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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原　著

日本母乳哺育学会雑誌　2巻1号　23～28（2008年）

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,b]
1	7	15	9.8 ± 2.0[a,b,c]
2	7	19	9.4 ± 2.5[a,b,c]
3	6	13	8.7 ± 2.2[b,c]
4	6	11	8.3 ± 1.6[b,c]
5	6	12	7.8 ± 1.8[c,d]

平均値±SDにおいて、一元配置の分散分析を行い、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
高井ら	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） 801ml（180日）
米山ら	5	372ml（5, 6日）			
				685ml（1～5）	
				719ml（残乳あり）	
米山ら	6			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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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

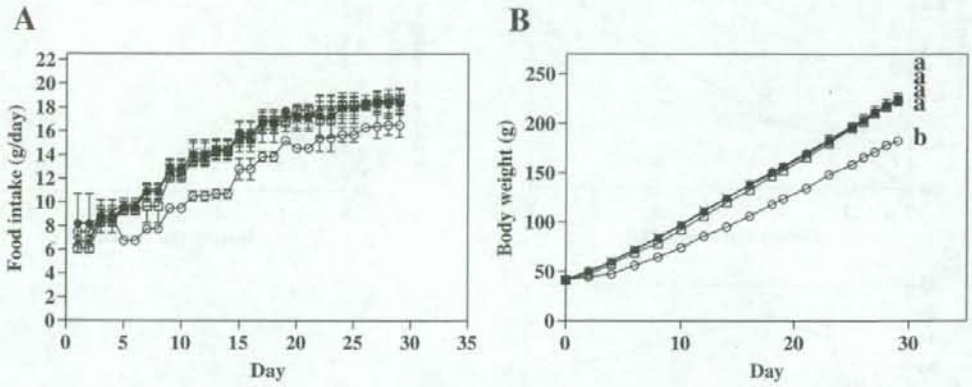

Fig. 1. Effect of Trp Intake on the Food Intake (A) and Body Weight (B) in Rats.

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

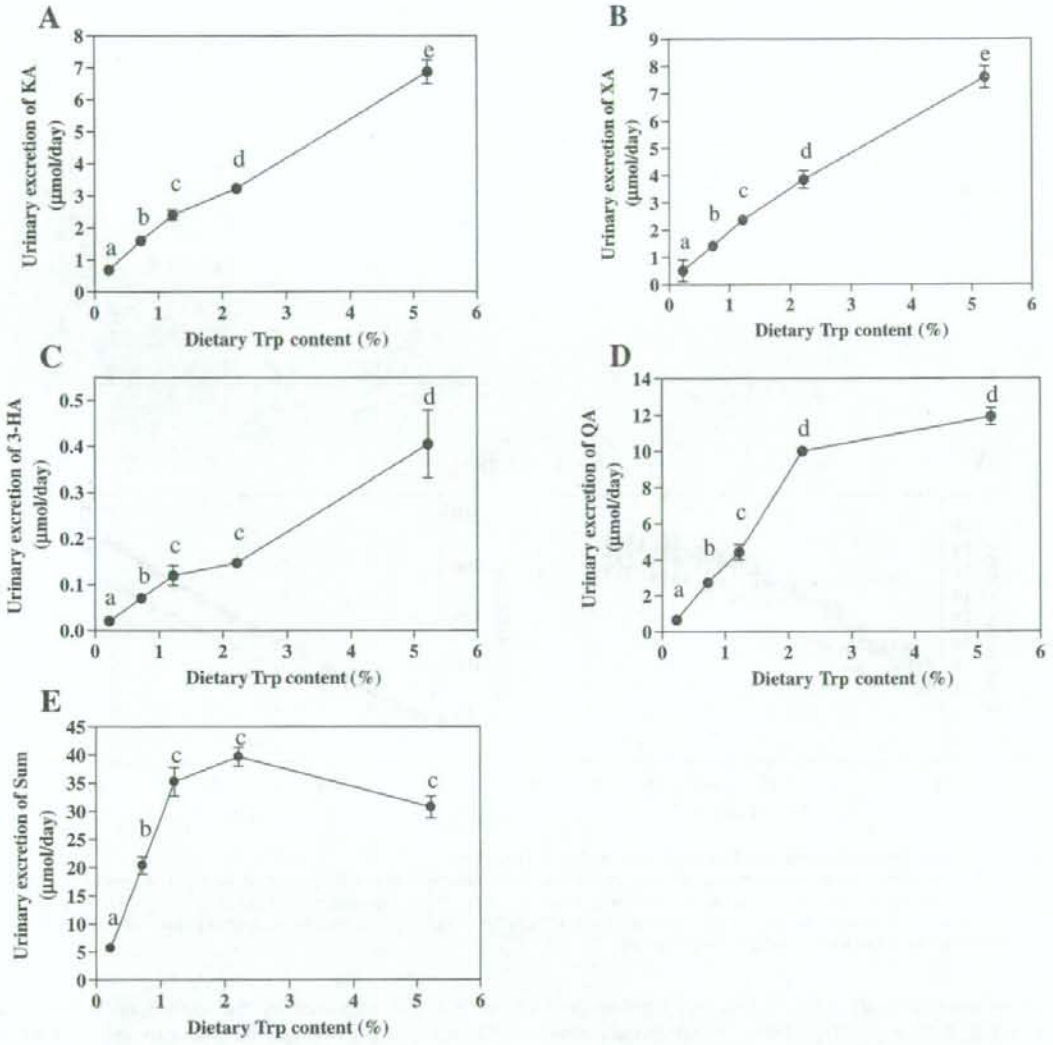

Fig. 2. Effect of Trp Intake on the Urinary Excretion of KA (A), XA (B), 3-HA (C), QA (D), and Sum = Nam + MNA + 2-Py + 4-Py (E) in Rats. A different letter on the curve means a significant difference at $p < 0.05$, as determined by the Tukey-Kramer multiple comparison test.

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

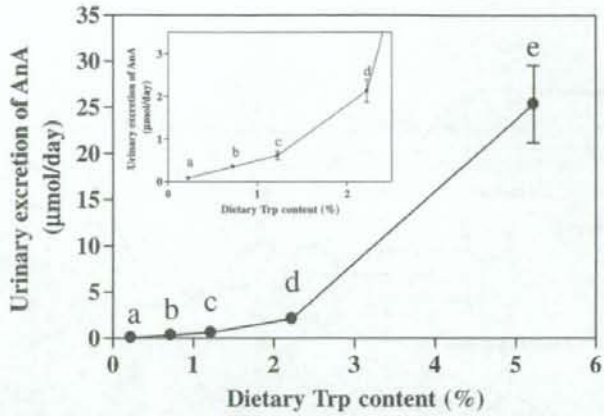

Fig. 3. Effect of Trp Intake on the Urinary Excretion of AnA in Rat.
The large graph shows the urinary excretion of AnA in the control group compared with the added 5% Trp supplemented group, and small graph shows that urinary excretion of AnA in control group to added 2% Trp group. A different letter on the curve means a significant difference at $p < 0.05$, as determined by the Tukey-Kramer multiple comparison test.

Table 2. Trp Intake and Metabolites Used for Indices

	Ctrl	+0.5%	+1%	+2%	+5%
Trp intake	213 ± 5	673 ± 25	1115 ± 43	2043 ± 32	4203 ± 256
(μmol/day)	(1)	(3.1)	(5.2)	(9.6)	(18.9)
AnA	82 ± 3	347 ± 20	614 ± 99	2126 ± 261	25420 ± 4207
(nmol/day)	(1)	(4.1)	(7.8)	(25)	(352)
KA	687 ± 57	1600 ± 80	2400 ± 160	3233 ± 118	6869 ± 364
(nmol/day)	(1)	(2.3)	(3.5)	(4.7)	(10)
XA	512 ± 39	1419 ± 142	2370 ± 157	3845 ± 321	7600 ± 400
(nmol/day)	(1)	(2.8)	(4.6)	(7.5)	(14.8)
3-HA	21 ± 3	71 ± 7	121 ± 22	148 ± 8	405 ± 7
(nmol/day)	(1)	(3.5)	(6.4)	(6.9)	(22)
Value of AnA/KA	0.12 ± 0.05	0.22 ± 0.07	0.26 ± 0.06	0.66 ± 0.10	3.7 ± 0.39
	(1)	(1.8)	(2.2)	(5.5)	(83.3)
Value of AnA/XA	0.16 ± 0.01	0.24 ± 0.08	0.26 ± 0.03	0.55 ± 0.04	3.3 ± 0.35
	(1)	(1.5)	(1.6)	(3.4)	(20.1)
Value of AnA/3-HA	3.9 ± 0.2	4.9 ± 0.3	5.1 ± 0.7	14.4 ± 1.0	62.8 ± 10.7
	(1)	(1.3)	(1.3)	(3.6)	(16.1)

Each value is expressed as the mean ± SEM of four rats, and in numbers parentheses are the relative to a control value of 1.

higher than the respective control group ratios. However, the excretion ratios were at the same level between the 0.5% and 1% Trp supplemented and the control groups.

Discussion

Trp has appeared on the market to treat sleep disorders,[14,15] and consequently, there is the risk of an excessive intake. It is therefore important to provide a useful index that will monitor an excessive tryptophan intake. Nutrient metabolite ratios change in urine in the case of an excessive nutrient intake. Our previous study has shown the effect of Nam when the nutrient intake exceeded the tolerable level. For example, when rats were fed with a diet containing different amounts of Nam, metabolic changes was observed; the urinary excretory ratio of (2-Py + 4-Py)/MNA was markedly reduced by a diet containing more than a tolerable intake of Nam.[16–18] We thought that an excessive Trp intake would also induce metabolic change which would be reflected in urinary excretion. Thus, we investigated the Trp-Nam metabolism in rats fed with a diet containing excessive Trp. Trp-Nam metabolites and metabolic changes might provide an index for the excessive intake of Trp.

In the present experiment, the urinary excretion of such Trp catabolites as KA, XA and 3-HA increased according to the intake of Trp (Fig. 2). Thus, the enzyme activity related with the conversion of Trp to 3-HA

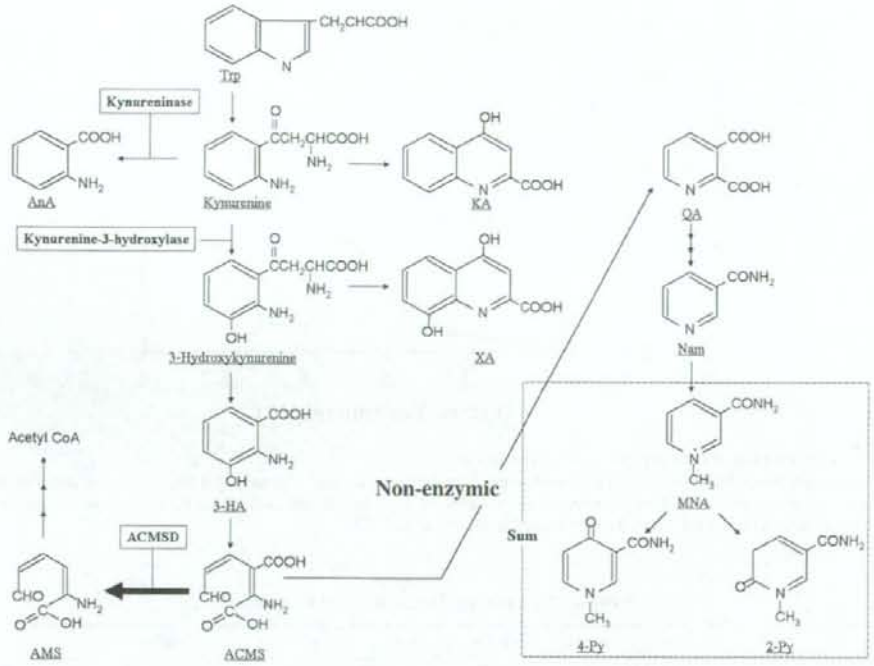

Fig. 4. Metabolism of Trp.
This whole metabolic pathway exists only in the liver.

might be sufficient to metabolize a diet containing up to 5% Trp.

QA is formed from ACMS and ACMS is formed from 3-HA. The reaction from 3-HA to ACMS is catalyzed by 3-hydroxyanthranilic acid oxygenase (3-HAO) whose enzyme activity is extremely high (715 ± 20 μmol/h/g of liver). In this study, we did not measure ACMS, although ACMS formation might increase because 3-HA was increased by an excessive Trp intake. The excretion of QA increased with increasing Trp intake up to 2%, but was almost the same form in the 2% and 5% Trp diet groups (Fig. 2D). This means that the activity of the enzyme, aminocarboxymuconate semialdehyde decarboxylase (ACMSD), was also increased which metabolizes α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) to α-aminomuconate-ε-semialdehyde (AMS) (the bold line in Fig. 4). The Trp–ACMS pathway branches at this metabolite; one branch is the reaction from ACMS to AMS catalyzed by ACMSD, and the other is the reaction from ACMS to QA by spontaneous autocyclization. The activity of ACMSD would be induced by Trp because it has been reported that ACMSD activity was altered by various nutritional factors and chemicals *in vivo*.[14,15,17,19]

The sum of the metabolites, Nam, MNA, 2-Py and 4-Py, was almost the same in the 1%, 2% and 5% Trp diet groups, which showed that the metabolism of QA to nicotinic acid mononucreotide was saturated in the 2% Trp diet group or the metabolite of ACMS to AMS was accelerated as already mentioned. Quinolinic acid phosphoribosyltransferase (QPRT) metabolized QA to NaMN. Rao *et al.*[18] have reported that QPRT was the limiting with the rate enzyme on the Trp–Nam pathway in rats.[20] Therefore, QPRT reached the limiting rate with the 2% Trp diet. We can explain these metabolic changes by enzymatic conversion.

Interestingly, the urinary excretion of AnA sharply increased above 2% Trp in the diet (Fig. 3), although KA, 3-HA and XA increased with increasing Trp intake. We cannot clearly explain why the excretion of AnA increased in this way. The Km for the value enzyme might be able to explain this result. The Km value for kynureninase, which catalyzes the reaction of kynurenine to AnA is 2.4×10^{-4} M. The Km value for kynurenine-3-hydroxylase, which catalyzes the reaction of kynurenine to 3-hydroxykynurenine, is 2.3×10^{-5} M, and the Km value for kynurenine amino transferase, which catalyzes the reaction kynurenine to KA, is 8.8×10^{-4} M (Fig. 4). Little AnA may be formed with intake of the in 1% Trp diet, but with intake of the 5% Trp diet, AnA formed might become extremely high (Fig. 4). Although the Km value is the same, the Kynurenine to KA reaction did not become extremely strong because this reaction may be complex. However, the synthesis of AnA increased by 25 and 308 times in the 2% and 5% Trp diet groups compared to the control

group although liver kynureninase, which is involved in the reaction of kynurenine to AnA did not change (data not shown). The reaction of kynurenine to 3-hydroxykynurenine is also involved in AnA formation, because a decrease in this reaction caused kynurenine to increase. Therefore, the increased urinary excretion of AnA was not attributable to any changes in the enzyme activities involved. We could not clearly explain why the excretion of AnA increased in the rats fed with the 2% and 5% Trp diets. But, Urinary excretion of AnA shows that extremely increase appeared lower level of adverse effect. We thought this might be able to be used as a bio marker.

Excess Trp intake is able to known by the daily amount excretion of AnA, but if we use metabolite of dependent on the Trp intake, it becomes easy. We calculated some ratios of AnA/some Trp metabolite. As Table 2 shows, the urinary excretory ratios of AnA/KA, AnA/XA and AnA/3-HA in the 2% and 5% Trp diet groups were higher than simple dose-dependence. When using the excretion ratio as a useful index to prevent excessive tryptophan intake, a spot test of urine can be used for the evaluation. Among the three ratios, the change in AnA/KA was the greatest. However this needs further examination because, in practice, we do not know when Trp metabolites appear in the urine.

In conclusion, we propose the urinary excretory ratio of AnA/KA, as an index to mark the excessive intake of Trp in rats. We want to study In the future whether it is possible to adapt this index.

Acknowledgment

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Communication

Molybdenum and Chromium Concentrations in Breast Milk from Japanese Women

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Molybdenum (Mo) and chromium (Cr) in 79 Japanese breast milk samples were measured by inductively coupled plasma-mass spectrometry. For Mo, 51 samples (64.6%) showed less than 5 ng/ml and only 12 samples (15.2%) showed more than 10 ng/ml. The range and median were <0.1 to 25.91 and 3.18 ng/ml respectively. For Cr, 38 samples (48.1%) showed less than 1 ng/ml, 20 samples (25.3%) showed 1 to 2 ng/ml, and only six samples (7.6%) showed more than 5 ng/ml. The range and median were <0.1 to 18.67 and 1.00 ng/ml respectively.

Key words: molybdenum; chromium; breast milk; dietary reference intake; inductively coupled plasma-mass spectrometry

Mo and Cr are essential trace elements in human nutrition, and deficiencies of them have been observed in patients with long-term total parenteral nutrition.[1,2] In Dietary Reference Intakes for Japanese in 2005 (DRI-J 2005), the recommended dietary allowances of Mo and Cr for adults were set at 20 to 25 μg/d and 25 to 40 μg/d respectively.[3]

Information on the secretion of trace elements in human milk is needed in order to estimate intake by breast-fed infants and, to establish the recommended intake for infants. In fact, adequate intake (AI) levels of several trace elements for infants (0 to 5 months) were set on the basis of the concentrations of those trace elements in breast milk of Japanese women in DRI-J 2005,[3] but, AI levels for Mo and Cr were not set in DRI-J 2005 because there was no available information on the concentration of these two trace elements in breast milk from Japanese women. In the present study, we measured Mo and Cr concentrations of breast milk samples from 79 Japanese women by inductively coupled plasma-mass spectrometry (ICPMS), and attempted to estimate AI levels for these two trace elements in Japanese infants.

The study was reviewed and approved by the Ethics Committee of the University of Shiga Prefecture, and it followed the Declaration of Helsinki. Seventy-nine healthy Japanese mothers who were breast-feeding exclusively and not taking vitamin or mineral supplements were recruited in several midwife clinics in Hokkaido, Chiba, Kanagawa, Kyoto, Hiroshima, and Nagasaki Prefectures in Japan from March 2005 to December 2006. The numbers of subjects recruited in the various prefectures were as follows: Hokkaido, 12; Chiba, 10; Kanagawa, 15; Kyoto, 30; Hiroshima, 2; and Nagasaki, 10. All the subjects had given birth to infants at term (gestational age 38 to 41 weeks). The mothers were 32.0 ± 4.1 years old (mean ± SD), with a range of 19 to 39 years. There were no health problems in their babies.

Breast milk was obtained from the subjects at an intermediate time during breast-feeding, placed in a nylon bag (Kaneson, Osaka, Japan) or a polypropylene centrifuge tube (Sumitomo Bakelite, Tokyo, Japan) and stored in a freezer at −20 °C until analysis. The postpartum day on which the sample was collected was 95.5 ± 46.8 d (mean ± SD) with a range of 5 to 191 d.

Two to 5 milliliters of breast milk was transferred to a ceramic melting pot (32ϕ × 24 mm), dried at 90 °C for 1 h in an electric oven, and then heated in an electric furnace (As One F-B1414M, Osaka, Japan) at 550 °C for 16 h. After dry incineration, the remaining ash was dissolved in 5 ml of 2% HNO_3. Mo and Cr in the sample solutions thus prepared were measured by ICPMS with

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Abbreviations: Mo, molybdenum; Cr, chromium; DRI-J 2005, Dietary Reference Intakes for Japanese in 2005; AI, adequate intake; ICPMS, inductively coupled plasma-mass spectrometry; Rh, rhodium; WHO, World Health Organization

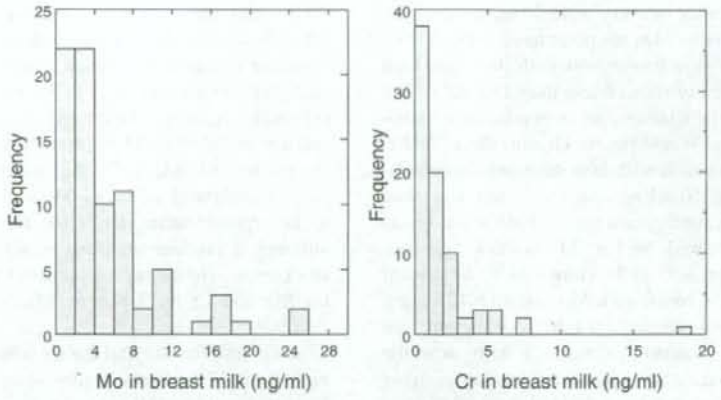

Fig. 1. Histograms of Mo and Cr Concentrations in Breast Milk from 79 Japanese Women.

direct nebulization. The ICPMS operating conditions were as follows: instrument, ICPM-8500 (Shimadzu, Kyoto, Japan); forward power, 1,200 W; coolant gas flow rate, 7.0 l/min; auxiliary gas flow rate, 1.5 l/min; nebulizer gas flow rate, 0.58 l/min; sampling depth, 5.0 mm; integration time, 2.0 s; number of runs, 20; mode of analysis, pulse; isotopes monitored, ^{52}Cr, ^{95}Mo, ^{97}Mo, and ^{98}Mo. A rhodium (Rh) isotope (^{103}Rh) was used as the internal standard. Since the three analytical values obtained from ion intensities at 95, 97, and 98 m/z were similar, the mean was used for Mo quantification. Mean values of triplicate analyses were used as Mo and Cr values for each subject. The detection limit was 0.1 ng/ml of breast milk for both elements.

Quadruplicate analyses of standard non-fat milk powder (SRM 1549, certified Cr content, 2.6 ± 0.7 ng/g; non-certified Mo content, 0.34 μg/g) showed values (mean ± SD) of 2.9 ± 0.6 ng/g as Cr content and 0.32 ± 0.04 μg/g as Mo content. On the other hand, quadruplicate analyses of pooled breast milk and a mixture of pooled breast milk with 1 ng/ml of standard Mo or Cr showed values (mean ± SD) of 5.22 ± 0.12 and 6.25 ± 0.10 ng/ml as Mo and 1.35 ± 0.11 and 2.26 ± 0.13 ng/ml as Cr respectively. In addition, quadruplicate analyses of pooled breast milk on another day showed 5.27 ± 0.08 ng/ml as Mo and 1.28 ± 0.09 ng/ml as Cr.

Among the 79 breast milk samples, only one sample had non-detectable Mo and 15 samples had non-detectable Cr. Figure 1 shows histograms of Mo and Cr concentrations in 79 breast milk samples. For Mo, 51 subjects (64.6%) showed less than 5 ng/ml, and only 12 subjects (15.2%) showed more than 10 ng/ml. This distribution of breast milk Mo is coincident with that observed in our preliminary study.[4] Similarly, for Cr, 38 subjects (48.1%) showed less than 1 ng/ml and 20 subjects (25.3%) showed values ranging from 1 to 2 ng/ml, while only six subjects (7.6%) showed more than 5 ng/ml. Except for samples with non-detectable Mo or Cr, skewness and kurtosis were calculated to be 0.210 ($z = -0.245$, NS) and −0.532 ($z = 6.853$, $p < 0.001$) for log Mo ($n = 78$) respectively, and −0.028 ($z = 0.094$, NS) and −0.101 ($z = 5.517$, $p < 0.001$) for log Cr ($n = 64$) respectively. These results indicate that both Mo and Cr show logarithmical normal distribution rather than normal distribution.

Table 1. Summary of Analyses of Molybdenum and Chromium Contents in Breast Milk from 79 Japanese Women

	Mo (ng/ml)	Cr (ng/ml)
Mean*	5.42	1.73
Standard deviation*	5.33	2.57
Minimum	<0.1	<0.1
Maximum	25.91	18.67
Geometric mean*	3.57	0.69
Median	3.18	1.00
25 percentile value	1.89	0.31
75 percentile value	7.16	2.32

*Non-detectable values were set to 0.05 ng/ml, which was half the detection limit.

Table 1 summarizes the analytical results for Mo and Cr in 79 breast milk samples. In the calculation of these statistical values, we set all non-detectable values to 0.05 ng/ml, which was half the detection limit. The arithmetical means for Mo and Cr in the 79 samples were 5.42 and 1.73 ng/ml respectively. Since both elements showed logarithmical normal distribution, their geometric mean and median values were lower than their arithmetical mean values. The ranges of geometric mean ± geometric standard deviation were 1.33 to 9.57 ng/ml for Mo and 0.17 to 3.34 ng/ml for Cr. There was no significant association between Mo or Cr concentrations and days postpartum on which samples were collected. In addition, no regional variation was observed in Mo or Cr.

There have been several reports on Mo in breast milk. Gunshin *et al.* found that the mean and range of Mo concentration in breast milk from 24 Japanese women

from 19 to 384 d after delivery were 24 ng/ml and 5 to 63 ng/ml respectively.[5] On the other hand, reports from the US found that most human milk collected more than 1 month after delivery showed less than 2 ng/ml of Mo concentration.[6,7] In addition, an international collaborative study by the World Health Organization (WHO) showed that most breast milk samples from Guatemala, Hungary, Nigeria, Sweden, and Zaire had less than 5 ng/ml Mo concentration, whereas Philippine breast milk samples showed higher Mo values (median, 16.36 ng/ml; range, 6.75 to 35.41 ng/ml).[8] The present analytical values of breast milk Mo (mean, 5.42 ng/ml; median, 3.18 ng/ml; range, <0.1 to 25.91 ng/ml) are lower than those obtained in Gunshin's study or in the Philippines, but somewhat higher than those in analyses performed in many countries outside of Asia. Since rice and soybeans are rich in Mo,[9] the dietary Mo intake of Asian people who eat large amounts of rice and soybean products is expected to be higher than that of Western people. In fact, we confirmed that dietary Mo intake and serum Mo concentrations in Japanese is somewhat higher than in Americans or Europeans.[9,10] Accordingly, it is likely that the Mo concentration in Japanese breast milk is somewhat higher than in breast milk collected in the US or Europe. The present analytical values for breast milk Mo are reasonable and representative values for Japanese breast milk, although the cause of high Mo values in breast milk in Gunshin's study[5] and in the Philippines[8] is unclear.

There have also been several reports on Cr concentrations in breast milk. In Japanese subjects, values of 6.5 ng/ml and of a non-detectable level to 20.9 ng/ml were reported as the mean and range respectively for 24 Japanese subjects.[5] Another recent Japanese study of a large number of subjects (n = 1,166) reported 59 ± 47 ng/ml (mean ± SD) as the breast milk Cr concentration,[11] but, the values in the latter study are not reliable, since no accuracy evaluation of analytical values using standard reference materials was performed. Similarly, the reliability of the former study is also insufficient, since accuracy was evaluated using only orchard leaves (SRM 1571), which contained about 1,000-fold higher amounts of Cr than breast milk. On the other hand, several recent reports indicate that the amounts of Cr in breast milk from most American mothers is less than 1 ng/ml.[12,13] Accordingly, the Dietary Reference Intakes of the US has adopted a value of 0.25 ng/ml as the average Cr value in breast milk from American mothers.[14] The present analytical values (mean, 1.73 ng/ml; median, 1.00 ng/ml; range, <0.1 to 18.67 ng/ml) were somewhat higher than the US averaged values, but are coincident with breast milk Cr values observed in an international collaborative study performed by the WHO;[15] the present Cr values are therefore reasonable and representative values for Japanese breast milk.

The main purpose of this study was to estimate the AI values for Mo and Cr for Japanese infants. In DRI-J 2005, since the content of each nutrient in breast milk taken by healthy infants is considered to be sufficient to maintain adequate nutritional status, the AI value for each nutrient for infants (0 to 5 months) was set by the following equation: [averaged concentration of each nutrient in breast milk] × [mean volume of milk intake in infants (0.78 l/d)].[16] As mentioned above, the present analytical values of Mo and Cr are considered to be representative values for Japanese breast milk, although a random sampling was not performed in a strict sense. Hence, we can attempt to estimate the AI for Mo and Cr for Japanese infants using the present results.

Since both the Mo and the Cr concentration in breast milk from 79 Japanese women showed a logarithmical normal distribution, the geometric mean is suitable for their averaged values. However, when the data include values below detection limit, the geometric mean may vary with the way of treating them. In Table 1, we set all non-detectable values to 0.05 ng/ml, which was half the detection limit. This treatment is the most convenient and has been adopted in many studies, but the estimated geometric mean varies with the setting of the detection limit. Other approaches to estimating the geometric mean of data including non-detectable values are to use Cohen's maximum likelihood estimator method,[17] the normal plot method,[18] and the robust method.[19] Following Cohen's method, we calculated the geometric means of the data excluding non-detectable values and adjusted those geometric means to those of all the data using a detection limit value (0.10 ng/ml) and Cohen's λ.[20] According to Cohen's method, the geometric means for Mo and Cr in the 79 breast milk samples were estimated to be 3.52 and 0.71 ng/ml respectively. Following the normal plot method, we depicted two probability plots, as shown in Fig. 2, and calculated a regression equation of log Mo or log Cr *versus* normal scores. Based on X intercepts in the equation, the geometric means for Mo and Cr were estimated to be 3.66 and 0.82 ng/ml respectively. Following the robust method, we substituted normal scores of the non-detectable values for Y in the regression equation of Fig. 2 to estimate extrapolated values below the detection limit. After this extrapolation, the geometric means for Mo and Cr in the 79 samples were estimated to be 3.66 and 0.82 ng/ml respectively.

These geometric means estimated by Cohen's method, the normal plot method, and the robust method are different from those described in Table 1 (Mo, 3.57; Cr, 0.69 ng/ml). Thus, since the geometric mean of the data including non-detectable values varied with the treatment of non-detectable values, we used medians as averaged values for Mo and Cr in the 79 Japanese breast milk samples to estimate AI. When the median is used in the estimation, the AI values for Mo and Cr for Japanese infants (0 to 5 months) are 2.5 μg/d (3.18 μg/l × 0.78 l/d = 2.48 μg/d) and 0.8 μg/d (1.00 μg/l × 0.78 g/l = 0.78 μg/d) respectively.

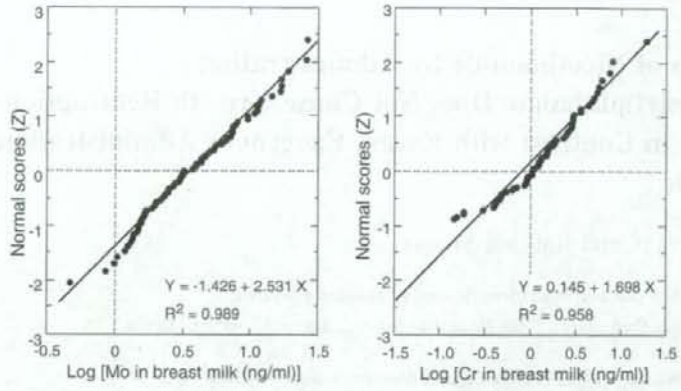

Fig. 2. Regression of Log Mo and Log Cr *versus* Normal Scores.

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Note

The Formation of Nicotinamide by Administration of Di(2-ethylhexyl)phthalate Does Not Cause Growth Retardation in Young Rats in Contrast with Excess Exogenous Administration of Nicotinamide

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We investigated the relationship between protein and tryptophan intake and the adverse-effect-level of di(2-ethylhexyl)phthalate (DEHP). Growth retardation of young rats due to DEHP was strengthened by increasing protein level. The addition of tryptophan to the diet caused extreme increases in the nicotinamide formation, but no growth retardation was observed.

Key words: nicotinamide; phthalic acid ester; tryptophan; toxicity; di(2-ethylhexyl)phthalate (DEHP)

DEHP is a ubiquitous environmental contaminant due to its extensive use as a plasticizer.[1] Since phthalate plasticizers are not chemically bound to polyvinylchloride plastics, they can leach, migrate, or evaporate into indoor air and atmosphere, foodstuffs, and other materials. These esters have been detected in animals and humans.[2–4] We have reported that the administration of phthalic acid esters such as dibutylphtahlate[5,6] and DEHP[7–12] disturbed the metabolism of *de novo* NAD biosynthesis. Concretely, the administration of phthalic acid esters increased the formation of QA (a key intermediate of the *de novo* NAD biosynthesis) and its metabolites.[7] For example, the conversion ratio of Trp to Nam was abnormally increased up to 30% by feeding of a diet containing 3% DEHP, while the conversion ratio of group on a non-DEHP diet was about 2%.[7] Handler *et al.*[13] and Shibata and Tanaka[14] have reported that the intake of an excess of Nam caused retarded growth in young rats. We have proposed that part of growth retardation in young rats due to phthalic acid esters is attributable to excess Nam formation. Objective of the present study was to test this hypothesis. Here we report that our hypothesis was clearly disconfirmed.

NAD^+ and $NADP^+$ were purchased from Sigma Chemical Company (St. Louis, MO). Vitamin-free milk casein, sucrose, L-methionine, Nam, and QA were purchased from Wako Pure Chemical Industries (Osaka, Japan). KA, XA, and MNA chloride were purchased from Tokyo Chemical Industry (Tokyo). 2-Py and 4-Py were synthesized by the methods of Pullman and Colowick[15] and Shibata *et al.*[16] respectively. Corn oil was purchased from Ajinomoto (Tokyo). Mineral and vitamin mixtures and gelatinized cornstarch were obtained from Oriental Yeast (Tokyo). All the other chemicals used were of the highest purity available from commercial sources.

The care and treatment of the experimental animals conformed to The University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. Male rats of the Wistar strain (3 weeks old with a body weight of about 40 g) were obtained from CLEA Japan (Tokyo). They were immediately placed in individual metabolic cages (CT-10; Clea Japan). In experiment 1, the rats were divided into five groups and fed *ad libitum* for 21 d on 40% casein diets[17] containing a niacin-free AIN-93 vitamin mixture with 0, 0.1, 0.5, 1.0, and 2.0% DEHP. In experiment 2, the rats were divided six groups and fed *ad libitum* for 21 d on niacin-free 20% casein diets,[7] with 0, 0.1, and 0.5% Trp with and without 0.1% DEHP. The room temperature was maintained at about 20 °C and about 60% humidity, and a 12-h light/12-h dark cycle was maintained. Body weight and food intake were measured daily at about 10:00 AM. In experiment 2, urine samples (24-h; 10:00 AM–10:00 AM) on the last day were collected in amber bottles containing 1 ml of 1 M HCl, stored at −20 °C until needed. The rats were killed by decapitation. To measure NAD (NAD^+ + NADH) and NADP ($NADP^+$ + NADPH), the

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Abbreviations: DEHP, di(2-ethylhexyl)phthalate; Trp, L-tryptophan; AnA, anthranilic acid; KA, kynurenic acid; XA, xanthurenic acid; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; Nam, nicotinamide; MNA, N^1-methylnicotinamide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide