我々は，女子学生にビタミンフリーの半精製食と遊離型ビタミン混合を7日間連続して与えたときの尿中水溶性ビタミン排泄量を報告した[13]．このときのチアミンの平均排泄率は19%であり，本研究の遊離型チアミンの排泄率とほぼ同じ値を示した．従って，本実験で得られた遊離型チアミン付加による尿中チアミン排泄量の増大は，付加した遊離型チアミン由来であると考えられる．

生細胞中のビタミン$B_1$のほとんどは補酵素型のチアミン二リン酸（TDP）として，酵素タンパク質と結合した状態で存在している．生細胞を加工して食する状態になったときに何%が遊離型となっているかは不明であるが，補酵素型の状態であるものは多く存在することが推定される．従って，吸収される前に遊離型への消化が必要である．つまり，食品中のビタミン$B_1$がどの程度消化されるかによって，生体が利用できるビタミン$B_1$の量を吸収できるかが決まる．酵素タンパク質から遊離したTDPは消化管内ではホスファターゼによりピロリン酸がはずれ，遊離型のチアミンとなったのち，吸収されるものと推定されている．しかしながら，この生体利用率を網羅的に検討した報告はみあたらない．

(2) ビタミン$B_2$

規定食摂取時のリボフラビン排泄率は12.2±3.2%（平均値±SD），遊離型リボフラビンの排泄率は19.4±4.8%，遊離型リボフラビンに対する規定食中のビタミン$B_2$の相対利用率は64±16%であった．相対利用率の最小値は41%，最大値は90%であった．

女子学生にビタミンフリーの半精製食と遊離型ビタミン混合を7日間連続して与えたときのリボフラビンの平均排泄率は22%であり，本研究の遊離型チアミンの排泄率とほぼ同じ値を示した[14]．

生細胞中のリボフラビンは，フラビンアデニンジヌクレオチド（FAD）あるいはフラビンモノヌクレオチド（FMN）として補酵素タンパク質に結合している．生細胞を加工して食する状態になったときに何%が遊離型となっているかは不明であるが，補酵素型の状態であるものは多く存在することが推定される．食品中の総リボフラビン含量を測定するときには，食品抽出液を光照射することによってリボフラビン，FMN，FADをルミフラビンに分解し，ルミフラビン量を測定する．実際の消化管内では，胃酸環境下で，酵素タンパク質の変性にともないFADは遊離するものと予想される．一部のFADはリン酸がとれてFMNとなるが，脱リン酸は小腸粘膜の非特異的ピロホスホリラーゼやホスファターゼにより小腸腔内でFMNを経由し加水分解され，遊離のリボフラビンとなり吸収されるものと考えられる．生体利用率については，特に参考となる資料はみあたらない．

(3) ビタミン$B_6$

規定食摂取時のビタミン$B_6$代謝産物4-PICの排泄率は55.3±6.6%（平均値±SD），遊離型ピリドキシンの排泄率は76.4±4.5%，遊離型ピリドキシンに対する規定食中のビタミン$B_6$の相対利用率は73±5%であった．相対利用率の最小値は66%，最大値は80%であった．

女子学生にビタミンフリーの半精製食と遊離型ビタミン混合を7日間連続して与えたときのビタミン$B_6$に関するデータはないため[14]，本研究の遊離型ピリドキシンの排泄率との比較はできなかった．

動物の生細胞中に含まれるビタミン$B_6$の多くは，リン酸化体であるピリドキサルリン酸（PLP）やピリドキサミンリン酸（PMP）である．この生細胞を加工して食する状態になったときに何%が遊離型となっているかは不明であるが，補酵素型の状態であるものは多く存在することが推定される．食品中の総ビタミン$B_6$量を測定するときには，塩酸酸性下で3時間，オートクレーブすることにより，PLPやPMPのリン酸基を切断して遊離型にし，遊離型の量を測定する．実際の消化管内では，これらは，小腸粘膜のホスファターゼにより遊離のピリドキサル（PL），ピリドキサミン（PM）になるものと考えられている．一方，植物に含まれるピリドキシン5′$\beta$-グルコシド（PNG）は，消化管内で一部が加水分解を受け，ピリドキシン（PN）を遊離する[2]．これら遊離のビタミン$B_6$は吸収された後PLキナーゼによりリン酸化型に変換されるピリドキシンリン酸（PNP）と，PMPはさらにPNP/PMPオキシダーゼによりPLPに変換される．PLPは血液中では，アルブミンに結合しておりホスファターゼによる脱リン酸化を免れているが余剰分については脱リン酸化を受けPLとなる．PLはアルデヒドオキシダーゼにより4-PICに変換される．4-PICはビタミン$B_6$効力を持たず，排泄されるのみである．PNGの生体利用率は，ヒトにおいては50%と見積もられている[2]．平均的な米国での食事におけるビタミン$B_6$の生体利用率は75%と報告されており，この値が一般的な生体利用率とされている[15]．本研究で得られた73±5%という値は米国での報告に近いもので

あった.

(4) ナイアシン

規定食摂取時の総ニコチンアミド代謝産物排泄率は37.9±4.8% (平均値±SD), 遊離型ニコチンアミドの排泄率は60.9±21.4%, 遊離型ニコチンアミドに対する規定食中のナイアシン当量の相対利用率は67±19%であった. 相対利用率の最小値は35%, 最大値は80%であった.

女子学生にビタミンフリーの半精製食と遊離型ビタミン混合を7日間連続して与えたときの総ニコチンアミド代謝産物の平均排泄率は79%であるが, これはナイアシンの供給源のほとんどは半精製食に含まれるトリプトファンに由来するものである[14]. 本研究の遊離型ニコチンアミドの排泄率に近い値を示したが, 摂取形態が異なるため直接比較はできない.

ナイアシンは生細胞内では主に補酵素型のNAD (P) として存在するが, 細胞の死にともない, 急激な速度で分解される. 食品として摂取するときにはNAD (P) が分解され, 動物性食品ではニコチンアミド, 植物性食品ではニコチン酸として存在する. 食品中の総ナイアシンを測定するときには, 食品抽出液を中性条件で10分間, オートクレーブしてニコチンアミドにし, ニコチンアミド量を測定する. 実際の消化管内では, 食品中にNAD (P) が残っていたとしてもニコチンアミドに分解される. ニコチンアミド, ニコチン酸は小腸で受動拡散によって吸収される. 植物性食品中のナイアシンの多くは難消化性の結合型ナイアシンとして存在し, 生体利用率が低いことが報告されている[4].

(5) パントテン酸

規定食摂取時のパントテン酸排泄率は34.4±4.8% (平均値±SD), 遊離型パントテン酸の排泄率は50.9±10.9%, 遊離型パントテン酸に対する規定食中のパントテン酸の相対利用率は69±11%であった. 相対利用率の最小値は51%, 最大値は78%であった.

女子学生にビタミンフリーの半精製食と遊離型ビタミン混合を7日間連続して与えたときのパントテン酸の平均排泄率は73%であり, 本研究の遊離型パントテン酸の排泄率の1.4倍と近い値を示した[14].

生細胞中のパントテン酸の存在形態は遊離型のパントテン酸よりコエンザイムA (CoA) やパンテテイン誘導体のような補酵素型が多い. 従って, 食事として摂取するパントテン酸は, 主としてCoAやパンテテイン誘導体の形が多い. 食品中の総パントテン酸を測定するときには, 食品抽出液をホスファターゼ・パンテテイナーゼ処理を行ったのち, 測定する. 実際の消化管内でも, ホスファターゼとパンテテイナーゼによって, パントテン酸に加水分解された後吸収される. 脂質のエネルギー比は40%であるが, 米国における一般的な食事でのパントテン酸の生体利用率は50%程度と報告されている[15]. しかしながら, 日本食におけるパントテン酸の生体利用率に関する報告がみあたらない. 本研究で得られた69±11%という値は米国における値よりも高い値であった.

個々の食品中に含まれるビタミンの生体利用性を調べた論文を文献検索すると, 動物性食品に含まれるB群ビタミンの利用性は高いが, 植物性食品は低い, というものが検索されてくる. たとえば, トウモロコシの「ぬか」に含まれるナイアシン, チアミン, パントテン酸の利用性が「ぬか」の製粉状態により異なる[16], 食物繊維の存在はビタミン$B_6$の吸収を阻害する[17], ヨーグルトの摂取はチアミン, リボフラビン, ビタミン$B_6$栄養状態を低下させる傾向がある[18], 牛乳の摂取が食品中の葉酸の消化・吸収率を高める[19], 植物食品中にはビタミンと糖類が結合したもの[1)20)], あるいはタンパク質と結合したもの[21]が報告されており, これらの結合型ビタミンは消化されにくいため, 吸収が悪いことが報告されている[22)23)]. さらに, まぐろ, パン, ピーナッツバター中のビタミン$B_6$の栄養有効性を相対的に求めた結果, まぐろ中のビタミン$B_6$が他の食品よりも高かったという報告もある[24].

しかしながら, これらの論文は, 著者らが知りたい「習慣的に食べている1日食事由来のビタミンがどの程度我々の体で消化・吸収され, かつ体内で利用されているか」に関する情報, すなわち生体利用率を定量的に与えてくれない. いずれも, 定性的な現象を報告しているにすぎない. 知りたい情報は, 1日に食べた食事由来のビタミンが, ① 消化, ② 吸収, ③ 臓器・組織中の細胞内への輸送, ④ 補酵素への合成, ⑤ アポ酵素との結合, ⑥ ホロ酵素機能を発揮, ⑦ ホロ酵素終末, ⑧ 補酵素の分解, ⑨ 遊離ビタミンの異化, ⑩ 尿中への排泄の各過程で, ともに摂取した食品成分がどのような影響を与え, 最終的に摂取したビタミンの何%が生体内で機能を発揮することができたのかである. これらの過程を一つずつ調べる技術はない. そこで, 現実的な生体利用率を求めるために, 遊離型のビタミンの利用率を100%とし, 食事に含まれるビタミンの利用率を相対利用率として求めることを本論

文では提案した．すなわち，規定食摂取時の尿中ビタミン排泄量と規定食中のビタミン含量を用い，規定食摂取時の尿中ビタミン排泄率を求める．規定食摂取時とビタミン混合付加時の尿中ビタミン排泄量の差から，遊離型ビタミン付加による増大量を求める．この排泄量の増大と付加した遊離型ビタミン量を用い，遊離型ビタミンの尿中ビタミン排泄率を求める．遊離型ビタミンの尿中排泄率に対する規定食摂取時の尿中ビタミン排泄率の相対比を求め，これを規定食に含まれるビタミンの相対利用率とするものである．B群ビタミン供給源として遊離型ビタミンのみを摂取したときの遊離型ビタミン排泄率と本研究の遊離型ビタミン排泄率はほぼ同じ値を示したことから[14]，本研究の遊離型ビタミン付加による尿中排泄量の増大は付加した遊離型ビタミン由来であると考えることは妥当である．以上のことから，本論文で提案する相対利用率の決定法は簡便な方法として利用されることが期待される．

## 4. 結　論

日本人女子学生が一般的な食事を摂取したときのビタミン$B_1$の相対利用率は，67±20%（平均値±SD，$n$=6），ビタミン$B_2$は64±16%，ビタミン$B_6$は73±5%，ナイアシンは67±19%，パントテン酸は69±11%であった．

本研究は，平成16年度～18年度厚生労働科学研究費補助金・循環器疾患等総合研究事業・日本人の食事摂取基準（栄養所要量）の策定に関する研究（主任研究者　柴田克己）を受けて行ったものである．関係各位に謝意を表する．

## 引　用　文　献


1) Tadera, K., Kaneko, T., and Yagi, F.: Isolation and Structural Elucidation of Three New Pyridoxine-Glycosides in Rice Bran, *J. Nutr. Sci. Vitaminol.*, **34**, 167-177 (1988)
2) Reynolds, R. D.: Bioavailability of Vitamin B-6 from Plant Foods, *Am. J. Clin. Nutr.*, **48**, 863-867 (1988)
3) Mason, J. B., and Kodicek, E.: The Metabolism of Niacytin in the Rat. Trigonelline as a Major Metabolite of Niacytin in the Urine, *Biochem. J.*, **120**, 515-521 (1970)
4) Das, M. L., and Guha, B. C.: Isolation and Chemical Characterization of Bound Niacin (Niacinogen) in Cereal Grains, *J. Biol. Chem.*, **235**, 2971-2976 (1960)
5) Food and Nutrition Board, Institute of Medicine: *Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin $B_6$, Folate, Vitamin $B_{12}$, Pantothenic Acid, Biotin, and Choline*, National Academy Press, Washington DC, 1-40 (1998)
6) 厚生労働省：『日本人の食事摂取基準（2005年版）』，日本人の栄養所要量—食事摂取基準—策定検討会報告書（2004）
7) 科学技術庁資源調査会（編）：『日本食品成分表の改定に関する調査報告—五訂日本標準食品成分表—』（2000）
8) Kimura, M., Fujita, T., and Itokawa, Y.: Liquid Chromatographic Determination of the Total Thiamin Content of Blood, *Clin. Chem.*, **28**, 29-31 (1982)
9) Ohkawa, H., Ohishi, N., and Yagi, K.: New Metabolites of Riboflavin Appear in Human Urine, *J. Biol. Chem.*, **258**, 5623-5628 (1983)
10) Gregory, J. F. Ⅲ, and Kirk, J. R.: Determination of Urinary 4-pyridoxic Acid Using High Performance Liquid Chromatography, *Am. J. Clin. Nutr.*, **32**, 879-883 (1979)
11) Shibata, K., Kawada, T., and Iwai, K.: Simultaneous Micro-Determination of Nicotinamide and Its Major Metabolites, $N^1$-Methyl-2-pyridone-5-carboxamide and $N^1$-Methyl-3-pyridone-4-carboxamide, by High-Performance Liquid Chromatography, *J. Chromatogr.*, **424**, 23-28 (1988)
12) Shibata, K.: Ultramicro-Determination of $N^1$-Methylnicotinamide in Urine by High-Performance Liquid Chromatography, *Vitamins*, **61**, 599-604 (1987)
13) Skeggs, H. R., and Wright, L. D.: The Use of *Lactobacillus arabinosus* in the Microbiological Determination of Pantothenic Acid, *J. Biol. Chem.*, **156**, 21-26 (1944)
14) Shibata, K., Fukuwatari, T., Ohta, M., Okamoto, H., Watanabe, T., Fukui, T., Nishimuta, M., Totani, M., Kimura, M., Ohishi, N., Nakashima, M., Watanabe, F., Miyamoto, E., Shigeoka, S., Takeda, T., Murakami, M., Ihara, H., and Hashizume, N.: Values of Water-Soluble Vitamins in Blood and Urine of Japanese Young Men and Women Consuming a Semi-Purified Diet Based on the Japanese Dietary Reference Intakes, *J. Nutr. Sci, Vitaminol.*, **51**, 319-328 (2005)
15) Tarr, J. B., Tamura, T., and Stokstad, E. L. R.: Availability of Vitamin $B_6$ and Pantothenate in an Average American Diet in Man, *Am. J. Clin. Nutr.*, **34**, 1328-1337 (1981)
16) Yu, B. H., and Kies, C.: Niacin, Thiamin, and Pantothenic Acid Bioavailability to Humans from Maize Bran as Affected by Milling and Particle Size, *Plant Foods Hum. Nutr.*, **43**, 87-95 (1993)
17) Reynold, R. D.: Bioavailability of Vitamin B-6 from Plant Foods, *Am. J. Clin. Nutr.*, **48**, 863-867 (1988)
18) Elmadfa, I., Heinzle, C., Majchrzak, D., and Foissy, H.: Influence of a Probiotic Yoghurt on the Status of Vitamins $B_1$, $B_2$ and $B_6$ in the Healthy Adult Human,

*An. Nutr, Met.*, **45**, 13-18 (2001)
19) Picciano, M. F., West. S. G., Ruch, A. L., Kris-Etherton, P. M., Zhao, G., Johnson, K. E., Maddox, D. H., Fishell, V. K., Dirienzo, D. B., and Tamura, T.: Effect of Cow Milk on Food Folate Bioavailability in Young Women, *Am. J. Clin. Nutr.*, **80**, 1565-1569 (2004)
20) Kodicek, E., and Wilson, P. W.: The Isolation of Niacytin, the Bound Form of Nicotinic Acid, *Biochem. J.*, **76**, 27-28 (1960)
21) Das, M. L., and Guha, B. C.: Isolation and Chemical Characterization of Bound Niacin (Niacinogen) in Cereal Grains, *J. Biol. Chem.*, **235**, 2971-2976 (1960)
22) Carter, E. G. A., and Carpenter, K. J.: The Bioavailability for Humans of Bound Niacin from Wheat Bran, *Am. J. Clin. Nutr.*, **36**, 855-861 (1982)
23) Gregory, J, F. Ⅲ, Trumbo, P. R., Bailey, L. B., Toth, J. P., Baumgartner, T. G., and Cerda, J. J.: Bioavailability of Pyridoxine-5′-β-D-glucoside Determined in Humans by Stable-Isotopic Methods, *J. Nutr.*, **121**, 177-186 (1991)
24) Kabir, H., Leklem, J. E., and Miller, L. T.: Comparative Vitamin B-6 Bioavailability from Tuna, Whole Wheat Bread and Peanut Butter in Humans, *J. Nutr.*, **113**, 2412-2420 (1989)

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# Urinary Water-Soluble Vitamins and Their Metabolite Contents as Nutritional Markers for Evaluating Vitamin Intakes in Young Japanese Women

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***Summary*** Little information is available to estimate water-soluble vitamin intakes from urinary vitamins and their metabolite contents as possible nutritional markers. Determination of the relationships between the oral dose and urinary excretion of water-soluble vitamins in human subjects contributes to finding valid nutrition markers of water-soluble vitamin intakes. Six female Japanese college students were given a standard Japanese diet in the first week, the same diet with a synthesized water-soluble vitamin mixture as a diet with approximately onefold vitamin mixture based on Dietary Reference Intakes (DRIs) for Japanese in the second week, with a threefold vitamin mixture in the third week, and a sixfold mixture in the fourth week. Water-soluble vitamins and their metabolites were measured in the 24-h urine collected each week. All urinary vitamins and their metabolite levels except vitamin $B_{12}$ increased linearly in a dose-dependent manner, and highly correlated with vitamin intake ($r$=0.959 for vitamin $B_1$, $r$=0.927 for vitamin $B_2$, $r$=0.965 for vitamin $B_6$, $r$=0.957 for niacin, $r$=0.934 for pantothenic acid, $r$=0.907 for folic acid, $r$=0.962 for biotin, and $r$=0.952 for vitamin C). These results suggest that measuring urinary water-soluble vitamins and their metabolite levels can be used as good nutritional markers for assessing vitamin intakes.

***Key Words*** biomarker, human, urine, vitamin

A nutritional marker can be an indicator of nutritional status with respect to intake or metabolism of dietary constituents. Nutritional markers can be designated into one or more of three categories, 1) a means of validation of dietary instruments, 2) surrogate indicators of dietary intakes, or 3) integrated measures of nutritional status for a nutrient (*1*). Nutritional markers may be interpreted more broadly as a biological consequence of dietary intake or dietary patterns, and contribute to setting recommendations, tolerable levels and guidelines. Recent validation studies have developed the urinary compounds as nutritional markers to estimate nutrient intakes. For example, 24-h urinary nitrogen has been established as a marker for protein intake (*2*), the same as urinary potassium for energy and potassium intake (*3*), and urinary sugars for sugar intake (*4*).

Water-soluble vitamins are absorbed from the digestive tract after ingestion, stored in the liver, delivered to peripheral sites and then excreted to urine. Urinary water-soluble vitamins or their metabolites decrease markedly as vitamin status declines, and they are affected by recent dietary intake. Urinary excretion of water-soluble vitamins such as thiamin, riboflavin and niacin has been used for setting Dietary Reference Intakes (DRIs) in the USA and Japan (*5*, *6*). However, only a single study investigated urinary vitamins as a possible marker for intake. Individuals' 30-d means of thiamin intake are highly correlated with their mean 24-h urine thiamin levels under strictly controlled conditions, showing 24-h urinary thiamin as a useful marker for thiamin intake under strictly controlled conditions (*7*). Although pharmacological doses of water-soluble vitamin intake such as vitamin $B_2$ (*8*), nicotinamide (*9*) and biotin (*10*) dramatically increase urinary vitamin levels, few studies have investigated the relationship between several oral doses and dietary intake and urinary excretion of vitamin C, to the best of our knowledge (*11*, *12*).

To determine whether urinary levels of water-soluble vitamins and their metabolites can be used as possible markers for estimating their intakes, six female Japanese college students were given a standard Japanese diet with or without a 1-, 3- and 6-fold vitamin mixture based on Dietary Reference Intakes (DRIs) for Japanese. The 24-h urinary excretion of water-soluble vitamins and their metabolites was measured, and the relationships between vitamin oral dose and urinary excretion were determined. This is the first report clearly to show that 24-h urinary vitamins and their metabolite levels were correlated to their intakes, and can be used as nutritional markers for their intakes.

## SUBJECTS AND METHODS

*Subjects.* Six healthy female Japanese college students participated in the present experiment. They did not have regular use of medications or dietary supple-

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Table 1. The composition of the diets.

| | Diet 1 | Diet 2 | Average | RDA[3] |
|---|---|---|---|---|
| Energy (kcal) | 1,708 | 1,618 | 1,663 | 1,750 |
| Protein (g) | 68.5 | 61.5 | 65 | 50 |
| Fat (g) | 50.8 | 45.1 | 48.0 | 40–50 |
| Carbohydrates (g) | 236 | 237 | 237 | — |
| Water-soluble vitamins[1] | | | | |
| Vitamin $B_1$ (mg as thiamin) | 0.59 | 0.46 | 0.53 (2.0 μmol) | 0.74 |
| Vitamin $B_2$ (mg as riboflavin) | 0.92 | 0.82 | 0.87 (2.3 μmol) | 1.05 |
| Vitamin $B_6$ (mg as pyridoxine) | 1.24 | 0.86 | 1.05 (6.2 μmol) | 1.15 |
| Vitamin $B_{12}$ (μg as cyanocobalamin) | 7.4 | 11.3 | 2.4 (1.77 nmol) | 2.4 |
| Niacin equivalent[2] (mg) | 30.4 | 24.8 | 27.6 (226 μmol) | 10.2 |
| Pantothenic acid (mg) | 9.3 | 9.3 | 9.3 (42 μmol) | 5 |
| Folates (μg as pteroyl monoglutamic acid) | 230 | 282 | 256 (0.58 μmol) | 200 |
| Biotin (μg) | 67 | 53 | 60 (246 nmol) | 30 |
| Vitamin C (mg as L-ascorbic acid) | 118 | 112 | 115 (0.65 mmol) | 100 |

[1] Water-soluble vitamins except for vitamin $B_{12}$ are measured. Other nutrients are calculated by using the Standard Tables of Food Composition in Japan (*15*).
[2] The niacin equivalent intake was calculated as follows: the average tryptophan content in food protein is 1.1% and the 1/60 (on a weight basis) of tryptophan taken was converted into niacin in the body.
[3] The Recommended Dietary Allowance (RDA) for vitamin $B_1$ is 0.42 mg/1,000 kcal as thiamin, vitamin $B_2$ is 0.60 mg/1,000 kcal, vitamin $B_6$ is 0.023 mg/g protein, niacin is 5.8 mg NE/1,000 kcal, folic acid is 240 μg/d and vitamin C is 100 mg/d for Japanese adults, and the Adequate Intake for pantothenic acid is 5 mg/d and biotin is 45 μg/d for Japanese adult women (*6*).
The subjects consumed Diet 1 on days 1 and 3 each week, and Diet 2 on days 2 and 4.

ments, or habitual alcohol or cigarette consumption. Their age, body weight, height and body mass index (mean±SD) are 21.0±0.0 y old, 161.7±1.7 cm, 51.2±2.8 kg and 19.6±1.2, respectively. This study was reviewed and approved by The Ethical Committee of the National Institute of Health and Nutrition (Tokyo, Japan).

*Chemicals.* Thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, nicotinamide, calcium pantothenate, pteroylmonoglutamic acid (folic acid), D(+)-biotin, L(+)-ascorbic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-Pyridoxic acid (4-PIC) was manufactured by ICN Pharmaceuticals (Costa Mesa, CA, USA) and obtained through Wako Pure Chemical Industries. $N^1$-Methylnicotinamide (MNA) chloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). $N^1$-Methyl-2-pyridone-5-carboxamide (2-Py) and $N^1$-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized (*13*, *14*). All the other chemicals used were of the highest purity available from commercial sources.

*Diet.* Two kinds of meals were given to the subjects. Diet 1 consisted of bread, margarine, ham, tomato, jelly and milk as breakfast; rice, miso-soup, Hamburg steak, cabbage, boiled spinach and Japanese tea as lunch; and rice, raw skipjack, laver, pan-fried vegetables and Japanese tea as dinner. Diet 2 consisted of bread, margarine, ham, tomato, jelly and milk as breakfast; rice, miso-soup, broiled chicken, cabbage, simmered hijiki and Japanese tea as lunch; and rice, raw scallop, laver, pan-fried vegetables and Japanese tea as dinner. The nutrient elements are shown in Table 1. The subjects consumed Diet 1 or days 4 and 6 in each week, and Diet 2 or days 5 and 7. Water-soluble vitamins, except for vitamin $B_{12}$, in the diets were measured by the procedures described in *Determination of vitamins and their metabolites in urine and diets*. Other nutrients were calculated by using the Standard Tables of Food Composition in Japan (*15*).

*Experimental design.* The subjects took the diet freely on days 1 to 3, and took the diet shown in Table 2 on days 4 to 7 in each week. Approximately 1, 3- and 6-fold of the synthesized water-soluble vitamin mixture as vitamin mixture A, B and C shown in Dietary Reference Intakes for Japanese, 2005, were made (Table 2) (*6*). The subjects did not take any vitamin mixture in the first week, and then took the vitamin mixture A in the second week, the vitamin mixture B in the third week, and the vitamin mixture C in the fourth week. One third of the dose was put into a small gelatinous cap-

Table 2. The vitamin contents in the vitamin mixtures for 3 capsules per day.

| | V. mix. A | V. mix. B | V. mix. C |
|---|---|---|---|
| Thiamin | 0.56 mg/d (2.1 μmol/d) | 1.78 mg/d (6.7 μmol/d) | 3.89 mg/d (14.7 μmol/d) |
| Riboflavin | 0.92 mg/d (2.4 μmol/d) | 2.95 mg/d (7.8 μmol/d) | 5.74 mg/d (15.3 μmol/d) |
| Pyridoxine | 0.96 mg/d (5.7 μmol/d) | 3.21 mg/d (19.0 μmol/d) | 6.61 mg/d (39.1 μmol/d) |
| Nicotinamide | 9.2 mg/d (75 μmol/d) | 36.4 mg/d (298 μmol/d) | 67.4 mg/d (552 μmol/d) |
| Pantothenic acid | 4.8 mg/d (22 μmol/d) | 15.0 mg/d (68 μmol/d) | 31.4 mg/d (143 μmol/d) |
| Pteroylmonoglutamic acid | 205 μg/d (0.46 μmol/d) | 530 μg/d (1.20 μmol/d) | 1,340 μg/d (3.04 μmol/d) |
| Biotin | 26 μg/d (107 nmol/d) | 84 μg/d (344 nmol/d) | 182 μg/d (746 nmol/d) |
| L-Ascorbic acid | 98 mg/d (0.56 mmol/d) | 296 mg/d (1.68 mmol/d) | 600 mg/d (3.41 mmol/d) |

sule, and the capsule was administered three times daily after breakfast, lunch and dinner. The 24-h urine samples were collected from the second urinary sample on the last day to the first sample on the next day in each week. The urine sample volumes were measured, and the samples were immediately treated as described below, to avoid destruction of water-soluble vitamins and their metabolites, and then stored at −20°C until needed.

*Determination of vitamins and their metabolites in urine and diets.* For analysis of urinary thiamin, riboflavin, 4-PIC, MNA, 2-Py and 4-Py, 1 mL of 1 mol/L HCl was added to 9 mL urine. For analysis of urinary pantothenic acid and biotin, urine samples were not treated. For analysis of urinary folic acid, 1 mL of 1 mol/L-ascorbic acid was added to 9 mL urine. For analysis of urinary ascorbic acid, 4 mL of 10% metaphosphate was added to 4 mL urine. Urinary thiamin was determined by the HPLC-post labeled fluorescence method (*16*). Urinary riboflavin was determined by the HPLC method (*17*). Urinary 4-PIC was determined by the HPLC method (*18*). Urinary 2-Py, 4-Py and MNA, nicotinamide metabolites, were determined by the HPLC method (*13*, *19*). Urinary pantothenic acid was determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014 (*20*). Urinary folic acid was determined by the microbioassay method using *Lactobacillus casei* ATCC 2733 (*21*). Urinary biotin was determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014 (*22*). Urinary reduced and oxidized ascorbic acid, and 2,3-diketogluconic acid, were determined by the HPLC method (*23*).

For analysis of water-soluble vitamins in the diets, Diet 1 and 2 were homogenized in water. Vitamin $B_1$ as sum of thiamin, TMP, TDP and TTP in the diets was determined by the HPLC-post labeled fluorescence method (*16*). Riboflavin, FMN and FAD in the diets were converted to lumiflavin by photolysis, and then determined by the HPLC method (*17*). Vitamin $B_6$ vitamer in the diets was converted to pyridoxine by autoclave under acidic condition, and total pyridoxine was determined by the microbioassay method using *Saccharomyces carlsbergenisis* strain 4228 ATCC 9080 (*24*). NAD and NADP in the diets were converted to nicotinamide by autoclave, and total nicotinamide was determined by the HPLC method (*13*). Bound pantothenic acid such as CoA and pantetheine in the diets was digested to free form by alkaline phophatase and pigeon liver amidase, and total pantothenic acid was determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014 (*20*). Folates in the diets were digested to pteroylmonoglutamic acid by conjugase and protease, and pteroylmonoglutamic acid as total folic acid was determined by the microbioassay method using *Lactobacillus casei* ATCC 2733 (*21*). Bound biotin in the diet was converted to free form by autoclave under acidic conditions, and total biotin was determined by the microbioassay method using *Lactobacillus plantarum* ATCC 8014 (*22*). Reduced and oxidized ascorbic acid, and 2,3-diketogluconic acid in the diets were determined by the HPLC method (*23*).

*Statistical analysis.* Linear regression analysis was carried out using a computer program, GraphPad Prism version 4.03 (GraphPad Software, Inc., San Diego, CA 92130, USA). Correlation coefficients were calculated using the method of Pearson product-moment correlation coefficient. The significance of the linear correlation coefficient was tested using Fisher's transformation test.

## RESULTS

*Vitamin $B_1$*

The urinary excretion of thiamin in the first week was 0.288±0.074 μmol/d to 0.53 mg/d (2.0 μmol/d) of thiamin intake (mean±SD, $n=6$), and the level increased linearly until the fourth week taking 4.42 mg/d (22.4 μmol/d) (Fig. 1A). The correlation between urinary and oral thiamin was significantly high ($y=0.281x-0.514$, $r=0.959$; $p<0.0001$). The urinary recovery of thiamin (mean±SD, $n=6$) was

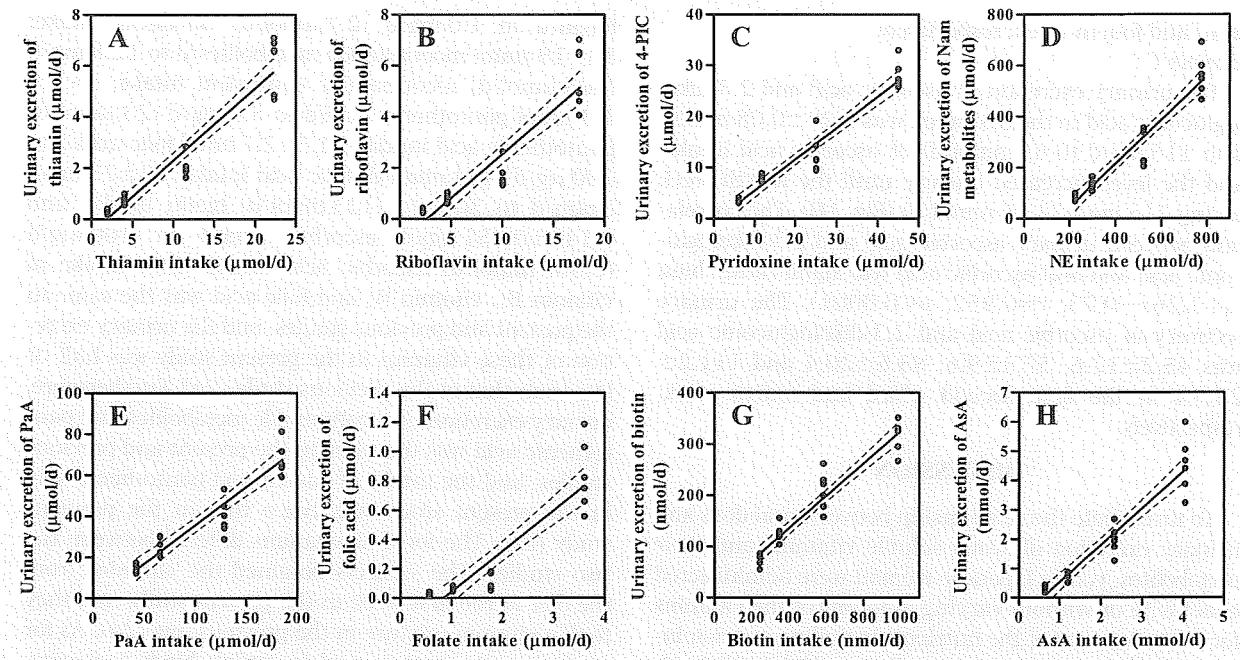


Fig. 1. Regression and 95% CI of oral dose and urinary excretion of vitamin $B_1$ (A), vitamin $B_2$ (B), vitamin $B_6$ (C), niacin (D), pantothenic acid (E), folate (F), biotin (G) and vitamin C (H). Values are individual points of six subjects in each dose. 4-PIC signifies 4-pyridoxic acid, a catabolite of vitamin $B_6$ vitamers, and the Nam metabolites signify the total amount of nicotinamaide metabolites, MNA, 2-Py and 4-Py.

14.4±3.7, 19.0±4.3, 17.6±3.6 and 27.2±4.7% in the first, second, third and fourth week, respectively.

*Vitamin $B_2$*

The urinary excretion of riboflavin in the first week was 0.283±0.073 μmol/d to 0.87 mg/d (2.3 μmol/d) of riboflavin intake, and the level increased linearly until the fourth week taking 6.61 mg/d (17.6 μmol/d) (Fig. 1B). The correlation between urinary and oral riboflavin was significantly high ($y=0.342x-0.901$, $r=0.926$; $p<0.0001$). The urinary recovery of riboflavin was 12.3±3.2, 16.1±3.5, 16.4±5.0 and 31.6±6.9% in the first, second, third and fourth week, respectively.

*Vitamin $B_6$*

The urinary excretion of 4-PIC, a metabolite of vitamin $B_6$, in the first week was 3.44±0.41 μmol/d to 1.05 mg/d (6.2 μmol/d) of pyridoxine intake, and the level increased linearly until the fourth week taking 7.66 mg/d (45.2 μmol/d) (Fig. 1C). The correlation between urinary 4-PIC and oral pyridoxine was significantly high ($y=0.611x-0.59$, $r=0.966$; $p<0.0001$). The urinary recovery of 4-PIC was 55.4±6.6, 65.1±5.5, 49.9±14.3 and 61.9±5.9% in the first, second, third and fourth week, respectively.

*Niacin*

The urinary excretion of nicotinamide metabolites in the first week was 85.6±10.8 μmol/d to 27.6 mg niacin equivalents (NE)/d (226 μmol/d) of niacin intake, and the level increased linearly until the fourth week taking 95.0 mg NE/d (779 μmol/d) (Fig. 1D). The correlation between urinary nicotinamide metabolites and oral niacin was significantly high ($y=0.852x-125.9$, $r=0.957$; $p<0.0001$). The urinary recovery of nicotinamide metabolites was 37.9±4.8, 43.6±6.2, 53.4±13.6 and 71.9±10.1% in the first, second, third and fourth week, respectively.

*Pantothenic acid*

The urinary excretion of pantothenic acid in the first week was 14.6±2.0 μmol/d to 9.3 mg/d (42 μmol/d) of pantothenic acid intake, and the level increased linearly until the fourth week taking 40.7 mg/d (186 μmol/d) (Fig. 1E). The correlation between urinary and oral pantothenic acid was significantly high ($y=0.378x-1.6$, $r=0.951$; $p<0.0001$). The urinary recovery of pantothenic acid was 34.4±4.8, 39.1±6.1, 30.5±6.7 and 38.4±5.9% in the first, second, third and fourth week, respectively.

*Folate*

The urinary excretion of folic acid in the first week was 0.022±0.009 μmol/d to 256 μg/d (0.58 μmol/d) of folate intake, and the level increased linearly until the fourth week taking 1.60 mg/d (3.62 μmol/d) (Fig. 1F). The correlation between urinary folic acid and oral folate was significantly high ($y=0.277x-0.235$, $r=0.907$; $p<0.0001$). The urinary recovery of folic acid was 3.8±1.5, 5.1±1.5, 5.5±3.3 and 22.9±6.5% in the first, second, third and fourth week, respectively.

*Biotin*

The urinary excretion of biotin in the first week was 74.5±12.0 nmol/d to 60 μg/d (246 nmol/d) of biotin intake, and the level increased linearly until the fourth week taking 242 μg/d (990 nmol/d) (Fig. 1G). The correlation between urinary and oral biotin was significantly high ($y=0.316x+8.2$, $r=0.962$; $p<0.0001$). The urinary recovery of biotin was 30.3±4.9, 35.6±4.8, 35.1±6.4 and 31.8±3.0% in the first, second,

third and fourth week, respectively.

*Vitamin C*

The urinary excretion of ascorbic acid and 2,3-diketogluconic acid in the first week was 0.29±0.08 mmol/d to 115 mg/d (0.65 mmol/d) of ascorbic acid intake, and the level increased linearly until the fourth week taking 715 mg/d (4.06 mmol/d) (Fig. 1H). The correlation between urinary ascorbic acid and 2,3-diketogluconic acid and oral ascorbic acid was significantly high ($y=1.26x-0.73$, $r=0.952$; $p<0.0001$). The urinary recovery of ascorbic acid and 2,3-diketogluconic acid was 45.2±12.6, 57.3±9.6, 83.6±20.4 and 111.2±23.5% in the first, second, third and fourth week, respectively.

## DISCUSSION

To investigate the relationship between oral dose and urinary excretion of water-soluble vitamins and their metabolites, young Japanese women were administered a diet with or without varying amounts of the vitamins for 1 wk. Amount of the nutrients including water-soluble vitamins in the diets were close to RDA in DRIs (*5*, *6*) and previous dietary assessment in free-living Japanese young women (*25*). The concentrations of all eight water-soluble vitamins and their metabolites in 24-h urine samples increased linearly in a dose-dependent manner, and strongly correlated with their intakes. These findings show that water-soluble vitamins and their metabolite levels in 24-h urine reflect the vitamin intakes under strictly controlled conditions, and suggest that vitamin intakes can be estimated from 24-h urinary vitamins and their metabolite contents.

In the present study, the correlations between urinary levels and their intakes for vitamin $B_2$ and folic acid were lower than those for other vitamins tested. The urinary riboflavin level linearly increased in a dose-dependent manner at 0.87 to 3.82 mg (2.3 to 10.1 μmol) vitamin $B_2$ intake, and then the level dramatically increased when the subjects took 6.61 mg (17.6 μmol) vitamin $B_2$. The urinary folic acid contents also showed a similar pattern to riboflavin: the contents linearly increased at 0.256 to 0.786 mg (0.58 to 1.78 μmol) folate intakes, and then dramatically increased at 1.60 mg (3.62 μmol) intake. The urinary vitamin levels may be affected by several factors such as absorption in the digestive tract, storage in the tissue, energy expenditure, tissue turnover and reabsorption in the kidney. However, no report has disclosed whether these factors change the urinary excretions of vitamins when humans take vitamins at the range used in the present study. Investigation of relationships for oral dose to urinary, blood and stored vitamin levels may explain what the dramatic increases in urinary rivoflabin and folic acid mean.

We previously reported the levels of water-soluble vitamins and their metabolites in 24-h urine samples from young Japanese women consuming a semi-purified diet with a vitamin mixture for 7 d (*26*). The levels were 0.665±0.114 μmol thiamin/d to 0.71 mg/d (2.7 μmol/d) thiamin intake; 0.580±0.145 μmol riboflavin/d to 1.0 mg/d (2.7 μmol/d) riboflavin intake; 83±19 μmol nicotinamide metabolites/d to 12.8 mg/d (105 μmol/d) nicotinamide equivalent intake; 16.9±1.3 μmol pantothenic acid/d to 5.0 mg/d (23 μmol/d) pantothenic acid intake; 22.7±2.7 nmol folic acid/d to 200 μg/d (454 nmol/d) folic acid intake; 83±23 nmol biotin/d to 30 μg/d (123 nmol/d) biotin intake; and 0.140±0.051 nmol ascorbic acid/d to 100 mg/d (0.568 mmol/d) ascorbic acid intake (*26*). Intake of vitamin $B_1$, vitamin $B_2$ and folic acid was the same in the present and previous studies, and the urinary excretion of these vitamins in the present study was half or less than that in the previous study (*26*). Furthermore, urinary excretion of nicotinamide metabolites and pantothenic acid was the same in the present and previous studies, and the intake of niacin and pantothenic acid in the present study was twice that in the previous study (*26*). The form of vitamins differed between the two studies. The subjects obtained the vitamins from the diet in the first week in the present study, and they took a vitamin mixture in the previous one (*26*). As for niacin, most nicotinic acid in cereals binds to sugars, and bioavailability of this form is less than half that of free nicotinic acid (*27*). Pyridoxine-5′-β-D-glucoside (PN-glucoside) is a major naturally occurring form of vitamin $B_6$ in fruits (*28*), vegetables and cereal grains, and the bioavailability of PN-glucoside is ~50% relative to pyridoxine (*29*). Bioavailability of pantothenic acid in food is also half that of free pantothenic acid (*30*). Supplements of folic acid are nearly 100% bioavailable under fasting conditions (*31*), and a long-term controlled dietary study indicated that the bioavailability of folate in a typical mixed diet was no more than 50% relative to that in a formula diet (*32*). A recent study showed that bioavailability of food folate was 78% of that of folic acid according to an isotope (*33*). Most water-soluble vitamins, except vitamin C, bind to proteins or sugars in food, and the bioavailability of these forms is considered to be lower than that of the free forms (*5*).

The primary indicators selected to determine water-soluble vitamin sufficiency are the levels in urine, blood and/or serum. However, blood pantothenic acid and plasma biotin concentrations are not sensitive indicators of inadequate intake of these vitamins (*34*, *35*). The present study shows the first evidence that urinary excretion of all eight water-soluble vitamins and their metabolites is highly correlated with vitamin intake when the subjects take a standard diet with or without 1, 3- and 6-fold vitamins based on DRIs. The next step in this type of study is to determine the number of days reflecting vitamin and metabolite contents in 24-h urine samples, and to determine whether urinary vitamins and their metabolites in spot urine samples reflect their intakes in everyday life. We propose that estimating urinary 24-h water-soluble vitamin and their metabolite excretion is a good approach for assessing vitamin intakes in individuals. Furthermore, these results will contribute to determine dietary guidelines and recommendations.

Some vitamin-vitamin interactions are well known for accumulating homocystein by a folate, vitamin $B_6$ or vitamin $B_{12}$ deficiency, and requiring vitamin $B_2$ and vitamin $B_6$ for conversion of nicotinamide from tryptophan (*36*). These vitamin-vitamin interactions can be seen in some vitamin deficiencies, and little is known about how administrations of large amounts of water soluble vitamins affect other vitamins' metabolism. However, 1 g of ascorbic acid administration for 7 d does not alter plasma pyridoxal 5′-phosphate level or urinary excretion of 4-PIC (*37*). We previously reported that 150 mg (1.22 mmol) of nicotinamide administration increased nicotinamide metabolites approximately 800 μmol in 24 h urine (*9*). Chronic administration of a multivitamin supplement containing 150 mg of nicotinamide (1.22 mmol/d), 5.45 mg of fursulthiamin hydrochloride (12.5 μmol/d), 3.5 mg of riboflavin (9.3 μmol/d), 4.5 mg of pyridoxine hydrochloride (22 μmol/d), 6.5 μg of cyanocobalamin (4.8 nmol/d), 15 mg of calcium pantothenate (63 μmol/d as pantothenic acid) and 125 mg of ascorbic acid (0.71 mmol/d) increased nicotinamide metabolites approximately 700 μmol in 24 h urine, showing that these doses of vitamin intake did not affect nicotinamide metabolism (*38*). Intestinal cells transport biotin, pantothenic acid and lipoate via a sodium-dependent multivitamin transporter (SMVT), and biotin uptake is inhibited by pantothenic acid at a micromolar range in vitro (*39*). This SMVT system is the major biotin uptake system in the intestinal cells, and physiological (nanomolar) concentrations of pantothenic acid have no effect on the biotin uptake in vitro (*40*). These reports and the present results that urinary excretions of biotin and pantothenic acid linearly or more increased with administration of vitamins mixtures suggest that biotin and pantothenic acid do not inhibit their absorption in the present study. Moreover, urinary excretions of other vitamins or their metabolites increased linearly in a dose-dependent manner, suggesting no major effect on water soluble vitamin metabolism or absorption because of vitamin administration.

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## REFERENCES

1) Potischman N, Freudenheim JL. 2003. Biomarkers of nutritional exposure and nutritional status: an overview. *J Nutr* **133**: 873S–874S.
2) Bingham SA. 2003. Urine nitrogen as a biomarker for the validation of dietary protein intake. *J Nutr* **133**: 921S–924S.
3) Tasevska N, Runswick SA, Bingham SA. 2006. Urinary potassium is as reliable as urinary nitrogen for use as a recovery biomarker in dietary studies of free living individuals. *J Nutr* **136**: 1334–1340.
4) Tasevska N, Runswick SA, McTaggart A, Bingham SA. 2005. Urinary sucrose and fructose as biomarkers for sugar consumption. *Cancer Epidemiol Biomarkers Prev* **14**: 1287–1294.
5) Food and Nutrition Board, Institute of Medicine. 1998. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin $B_6$, Folate, Vitamin $B_{12}$, Pantothenic Acid, Biotin, and Choline. National Academy Press, Washington DC.
6) The Ministry of Health, Labour, and Welfare. 2004. Dietary Reference Intakes for Japanese, 2005. Tokyo (in Japanese).
7) Tasevska N, Runswick SA, McTaggart A, Bingham SA. 2007. Twenty-four-hour urinary thiamine as a biomarker for the assessment of thiamine intake. *Eur J Clin Nutr* DOI: 10.1038/sj.ejcn.1602829.
8) Zempleni J, Galloway JR, McCormick DB. 1996. Pharmacokinetics of orally and intravenously administered riboflavin in healthy humans. *Am J Clin Nutr* **63**: 54–66.
9) Shibata K, Matsuo H. 1990. Changes in blood NAD and NADP levels, and the urinary excretion of nicotinamide and its metabolism in women student after nicotinamide administration. *Vitamins* **64**: 301–306.
10) Zempleni J, Mock DM. 1999. Bioavailability of biotin given orally to humans in pharmacologic doses. *Am J Clin Nutr* **69**: 504–508.
11) Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR. 1996. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* **93**: 3704–3709.
12) Levine M, Wang Y, Padayatty SJ, Morrow J. 2001. A new recommended dietary allowance of vitamin C for healthy young women. *Proc Natl Acad Sci USA* **98**: 9842–9846.
13) Pullman ME, Colowick SP. 1954. Preparation of 2- and 6-pyridones of $N^1$-methylnicotinamide. *J Biol Chem* **206**: 121–127.
14) Shibata K, Kawada T, Iwai K. 1988. Simultaneous micro-determination of nicotinamide and its major metabolites, $N^1$-methyl-2-pyridone-5-carboxamide and $N^1$-methyl-3-pyridone-4-carboxamide, by high-performance liquid chromatography. *J Chromatogr* **424**: 23–28.
15) Resources Council, Science and Technology Agency. 2000. Standard Tables of Food Composition in Japan, Fifth revised edition —2000—. Tokyo (in Japanese).
16) Fukuwatari T, Suzuura C, Sasaki R, Shibata K. 2004. Action site of bisphenol A as metabolic disruptor lies in the tryptophan-nicotinamide conversion pathway. *J Food Hyg Soc Japan* **45**: 231–238.
17) Ohkawa H, Ohishi N, Yagi K. 1983. New metabolites of riboflavin appear in human urine. *J Biol Chem* **258**: 5623–5628.
18) Gregory JF 3rd, Kirk JR. 1979. Determination of urinary 4-pyridoxic acid using high performance liquid chromatography. *Am J Clin Nutr* **32**: 879–883.
19) Shibata K. 1987. Ultramicro-determination of $N^1$-methylnicotinamide in urine by high-performance liquid chromatography. *Vitamins* **61**: 599–604.
20) Skeggs HR, Wright LD. 1944. The use of Lactobacillus arabinosus in the microbiological determination of pantothenic acid. *J Biol Chem* **156**: 21–26.
21) Aiso K, Tamura T. 1998. Trienzyme treatment for food folate analysis. Optimal pH and incubation time for α-

amylase and protease treatment. *J Nutr Sci Vitaminol* **44**: 361–370.
22) Fukui T, Iinuma K, Oizumi J, Izumi Y. 1994. Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. *J Nutr Sci Vitaminol* **40**: 491–498.
23) Kishida K, Nishimoto Y, Kojo S. 1992. Specific determination of ascorbic acid with chemical derivatization and high-performance liquid chromatography. *Anal Chem* **64**: 1505–1507.
24) Association of Official Analytical Chemists. 2000. Official Methods of Analysis, 17th ed. AOAC Inc, Arlington, VA.
25) Kimura N, Fukuwatari T, Sasaki R, Hayakawa F, Shibata K. 2003. Vitamin intake in Japanese women college students. *J Nutr Sci Vitaminol* **49**: 149–155.
26) Shibata K, Fukuwatari T, Ohta M, Okamoto H, Watanabe T, Fukui T, Nishimuta M, Totani M, Kimura M, Ohishi N, Nakashima M, Watanabe F, Miyamoto E, Shigeoka S, Takeda T, Murakami M, Ihara H, Hashizume N. 2005. Values of water-soluble vitamins in blood and urine of Japanese young men and women consuming a semi-purified diet based on the Japanese Dietary Reference Intakes. *J Nutr Sci Vitaminol* **51**: 319–328.
27) Carter EG, Carpenter KJ. 1982. The bioavailability for humans of bound niacin from wheat bran. *Am J Clin Nutr* **36**: 855–861.
28) Gregory JF 3rd, Sartain DB. 1991. Improved chromatographic determination of free and glycosylated forms of vitamin B-6 in foods. *J Agric Food Chem* **39**: 899–905.
29) Nakano H, McMahon LG, Gregory JF 3rd. 1997. Pyridoxine-5′-β-D-glucoside exhibits incomplete bioavailability as a source of vitamin B-6 and partially inhibits the utilization of co-ingested pyridoxine in humans. *J Nutr* **127**: 1508–1513.
30) Tarr JB, Tamura T, Stokstad ELR. 1981. Availability of vitamin $B_6$ and pantothenate in an average American diet in man. *Am J Clin Nutr* **34**: 1328–1337.
31) Gregory JF 3rd. 1997. Bioavailability of folate. *Eur J Clin Nutr* **51**: S54–S59.
32) Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL, Taylor PC. 1987. Folate requirement and metabolism in nonpregnant women. *Am J Clin Nutr* **46**: 1016–1028.
33) Winkels RM, Brouwer IA, Siebelink E, Katan MB, Verhoef P. 2007. Bioavailability of food folates is 80% of that of folic acid. *Am J Clin Nutr* **85**: 465–473.
34) Eissenstat BR, Wyse BW, Hansen RG. 1986. Pantothenic acid status of adolescents. *Am J Clin Nutr* **44**: 931–937.
35) Mock NI, Malik MI, Stumbo PJ, Bishop WP, Mock DM. 1997. Increased urinary excretion of 3-hydroxyisovaleric acid and decreased urinary excretion of biotin are sensitive early indicators of decreased biotin status in experimental biotin deficiency. *Am J Clin Nutr* **65**: 951–958.
36) McCormick DB. 1989. Two interconnected B vitamins: riboflavin and pyridoxine. *Physiol Rev* 69: 1170–1198.
37) Shultz TD, Leklem JE. 1982. Effect of high dose ascorbic acid on vitamin $B_6$ metabolism. *Am J Clin Nutr* **35**: 1400–1407.
38) Shibata K, Onodera M, Shimada S, Yasuda K. 1992. Changes in nicotinamide metabolism and its bioavailability during long-term administration of a multivitamin preparation. *Vitamins* **66**: 309–314.
39) Chatterjee NS, Kumar CK, Ortiz A, Rubin SA, Said HM. 1999. Molecular mechanism of the intestinal biotin transport process. *Am J Physiol* **277**: C605–C613.
40) Balamurugan K, Ortiz A, Said HM. 2003. Biotin uptake by human intestinal and liver epithelial cells: roles of the SMVT system. *Am J Physiol* **285**: G73–G77.

原　著

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# 日本人母乳栄養児（0～5ヵ月）の哺乳量

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## 要　旨

わが国における離乳開始前の完全母乳栄養児の哺乳量を明らかにするために、生後4日目、15日目および5ヵ月齢までの各満月齢日の1日哺乳量を測定した。1日哺乳量の平均値は、生後4日目は424±156ml(n=15)、15日目は673±256ml(n=14)、月齢1では745±171ml(n=21)、月齢2では842±192ml(n=28)、月齢3では820±158ml(n=26)、月齢4では781±190ml(n=22)、月齢5では786±179ml(n=22)であった。15日目～月齢5では、哺乳量に有意な差が見られなかったため、平均値をとると、785±191mlであり、この値は、日本人の食事摂取基準(2005年版)で採用された値を支持するものであった。

## 緒　言

生後0～5ヵ月の乳児の発育に必要な食事は、基本的に母乳のみである。従って、生後0～5ヵ月の乳児の栄養素摂取量は、母乳中の栄養素濃度と哺乳量の積から得られる。日本人の食事摂取基準(2005年版)においても、0～5ヵ月の乳児の栄養素必要量は、この考え方に基づいて策定されている[1]。しかしながら、栄養素濃度のみならず、哺乳量に関するデータも十分に検討されていないのが現状であった。

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そこで、より精度の高い日本人乳児の哺乳量を調査するために、平成13年度～15年度の厚生労働科学研究費補助金「日本人の水溶性ビタミン必要量に関する基礎的研究」班（主任研究者、柴田克己）において、分担研究者である戸谷がはじめて、日本人の0～5ヵ月の乳児を対象とした精度の高い哺乳量調査を行った。この成果報告[2]に基づいて、「日本人の食事摂取基準（2005年版）」[1]の哺乳量、780ml/日が策定された。

哺乳量は、調査の行われる社会背景によって影響をうける可能性があり、精度の高い調査を継続して行う必要がある。そこで、引き続いて、戸谷がおこなった哺乳量の調査報告[2]をさらに強固にするために、平成16年度～18年度厚生

労働科学研究費補助金において「日本人の食事摂取基準（栄養所要量）の策定に関する研究」を行った。本研究においては、哺乳量に関する成果を報告する。

## 対象・方法

1．調査対象

本研究は、滋賀県立大学倫理審査委員会の承認を得た上で行った。本研究の主旨について文書で説明し、参加同意が得られた母親を対象とした。尚、最終調査終了まで完全母乳栄養であったことを、母親に口頭および摂取状況調査票を用いて確認した。母親36名（のべ148名）を調査対象とした。対象者はすべて単胎児出産で、在胎期間37週以上、妊娠中および分娩経過は正常であった。母親の第一回測定開始日の年齢は32±3歳であった。対象者の在住地域は、関西在住者19名、北陸在住者17名であった。調査対象児は男児19名、女児17名で、各月齢における内訳を表1に示した。

表1　調査対象者（乳児）の内訳

| 月齢 | 0（4日） | 0（15日） | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|
| 男児（人） | 7 | 7 | 11 | 16 | 15 | 13 | 12 |
| 女児（人） | 8 | 7 | 10 | 12 | 11 | 9 | 10 |

2．哺乳量の測定方法と調査の実施方法

調査は、平成17年6月から平成19年2月にかけて、生後4日目、15日目、および生後1～5ヵ月については各満月齢日から3日前後の間に乳児の24時間哺乳量（1日哺乳量）の測定と母子の基礎情報（健康状態など）の調査を行った。尚、生後4日目は産院での測定、それ以降は郵送による紙面調査を行った。

体重測定には、全対象者にタニタベビースケールBLB-2（株式会社タニタ（東京）：最小表示値0～6kgは2g、6～12kgは5g、自動補正機能つき）を無償貸与し、測定値の均一化をはかった。

3．統計処理

統計処理はMicrosoft ExcelおよびGraph-Pad Prism 4(Graph Pad Software, Inc. San Diego、California、USA）を用いて行った。検定方法は一元配値の分散分析を行い、有意差が認められた場合、各々の比較はKruskal Wallis testで行った。その結果、$p<0.05$を有意差が認められたとした。

## 結　果

1．乳児の身体状況

厚生労働省による平成12年乳幼児身体発育調査の体重[3]と比較した結果、本調査対象児は、平成12年乳幼児身体発達調査の3～97パーセンタイル値から大きく外れるものはなかった。

2．1日哺乳量の分布と平均値

各月齢平均哺乳量±標準偏差（SD）を表2に示した。生後4日目の1日哺乳量は、他の月齢に比べ有意に低かった（$p<0.01$）。また、有意な差が認められなかった生後15日目～月齢5の1日哺乳量の平均哺乳量±標準偏差（SD）は785±191ml/日であった。

表2　1日哺乳量の月齢別平均値

| 月齢 | 0（4日） | 0（15日） | 1 | 2 | 3 | 4 | 5 | 0（15日）～5の平均 |
|---|---|---|---|---|---|---|---|---|
| n | 15 | 14 | 21 | 28 | 26 | 22 | 22 | 133 |
| 平均（ml） | 424 | 673 | 745 | 842 | 820 | 781 | 786 | 785 |
| SD（ml） | 156 | 256 | 171 | 192 | 158 | 190 | 179 | 191 |

＊月齢1～5については各満月齢日から3日前後の間に測定した。

3．哺乳量に影響を及ぼす因子

（1）　哺乳回数と哺乳量との関係

生後4日目、15日目、および月齢1～5において、哺乳回数と哺乳量との間には相関関係は認められなかった。

（2）　月齢と哺乳回数との関係

哺乳回数は月齢に従って増えるのか、あるいは成長すると1回の哺乳量が増大し、

表３　月齢ごとの哺乳回数

| 月齢 | 最小値(回) | 最大値(回) | 平均値±SD (回) |
|---|---|---|---|
| 0( 4日) | 7 | 16 | 11.7 ± 2.6 [a] |
| 0(15日) | 6 | 16 | 10.9 ± 2.9 [a,c] |
| 1 | 7 | 15 | 9.8 ± 2.0 [a,c,d] |
| 2 | 7 | 19 | 9.4 ± 2.5 [a,c,d] |
| 3 | 5 | 13 | 8.7 ± 2.2 [b,c] |
| 4 | 5 | 11 | 8.3 ± 1.6 [b,c] |
| 5 | 5 | 12 | 7.8 ± 1.8 [b,d] |

平均値±SDにおいて、一元配値の分散分析を行い、有意差が認められた場合、各々の比較はKruskal-Wallis testで行った。同じ添え字を有する平均値±SD間では有意差が認められなかったことを示す。

哺乳回数が減るのかを明らかにするため、月齢と哺乳回数との関係を調べた。その結果を表３に示した。生後４日目と15日目の哺乳回数を比較したが、15日目において、哺乳回数が多くなる、あるいは少なくなるという調査結果は得られず、差異は認められなかった。さらに、月齢１、２、３、４、５の各月齢における哺乳回数も調べてみたところ、月齢に依存して哺乳回数が低下する傾向が認められた。生後15日目～月齢５では哺乳量には差異は認められなかったので、成長に従い、１回の哺乳量が増大していることが明らかとなった。

（３）　哺乳時間帯と哺乳量との関係

哺乳時間帯と哺乳量との関係を、生後４日目、15日目、および月齢１～５において、調べてみたが、どの時点においても、規則だった傾向はみられなかった。

## 考　察

わが国の栄養に関する政策などを決定する基となる食事摂取基準は５年ごとの改定が行われ、最新のデータに基づく策定が行われてきた。そこで、昭和35年改定からの栄養所要量（食事摂取基準）の哺乳量（０～５ヵ月）の策定の根拠となったデータをみてみると、昭和35年改定では根拠が示されず、昭和44年と昭和50年改定で使用された高井ら[4]のデータでは、生後満２週～13週の母乳栄養児68例について、３日間の平均値を１日哺乳量とし、生後２～３週の哺乳量は平均631±121ml/日、10～11週では914±174ml/日としている。それ以降、第六次改定まで哺乳量は850mlのままで変更されていなかった。第六次改定では根拠となるデータの記載はないが、それまでの採用値850mlから100ml少ない750mlとなった。この100ml少ない値となった理由として、米国でのNevilleら[5]の報告とAllenら[6]の報告、および日本人の報告として、米山ら[7-9]の報告が考えられる。Nevilleら[5]は600～750ml/日程度、Allenら[6]は700～800ml/日程度と報告している。米山ら[7-9]は、700ml/日台前半の哺乳量の値を報告している。

生後０～５ヵ月の乳児の栄養素必要量は、母乳中の栄養素濃度×哺乳量から求められている。さらに、生後０～５ヵ月の栄養素必要量の値は、それ以降の年齢の栄養素必要量を算定するための、外挿の基準値としても利用されている。特に、必要量の算定の基本的なデータが存在しない６～11ヵ月の乳児の栄養素必要量算定において、重要である。従って、精度の高い哺乳量の調査の継続が必要である。

また、我々は哺乳量を測定する方法として、哺乳量に関する論文[2,4-9]を参考にして、授乳ごとに哺乳前後の乳児の体重を測定し、その差を哺乳量とする方法を採用した。授乳期には母親は子育てのストレスを強く感じる時期であることから、被験者の負担をできるだけ軽くする必要がある。現在、乳児用体重計の精度が上がり、乳児の動きに対応した自動補正機能付のものもあり、測定者による個人的な誤差が低くなった。そのため、本調査で使用した方法が最も適切なものであると考える。なお、鈴木・戸谷ら[2]も授乳直前直後の乳児の体重測定法で行い、食事摂取基準の表記方法に合わせるために、五訂増補日本食品標準成分表[10]の母乳の比重の値を用いて算出している。

本研究での１日哺乳量の結果では、生後４日

表4　哺乳量に関する報告の月齢別一覧

| | 文献番号 | 生後の月齢 | | | |
|---|---|---|---|---|---|
| | | 0～1(～14日) | 0～1(14日～) | 1～2 | 2～6 |
| 髙井ら | 2 | | 631ml (2から3週) | | 914ml (10～11週) |
| Nevilleら | 3 | 615g (7～14日) | 689g (15～28日) | 707g (30～59日) | 753g (60～150日) |
| Allenら | 4 | | 717ml (21日) | 713ml (45日) | 700ml (90日)<br>801ml (180日) |
| 米山ら | 5 | 372ml (5, 6日) | | | |
| 米山ら | 6 | | | 685ml (1～5)<br>719ml (残乳あり)<br>662ml (1～5, 残乳なし) | |
| 鈴木ら | 8 | | | 778ml (1～5) | |
| Kentら | 13 | | | 788±169g (1～6) | |
| 本調査 | | 420ml (4日) | 673ml (14日) | 801ml (1～5) | |

かっこ内の数字で記載のないものは，月齢を示す．

目の哺乳量は、他の月齢と比べて有意に低い値となった。一方、生後15日目～月齢5では、哺乳量に有意差は見られなかった。この期間の平均値は、785±191mlであった。表4に今回の結果と今までに報告されている哺乳量と生後日数との関係をまとめたが、生後2週間までは哺乳量が安定していないことがわかる。産褥の乳汁分泌は産褥2日目ごろより開始し、7日目ごろにほぼ確立する[11]。生後4日目は安定した乳汁分泌確立までのちょうど中間的時期にあると考えられる。つまり、哺乳量については生後2週ごろまでは特別にわけて考慮する必要があると考えられる。鈴木・戸谷ら[2]の報告では哺乳量調査開始を生後1ヵ月としているが、今回測定した結果では15日目でも1ヵ月以降とほぼ同様の値が得られた。このように戸谷らの報告と近い哺乳量が得られたのは、比較的近い時期に実施したので食環境や社会環境などが変化していないためではないかと推測する。また、どの月齢においても哺乳量は2～3倍程度の個人差が見られ、哺乳回数においても、2倍程度の幅が見られた。

山内[12]は月齢1では哺乳回数が5～11回と報告しているが、本調査では少し高めの7～15回であった（表3）。なお、オーストラリアからの報告であるが、Kentら[13]は、月齢1～6で11±3回（6～18回）と報告している。哺乳回数については、時代ごとの社会背景の違いによる母乳哺育への取り組み方の違いや、母乳中の成分の違いによる影響も考えられるので、更なる解析が必要であると考える。哺乳回数は、4日目、15日目では乳児によるバラツキが、他の月齢よりも大きかった。一方、4、5ヵ月齢になると乳児による哺乳回数の差異は小さくなった。4日目の哺乳回数は他の月齢よりも多かったが、哺乳量は逆に少なかった。このことは、乳児の哺乳能力が未発達なためと母親が授乳に不慣れなことが重なり、その結果、乳頭への吸啜刺激が上手く行われないため母乳産生が少なくなったものと思われた[14]。しかしながら、生後4日目の乳児の体重増加量をみると、今回の哺乳量で充足していると考えられた。

授乳時間帯と哺乳量に関して興味が持たれるが、本調査において、哺乳量はどの時間帯においても、大きな差異は認められなかった。

月齢と哺乳時間との関係であるが、特に生後間もない4日目、15日目および月齢1では0時～6時までのヒトの一般的な就寝時間(夜間)においても哺乳が多いため、月齢1までの乳児においては、エネルギーおよび栄養素摂取には夜間の哺乳も重要であると考えられる。Kentら[13]も同様なことを報告しており、乳児の要求に基づいて時間を気にせずに哺乳させることを勧めている。

以上のことから、月齢1～5の乳児の哺乳量

は、月齢、哺乳回数、哺乳時間帯に影響されにくいことがわかった。また、これらの各項目については、個人差が大きいことから、平均値を使用して栄養指導などを行う場合には、取り扱いに注意が必要であり、今後、母乳中の栄養素濃度との関係についてもあわせて調査が必要であると考えられる。

日本人の食事摂取基準(2005年版)[1]は、鈴木・戸谷ら[2]が調査した値、すなわち、日本人乳児(1～5ヵ月)の1日平均哺乳量は780mlである、という報告値を採用したが、我々の今回の報告も、この値を支持するものであった。なお、生後まもない4日目の哺乳量は、この値よりも低いものであったことも強調しておきたい。

## 結　論

1日哺乳量の平均値は、生後4日目は424±156ml、15日目～5ヵ月齢では785±191mlであった。「日本人の食事摂取基準(2005年版)[1]」の哺乳量は策定に使用された780mlを支持する結果となった。

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## 文　献

1．厚生労働省、日本人の食事摂取基準2005年版
2．鈴木久美子、佐々木晶子、新澤佳代他、離乳前乳児の哺乳量に関する研究、栄養学雑誌、2004；62：369-372
3．厚生労働省、平成12年乳幼児身体発育調査報告書、平成13年
4．高井俊夫、久原良躬、合瀬徹他、母乳ならびに粉乳をad libitumに与えた場合の観察(第Ⅱ報)、日本小児科学会雑誌、1968；72：1583
5．Neville MC, Keller R, Seacat J, *et al*. Studies in human lactation: milk volumes in lactating women during the onset of lactation and full lactation. Am J Clin Nutr, 1988;48:1375-1386
6．Allen JC, Keller RP, Archer P, *et al*. Studies in human lactation: milk composition and daily secretion rates of macronutrients in the first year of lactation. Am J Clin Nutr, 1991;54:69-80
7．米山京子、池田順子、永田久紀、母乳中の総蛋白質量、母乳分泌に関する研究（第2報)、食生活との関連、日本公衛誌、1991；38：173-181
8．米山京子、中本雅子、母乳分泌量、母乳中成分の日内および授乳月数に伴う変動－総蛋白質、sIgA、カルシウムについて、小児保健研究、1993；52：510-517
9．米山京子、母乳栄養児の発達と母乳からの栄養素摂取量、小児保健研究、1998；57：49-57
10．五訂増補　日本食品標準成分表、2005：文部科学省科学技術・学術審議会資源調査分科会編集、独立行政法人　国立印刷局
11．山中美智子、多賀理吉、母乳分泌のメカニズム、周産期医学、1996;26:477-480
12．山内芳忠、乳児肥満（母乳哺育児）進みすぎの対応について、周産期医学増刊、2005；35：340-343
13．Kent JC, Mitoulas LR, Cregan MD, *et al*. Volume and frequency of breastfeeding and fat content of breast milk throughout the day. Periatrics, 2006;117:e387-e395
14．木下史子、吉永宗義、母乳栄養（基礎編）－分泌機能、成分．2001;31:331-334

# Amount of Breast Milk Sucked by Japanese Breast Feeding Infants

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To clarify the amount of breast milk sucked by infants who were completely breast fed before weaning, a daily intake of breast milk was measured. A total of 148 Japanese infants, who were completely breast feeding were subjected to the experiment. The mean ± SD (n) of breast milk sucked daily was 424±156 ml (n=15) at 4 days of age, 673±256 ml (n=14) at 15 days, 745±171 ml (n=21) at one month, 842±192 ml (n=28) at 2 months, 820±158 ml (n=26) at 3 months, 781±190 ml (n=22) at 4 months, and 786±179 ml (n=22) at 5 months. There was no statistically significant difference between the value at 15 days and 5 months. The mean value during the period (15 days to 5 months) of 785±191 ml, supported that of 780 ml determined in the Dietary Reference Intakes for Japanese (2005).

Key Words : Japanese infants, breast milk intake, Dietary Reference Intakes

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# Urinary Excretory Ratio of Anthranilic Acid/Kynurenic Acid as an Index of the Tolerable Amount of Tryptophan

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**Some people may take excessive tryptophan as a supplement in the expectation that the tryptophan metabolite, melatonine, will help to induce sufficient sleep. We investigated the basis for a useful index to assess the risk of a tryptophan excess. Young rats were fed on a 20% casein diet with 0, 0.5, 1.0, 2.0 or 5.0% added tryptophan for 30 d the apparent toxicity and growth retardation was observed in the 5.0% tryptophan-added group. Metabolites of the Tryptophan-nicotinamide pathway and such intermediates as kynurenic acid (KA), anthranilic acid (AnA), xanthurenic acid, 3-hydroxyanthranilic acid and quinolinic acid in 24-h urine increased in a dose-dependent manner. Of those metabolites and intermediates, the urinary excretion of KA progressively increased, and that of AnA dramatically increased in the 2.0 and 5.0% tryptophan-added groups. The urinary excretory ratio of AnA/KA was a high value for both the groups. These results suggest that the urinary ratio of AnA/KA could be a useful index to monitoran excessive tryptophan intake.**

**Key words:** tryptophan; anthranilic acid; kynurenic acid; tolerance; urine

Nicotinamide (Nam), serotonin and melatonin are important bioactive compounds derived from the essential amino acid, tryptophan (Trp).[1–3] Nicotinamide is concerned with metabolism as a coenzyme; serotonin is involved in relieving pain, in hypnosis, and in tranquilizing as a neurotransmitter; and melatonin is a pineal hormone that is involved in the rhythm of sleep. Trp is widely available on the market as a supplement for its metabolites' effects on maintenance of health. Taking such a supplement too much may result in an excessive intake, and Trp is one of the most toxic amino acids.[4] Its LD50 value was 1.6 g/kg of body weight when injected intraperitoneally into rat.[5] Its adverse effects are ataxia, tremors, diaphoresis, blurred vision, dry mouth, muscle stiffness, palpitations, and urticaria.[6] The tolerable upper intake level (UL) of Trp by humans is not well known, and this level in humans cannot be determined with human subject due to ethical considerations. The establishment of a biomarker for showing a large amount of Trp intake will be useful to prevent excessive Trp intake and its adverse effects. We investigated in the present study the effects of an excessive Trp intake on Trp–nicotinamide (Nam) metabolism and Trp degradation metabolism to identify the metabolic change. We also show the urinary excretory ratio of anthranilic acid (AnA)/kynurenic acid (KA) as an index for a large amount of tryptophan intake.

## Materials and Methods

*Chemicals.* Vitamin-free milk casein, sucrose, L-methionine, gelatinized cornstarch, Trp, Nam, and quinolinic acid (QA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). KA, xanthurenic acid (XA), 3-hydroxyanthranic acid (3-HA), and $N_1$-methylnicotinamide chloride (MNA) 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 synthesized by the methods of Pullman and Colowick[7] and Shibata *et al.*,[8] respectively. The mineral (AIN-93-G-MX) and nicotinic acid-free vitamin (AIN-93-VX) mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan). All other chemicals used were the highest purity available from commercial sources.

*Animals and diet.* The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. Twenty male Wistar rats (3 weeks old obtained from Clea, Japan) were divided into five groups of four rats each, and placed in an individual metabolic cage (CT-10 for rats; Clea). One of the groups was fed with a 20% casein diet as a control, and the

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*Abbreviations*: Trp, tryptophan; AnA, anthranilic acid; KA, kynurenic acid; XA, xanthurenic acid; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; ACMS, α-amino-β-carboxymuconate-ε-semialdehyde; AMS, α-aminomuconate-ε-semialdehyde; Nam, nicotinamide; MNA, $N^1$-methylnicotinamide; 2-Py, $N^1$-methyl-2-pyridone-5-carboxamide; 4-Py, $N^1$-methyl-4-pyridone-3-carboxamide; Sum, Nam+MNA+2-Py+4-Py; ACMSD, aminocarboxymuconate semialdehyde decarboxylase; QPRT, quinolinic acid phosphoribosyltransferase

**Table 1.** Compositions of the Diets

| | Ctrl diet | Test diets (Control diet + Trp) | | | |
|---|---|---|---|---|---|
| | | +0.5% Trp | +1% Trp | +2% Trp | +5% Trp |
| | | | (%) | | |
| Casein | 20 | 20 | 20 | 20 | 20 |
| L-methionin | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Gelatinized cornstarch | 45.9 | 45.4 | 44.9 | 43.9 | 40.9 |
| Sucrose | 24.4 | 24.4 | 24.4 | 24.4 | 24.4 |
| Corn oil | 5 | 5 | 5 | 5 | 5 |
| Mineral mixture (AIN-93-G-MX) | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Vitamin mixture (AIN-93-VX, nicotinic acid-free) | 1 | 1 | 1 | 1 | 1 |
| Trp | 0 | 0.5 | 1 | 2 | 5 |

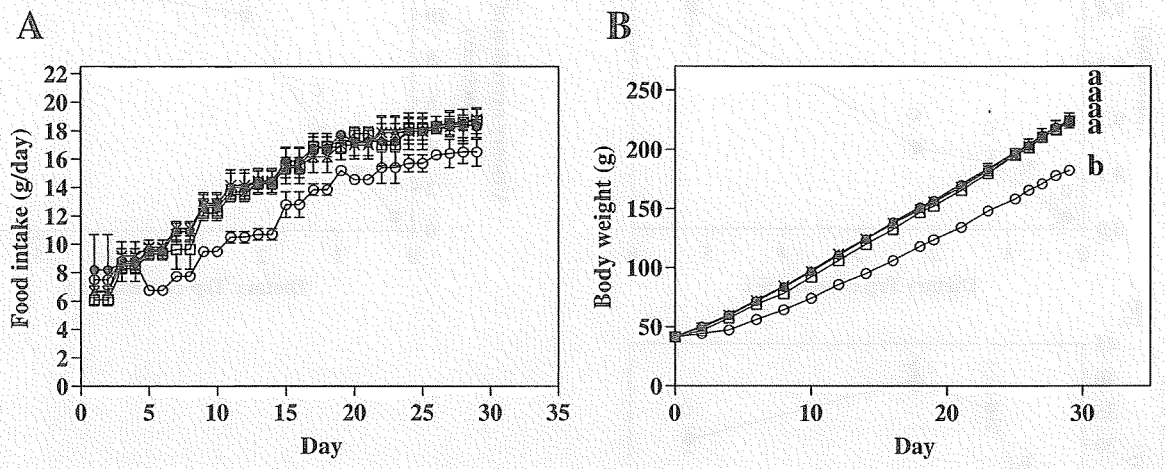


**Fig. 1.** Effect of Trp Intake on the Food Intake (A) and Body Weight (B) in Rats.

Male rats of the Wistar strain (3 weeks old) were obtained and immediately placed in individual metabolic cages. ●, control diet group; ×, 0.5% Trp supplemented diet group; ▲, 1% Trp supplemented diet group; □, 2% Trp supplemented diet group; ○, 5% Trp supplemented diet group. Each value is the mean ± SEM of four rats. A different letter on the curve indicates a significant difference at $p < 0.05$, as determined by Tukey-Kramer multiple comparison test.

others were fed with a 20% casein diet supplemented with 0.5, 1, 2 or 5% Trp (Table 1). All animals were allowed free access to food and water. The animal room was maintained at a temperature of about 20 °C with 60% humidity and a 12-h light/12-h dark cycle (light on at 6:00 a.m.). The body weight and food intake were measured daily at around 9:00 a.m., and food and water were renewed daily. The experimental period was for 30 d. Urine samples (10:00 a.m.–10:00 a.m.; 24-h urine) were collected in a conical beaker containing 1 ml of 1 M HCl on the last day of the experiment. The urine samples were stored at −20 °C until needed.

*Analysis.* The urinary content of Nam, 2-Py and 4-Py was simultaneously measured by the HPLC method of Shibata *et al.*,[8] and that for MNA by the method of Shibata.[9] Urinary concentration of 3-HA was measured by the HPLC method of Shibata and Onodera,[10] while the urinary concentration of KA was measured by the method of Shibata.[11] The urinary concentration of XA was measured by the method of Shibata and Onodera,[12] and QA was measured by the method of Mawatari *et al.*[13]

*Statistical analysis.* Each value is expressed as the mean ± SEM. The statistical significance was determined by ANOVA and subsequent Tukey–Kramer multiple-comparison tests. Differences of $P < 0.05$ were considered to be statistically significant. Prism 4.0 (Graph Pad Software, San Diego, CA, USA) was used for all analyses.

## Results

*Effect of excessive Trp intake on the body weight gain and food intake*

The food intake and body weight gain are shown in Fig. 1. The food intake and body weight gain of the rats fed with the 5% Trp supplemented diet were lower than the other groups from day 4, The value for the other Trp

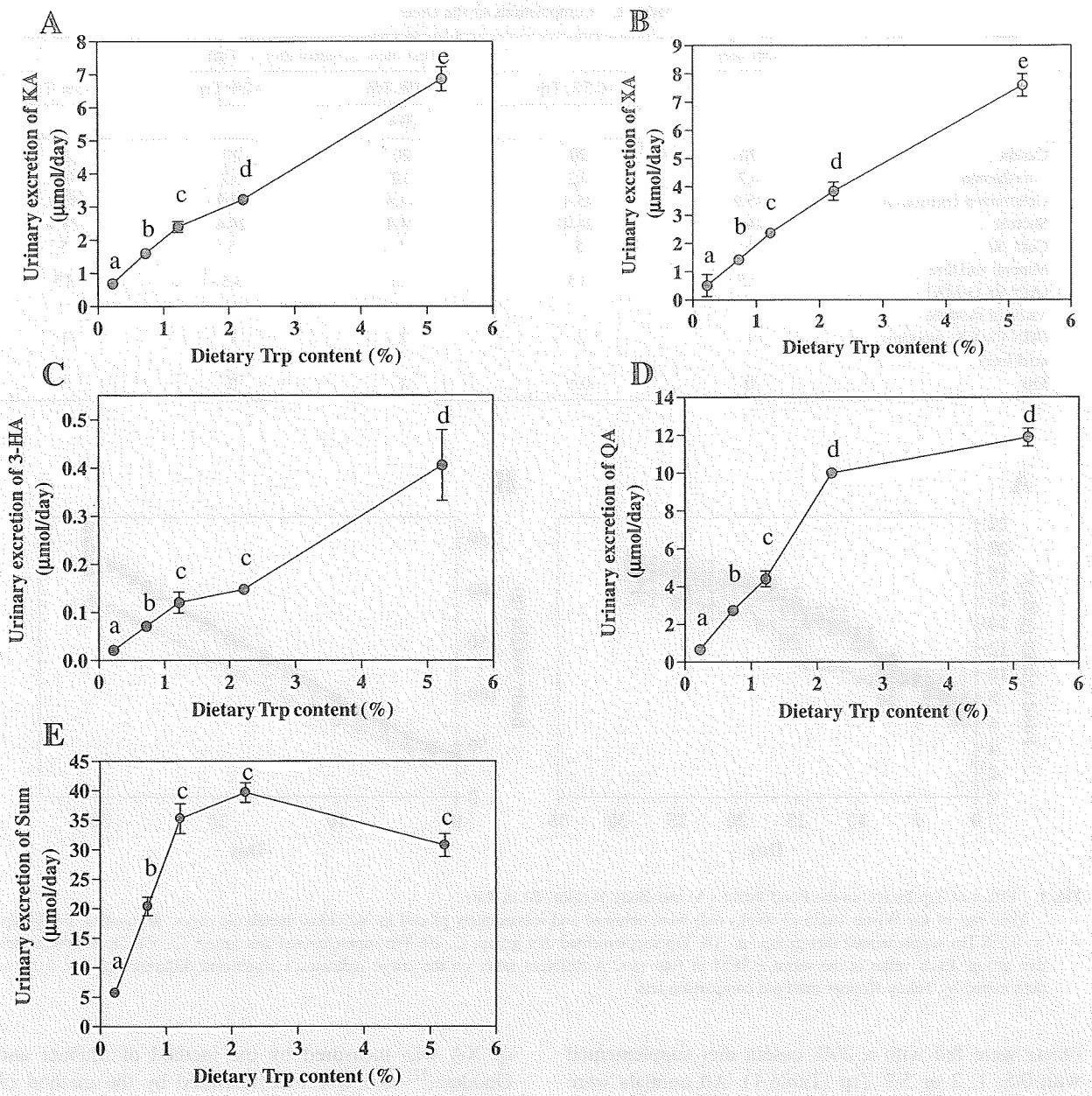


**Fig. 2.** Effect of Trp Intake on the Urinary Excretion of KA (A), XA (B), 3-HA (C), QA (D), and Sum = Nam + MNA + 2-Py + 4-Py (E) in Rats. A different letter on the curve means a significant difference at $p < 0.05$, as determined by the Tukey-Kramer multiple comparison test.

supplemented groups being no different from the control group throughout the experiment.

*Effect of an excessive Trp intake on Trp–Nam metabolism*

The effect of excess Trp on Trp–Nam metabolism is shown in Fig. 2. The urinary excretion of such Trp catabolites as KA, XA and 3-HA increased according to the intake of Trp. Although the excretion of QA was also increased in a dose-dependent manner, its content was at the same level in the 2% and 5% Trp supplemented groups. The sum of the metabolites Nam, MNA, 2-Py and 4-Py was the same in the 1%, 2% and 5% Trp supplemented groups.

Interestingly, the urinary excretion of AnA dramatically increased in the 2% and 5% Trp supplemented groups (Fig. 3). This increased level of AnA was 308 times with the 5% Trp supplemented group compared to the control group. Kynureninase, which is converted to AnA from kynurenine, did not change (data not shown).

*Effect of excessive Trp intake on urinary excretory ratio of AnA/KA, AnA/XA and AnA/3-HA*

The urinary excretory ratios of AnA/KA, AnA/XA and AnA/3-HA for the 5% Trp supplemented group were 83, 20 and 16 times higher than the respective control group ratios (Table 2). These ratio for the 2% Trp supplemented group were 5.5, 3.4 and 3.6 times