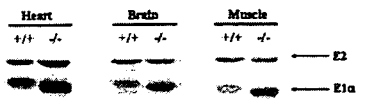
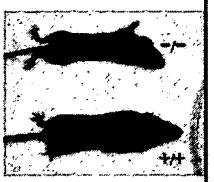
BCKDH kinase knockout mouse



Leucine concentrations in blood and brain

	Wild type	BDK knockout
Blood (μM)	149 ± 12	71 ± 9
Brain (μmol/g wet wt.)	62 ± 6	20 ± 2

Means ± SEM for n=5.



BCAAは脳機能を正常に保つために重要

From Dr. Robert A. Harris (Indiana Univ. School of Med.)

アミノ酸輸液のフィッシャー比と脳症改善効果

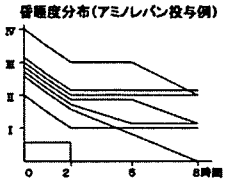
肝性脳症患者にアミノレバン又はGO-80Ⅲを500mL投与

アミノ酸組成	アミノレバン	GO-80Ⅲ*
イソロイシン	0.90g/dL	0.90g/dL
ロイシン	1.10g/dL	1.10g/dL
バリン	0.84g/dL	0.84g/dL
総分枝鎖	2.84g/dL	2.84g/dL
フェニルアラニン	0.10g/dL	–
フィッシャー比	37.05	∞

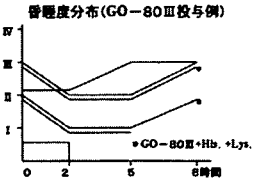
*アミノレバンのBCAAのみ同じ含量を含み他のアミノ酸を含まない輸液

アミノレバン投与例では、昏睡度の改善が持続的に認められたが、GO－80Ⅲでは、投与中の一過性の効果のみで悪化傾向がみられた。

昏睡度分布(アミノレバン投与例)



昏睡度分布(GO－80Ⅲ投与例)



川出健彦ほか：救急医学, 2, 689, 1978

BCAAと食欲

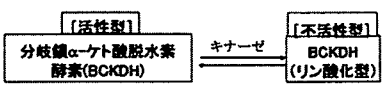
BCAAと食欲の関係

- BCAA不足は拒食症の原因となる (J. Nutr. 131: 851S (2001))。
- 血液人工透析患者(血中BCAA低下)の食欲低下の改善にBCAAは有効である (Nephrol. Dial. Transplant. 16: 1856 (2001))。
- 肝硬変患者(血中BCAA低下)の食欲低下の改善にBCAA投与は有効 である(Gastroenterology 124: 1792 (2003))。
- 癌患者の食欲低下の改善にBCAA投与は有効である(J. Natl. Cancer Inst.88: 550 (1996))。
- 暑い日の食欲低下ーサッパリした食事。
- タンパク質・アミノ酸(BCAA)不足。
- 筋肉タンパク質の分解促進。
- 夏の食欲低下防止にBCAAは有効？！

分岐鎖アミノ酸
(バリン、ロイシン、イソロイシン)

過剰血漿BCAAモデル動物
BCATmノックアウト
マウス

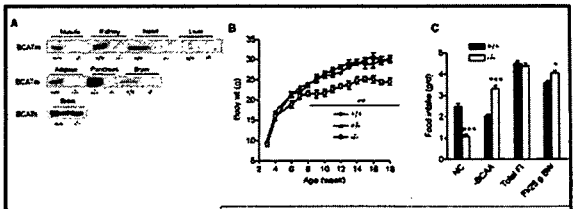
分岐鎖α-ケト酸

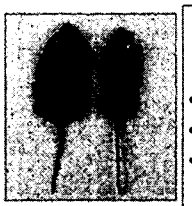


CoA-化合物

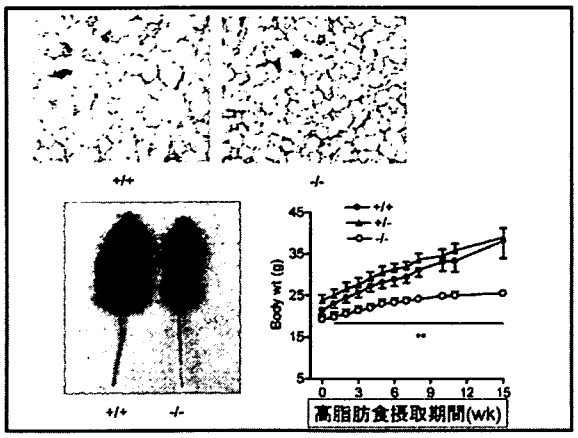
完全分解
(クエン酸回路)

P. She et al., Dr. Susan M. Hutson's group,
Pen. St. Univ. & Wake Forest Univ. School of Medicine
Cell Metabolism 6: 181-194, September 2007





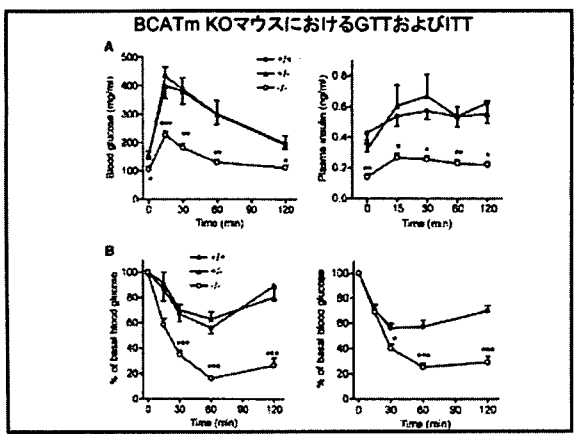
[BCATm$^{-/-}$マウスの特徴]
- タンパク質合成促進
- タンパク質分解促進
- エネルギー消費の増大



Plasma amino acids (mM)

		BCATm+/+	BCATm-/-
Leu	fed	115.4 ± 9.0	1621 ± 381***
Ile	fed	57.1 ± 4.9	1236 ± 301***
Val	fed	139.0 ± 7.7	4243 ± 700***
Asp	fed	9.6 ± 1.3	5.7 ± 0.7*
Gly	fed	347.5 ± 55.6	522.9 ± 53.1*
Thr	fed	189.0 ± 26.0	328.4 ± 42.7**
Cit	fed	44.6 ± 1.9	74.7 ± 7.1**
Arg	fed	118.5 ± 12.4	244.6 ± 36.1**
b-Ala	fed	6.3 ± 0.5	3.5 ± 0.5***
Ala	fed	438.5 ± 44.7	245.4 ± 25.9**

(その他の血漿アミノ酸濃度に変化なし)

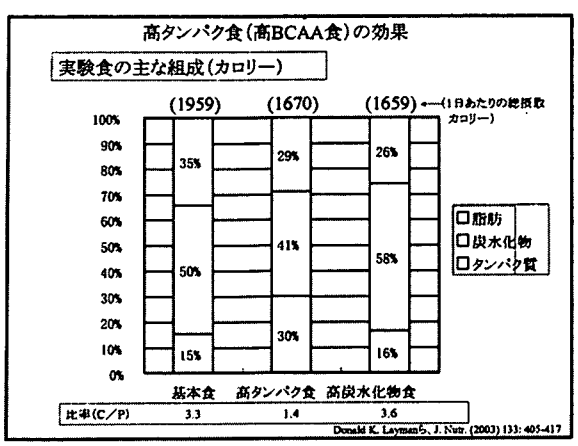


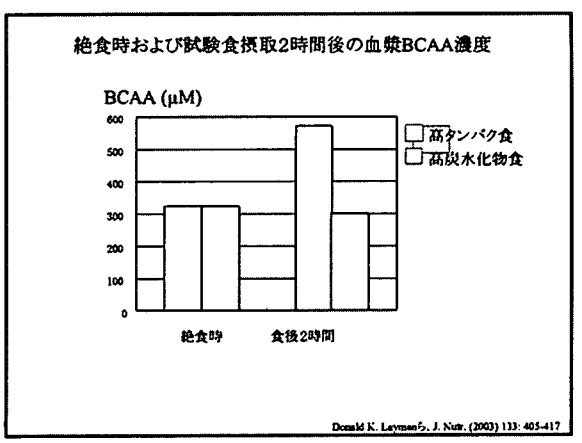
ダイエットとBCAA

Donald K. Laymanら, J. Nutr. (2003) 133: 405-410-417

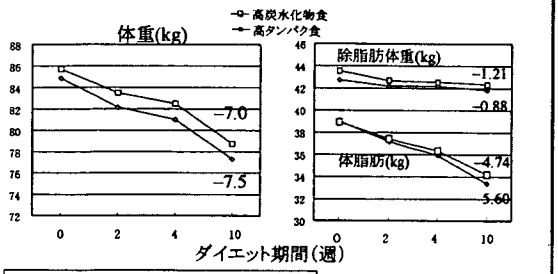
被験者:24名女性、45~56歳、平均体重約85 kg、
平均体脂肪率46%

1週間の基本食摂取の後、10週間の試験食摂取
試験食:高タンパク食 vs 高炭水化物食





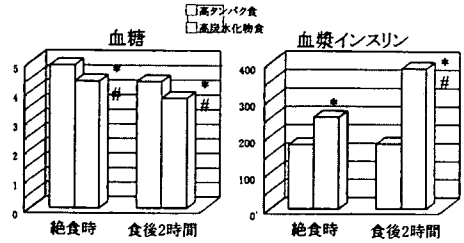
高タンパク食(高BCAA食)の効果:
体重、除脂肪体重、体脂肪量



	10週間の減少量(kg)		
実験食	体重	筋肉	体脂肪
高炭水化物食	7.0	1.21	4.74
高タンパク食	7.5	0.88	5.60

Donald K. Laymanら、
J. Nutr. (2003) 133: 405-417

絶食時および試験食摂取2時間後の血糖と血漿インスリン反応



摂食2時間後の測定は、400 kcalの朝食(試験食)摂取後に測定。
*P <0.05 (vs絶食時), #P < 0.05 (vs高タンパク食)

食後のインスリン反応は、高炭水化物食ダイエットにより上昇したが、高タンパク食ダイエットでは上昇しなかった。

Donald K. Laymanら、J. Nutr. (2003) 133: 405-417

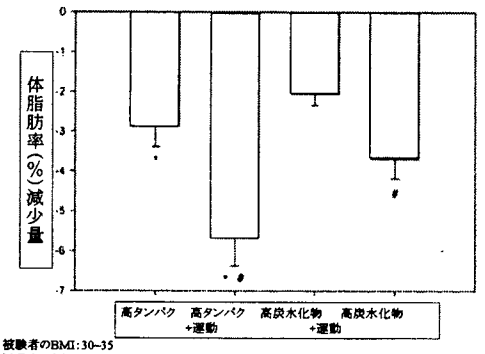
高タンパク(BCAA)／低炭水化物食ダイエットの効果

- 筋肉減少の抑制
- 体脂肪減少の促進
- 食後体熱産生の増加
- グルコース代謝の改善

これらの効果はBCAA(特にロイシン)により
もたらされると考えられる。

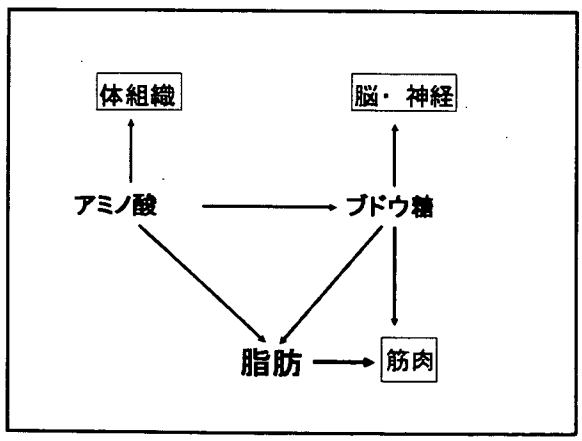
Donald K. Laymanら、J. Nutr. (2003) 133: 405-417

減量による体脂肪減少に対する高タンパク食と運動の相乗効果



被験者のBMI: 30–35
減量食: 通常(約2000kcal)の65%前後
期間: 16週間

D.K. Layman, et al. (2005) J. Nutr. 135: 1903-1910.



Ⅴ．研究成果の刊行に関する一覧表

発表者氏名	論文タイトル	発表誌名	巻	頁	出版年
Fukuwatari T, Shibata K	Effect of nicotinamide administration on the tryptophan-nicotinamide pathway in humans.	*Int. Vitam. Nutr. Res.*	77	255-262	2007
Sawamura H, Fukuwatari T, Shibata K	Effects of excess biotin administration on the growth and urinary excretion of water-soluble vitamins in young rats.	*Biosci. Biotechnol. Biochem.*	71	2977-2984	2007
Okuno A, Fukuwatari T, Shibata K	Characterization of tryptophan-niacin metabolism in rats fed with an excessive tryptophan diet.	*International Congress Series*	1304	171-174	2007
柴田克己，福渡努	生理活性ミネラルとB群ビタミンの生体利用率との関係	*ビタミン*	82	115-125	2008
Yoshida M, Okada T, Namikawa Y, Matsuzaki Y, Nishiyama T, Fukunaga K	Evaluation of nutritional availability and anti-tumor activity of selenium contained in selenium-enriched *Kaiware* radish sprouts.	*Biosci. Biotechnol. Biochem.*	71	2198-2205	2007
吉田宗弘	亜鉛の栄養的意義と亜鉛含有食品素材	*食品加工技術*	27	141-149	2007
吉田宗弘，生田剛	食品および飲料水中のバラジウム含量と日本人のバナジウム摂取量（予報）	*Trace Nutrients Reserach*	24	65-70	2007
吉原花織，福永健治，吉田宗弘	試料中モリブデン濃度がラット臓器および血清モリブデン濃度に及ぼす影響	*Trace Nutrients Reserach*	24	120-123	2007
吉田宗弘	微量元素(5) マンガン摂取－過不足と茶の影響－.	臨床栄養	112	14-15	2008

Ⅵ. 研究成果の刊行物・別刷

Int. J. Vitam. Nutr. Res., 77 (4), 2007, 255–262

Effect of Nicotinamide Administration on the Tryptophan-Nicotinamide Pathway in Humans

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Received for publication: July 24, 2006; Accepted for publication: December 11, 2006

Abstract: The vitamin nicotinamide is synthesized in the liver from tryptophan, and distributed to non-hepatic tissues. Although it is generally accepted that 60 mg tryptophan is equivalent to 1 mg nicotinamide in humans, the conversion ratio of tryptophan to nicotinamide is changeable. To determine if *de novo* nicotinamide synthesis from tryptophan is influenced by nicotinamide intake itself, six young women consumed controlled diets containing 30.4 or 24.8 mg niacin-equivalent nicotinamide supplements with 0, 89, 310, or 562 μmol/day (0, 10.9, 37.8, or 68.6 mg/day, respectively), and urinary excretion of intermediates and metabolites of the tryptophan-nicotinamide pathway were measured. Urinary excretion of nicotinamide metabolites increased linearly in a dose-dependent manner. None of the intermediates, including anthranilic acid, kynurenic acid, xanthurenic acid, 3-hydroxyanthranilic acid, and quinolinic acid, changed at all, even when up to 562 μmol/day nicotinamide was given. That is, exogenous nicotinamide did not affect *de novo* nicotinamide synthesis. Therefore, when niacin equivalent is calculated, the intake of nicotinamide itself need not be considered as a factor that changes the tryptophan-nicotinamide conversion ratio.

Key words: Feedback inhibition, human, kynurenine pathway, NAD, nicotinamide, tryptophan, urine

Introduction

One of the general regulatory mechanisms in biosynthetic pathways is feedback inhibition by the end product. With regard to vitamins, mammals, including humans, can synthesize nicotinamide derivatives from the essential amino acid tryptophan. The conversion of tryptophan to nicotinamide is important for determining the niacin equivalent. The tryptophan-nicotinamide pathway exists in several tissues such as the liver, kidney, spleen, and brain, and the liver primarily contributes to the supply of nicotinamide to non-hepatic tissues [1]. Generally, 60 mg tryptophan is estimated to be equivalent to 1 mg nicotinamide in humans [2–10], although the conversion ratio of tryptophan to nicotinamide is altered by numerous factors such as vitamin deficiency [11, 12], high protein intake [13], hormones [14, 15], food restriction [16], exercise [17], and chemicals [18–21]. Since the amount of *de novo* synthesized nicotinamide derivatives from tryptophan is almost the same as intakes of nicotinamide and nicotinic acid in the Japanese population [22], using this conversion ratio to calculate niacin equivalent is important. Rat liver tryptophan 2,3-dioxygenase activity, which catalyzes the first irreversible step in the degradative me-

DOI 10.1024/0300-9831.77.4.255

tabolism of tryptophan to nicotinamide, is inhibited by pyridine nucleotide coenzymes in *in vitro* experiments [23]. We have previously reported that excess nicotinamide administration does not influence the upper part of the tryptophan-nicotinamide pathway in rats [24]. However, there are no reports on whether nicotinamide intake influences the tryptophan-nicotinamide pathway in humans. In the present study, we assessed the influence that administration of nicotinamide exerted on the production of an intermediary metabolite of the tryptophan-nicotinamide pathway.

Materials and Methods

Chemicals

Quinolinic acid (QA) and anthranilic acid (AnA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). N^1-methylnicotinamide (MNA) chloride, xanthurenic acid (XA), kynurenic acid (KA), and 3-hydroxyanthranilic acid (3-HA) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). N^1-methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick [25] and Shibata *et al* [26]. Other chemicals used were of the highest purity available from commercial sources.

Subjects

Six healthy female Japanese college students who were not regularly using medication or dietary supplements, and who were not habitually consuming alcohol or cigarettes, participated in the experiment. Their age, body weight, height, and body mass index (BMI) are shown in Table I. This study was reviewed and approved by The Ethical Committee of the National Institute of Health and Nutrition (Tokyo, Japan).

Table I: Characteristics of the Subjects.

Subjects	Age (Yr)	Height (cm)	Body weight (kg)	BMI
Woman 1	21	161.0	50.0	19.3
Woman 2	21	161.0	52.5	20.3
Woman 3	21	162.0	46.0	17.5
Woman 4	21	160.7	53.0	20.5
Woman 5	21	160.5	53.0	20.6
Woman 6	21	165.0	52.5	19.3
Mean	21.0	161.7	51.2	19.6
SD	0.0	1.7	2.8	1.2

Diet

Two kinds of meals were given to the subjects. The nutrient elements in each meal are shown in Tables II and III. Other nutrients were calculated by using the Standard Tables of Food Composition in Japan (5th edition) [27].

Experimental design

The outline of the experiment is shown in Figure 1. The subjects spent their time as usual except for diets, on the following schedule: meal-times (breakfast 07:00–07:30 h, lunch 12:00–12:30 h, and dinner 18:00–18:30 h), wake-up time (06:30 h), and bed time (23:00 h). The experimental period was about 4 weeks. Only the diets shown in Tables II and III were given to the subjects during the first week. The subjects were given the diets with 89 μmol/day (10.9 mg/day) nicotinamide in the second week, 310 μmol/day (37.8 mg/day) in the third week, and 562 μmol/day (68.6 mg/day) in the fourth week. Nicotinamide was divided into a ratio of 3:4:3 and administered daily after breakfast, lunch, and dinner. The 24-hour urine samples were collected from the second urinary sample on the fourth day to the first urinary sample after 06:30 h (wake-up time) on the fifth day in each week. The urine sample volumes were measured, and 1 mL of 1 mol/L HCl was added to 9 mL urine samples to stabilize the metabolites in the tryptophan-nicotinamide pathway. The acidified urine samples were stored at –20° C until needed.

Analyses

Urinary MNA was measured using the high-performance liquid chromatography (HPLC) method of Shibata [28]. The 2-Py and 4-Py contents in the urine were simultaneously measured using the HPLC [26]. The contents of KA [29], XA [30], 3-HA [30], AnA [31], and QA [32] in the urine were measured using HPLC.

Results

Relationship between nicotinamide intake and urinary excretion of nicotinamide metabolites

The increased administration of nicotinamide led to a significant increase in the urinary excretion of nicotinamide metabolites, which are the sum of MNA, 2-Py, and 4-Py (Figure 2). The relationship between nicotinamide intake and urinary excretion was linear. This result suggests that exogenous nicotinamide is well absorbed into the body,

Table II: The Composition of Diet 1.

	Breakfast	Lunch	Dinner	Total
Energy (kcal)	402	689	617	1784
Protein (g)	19.5	23.8	25.2	68.6
Fat (g)	15.7	25.5	9.6	50.8
Carbohydrates (g)	46.0	85.8	104.4	248.8
Fat-soluble vitamins				
Vitamin A (μg)	150	309	419	878
Vitamin D (μg)	1	0	2	3
Vitamin E (mg)	1.1	2.1	2.4	5.6
Vitamin K (μg)	8	204	98	311
Water-soluble vitamins[1]				
Vitamin B_1 (mg as thiamin)	0.35	0.17	0.07	0.59
Vitamin B_2 (mg as riboflavin)	0.47	0.20	0.25	0.92
Vitamin B_6 (mg as pyridoxine)	0.20	0.36	0.68	1.24
Vitamin B_{12} (μg as cyanocobalamin)	0.7	0.5	6.2	7.4
Niacin equivalent[2] (mg)	3.5	8.4	18.5	30.4
Pantothenic acid (mg)	1.97	4.21	3.14	9.32
Folic acid (μg as pteroyl monoglutamic acid)	52	134	44	230
Biotin (μg)	21	20	26	67
Vitamin C (mg as L-ascorbic acid)	34	34	50	118
Minerals				
Na (mg)	794	1175	850	2845
K (mg)	592	601	625	1993
Ca (mg)	249	142	85	479
Mg (mg)	47	71	74	192
P (mg)	380	293	317	1071
Fe (mg)	0.8	3.4	2.6	6.7
Zn (mg)	1.8	3.7	2.5	8.0
Cu (mg)	0.15	0.44	0.43	1.02

[1] Water-soluble vitamins except for vitamin B_{12} were measured. Other nutrients were calculated by using the Standard Tables of Food Composition in Japan, fifth revised edition -2000-, Resources Council, Science and Technology Agency, Japan.
[2] The niacin equivalent intake was calculated as follows: The average tryptophan content in food protein is 1.1% and the 1/60 (in weight basis) of tryptophan taken was converted into niacin in the body.

and excess amount of nicotinamide is catabolized to MNA and its further metabolites 2-Py and 4-Py.

Relationship between nicotinamide intake and urinary excretion of the intermediates in the tryptophan-nicotinamide pathway

If nicotinamide inhibits the tryptophan-nicotinamide pathway in the liver, formation of one or more of the intermediates such as AnA, KA, XA, 3-HA, and QA should be reduced. Thus, the effects of nicotinamide administration on the urinary excretion of the intermediates were investigated, regardless of whether or not nicotinamide inhibits *de novo* nicotinamide synthesis. As shown in Figure 3, urinary excretion of AnA (Fig. 3A), KA (Fig. 3B), XA (Fig. 3C), 3-HA (Fig. 3D), and QA (Fig. 3E) was little affected by the increased intake of nicotinamide. In other words, the tryptophan-nicotinamide pathway is not affected, even when taking a large amount of nicotinamide.

Discussion

Humans can synthesize the vitamin nicotinamide from tryptophan in the liver, and the resultant nicotinamide is distributed to non-hepatic tissues. The purpose of the synthetic pathway in the liver is not the supply of NAD^+ but the supply of nicotinamide for non-hepatic tissues. We have clarified that the conversion pathway of nicotinamide from tryptophan is affected by various nutrients [11–13, 16], hormones [14, 15], exercise [17], and drugs [18–21] based on data concerning the urinary excretion of metabolic intermediates in the tryptophan-nicotinamide pathway. Furthermore, we have clarified that excessive nicotinamide administration does not influence the upper part of tryptophan-nicotinamide pathway in rats [24]. However, we still did not know whether nicotinamide itself affects this conversion pathway in humans. The present study was carried out to answer this question because the value of the conversion ratio of tryptophan to nicotinamide is important in deciding the niacin requirement.

Table III: The Composition of Diet 2.

	Breakfast	Lunch	Dinner	Total
Energy (kcal)	463	549	606	1693
Protein (g)	19.6	21.4	20.5	61.5
Fat (g)	22.3	12.8	10.0	45.0
Carbohydrates (g)	46.1	85.6	105.5	249.8
Fat-soluble vitamins				
Vitamin A (μg)	294	144	444	882
Vitamin D (μg)	1	0	0	1
Vitamin E (mg)	2.7	0.6	2.9	6.2
Vitamin K (μg)	12	98	100	210
Water-soluble vitamins[1]				
Vitamin B_1 (mg as thiamin)	0.35	0.09	0.02	0.46
Vitamin B_2 (mg as riboflavin)	0.47	0.18	0.17	0.81
Vitamin B_6 (mg as pyridoxine)	0.20	0.35	0.31	0.86
Vitamin B_{12} (μg as cyanocobalamin)	0.7	0.3	10.3	11.3
Niacin equivalent[2] (mg)	7.0	8.1	9.7	24.8
Pantothenic acid (mg)	1.97	3.73	3.55	9.25
Folic acid (μg as pteroyl monoglutamic acid)	52	125	105	282
Biotin (μg)	21	12	20	53
Vitamin C (mg as L-ascorbic acid)	34	25	53	112
Minerals				
Na (mg)	833	1237	1080	3177
K (mg)	594	851	615	2235
Ca (mg)	250	173	96	523
Mg (mg)	47	113	96	257
P (mg)	381	253	317	1032
Fe (mg)	0.8	6.2	3.2	10.2
Zn (mg)	1.9	2.8	4.2	8.9
Cu (mg)	0.15	0.33	0.47	0.95

[1] Water-soluble vitamins except for vitamin B_{12} were measured. Other nutrients were calculated by using the Standard Tables of Food Composition in Japan, fifth revised edition -2000-, Resources Council, Science and Technology Agency, Japan.
[2] The niacin equivalent intake was calculated as follows: The average tryptophan content in food protein is 1.1% and the 1/60 (in weight basis) of tryptophan taken was converted into niacin in the body.

Nicotinamide administration led to a significant increase in the urinary excretion of the nicotinamide metabolites MNA, 2-Py, and 4-Py in a dose-dependent manner (Figure 2). This result means that the exogenous nicotinamide was equally well absorbed and utilized in the body. We did not measure the contents of nicotinamide and NAD in the blood, because the objective of the experiment was simply to know whether or not large amounts of exogenous nicotinamide affect the tryptophan-nicotinamide pathway. For this purpose, the most appropriate method is to measure the metabolites excreted into the urine, as reported previously [11–21, 24]. Therefore, six young women were recruited for our study, and the influence that nicotinamide administration exerted on the production of intermediates in the tryptophan-quinolinic acid pathway, which is in the upper part of the tryptophan-nicotinamide pathway, was examined to measure the urinary excretion of the metabolites. The present data clearly show that excessive nicotinamide intake does not influence the tryptophan-quinolinic acid metabolism. This result means that we can calculate the niacin equivalent regardless of the intake of nicotinamide.

The tryptophan-nicotinamide metabolic pathway is divided into two parts: one involves tryptophan-quinolinic acid metabolism and the other quinolinic acid-nicotinamide metabolism. Cho-Chung *et al* [23] have reported that rat liver tryptophan 2,3-dioxygenase activity, which catalyzes the first irreversible step in the degradative metabolism of tryptophan to nicotinamide, is inhibited by pyridine nucleotide coenzymes in *in vitro* experiments. However, the present study shows that excessive nicotinamide administration does not decrease the formation of intermediates from tryptophan (Figure 3). NAD^+ synthesis from nicotinamide is well regulated by NAD^+ through inhibiting the activity of nicotinamide phosphoribosyltransferase [33], which catalyzes the formation of nicotinamide mononucleotide from nicotinamide and 5-phosphoribosyl-1-pyrophosphate. Therefore, *in vivo*, even when excessive nicotinamide is given, the liver pyridine nucleotide concentrations do not increase, since the cel-

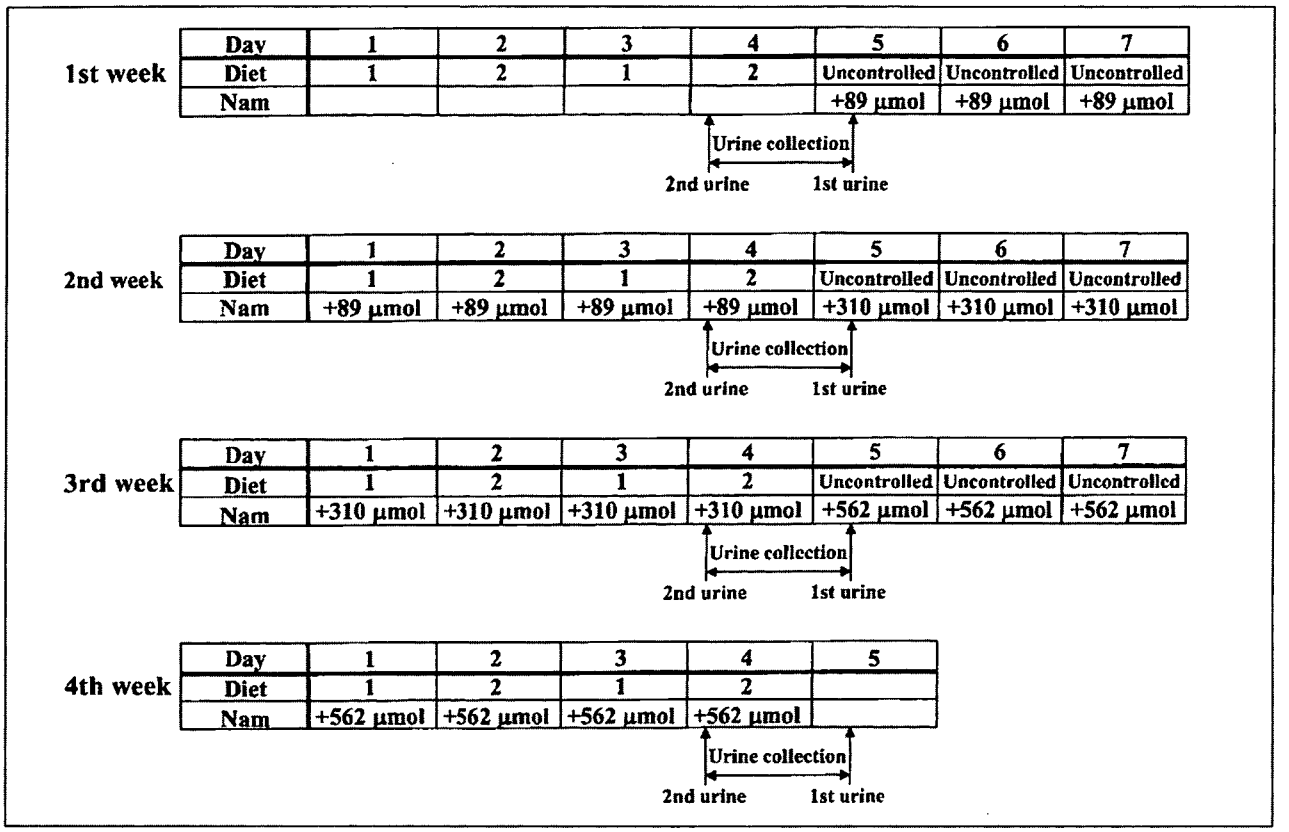

Figure 1: Scheme of the experimental design. Subjects were given the diet and nicotinamide (Nam) as indicated. The compositions of diets 1 and 2 are shown in Tables II and III, respectively. Nicotinamide was divided into a ratio of 3:4:3 and administered daily after breakfast, lunch, and dinner. The 24-hour urine samples were collected from the second urinary sample on the fourth day to the first sample on the fifth day in each week. The urine sample volumes were measured, and 1 mL of 1 mol/L HCl was added to 9 mL urine samples to stabilize the metabolites in the tryptophan-nicotinamide pathway. The acidified urine samples were stored at –20° C until needed.

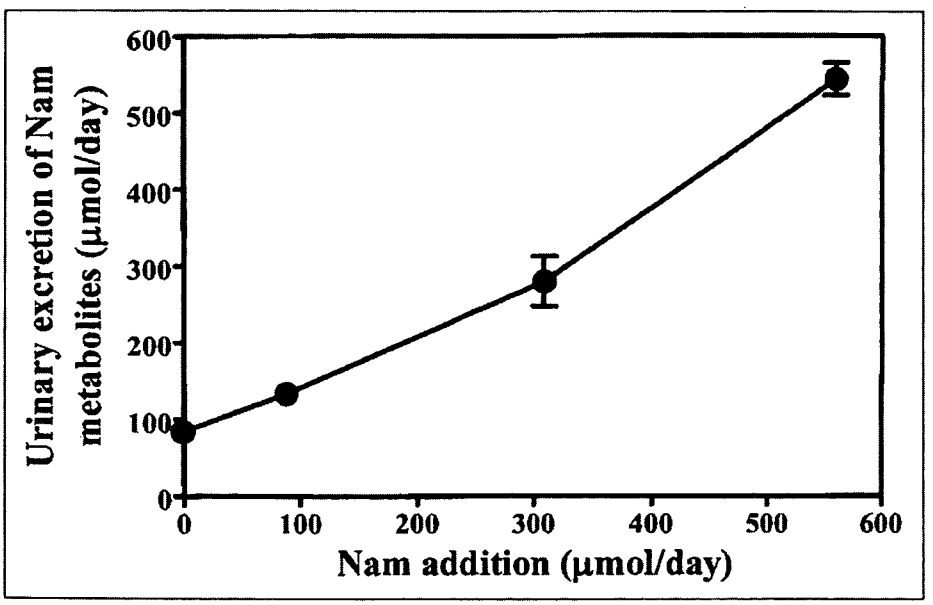

Figure 2: Effect of nicotinamide (Nam) administration on urinary excretion of its metabolites. Values are presented as the mean ± SEM for six subjects. Nam metabolites signify the total amount of nicotinamide metabolites MNA, 2-Py, and 4-Py. The experimental conditions were as outlined in Figure 1 and in the Materials and Methods section.

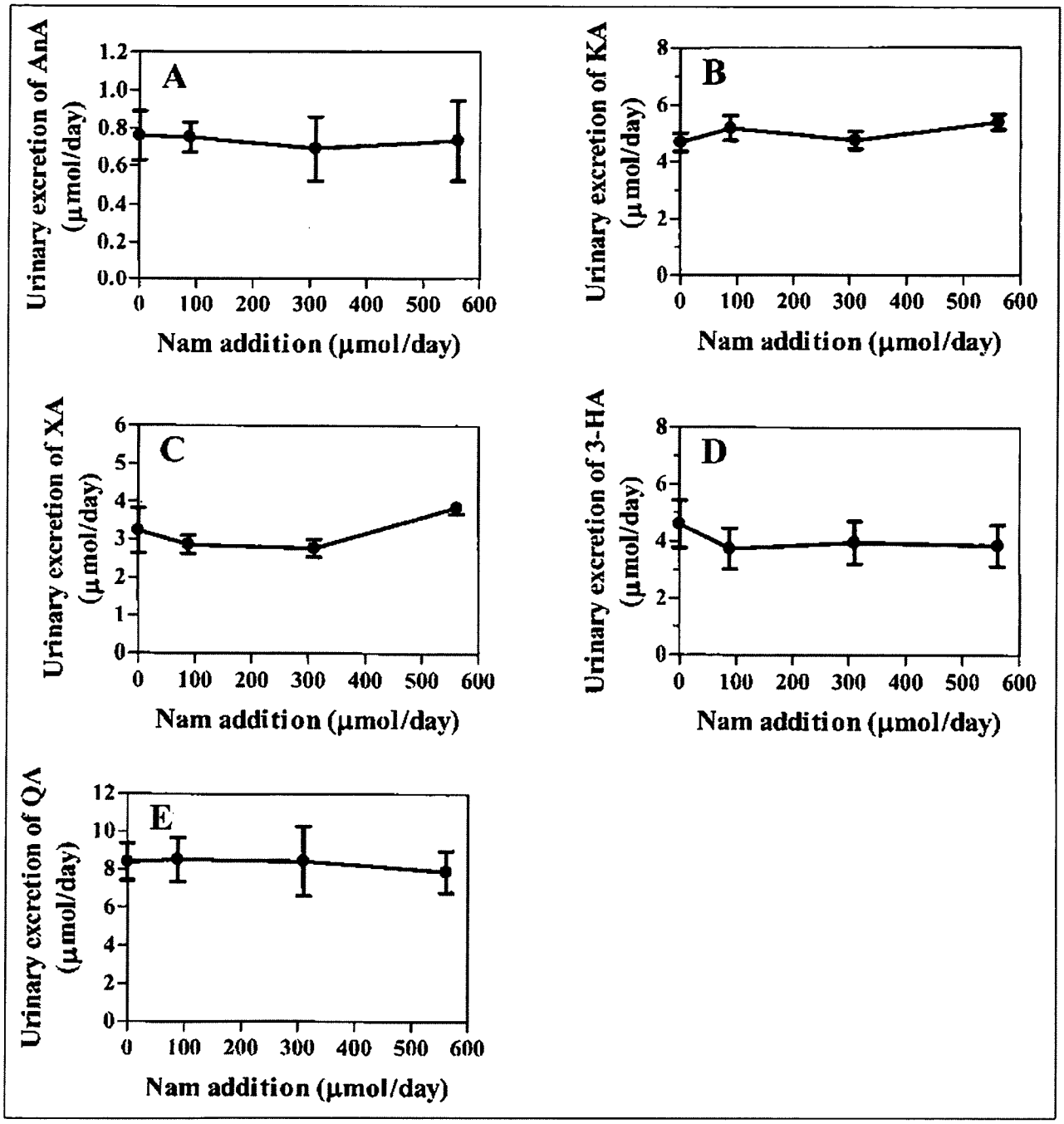

Figure 3: Effect of nicotinamide (Nam) administration on urinary excretion of anthranilic acid (A), kynurenic acid (B), xanthurenic acid (C), 3-hydroxyanthranilic acid (D), and quinolinic acid (E).
Values are presented as the mean ± SEM for six subjects. The experimental conditions were as outlined in Figure 1 and in the Materials and Methods section.

lular pyridine nucleotide coenzyme concentrations are well controlled, as mentioned above.

In conclusion, we have shown that excessive nicotinamide does not influence the conversion pathway of tryptophan to nicotinamide in humans. This result means that the conversion ratio of tryptophan to niacin is not changed with either more or less nicotinamide intake. Thus, the niacin equivalent intake is calculated as usual: niacin intake (mg) = nicotinamide (mg) + nicotinic acid (mg) + 1/60 tryptophan (mg).

Acknowledgments

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Effects of Excess Biotin Administration on the Growth and Urinary Excretion of Water-Soluble Vitamins in Young Rats

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To determine the effects of excess biotin administration on growth and water-soluble vitamin metabolism, weaning rats were fed on a 20% casein diet containing 0.00002% biotin, or same diet with 0.04, 0.08, 0.10, 0.20, 0.50, 0.80 or 1.0% added biotin for 28 days. More than 0.08% biotin administration decreased the food intake and body weight gain compared with the levels in control rats. An accumulation of biotin in such tissues as the liver, brain and kidney increased in a dose-dependent manner, and the both bound and free biotin contents in the liver also increased in a dose-dependent manner. An excess administration of biotin did not affect the urinary excretion of other water-soluble vitamins, suggesting no effect on the metabolism of other water-soluble vitamins. The results of the food intake and body weight gain indicated that the lowest observed adverse effect level for young rats was 79.2 mg/kg body weight/day, while the no observed adverse effect level was 38.4 mg/kg/day. These results suggested immediately setting a tolerable upper intake level for biotin.

Key words: no observed adverse effect level (NOAEL); lowest observed adverse effect level (LOAEL); tolerable upper intake level (UL); urine; blood

Biotin is a water-soluble vitamin classified among the B-group of vitamins. In humans, biotin serves as a coenzyme for four carboxylases: pyruvate, acetyl-CoA, propionyl-CoA, and β-methylcrotonyl-CoA.[1] These carboxylases have important roles in fatty acid synthesis, branched-chain amino acid catabolism, odd-chain fatty acid metabolism, and gluconeogenesis. Although dietary biotin deficiency has not been reported in humans, biotin deficiency has caused growth retardation, alopecia, dermatitis and neurological impairment in experimental animals and humans.[2] In addition, biotin is important in the normal reproductive performance and embryonic growth and development of mammals.[3–5]

Some people have recently been taking 1–10 mg/d of biotin as a medical treatment because biotin has been found to be correlated with certain diseases such as diabetes mellitus,[6,7] liver[8] and skin[9] disorders, neurological abnormality,[10] and epilepsy.[11]

Biotin is a heterocyclic compound, an imidazolidone ring joined to a tetrahydrothiophene ring. The latter possesses a valeric acid side chain. The structure is unique, and biotin is more toxic than would be expected if a repeated excess dosage is administered. Indeed, single or repeated doses of biotin (total doses of 50 and 100 mg/kg body weight by subcutaneous injection) given to rats resulted in irregularities of the estrus cycle[12,13] and fetal and placental resorption in pregnant rats,[13] accompanied by decreased uterine weight, reduced glycogen and protein in the uterus, and reduced protein in the liver. However, these studies cannot be regarded as conclusive for human dietary biotin uptake, because of the route of administration. The administration of oral biotin in doses up to 100 mg/day to patients with holocarboxylase synthetase and biotinidase deficiency has not resulted in adverse effects,[14] although the metabolic defect may prevent or mask toxicity. The Japanese Dietary Reference Intake recommendation presents no data on the tolerable upper intake level (UL) for biotin.[15] Biotin toxicity in healthy humans has not been studied, and performing such a study with the risk of an adverse effect would not be permitted. In the present study, we investigated the effects of excess orally administered biotin on the food intake, body weight gain, tissue weight and water-soluble vitamin metabolism in young rats.

Materials and Methods

Chemicals. Vitamin-free milk casein, sucrose, and L-methionine were purchased from Wako Pure Chemical

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Abbreviations: 4-PIC, 4-pyridoxic acid; Nam, nicotinamide; PaA, pantothenic acid; UL, tolerable upper intake level; MNA, N^1-methylnicotinamide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide; NOAEL, no observed adverse effect level; LOAEL, lowest observed adverse effect level

Table 1. Composition of the Diets

	Control	+0.04%	+0.08%	+0.10%	+0.20%	+0.50%	+0.80%	+1.00%
Casein	20	20	20	20	20	20	20	20
L-Methionine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
α-Cornstarch	46.9	46.86	46.82	46.8	46.7	46.5	46.3	46.2
Sucrose	23.4	23.4	23.4	23.4	23.4	23.3	23.2	23.1
Corn oil	5	5	5	5	5	5	5	5
Mineral mixture[a]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mixture[a]	1	1	1	1	1	1	1	1
D-Biotin	0	0.04	0.08	0.10	0.20	0.50	0.80	1.00

Each value is expressed as g/100 g of diet.
[a]The compositions of the mineral and vitamin mixtures are described in Ref. 16.

Industries (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized corn starch, the mineral mixture (AIN-93M)[16] and vitamin mixture (AIN-93-VX containing 25% choline bitartrate)[16] were obtained from Oriental Yeast (Tokyo, Japan). Thiamin hydrochloride ($C_{12}H_{17}ClN_4OS$-HCl, 337.27), riboflavin ($C_{17}H_{20}N_4O_6$, 376.37), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$, 1355.40), nicotinamide (Nam; $C_6H_6N_2O$, 122.13), calcium pantothenate ($C_{18}H_{32}N_2O_{10}$-Ca, 476.54), folic acid (pteroylmonoglutamic acid; $C_{19}H_{19}N_7O_6$, 441.40), D(+)-biotin ($C_{10}H_{16}N_2O_3S$, 244.31), and L(+)-ascorbic acid ($C_6H_8O_6$, 176.13) were purchased from Wako Pure Chemical Industries. 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[17] and Shibata *et al.*,[18] respectively. All other chemicals used were of the highest purity available from commercial sources.

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

Male Wistar rats (3 weeks old) were obtained from CLEA Japan (Tokyo, Japan) and placed in individual metabolic cages (CT-10; CLEA). They were divided into eight groups (such group consisting of four rats) and fed *ad libitum* for 28 days: one group with a 20% casein diet (used as a control group containing 0.00002% biotin), and the others with the same diet plus 0.04, 0.08, 0.10, 0.20, 0.50, 0.80 or 1.0% biotin (Table 1).

The room temperature was maintained at around 22 °C and 60% humidity, and a 12-h light (06:00–18:00)/12-h dark (18:00–06:00) cycle was maintained. The body weight and food intake were measured every 2 days at around at 10:00. Urine samples (24 h; 10:00–10:00) were collected in amber bottles containing 1 ml of 1 M HCl on the last day of the experiment, and were stored at −20 °C until needed.

The rats were killed by decapitation at around 10:00 on the last day (day 28), after the urine sample had been collected. Serum was collected to measure biotin, and was stored at −20 °C until needed. The liver, spleen, kidney, heart, lung, brain, testis and thigh muscle of each animal were removed, and a portion (about 0.5 g) was immediately treated as described next to measure biotin.

Analyses. Vitamin B_1: One milliliter of 1 M HCl was added to 9 ml of the 24-h urine sample. The urinary concentration of thiamin was determined by the HPLC post-labeled fluorescence method.[19]

Vitamin B_2: One milliliter of 1 M HCl was added to 9 ml of the 24-h urine sample. The urinary concentration of riboflavin was determined by the HPLC method.[20]

Vitamin B_6: One milliliter of 1 M HCl was added to 9 ml of the 24-h urine sample. The urinary concentration of the vitamin B_6 catabolite, 4-pyridoxic acid (4-PIC), was determined by the HPLC method.[21]

Vitamin B_{12}: Part of the 24-h urine samples was stored at −20 °C. The urinary vitamin B_{12} concentration was assayed by a microbiological method with *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830.[22]

Niacin: One milliliter of 1 M HCl was added to 9 ml of the 24-h urine sample. The quantity of Nam, 2-Py and 4-Py in the urine was measured simultaneously by the HPLC method of Shibata *et al.*[18] The content of MNA was measured by the method of Shibata.[23] The sum of Nam, MNA, 2-Py and 4-Py was used to represent the niacin catabolites.

Pantothenic acid: Part of the 24-h urine samples was stored at −20 °C. The content of pantothenic acid in the urine was directly measured by using *Lactobacillus plantarum* ATCC 8014.[24]

Folates: One milliliter of 1 M ascorbic acid was added to 9 ml of the 24-h urine sample. The urinary concentration of folates was determined by the microbioassay method with *Lactobacillus casei* ATCC 7469.[25]

Biotin: Part of the 24-h urine samples was stored at −20 °C. The content of biotin in the urine was directly measured by using *Lactobacillus plantarum* ATCC 8014.[26] To measure the free serum biotin content, 0.05 ml of serum was added to 1 ml of distilled water, and the mixture was heated for 5 min in a water bath at 100 °C. After cooling to room temperature, the solution was centrifuged at 9000 *g* for 10 min at 4 °C, and the resulting supernatant was used to measure biotin. To

Table 2. Effects of Biotin Intake on the Body Weight Gain and Food Intake

	Control	+0.04%	+0.08%	+0.10%	+0.20%	+0.50%	+0.80%
Initial body weight (g)	37.8 ± 0.5	37.6 ± 0.5	37.8 ± 1.2	37.9 ± 0.6	37.7 ± 0.8	37.6 ± 1.3	37.5 ± 0.6
Final body weight (g)	219.7 ± 4.2^{a}	208.1 ± 1.9^{ab}	179.6 ± 7.7^{bc}	165.2 ± 5.2^{c}	131.7 ± 9.7^{d}	120.9 ± 8.8^{d}	101.0 ± 0.9^{d}
Body weight gain (g/28d)	181.9 ± 4.1^{a}	170.5 ± 1.5^{ab}	141.8 ± 7.3^{bc}	127.3 ± 4.6^{c}	94.1 ± 9.8^{d}	83.3 ± 9.0^{d}	63.8 ± 0.1^{d}
Food intake (g/28d)	380.1 ± 6.9^{a}	360.6 ± 4.4^{ab}	323.4 ± 10.0^{b}	315.4 ± 9.9^{b}	256.4 ± 15.1^{c}	236.9 ± 16.6^{c}	203.9 ± 4.7^{c}

Each value is expressed as the mean ± SEM.
A different superscript letter means significant difference at $p < 0.05$.

Table 3. Effects of Biotin Intake on the Tissue Weights of Rats

	Control	+0.04%	+0.08%	+0.10%	+0.20%	+0.50%	+0.80%
Brain	1.05 ± 0.05	1.05 ± 0.01	1.03 ± 0.00	1.02 ± 0.01	0.96 ± 0.03	1.06 ± 0.02	1.00 ± 0.05
Liver	10.23 ± 0.58^{a}	10.64 ± 0.15^{a}	8.53 ± 0.52^{ab}	7.54 ± 1.03^{bc}	6.94 ± 0.53^{bc}	5.95 ± 0.55^{bc}	5.34 ± 0.29^{c}
Heart	0.85 ± 0.01^{a}	0.76 ± 0.02^{ab}	0.66 ± 0.02^{bc}	0.62 ± 0.04^{cd}	0.54 ± 0.04^{dc}	0.49 ± 0.01^{c}	0.42 ± 0.02^{c}
Kidney	1.84 ± 0.03^{a}	1.72 ± 0.02^{ab}	1.53 ± 0.04^{b}	1.46 ± 0.10^{bc}	1.19 ± 0.08^{cd}	1.22 ± 0.06^{cd}	1.04 ± 0.03^{d}
Lung	1.61 ± 0.12^{a}	1.30 ± 0.07^{ab}	1.14 ± 0.05^{bc}	1.13 ± 0.17^{bc}	0.78 ± 0.09^{c}	0.73 ± 0.06^{c}	0.67 ± 0.00^{c}
Spleen	0.68 ± 0.01^{a}	0.69 ± 0.01^{a}	0.59 ± 0.01^{a}	0.53 ± 0.07^{ab}	0.41 ± 0.05^{bc}	0.29 ± 0.02^{c}	0.26 ± 0.02^{c}
Testis	2.32 ± 0.04^{a}	2.29 ± 0.03^{a}	2.24 ± 0.01^{a}	2.15 ± 0.16^{a}	1.91 ± 0.17^{a}	1.41 ± 0.10^{b}	0.81 ± 0.10^{c}

Each value is expressed in g as the mean ± SEM.
A different superscript letter means significant difference at $p < 0.05$.

measure the total biotin content in the tissues, a portion (about 0.5 g) of each tissue (liver, spleen, kidney, heart, lung, brain, testis and skeletal muscle) was homogenized with two volumes of 2.25 M H_2SO_4 and then hydrolyzed by autoclaving for 1 h at 121 °C and 2 atm. After cooling, the hydrolysate was centrifuged at 9000 *g* for 10 min at 4 °C, and the resulting supernatant was used to measure biotin. To measure the free biotin content in the liver, a portion of the liver was homogenized with two volumes of a 0.05 M potassium phosphate buffer (pH 7.0), the homogenate was centrifuged at 9000 *g* for 10 min at 4 °C, and the resulting supernatant was used to measure biotin.

Statistical analysis. Each value is expressed as the mean ± SEM. The statistical significance was determined by ANOVA, this being followed by Tukey's multiple-comparison test. $P < 0.05$ was considered to be statistically significant. Graph Pad Prism4.0 (Graph Pad Software, San Diego, CA, USA) was used for all the analyses.

Results

Effect of excessive biotin administration on the food intake and body weight gain in young rats

The 0.00002% biotin diet was set as the control because the AIN-93 diet recommended by AIN contains 0.00002% biotin.[16] The food intake and body weight gain were not significantly different between the 0.04% biotin-added and control groups, whereas the food intake and body weight gain in the group with the >0.08% biotin-added diets were significantly lower than those in the control group (Table 2). Diarrhea was observed in the young rats fed with the >0.50% biotin-added diets. One rat in four died with the 0.80% biotin-added diet, and two in four rats died with the 1.0% biotin-added diet. Therefore, the data for the 1.00% biotin-added diet group are not shown in Table 2.

Effect of excess biotin administration on the tissue weight of young rats

Table 3 shows the tissue weight of the rats fed on the biotin diets. The brain weight was not significantly different among the seven groups. The weights of other tissues, including the liver, heart, kidney, lung, spleen and testis, showed increasingly lower values in a dose-dependent manner. The tissue weights in the 0.04% biotin-added group were the same as those in the control group, and all tissue weights except the brain in the 0.50% and 0.80%-added groups were lower than those in the control group. The heart and kidney weights in the groups with the >0.08% biotin-added diets were lower than those in the control group, the liver and lung weights in the groups with the >0.10% biotin-added diets were lower, and the testis weights in the group with the >0.20% biotin-added diets were lower those in the control group.

Effect of excess biotin administration on the biotin concentration in the urine, tissues and blood

The effect of excess biotin on the concentration of biotin in the urine is shown in Fig. 1A. The urinary excretion of biotin increased with increasing to dietary intake of biotin. The urinary biotin excretion rate to biotin intake was 54.1 ± 5.8 in the control group, and 82.7 ± 2.5, 68.9 ± 5.7, 63.3 ± 2.5, 60.0 ± 2.6, 31.4 ± 6.8 and 29.8 ± 6.8% in the 0.04, 0.08, 0.10, 0.20, 0.50 and 0.80% biotin-added groups, respectively. The serum free biotin content also increased with increasing intake of biotin (Fig. 1B).

The liver total, bound and free biotin contents are shown in Fig. 2. These biotin contents in the liver increased in a dose-dependent manner, although the

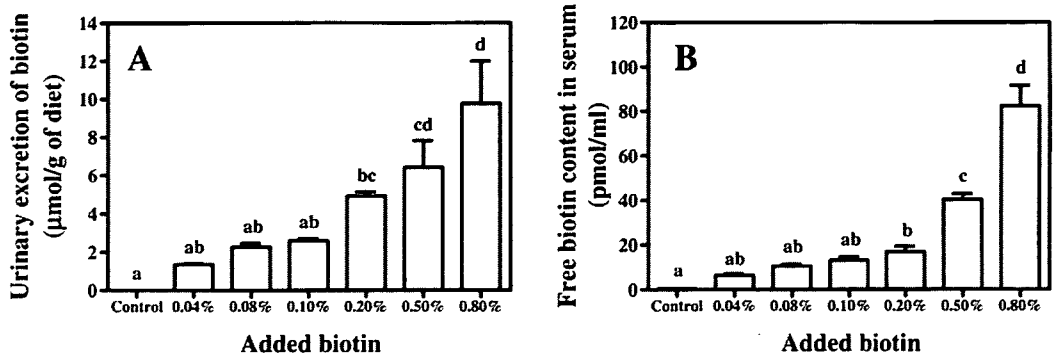

Fig. 1. Effects of Excessive Administration of Biotin on the Urinary Excretion of Biotin (A) and Free Biotin Content in the Serum (B).
The 24-hr urine samples were collected on the last day of the experiment, and then the serum was collected. Each bar is the mean ± SEM for 3 or 4 rats. A different superscript letter means significant difference at $p < 0.05$.

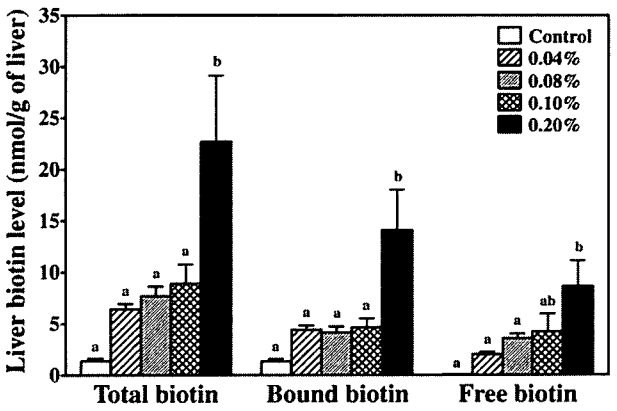

Fig. 2. Effects of Excessive Administration of Biotin on the Total, Bound and Free Biotin Contents in the Liver.
The serum and liver were collected on the last day of the experiment. Each bar is the mean ± SEM for 3 or 4 rats. A different superscript letter means significant difference at $p < 0.05$.

liver bound biotin contents were at the same level in the 0.04, 0.08 and 0.10% biotin-added groups. Ninety seven percent of the liver total biotin existed as bound biotin in the control group, and 30–50% of total biotin was of the bound type in the 0.04, 0.08, 0.10 and 0.20% biotin added-groups.

The concentrations of biotin in the skeletal muscle, brain, heart, kidney, lung, spleen and testis also increased with increasing intake of biotin (Fig. 3). The levels of biotin in the skeletal muscle, brain, heart, kidney, lung and testis in the 0.04, 0.08, 0.10 and 0.20% biotin-added groups were significantly higher than those in the control groups. The spleen biotin content in the 0.04% biotin-added group was not significantly higher than that in the controls, however, the biotin contents in the 0.08, 0.10 and 0.20% biotin adding groups were significantly higher than that in the control group.

Effect of excess biotin administration on the urinary excretion of other water-soluble vitamins

A mega-dose of biotin did not greatly affect the urinary excretion of other water-soluble vitamins (Fig. 4). Only the urinary excretion of folates was significantly increased by feeding a diet containing up to 0.10% biotin. The urinary excretion of ascorbic acid tended to increase with increasing intake of biotin, but a small number of rats in each group failed to show any significant difference.

Discussion

We have previously reported that a 0.3% nicotinamide diet and 1.0% calcium pantothenate diet did not show any effect on the growth of young rats.[27,28] In the present study, an extremely high dose of biotin representing more than a 0.80% in the diet caused death, and more than 0.08% biotin-added diet retarded the growth of young rats. These results suggest that an excess biotin intake might cause some adverse effects on humans, and that setting UL for biotin would be important to prevent such dietary biotin-induced adverse effects. Although no adverse effects of biotin on humans have been reported, two studies have reported that subcutaneously administered biotin (50 and 100 mg/kg) to pregnant rats inhibited fetal and placental growth and resorption of fetuses and placentae.[12,13] The effects of excess biotin intake on the reproductive organs of male rats were not investigated in the present study, although the testis weights in the young rats fed with the diets containing more than 0.50% biotin were lower than those in the other groups. Whether an oral intake of high biotin by pregnant rats would affect the sex hormones, reproductive organs and fetal growth remains to be elucidated.

For increasing accumulation of biotin in the tissues was observed as the amount of biotin administered was increased. This phenomenon might have been due to too great an amount of biotin than was possible to metabolize and excrete. It is suggested that this accumulation was associated with the retardation of growth. The bound biotin content in the liver increased in the present study in a dose-dependent manner, and biotin quantification after SDS–PAGE separation showed that 40% of the accumulated biotin in the rat

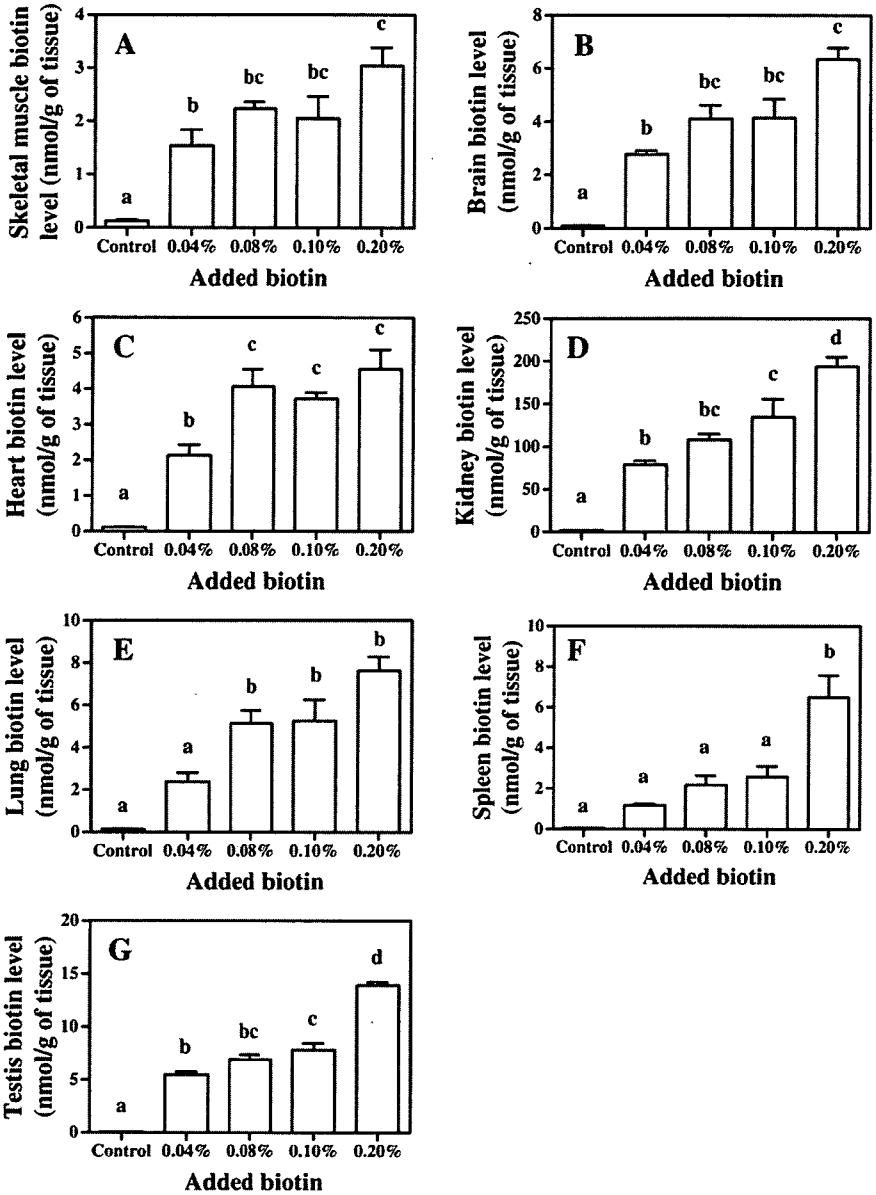

Fig. 3. Effects of Excessive Administration of Biotin on the Biotin Contents in the Muscle (A), Brain (B), Heart (C), Kidney (D), Lung (E), Spleen (F), and Testis (G).

The tissues were collected the last day of the experiment. Each bar is the mean ± SEM for 3 or 4 rats. A different superscript letter means significant difference at $p < 0.05$.

liver that have been overdosed with biotin was bound to protein (data not shown). Hymes *et al.* have proposed a reaction mechanism by which the enzyme, biotinidase (EC 3.5.1.12), mediates covalent binding of biotin to histones.[29] Biotinylation of histones might play a role in gene silencing,[30] cell proliferation,[31,32] and DNA repair or apoptosis.[30] Treatment of cell lines with a pharmacological concentration of biotin (10 pmol/ml) for several weeks had only a moderate impact on biotinylation of histones, whereas the biotinylation of carboxylases was strongly correlated with the biotin concentration in the culture media.[33–35] A pharmacological dose of dietary biotin (100 mg/kg) has decreased the abundance of biotinylated carboxylase in rat liver.[36] It is unclear whether an excess biotin intake wuold affect the biotinylation of histones, and how these changes to histones and some carboxylases are related to the detrimental effect of an excess biotin intake.

The present experiment using young rats clearly indicated that an excessive oral intake of biotin retarded the body weight gain and food intake. Judging from the results of the body weight gain and food intake, the no observed adverse effect level (NOAEL) in young rats was 0.04% in the diet, and the lowest observed adverse effect level (LOAEL) was 0.08% in the diet. Young rats in the 0.04% biotin group consumed about 6.83 g/day of their diet during days 0 to 28, the mean body weight during that period being about 177.8 g. Therefore, the

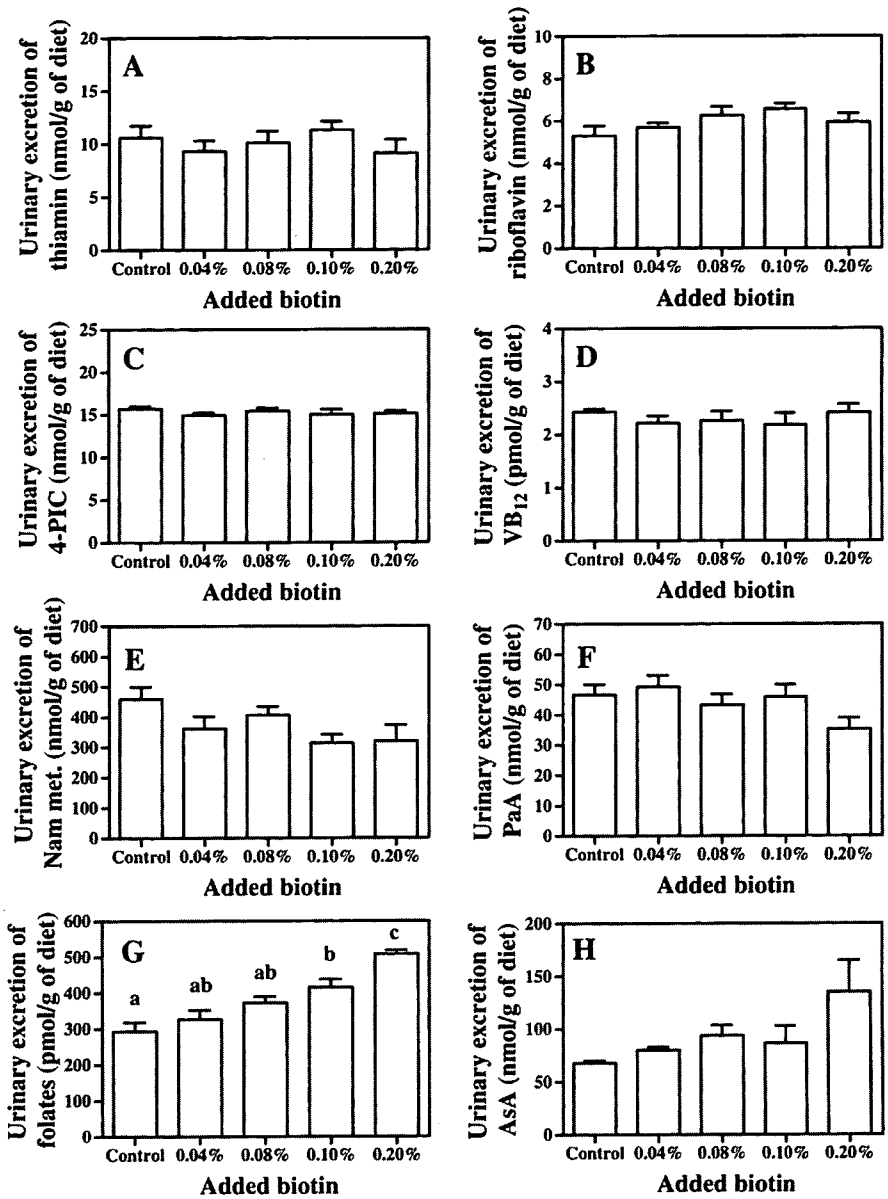

Fig. 4. Effects of Excessive Administration of Biotin on the Urinary Excretion of Thiamin(A), Riboflavin (B), 4-Piridoxic Acid (4-PIC), a Metabolite of Vitamin B_6 (C), Vitamin B_{12} (D), Sum of the Nicotinamide Metabolites, MNA, 2-Py and 4-Py (E), Pantothenic Acid (PaA) (F), Folates (G), and Ascorbic Acid (AsA) (H).

The 24-hr urine samples were collected the last day of the experiment. Each bar is the mean ± SEM for 3 or 4 rats. A different superscript letter means significant difference at $p < 0.05$.

biotin intake was calculated as 38.4 mg/kg body weight/day. Young rats in the 0.08% biotin group consumed about 11.76 g/day during days 0 to 28, the mean body weight during that period was being about 149.7 g. Therefore, the biotin intake was calculated as 79.2 mg/kg body weight/day. Although the present study clearly showed that the 79.2 mg/kg body weight/day oral intake of biotin caused adverse effects, the present study investigated the acute, but not chronic, effects of excess biotin intake on the body weight gain, food intake, tissue weight, tissue biotin content and urinary excretion of water-soluble vitamins, and not the histopathology nor production toxicity. Furthermore, the results of the present study were obtained from a limited number of animals, four rats in each group. A further study is needed to set more accurate NOAEL and LOAEL.

A single oral administration of 20 mg of biotin or 4.5 mg intravenously to healthy adults caused no adverse effect.[37] An oral intake of 1.2 mg/day of biotin by healthy adults for 14 days also did not cause any adverse effect.[38] Since the data on adverse effects from a high biotin intake are not sufficient for a quantitative risk assessment, UL for biotin has not been derived in USA and Japan.[15,39] The data from human studies plausibly show the low risk of several mg of biotin intake, but our