADH and after 40 min most of the NADH was converted into an unknown product (Fig. 2). Although degradation of NADH in the gastric juice may be more complex, structural analyses of this product is important, because oral NADH-induced improvement of some symptoms might be attributable to this compound. Thus at the present time the fate of NADH orally given is poorly understood and therefore recommendation of its use as a therapeutic or supplement appears to be premature.

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# Comparison of the Effects of Di(2-ethylhexyl)phthalate, a Peroxisome Proliferator, on the Vitamin Metabolism Involved in the Energy Formation in Rats Fed with a Casein or Gluten Diet

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**In order to find an alleviation method for the adverse effect of environmental endocrine disrupters, we studied the effects of the putative endocrine disrupter and peroxisome proliferator, di(2-ethylhexyl)phthalate (DEHP), on animal growth and vitamin metabolism. It is known that the effects of chemical compounds such as xenobiotics differ according to the dietary protein source. We compared the effects of dietary DEHP administration on rats fed with a diet containing milk casein or wheat gluten. The increased conversion ratio of tryptophan to nicotinamide by DEHP administration was significantly higher in the casein group than in the gluten group. We also investigated the effects of DEHP on the urinary excretion of other vitamins. DEHP administration resulted in decreased urinary excretion of vitamin $B_1$, vitamin $B_2$, and pantothenic acid.**

**Key words:** nicotinamide; tryptophan; di(2-ethylhexyl)-phthalate (DEHP); dietary protein

Phthalic acid esters, which are known to cause malformation of the mice fetus,[1–3] are used in a variety of industrial applications.[1–3] They are constituents of such diverse products as paint, adhesive, cosmetics and polyvinyl chloride plastic.[2,3] These esters are widely distributed throughout the environment and have been detected in animals and humans.[4] We have already reported that the administration of phthalic acid the esters such as di-*n*-butylphthalate[5] and di(2-ethylhexyl)phthalate (DEHP)[6–10] disturbed the *de novo* nicotinamide (Nam) synthesis from tryptophan (Trp).

Handler and Dann[11] and Shibata and Tanaka[12] have reported that an intake of excess Nam retarded the growth of young rats. We have proposed that part of the toxicity of phthalic acid esters was attributable to excess Nam formation.[5,6] The conversion ratio of Trp to Nam varies according to the amino acid composition of dietary proteins.[13] Furthermore, there are some reports that dietary grain proteins alleviated the adverse effect of a toxin[14,15] and reduced the toxicity with an excessive intake of nutrients[16] compared to dietary milk casein. In the present experiment, we report a comparison of the effects of phthalic acid esters on the vitamin metabolism in rats fed with a casein or gluten diet.

## Materials and Methods

*Chemicals.* Vitamin-free milk casein, wheat gluten, DEHP, sucrose, L-methionine, L-lysine, L-threonine, anthranilic acid, nicotinic acid, thiamin hydrochloride, riboflavin, calcium pantothenate, Nam and quinolinic acid (QA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Kynurenic acid (KA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HA) and $N^1$-methylnicotinamide (MNA) chloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). $N^1$-Methyl-2-pyridone-5-carboxamide (2-Py) and $N^1$-methyl-4-pyridone-3-carboxamide (4-Py) were respectively synthesized by the methods of Pullman and Colowick[17] and Shibata *et al.*[18] Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized cornstarch, and the mineral (AIN-93-G-MX)[19] and Nam-free vitamin (AIN-93-VX)[19] mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan), all the other chemicals used being of the highest purity available from commercial sources.

*Animal and diets.* The care and treatment of the experimental animals conformed with The University of

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**Table 1.** Composition of the Diets

| | 20% Gluten diet*1 (%) | | 20% Casein diet*2 (%) | |
|---|---|---|---|---|
| | Control | Test 0.5% DEHP | Control | Test 0.5% DEHP |
| Gluten | 20 | 20 | 0 | 0 |
| Casein | 0 | 0 | 20 | 20 |
| L-Lysine | 0.76 | 0.76 | 0 | 0 |
| L-Threonine | 0.25 | 0.25 | 0 | 0 |
| L-Methionine | 0 | 0 | 0.2 | 0.2 |
| Gelatinized cornstarch | 46.83 | 46.33 | 46.9 | 46.4 |
| Sucrose | 22.66 | 22.66 | 23.4 | 23.4 |
| Corn oil | 5 | 5 | 5 | 5 |
| Mineral mixture (AIN-93G-MX) | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mixture (AIN-93-VX) (nicotinic acid-free) | 1 | 1 | 1 | 1 |
| DEHP | 0 | 0.5 | 0 | 0.5 |

*1The amino acid contents (total content was 18,293 mg) of 100 g of the diet were 606.2 mg of isoleucine, 1,086.2 mg of leucine, 1,797.8 mg of lysine, 252.6 mg of methionine, 328.4 mg of cysteine, 808.4 mg of phenylalanine, 505.2 mg of tyrosine, 904.2 mg of threonine, 156.6 mg of tryptophan, 656.8 mg of valine, 353.6 mg of histidine, 555.6 mg of arginine, 404.2 mg of alanine, 555.6 mg of aspartic acid, 5,810.4 mg of glutamic acid, 530.4 mg of glycine, 2,273.6 mg of proline, and 707.2 mg of serine.

*2The amino acid contents (total content was 19,194.6 mg) of 100 g of the diet were 972.6 mg of isoleucine, 1,675.2 mg of leucine, 1,432.0 mg of lysine, 940.4 mg of methionine, 86.40 mg of cysteine, 918.6 mg of phenylalanine, 999.8 mg of tyrosine, 729.4 mg of threonine, 226.8 mg of tryptophan, 1,188.8 mg of valine, 540.4 mg of histidine, 648.4 mg of arginine, 540.4 mg of alanine, 1,243.0 mg of aspartic acid, 3,783.0 mg of glutamic acid, 324.2 mg of glycine, 2,026.6 mg of proline, and 918.6 mg of serine.

Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

Male rats of the Wistar strain (6 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; Clea Japan).

The rats were fed *ad libitum* for 21 days with a Nam-free casein or gluten diet with or without 0.5% DEHP. The composition of each diet is shown in Table 1. The diets used did not contain the preformed vitamin, niacin (Nam and nicotinic acid), so that Nam and such metabolites as MNA, 2-Py and 4-Py originated from Trp. Mammals such as rats and humans cannot produce nicotinic acid from Trp.[20]

The room temperature was maintained at around 20 °C and about 60% humidity, and a 12 h light/12 h dark cycle was maintained. The body weight and food intake were measured daily at around 10:00 a.m. Urine samples (24 h; 10:00 a.m.–10:00 a.m.) on the last day were collected in amber bottles containing 1 ml of 1 mol/l of HCl, and were stored at −25 °C until needed. The rats were killed by decapitation at around 10:00 a.m. on the last day of the experiment.

*Analyses.* The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the HPLC method of Shibata *et al.*,[18] while the content of MNA in the urine was measured by the HPLC method of Shibata.[21]

The contents of KA,[22] XA,[23] 3-HA,[24] AnA,[24] QA,[25] thiamin,[26] and riboflavin[27] in the urine were measured by the HPLC method. The urine content of pantothenic acid was measured by a microbiological method.[28]

## Results

*Effects of DEHP administration on the body weight gain, food intake, and liver weight of the rats fed with the gluten and casein diets*

We have previously reported that an adverse effect of DEHP on rats fed on a casein diet was observed with 1% addition to the casein diet but not with up to a 0.5% addition.[6] As expected, the body weight gain and food intake of all groups (20% casein, 20% casein + 0.5% DEHP, 20% gluten, and 20% gluten + 0.5% DEHP diets) were almost the same as shown in Fig. 1. The characteristic phenomenon that the administration of DEHP increased the liver weight has been reported.[6] Enlargement of the liver by the administration of DEHP was also observed in the present experiment (Table 2). The degree of enlargement of the liver was almost the same between the casein and gluten groups.

*Comparison of the effect of DEHP on the metabolism of Trp to Nam between the rats fed with the gluten and casein diets*

The DEHP intake had no significant effects on the Trp to 3-HA metabolism when comparing the urinary excretion. However, the urinary excretion of KA was increased and that of XA decreased by the DEHP intake (Table 2).

We have previously reported that a target for the disturbance of Trp metabolism was the reaction of α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) → α-aminomuconate-ε-semialdehyde (AMS),[10] which resulted in the increased formation of QA. As shown in Table 2, the QA formation was significantly increased by the DEHP intake in the experiments with both the

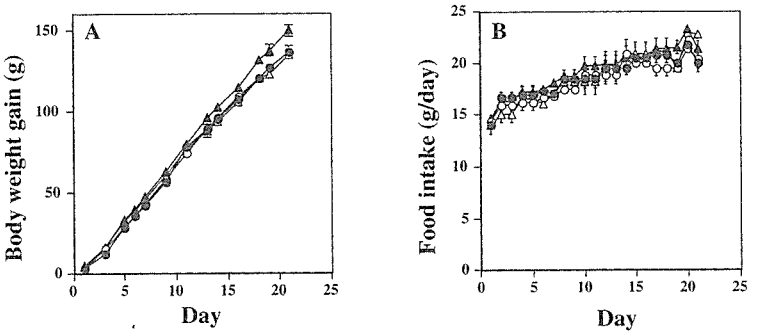


**Fig. 1.** Effect of DEHP on the Body Weight Gain (A) and Food Intake (B) of Rats Fed with the Gluten or Casein Diet.
Young rats of the 6 weeks olds were fed on respective diet for 21 days. Each point is the mean ± SEM for 5 rats. ●, Gluten diet; ○, gluten diet + 0.5% DEHP; ▲, casein diet; △, casein diet + 0.5% DEHP.

**Table 2.** Effect of DEHP on the Liver Weight and Urinary Excretion of Metabolites on the Trp-Niacin Pathway

| | 20% Gluten diet (%) | | 20% Casein diet (%) | |
|---|---|---|---|---|
| | Control | Test 0.5% DEHP | Control | Test 0.5% DEHP |
| Liver weight | | | | |
| (g/rat) | 11.5 ± 0.4[a] | 15.5 ± 0.4[b] | 11.3 ± 0.3[a] | 16.1 ± 0.8[b] |
| (g/100 g b.w.) | 4.0 ± 0.1[a] | 5.9 ± 0.2[b] | 4.0 ± 0.2[a] | 6.1 ± 0.2[b] |
| Urinary excretion (mmol/mol Trp intake) | | | | |
| AnA | 0.49 ± 0.03 | 0.57 ± 0.03 | 0.44 ± 0.02 | 0.42 ± 0.03 |
| KA | 5.62 ± 0.31[a] | 7.95 ± 0.90[b] | 5.36 ± 0.20[a] | 10.52 ± 0.87[b] |
| XA | 4.07 ± 0.35 | 2.68 ± 0.37 | 4.06 ± 0.04 | 2.92 ± 2.1 |
| 3-HA | 0.34 ± 0.05 | 0.28 ± 0.2 | 0.28 ± 0.04 | 0.25 ± 0.03 |
| QA | 1.80 ± 0.34[a] | 12.26 ± 1.33[c] | 2.50 ± 0.16[a] | 25.28 ± 2.80[b] |
| Nam | N.D.* | 4.41 ± 0.97[b] | N.D.* | 7.57 ± 0.25[a] |
| MNA | 1.14 ± 0.24[a] | 12.14 ± 1.29[c] | 1.08 ± 0.18[a] | 62.95 ± 8.41[b] |
| 2-Py | 0.57 ± 0.16[a] | 5.49 ± 1.09[c] | 0.68 ± 0.09[a] | 19.53 ± 2.60[b] |
| 4-Py | 9.00 ± 1.35[a] | 34.87 ± 4.11[b] | 10.53 ± 1.08[a] | 39.39 ± 3.74[b] |

Each value is expressed as the mean ± SEM (n = 5); a different superscript letter in the same row means significantly different at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test.
*N.D., not detected

gluten and casein diets, although the effect of DEHP was significantly lower with the gluten diet than with the casein diet. The subsequent metabolites beyond QA were also increased by the DEHP intake with both the gluten and casein diets. Figure 2 shows the conversion ratio of Trp to Nam. The values were increased by the DEHP intake in both groups. In the present study, Nam and its catabolites such as MNA, 2-Py, and 4-Py were synthesized only from Trp, because the diets do not contain any Nam. The conversion ratio of Trp to Nam was calculated by the following equation: sum of the urinary excretion of Nam, MNA, 2-Py, and 4-Py (μmol/day)/Trp intake during urine collection (μmol/day) × 100. The conversion ratio with the casein and gluten diets when rats were not given DEHP were not statistically different (Fig. 2), but the administration of DEHP caused a significant difference in the conversion ratio of Trp to Nam between the gluten and casein diets.

*Effects of DEHP on the urinary excretion of thiamin, riboflavin, and pantothenic acid in the rats fed with the gluten and casein diets*

Treatment of rats with DEHP increases the induction of several metabolic enzymes, including those involved in peroxisomal β-oxidation.[29] We therefore compared the effects of DEHP administration on the vitamins involved in the β-oxidation pathway such as riboflavin and pantothenic acid in the rats fed with the diets of gluten and casein.

The urinary excretion of both riboflavin and pantothenic acid was decreased by the administration of DEHP with both protein diets as shown in Figs. 3 and 4, the effect with the gluten diet being more than that with the casein diet.

Thiamin is not required in fatty acid metabolism, but is in glucose metabolism. Thus, the effect of DEHP administration on the urinary excretion of thiamin was also investigated. As shown in Fig. 5, the urinary

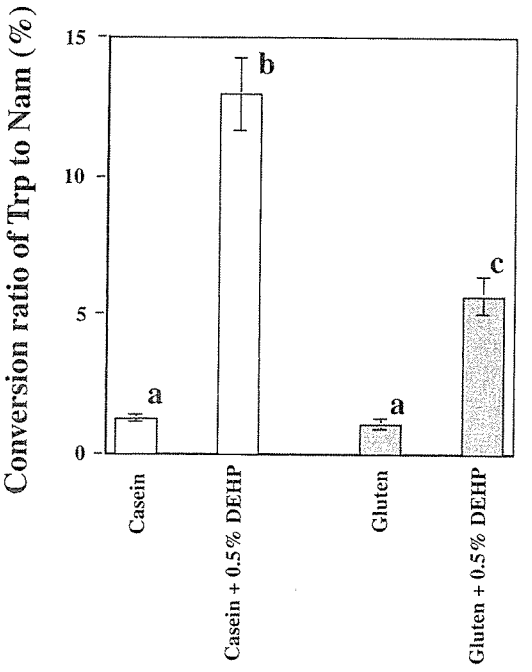


**Fig. 2.** Effect of DEHP on the Conversion Ratio of Trp to Nam in Rats Fed with the Gluten or Casein Diet.

Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column, casein diet group; hatched column, gluten diet group.

excretion of thiamin was lower in the DEHP group than in the non-DEHP group with both protein diets, the effect on the casein group being more severe than that on the gluten group.

## Discussion

We have already reported that the administration of DEHP significantly increased the formation of Nam from Trp by inhibiting the activity of ACMSD[10] and that feeding a casein diet containing over 1% DEHP retarded the growth of young rats.[6] Handler and Dann[11] and Shibata and Tanaka[12] have reported that the intake of excess Nam retarded the growth of young rats. It is therefore considered that part of the toxicity of phthalic acid esters is attributable to excess Nam formation. In a previous report,[5] we stated that the degree of conversion of Trp to Nam differed according to the dietary casein level; the increased conversion was significantly lower in the group fed with the 10% casein diet than in the group fed with the 20% casein diet when the diets contained a phthalic acid ester. Shibata[13] has clarified that the conversion ratio varied according to the amino acid composition of dietary proteins. Treating the rats with DEHP increased the induction of several metabolic enzymes, including those involved in peroxisomal $\beta$-oxidation.[29] Furthermore, some reports have revealed that dietary grain protein alleviated the adverse effect of a toxin.[14,15] We therefore compared the effect of DEHP on the vitamin metabolism involved in energy formation between the casein and gluten diets.

In the present experiments, the limiting amino acids were appropriately supplemented to the two diets to maintain an equal growth rate (Table 1). However, the effects of DEHP on the metabolism of Trp to Nam differed between the groups fed with the gluten and casein diets (Fig. 2 and Table 2). The difference in the effect of DEHP would have been due to the reaction of ACMS → AMS. This reaction is catalyzed by ACMSD, although the liver ACMSD activity was almost the same between the groups fed with the gluten and casein diets (data not shown). We have reported that the mono-ethylhexyl phthalic acid ester was an inhibitor of ACMSD.[10] It is known that the enzyme activities[16,29] and gene expression[30–33] are affected by the kind of dietary protein. Therefore, the enzyme activity catalyzing the reaction of DEHP → mono(2-ethylhexyl)phthalate and/or its mRNA level might be expected to differ between the gluten and casein diets. It is known that the amino acid score is higher in casein than in gluten. In the present experiment, the limiting amino acids were added to the gluten diet to give the same body weight gain between the two dietary groups (Fig. 1). The effect of DEHP on the metabolism of Trp to Nam (Fig. 2 and Table 2), riboflavin (Fig. 3), pantothenic acid (Fig. 4), and thiamin (Fig. 5) was significantly different between the gluten and casein diets. A decreased urinary excretion of riboflavin, pantothenic acid, and thiamin generally means an increased requirement of these vitamins in the body when the intake of these vitamins is the same. Additionally, increasing the catabolism of these vitamins by enhancing the drug metabolizing system could be considered as the reason for decreased urinary excretion of these vitamins by the DEHP intake. 7$\alpha$-Hydroxyriboflavin and 8$\alpha$-hydroxyriboflavin have been reported as catabolic metabolites of riboflavin,[27] although we were not able to confirm the peaks that corresponded to 7$\alpha$-hydroxyriboflavin and 8$\alpha$-hydroxy-riboflavin in the HPLC data. Hydroxylated compounds of pantothenic acid and thiamin have not been reported, so the main reason for the decreased amounts of these vitamins would be attributable to accentuation of the $\beta$-oxidation pathway. In other words, the requirement for riboflavin, pantothenic acid, and thiamin might be increased when $\beta$-oxidation pathway is accentuated by the DEHP intake. It could be expected that the necessity for Nam would rise as well and that the urinary excretion of Nam and its metabolites would decrease with accentuation of the $\beta$-oxidation pathway when the intake of Nam was the same and the conversion ratio of Trp to Nam was also the same. However, the administration of DEHP significantly increased the formation of QA by inhibiting the ACMSD activity. This increased formation resulted in metabolites beyond QA such as Nam, MNA, 2-Py, and 4-Py. Therefore, the increased urinary excretion of Nam and its metabolites did not

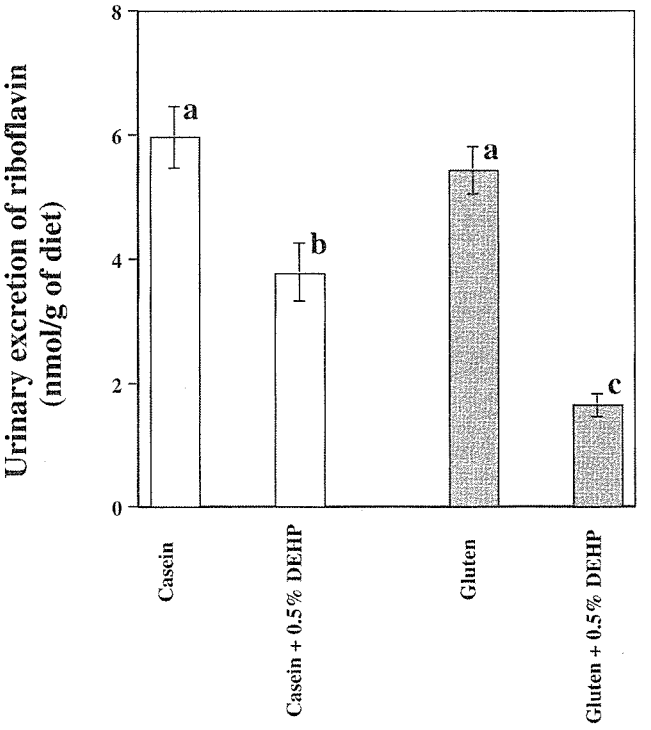


**Fig. 3.** Effect of DEHP on the Urinary Excretion of Riboflavin in Rats Fed with the Gluten or Casein Diet.
Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column, casein diet group; hatched column, gluten diet group.

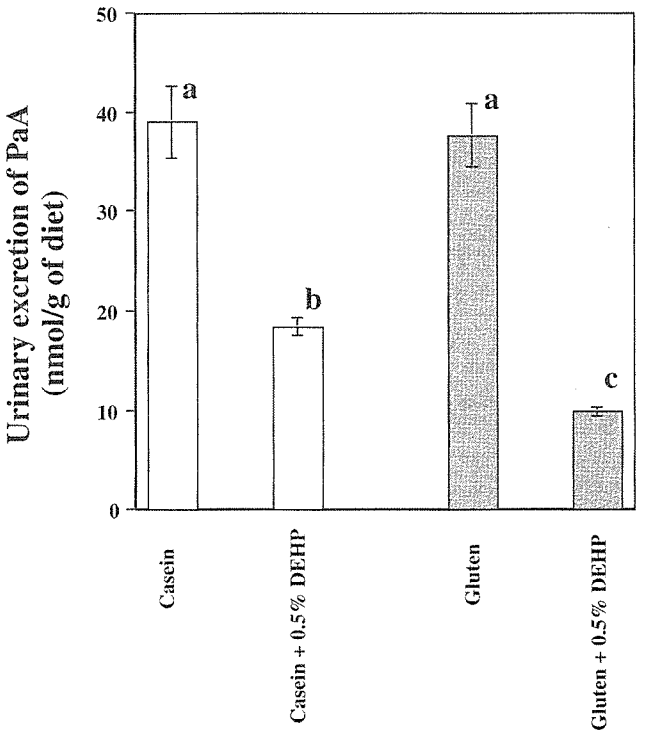


**Fig. 4.** Effect of DEHP on the Urinary Excretion of Pantothenic Acid (PaA) in Rats Fed with the Gluten or Casein Diet.
Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column, casein diet group; hatched column, gluten diet group.

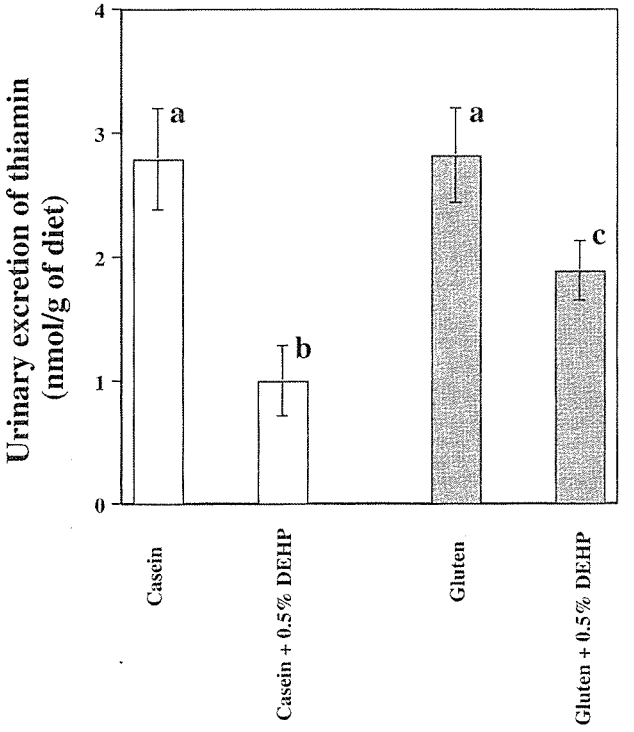


**Fig. 5.** Effect of DEHP on the Urinary Excretion of Thiamin in Rats Fed with the Gluten or Casein Diet.
Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column, casein diet group; hatched column, gluten diet group.

mean that the DEHP administration resulted in a lower need for Nam, because the conversion ratio of Trp to Nam was significantly increased (Fig. 2). It is likely that the need for Nam was increased by the DEHP administration. Therefore, when the toxicity of DEHP is being discussed, it is necessary to include the increased requirement of such vitamins as thiamin, riboflavin, and pantothenic acid. We therefore recommend when DEHP is being administered that it is necessary to increase the dietary intake of vitamin $B_1$, vitamin $B_2$, and pantothenic acid.

## Acknowledgments

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報　文

# 健常成人における葉酸の必要量についての検討

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（2004年11月8日受付；2006年1月14日受理）

**要旨**：第六次改定日本人の栄養所要量―食事摂取基準―において，葉酸の所要量は食事から摂取可能な量として，成人で200 μg/日と策定された。また日本人の食事摂取基準（2005年版）では240 μg/日と改定された。しかしながら，この根拠となるわが国での科学的エビデンスは必ずしも十分でないので，私たち自身の新しいデータの蓄積と解析が求められている。そこで，健常な成人男女を対象にして，所要量に見合った水溶性ビタミンをサプリメントとして与え，葉酸の必要量を検討した。成人女性では，血清および尿中葉酸レベルが増加した。一方成人男性では血清葉酸の減少がみられたが，尿中葉酸排泄量は変化しなかった。このように，葉酸の必要量に性差がみられ，今回使用した葉酸量（200 μg/日）は，男性では不足している可能性があるが，女性では必要量を十分に満たしているものと考えられる。

**キーワード**：葉酸，食事摂取基準，尿，血清，必要量

葉酸は，N-ヘテロ環のプテリジンと*p*-アミノ安息香酸からなるプテロイル基，つまり4-[((2-アミノ-4(3H)-オキソプテリジン-6-イル）メチル）アミノ］安息香酸に1-7個のL-グルタミン酸が結合したプテロイル（ポリ）グルタミン酸である。食品に含まれるのは，プテロイルポリグルタミン酸型であり，小腸粘膜でモノグルタミン酸型となり，吸収される[1)2)]。血漿や尿中では，モノグルタミン酸型，組織中ではポリグルタミン酸型としてタンパク質と結合した形で機能している。なお，小腸粘膜では，プテリジン環が還元されてテトラヒドロ型となり，さらにメチルテトラヒドロ型となる。還元型葉酸は，細胞内では補酵素として，ヌクレオチド類の生合成やメチル基の生成転換系などに関与している。また，アミノ酸やタンパク質の代謝などにも不可欠であり，グリシン，セリン，メチオニンの代謝やビタミン$B_{12}$とともにホモシステインからメチオニンの生成などにも関与している。

葉酸の生理機能としては，正常な造血機能を保つために重要であるばかりでなく，成長や妊娠の維持にも欠かせないビタミンである。このため，欠乏症状としては，造血機能に異常が生じ，巨赤芽球性貧血や神経障害が知られている。最近，多くの疫学調査によって，葉酸が，胎児における神経管閉鎖障害の発症リスクの低減に効果があることが認められている[3-5)]。また，葉酸の摂取量が低下すると，血漿ホモシステインの上昇がみられ，動脈硬化症と関連がある血液凝固因子や血管内皮細胞に影響している[6-8)]。

葉酸の「所要量」は，わが国では食事から摂取可能な量として，成人で200 μg/日であるが，米国では400 μg/日となっている[9)10)]。なお，妊婦および授乳婦での付加量は，それぞれ200および80 μg/日となっている。葉酸の摂取量が，1日当たり320 μg以上であれば，血漿のホモシステインレベルを一定に保つことができる。このことから，米国では穀類に葉酸を140 μg/100 g添加するように勧告している。わが国でも，1日に350 gの野菜を摂取するように勧めている。また日本人の食事摂取基準（2005年版）では，葉酸の「推奨量」が240 μg/日に改定された[11)]。許容上限摂取量は1 mg/日となっているが，これ以上摂取すると葉酸過敏症（発熱や蕁麻疹など）を起こすことがある。

このように，葉酸の推奨量を策定するために，その根拠となる科学的エビデンスは必ずしも十分でなく，わが国での新しいデータの蓄積と解析が求められている。そこで，本研究では健常な成人男女を対象にして，所要量200 μg/日に見合った水溶性ビタミンをサプリメントとして与え，葉酸の出納を検討した。なお，葉酸が亜鉛の吸収を阻害するという報告があることから，血清亜鉛の測定も合わせて行った。

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## 方　　法

### 1. 対　　象

被験者として，喫煙，飲酒の習慣がなく，朝食を食べるなどの規則正しい食習慣をもつ大学生のうち，血液検査をうけ，健康であることが確認できた成人女性20名（20.6±1.11歳），成人男性10名（20.4±1.26歳）を選択した。血液検査においては，一般生化学的項目として，白血球，赤血球，ヘモグロビン，総タンパク，アルブミン，A/G，GOT，GPT，γ-GTP，クレアチニン，総脂質，総コレステロール，中性脂肪，HDL-コレステロール，LDL-コレステロールを調べ，本試験に問題のないことを確認している。なお，本研究は独立行政法人国立健康・栄養研究所倫理委員会において承認を受け，対象者に内容を十分説明し，理解していただいた上で書面にて同意を得て，ヘルシンキ宣言の精神に則って行われたものである。

### 2. 試験期間

葉酸の出納試験として，成人女性を対象としたもの2回，成人男性を対象としたもの1回の計3回行った。成人女性では2002年3月1日-2002年3月8日の期間と2003年3月4日-2003年3月11日の期間，成人男性では2002年8月27日-2002年9月3日の期間に実施した。それぞれ2002年女性，2003年女性および2002年男性とした。

### 3. 生活条件

被験者の快適な生活環境の確保等に留意した。大学生の一般的な日常生活をモデルとした通学，講義，実験，運動等の活動から構成された1日のスケジュールを作成した。被験者はこのスケジュールに従って試験期間中生活をした。

### 4. 食　　事

食事は，第六次改定日本人の栄養所要量の生活活動強度IIに従い，女性は1,800 kcal/日，男性は2,300 kcal/日を摂取した。食事の組成は表1に示したように，通常の食品を使用せずに，栄養素の含有量がはっきりしている半精製食品を利用した。ビタミンおよびミネラルの混合物の組成は表2および表3に示したとおりである。なお，2002年の結果から，2003年女性においては，小麦粉（薄力粉・1等）を除いたほか，ビタミン$B_1$，ビタミン$B_{12}$およびビタミンCの摂取量を所要量の1.5倍量とした。

葉酸摂取量は，ビタミン混合物から1日当たり200 μgであるが，小麦粉に葉酸化合物が9 μg/100 g（五訂

表1　半精製食品の組成

| 食材名 | 2002年女性 | 2002年男性 | 2003年女性 |
|---|---|---|---|
| 小麦粉（薄力粉・1等）(g) | 315 | 315 | — |
| カゼイン (g) | — | — | 39.5 |
| グルテン (g) | 37.3 | 56 | 25 |
| ダイズ油 (g) | 4.8 | 7 | 10.1 |
| なたね油 (g) | 16.8 | 21 | 13.8 |
| やし油 (g) | 7.1 | 8.5 | 6.2 |
| ラード (g) | 5.9 | 8.4 | 8.9 |
| グラニュー糖 (g) | 50 | 30 | 50 |
| コーンスターチ (g) | 33 | 118 | 274 |
| ファイバゾル[a] (g) | 0 | 0.8 | 3.6 |
| 麻セルロースパウダー[a] (g) | 10.1 | 14.3 | 14.4 |
| ビタミン混合[b,c] (g) | 1 | 1 | 1 |
| ミネラル混合[b,d] (g) | 12 | 14.6 | 18 |
| エネルギー量 (kcal) | 1800 | 2300 | 1800 |

—：不含，[a]水溶性食物繊維：不溶性食物繊維＝1：4，[b]所要量に従った（ミネラル，ビタミン），[c]表2参照，[d]表3参照（Se，Cr，Moは含まない）。

表2　ビタミン混合物の組成

| ビタミン名 | 所要量[a] | | 混合量 | | |
|---|---|---|---|---|---|
| | 18-29（女性） | 18-29（男性） | 2002年女性 | 2002年男性 | 2003年女性 |
| ビタミンA (μg) | 540 | 600 | 540 | 600 | 540 |
| ビタミンD (μg) | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| ビタミンE (mg) | 8 | 10 | 8 | 10 | 8 |
| ビタミンK (μg) | 55 | 65 | 55 | 65 | 55 |
| ビタミン$B_1$ (mg) | 0.8 | 1.1 | 0.8 | 1.1 | 1.2[c] |
| ビタミン$B_2$ (mg) | 1.0 | 1.2 | 1.0 | 1.2 | 1.0 |
| ビタミン$B_6$ (mg) | 1.2 | 1.6 | 1.2 | 1.6 | 1.2 |
| ビタミン$B_{12}$ (μg) | 2.4 | 2.4 | 2.4 | 2.4 | 3.6[c] |
| ニコチンアミド (mgNE[b]) | 13 | 17 | 13 | 17 | 11.2 |
| パントテン酸 (mg) | 5 | 5 | 5 | 5 | 5 |
| 葉酸 (μg) | 200 | 200 | 200 | 200 | 200 |
| ビオチン (μg) | 30 | 30 | 30 | 30 | 30 |
| ビタミンC (μg) | 100 | 100 | 100 | 100 | 150[c] |
| グラニュー糖 (mg) | 883 | 877 | 883 | 877 | 832 |

[a]第六次改定日本人の栄養所要量，[b]NE：ナイアシン当量，[c]所要量の1.5倍。

表 3 ミネラル混合物の組成[a]

| 化合物 | 女性 | | | 男性 | | |
|---|---|---|---|---|---|---|
| | 混合量 | 含有量 | | 混合量 | 含有量 | |
| $CaHPO_4$-$2H_2O$ (mg) | 1100 | Ca (mg) | 253 | 1200 | Ca (mg) | 280 |
| | | P (mg) | 198 | | P (mg) | 216 |
| $CaCO_3$ (mg) | 900 | Ca (mg) | 360 | 1049 | Ca (mg) | 420 |
| $K_2PO_4$ (mg) | 2200 | K (mg) | 631 | 2124 | K (mg) | 610 |
| $KHCO_3$ (mg) | 3500 | P (mg) | 501 | | P (mg) | 484 |
| | | K (mg) | 1365 | 3558 | K (mg) | 1390 |
| $MgCl_2$-$6H_2O$ (mg) | 2083 | Mg (mg) | 250 | 2594 | Mg (mg) | 310 |
| $FeSO_4$-$7H_2O$ (mg) | 60 | Fe (mg) | 12 | 49.8 | Fe (mg) | 10 |
| $MnSO_4$-$4H_2O$ (mg) | 12.2 | Mn (mg) | 3 | 17.6 | Mn (mg) | 4 |
| $ZnCl_2$ (mg) | 19 | Zn (mg) | 9.1 | 22.9 | Zn (mg) | 11 |
| $CuSO_4$-$5H_2O$ (mg) | 6.3 | Cu (mg) | 1.6 | 7.1 | Cu (mg) | 1.8 |
| KI (mg) | 0.2 | I (mg) | 0.153 | 0.2 | I (mg) | 0.153 |
| NaCl (mg) | 2120 | Na (mg) | 833 | 4000 | Na (mg) | 1575 |
| 総重量 (g) | 12 | | | 14.6 | | |

[a] Se, Cr, Mo：不含。

日本食品標準成分表）含まれていることを考慮すると，2002年男女の葉酸摂取量は1日当たり228 μg/日となる。また，葉酸摂取量を考える場合には，食事性葉酸と比較して，葉酸サプリメントの体内利用率を1.7倍として，換算する必要がある。生体利用率を考慮して食事性葉酸当量（Dietary folate equivalents：DFEs）として考えると，今回の葉酸摂取量は2002年男女で368 μg DFEs/日，2003年女性で340 μg DFEs/日となる[10]。

ビタミン混合剤は，食事終了後，水で服用させた。給食比率は朝：昼：夕＝3：4：3とした。水分は市販ペットボトル詰め軟水とし，自由に摂取させた。平均的な1日摂取量は2 Lである。

**5. 試料の採取方法**

**5.1 尿** 尿は，第2尿（2002年男性は6時31分以降，2002年女性および2003年女性は7時01分以降）から翌朝第1尿（2002年男性は6時30分に排尿，2002年女性および2003年女性は7時00分に排尿）までの24時間尿を採取した。2002年女性は試験6日目のみ，2003年女性と2002年男性は試験1日目から7日目まで毎日採尿を行った。なお，2002年男性の7日目は，朝6時30分から翌日の8時30分までの間，飲料水をできるだけ多く摂取させ，6回（6：30-8：30，8：30-12：30，12：30-17：30，17：30-22：30，22：30-6：30，6：30-8：30）に分けて採尿した。尿は，採尿中は冷蔵し，採尿後は−4°Cで氷結保存した。

**5.2 血液** 2002年女性は試験1，3，8日目の朝食摂取前9時00分に，2003年女性は試験1，3，5，8日目の朝食前9時00分に，2002年男性は試験1，3，5，8日目の朝食摂取前8時30分に採血を行った。採取後は遠心し，血清を分離した。血清は，−40°Cで分析直前まで氷結保存した。

**6. 葉酸の測定**

血清および尿中の葉酸は，化学発光免疫測定法で分析した。測定機器としては，Centaur ケミルミアナライザーACS180およびIMULIZE 2000を使用した。

サンプルをジチオスレイトールで処理し，タンパクから遊離させた後，分析に供した。葉酸の分析には，葉酸測定キット（DPC・イムライズ葉酸-LKF01）を使用した。本法の最小検出感度は0.5 ng/mL（1.1 pmol/mL）であり，プール血清で測定した葉酸値は5.77 ng/mL（13.1 pmol/mL）であった。尿中の葉酸については，正常参考値となるような参考資料などがないので，血清と同じ測定条件で行った。

**7. 亜鉛の測定**

血清250 μLに6.25%トリクロロ酢酸を2.0 mL加え，混和した後，室温で10分間放置した。その後，3,000 rpmで10分間遠心をかけ，上清1 mLをとり，亜鉛を原子吸光光度計で測定した。

**8. 統計処理**

実験のデータについては，各群の分析値を平均値±標準偏差（means±SD）で表した。データの統計処理には，統計ソフトはStatView (SAS Institutes Inc., Cary) Ver. 5.0およびエクセル統計（オーエムエス出版，所沢）を使用した。同性および異性間の比較は，二元配置分散分析（Two-way repeated ANOVA）で，それ以外は一元配置分散分析（One-factor ANOVA）で比較した。各分散分析後，有意差をFisher's PLSD (Protected least significance test) にて検定した。いずれの場合にも，有意水準は，危険率5%未満を有意とし，5%未満（$^{**}p<0.05$）と1%未満（$^{***}p<0.01$）に分けて表示した。

## 結　　果

血清葉酸量を比較したものが図1である。2002年女性では，試験期間を通して平均11.2-13.5 ng/mL（25.4-30.5 pmol/mL）の範囲にあり，変化は認められなかった。しかし，2003年女性においては，平均9.2-13.5 ng/mL（20.8-30.5 pmol/mL）の範囲にあり，経日的に増加する傾向がみられた。試験1日目と比べ，すべての日で増加がみられ，3日目と8日目では有意であった。2002年女性と2003年女性との間には有意な差はみられなかった。一方，2002年男性では，血清葉酸量は平均6.9-9.8 ng/mL（15.6-22.2 pmol/mL）の範囲にあり，試験3日目以降は減少傾向にあり，試験8日目では有意に減少した。なお，2002年男性と女性との間には，差異がみられた。

図2は，1日当たりの尿中葉酸排泄量およびクレアチニンで補正した排泄量を示したものである。2003年女性では，尿中葉酸排泄量に有意な増加が認められ，クレアチニン補正値でみても，経日的に増加する傾向がみられた（図2b）。しかし，2002年男性ではほとんど変化がみられなかった。

摂取量200 μgとして尿中葉酸排泄率をみると，2002年男性で平均4.5±0.42%，2003年女性で平均4.6±0.47%，2002年女性は5.0%（試験6日目）であった。2003年女性では経日的に有意な変化がみられ，試験1日目と比べ，試験6日目で有意な増加がみられた（図3）。しかし，2002年男性では変化は認められなかった。また2002年男性と2003年女性の間には，差異はみられなかった。

図4aは，2002年男性における尿中葉酸排泄量の日内変動を示したものである。1日をとおして大きな変化がみられ，午前中に高い傾向を示したが，これ以外ではあまり変化は観察されなかった。また，クレアチニン補正した場合にも，日内変動を示し，午前中に高いピークを示した（図4b）。

試験期間中の血清亜鉛の変化を示したものが表4である。各群とも経日的な変化はみられなかった。しかし，2003年女性では，2002年男性と比較して，有意な差異

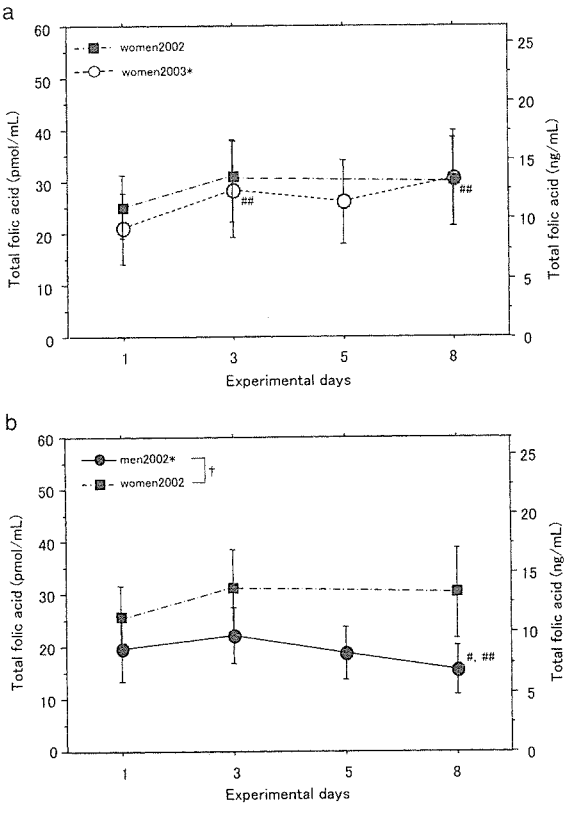


**図1**　半精製食品を摂取した成人の血清葉酸量の変化
(a) 血清葉酸濃度（2002年女性と2003年女性）。$^{*}p=0.083$, $^{\#\#}p<0.05$（試験1日目との比較）。(b) 血清葉酸濃度（2002年男性と2002年女性）。$^{*}p=0.080$, $^{\dagger}p<0.01$, $^{\#}p<0.1$, $^{\#\#}p<0.05$（それぞれ試験1日目および試験3日目との比較）。

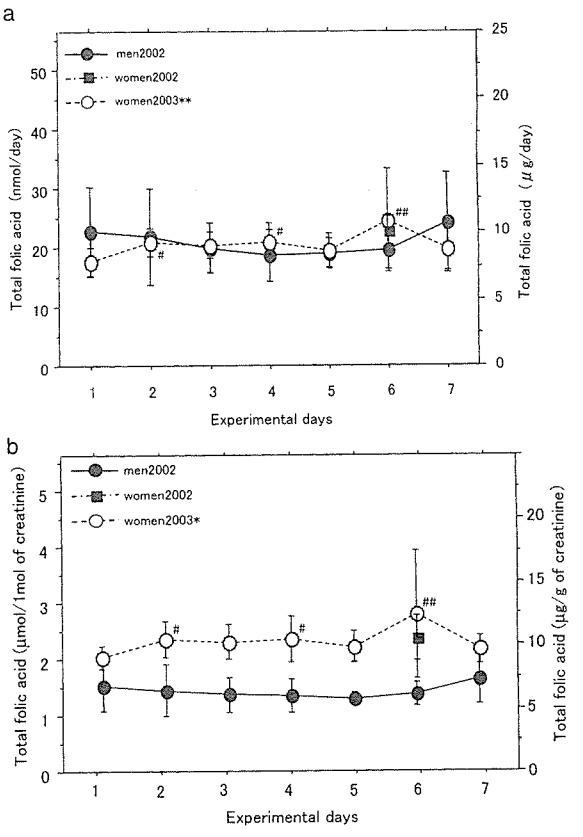


**図2**　半精製食品を摂取した成人の葉酸排泄量の変化
(a) 尿中葉酸（1日当たり）の排泄量。$^{**}p=0.029$, $^{\#}p<0.1$, $^{\#\#}p<0.05$（試験1日目との比較）。(b) 尿中葉酸濃度（クレアチニン補正）。$^{*}p=0.059$, $^{\#}p<0.1$, $^{\#\#}p<0.05$（試験1日目との比較）。

表 4　血清亜鉛

| 試験期間（日） | 血清亜鉛濃度 | | | | | |
|---|---|---|---|---|---|---|
| | 2002 年女性 | | 2002 年男性 | | 2003 年女性 | |
| | nmol/mL | μg/mL | nmol/mL | μg/mL | nmol/mL | μg/mL |
| 1 | 13.1±2.2 | 0.9±0.1 | 13.6±1.4 | 0.9±0.1 | 12.6±1.7 | 0.8±0.1 |
| 3 | 12.9±1.7 | 0.8±0.1 | 13.8±1.6 | 0.9±0.1 | 12.0±1.2 | 0.8±0.1 |
| 5 | — | — | 13.4±1.3 | 0.9±0.1 | 11.7±1.4 | 0.8±0.1 |
| 8 | 12.9±1.5 | 0.8±0.1 | 13.2±1.7 | 0.9±0.1 | 11.4±1.4 | 0.7±0.1 |

—：nd, † $p=0.003$.

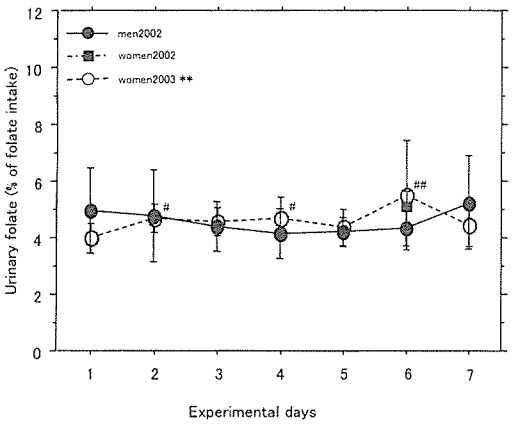


図 3　半精製食品を摂取した成人の葉酸排泄率（摂取量 200 μg とした場合）
**$p=0.029$, # $p<0.1$, ## $p<0.05$（試験 1 日目との比較）。

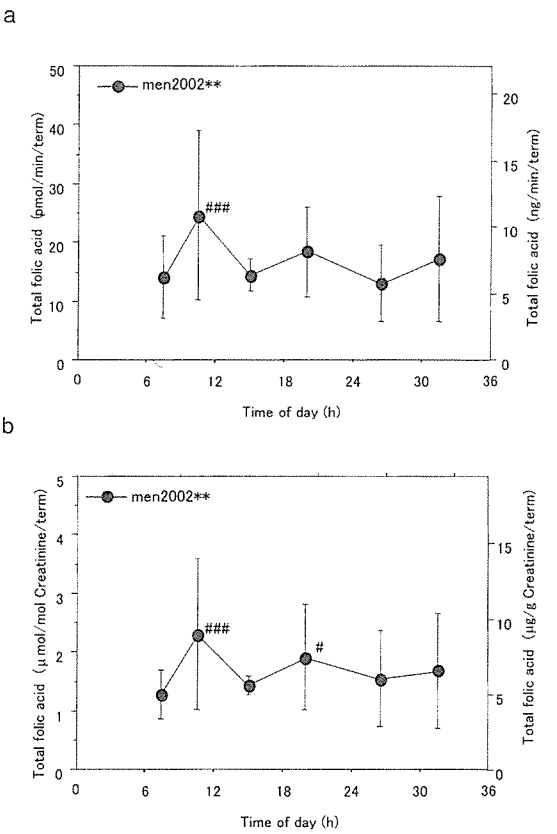


図 4　成人男性（2002 年）の尿中葉酸日内変動
(a) 尿中葉酸排泄量（単位時間当たり）の日内変動。**$p=0.020$, ### $p<0.01$（試験 1 日目との比較）。
(b) 尿中葉酸濃度（クレアチニン比）の日内変動。**$p=0.041$, # $p<0.1$, ### $p<0.01$（試験 1 日目との比較）。

がみられた。

## 考　　察

食事性葉酸の摂取量について，これまでの報告された栄養調査では，平岡・安田[12]は女子学生を対象として食物摂取状況調査から，葉酸摂取量は平均 190.6 μg/日で，所要量（Recommended Dietary Allowance：RDA）（200 μg/日）に対する充足率は 40.2%であると報告している。摂取量が 200 μg 以下と以上では，血清葉酸量に有意な差異が認められている。この結果は，オランダの DNFCS 調査（1992 年）の結果と一致している[13]。1-92 歳を対象とした食品分析結果から算出した葉酸摂取量は 189 μg/日である[14]。サプリメントを摂取している場合には 344 μg/日と高値である。なお，RDA（200 μg/日）に対する充足率は，男女それぞれ 42%および 54%である。また成人（20-65 歳）を対象にした調査では，食事からの葉酸摂取量は男性 232 μg/日，女性 186 μg/日と男女差がみられている。

著者らが行った東北地区における中高齢者 120 名の食事記録調査では，葉酸摂取量は平均 447 μg/日で季節変動がみられているが，男女差は観察されていない（未発表）。また平成 13 年度の国民栄養調査では，葉酸の摂取量は全平均で 313 μg/日で，年齢に依存して高く，女性で低値である[15]。女子学生を対象に行った食事調査でも，葉酸摂取量は五訂成分表を用いた場合には 334 μg/日となるのに対して，米国の成分表では 197 μg/日となる[16]。このように葉酸摂取量が高値であるが，これは日本食品標準分析表に掲載されている葉酸値が高いことによる可能性が示唆されている。なお，米国 Framingham

調査の高齢者（67-90歳）では，葉酸摂取量が253 μg/日以下では血漿ホモシステイン濃度が有意に増加するとの報告がある。しかしながら，ホモシステイン濃度は基準値14 μmol/L以下である[17]。これらの栄養調査から，摂取されている食事性葉酸の摂取量は，1日当たり200-300 μgである。食物摂取頻度調査の結果，食品を基礎として算出された非妊娠女性の所要量は，250 μg/日とされている[18]。

本研究においては，使用した葉酸量は1日当たり200 μgであるが，半精製食品の原料となっている小麦粉315 gに含まれる28 μgも関与しているため，総葉酸量は228 μgとなっている。この食事をした結果，血清葉酸量は男女ともにすべて基準値内にあった。女性成人では，試験期間後期において，開始日と比べて有意な増加がみられたが，男性成人では最終日に有意な減少がみられた。また尿中葉酸排泄量に男女差は認められなかったが，女性では試験期間中に有意な増加が観察された。男性では葉酸排泄量に変化はみられなかった。このように，今回の葉酸摂取量については，男性では不足している可能性があるが，女性では必要量を十分に満たしている，と考えられる。また，女性においては，2002年と2003年で血清葉酸量とその変化，尿中葉酸排泄量および排泄率に相違がみられなかった。このことは葉酸摂取量200 μgと228 μgに差異がないことを示している。

葉酸摂取量の指標をみると，米国においては，血清葉酸では<6.8 nmol/L，赤血球葉酸では<362 nmol/L，および血漿ホモシステインでは>16 μmol/Lが基準値として用いられている[10]。しかしながら，他の国においては，ホモシステインの下限値として，12および14 μmol/Lも使用されている。これらの指標の中で，これまで一般的に用いられてきたものは血清葉酸レベルである。多くの分析データがあり，血液生化学的指標として用いられている。葉酸が不足すると，血清葉酸レベルが低下するとともに，ホモシステインが蓄積する。このため血清ホモシステインレベルが高くなり，尿中のホモシステイン排泄が多くなる。これらのことから，ホモシステインは感度の良い指標であることが示されている。

血清ホモシステインレベルは，血清葉酸レベルあるいは葉酸の摂取量と関連があるとの報告がある。またホモシステインは，詳細な発症メカニズムは不明であるが，動脈硬化症や神経管閉鎖障害の誘発にかかわっている[8]。このため，ホモシステインを葉酸必要量の指標として考えることは，科学的根拠に基づくもので，非常に有用かつ重要である。しかしながら，ホモシステイン-メチオニン代謝経路においては，葉酸以外にもビタミン$B_{12}$およびビタミン$B_6$がかかわっているため，これらのビタミンのうち一つでも不足していると，ホモシステインが増加することが考えられる。このようなことから，葉酸の必要量を考える場合には，ホモシステインのみでなく血清葉酸レベルも考慮して，評価する必要がある。

米国の食事摂取基準の策定では，葉酸の平均必要量は血清葉酸レベル，赤血球葉酸濃度，血漿ホモシステイン濃度，血液学的検査（赤血球数，網赤血球数，平均赤血球容積値，ヘマトクリット値，ヘモグロビン濃度）を基準範囲に維持できる食事と定めている[10]。わが国の第六次改定の所要量や食事摂取基準の推奨量の策定で用いられた根拠は，欧米の多くの報告を基にしている。

ヒトを対象として葉酸の必要量を検討した代表的な論文として，著者らの知る限りでは，4編ある。これらのヒト試験では，食事由来の葉酸と葉酸サプリメントを利用して，検討を行っている。Milne *et al.*[19]は，成人男性40名を対象に代謝室で食事性葉酸（200 μg/日）の影響を調べている。この結果，血清葉酸量の変化は，試験開始時の葉酸の状態に依存している。つまり，血清葉酸量が10 ng/mL以下であれば，食事によって葉酸レベルの減少はみられなかった。このようなことから，体内の葉酸レベルを維持するためには，200 μg/日（150-250 μg/日）以上の葉酸摂取があれば，十分なようである。わが国では，葉酸が食品に強化されていないので，このデータはわが国で葉酸の食事摂取基準を考えるために重要な知見である。

食事とサプリメントの組み合わせを利用したものとして，O'Keefe *et al.*[20]は，成人女性に低葉酸含量（30 μg/日）の食事と葉酸サプリメント200，300，400 μg/日を70日間与えて，葉酸の必要量を検討している。血清の葉酸レベルをみると，試験開始時に比べ，200 μg群では有意に減少したが，400 μg群では有意に増加した。赤血球葉酸量についても，試験開始時に比べ，300 μgおよび400 μg群では増加したが，200 μg群では減少した。一方，試験終了時の血漿ホモシステイン量については，300 μg群や400 μg群と比べ200 μg群で有意に増加した。200 μg群では血漿ホモシステインと血漿や赤血球の葉酸レベルとの間に負の相関がみられた。この結果，これらの女性においては，葉酸200 μgの摂取量では，葉酸状態を維持するためには不十分である，と示唆している。米国における食事摂取基準の策定においては，この結果が基準となっている。

このほか，葉酸欠乏状態にした後，各種濃度の葉酸を摂取させて必要量を検討する欠乏-添加試験を行った報告がある。Sauberlich *et al.*[21]は，成人女性10名を3群に分けて代謝室で92日間検討した。まず28日間葉酸欠乏食を摂取した後，各種濃度の葉酸含有食を摂取し，血漿および赤血球中の葉酸の変化を調べた。欠乏食を摂取すると血漿葉酸は60%まで減少する。その後，天然由来の葉酸200 μgを摂取すると血漿葉酸は減少せず，300 μgではわずかながら上昇する。しかし，これらの濃度でも，赤血球葉酸は減少し続ける。このようなことから，成人女性の葉酸要求量は200-250 μg/日と推定される。Jacob *et al.*[22]は，成人男性10名を対象に代謝室で葉酸欠乏食と葉酸サプリメントを与えて，葉酸必要量を