表4　水溶性ビタミンの生体利用率の計算方法（パントテン酸を例として）

被験者番号	No.1
食事からのパントテン酸摂取量（μmol/日）	43.79
データ1＝尿中パントテン酸排泄量（μmol/日）	26.47
1 mol の食事性パントテン酸を摂取した時に尿中に排泄されるパントテン酸量（mol/日）＝26.47（尿中パントテン酸排泄量，μmol/日）/43.79（食事性パントテン酸摂取量，μmol/日）	0.60
データ2＝尿中パントテン酸排泄量（μmol/日）	81.60
食事性パントテン酸＋服用した水溶性ビタミン混合中のパントテン酸量（μmol/日）	124.99
水溶性ビタミン混合からのパントテン酸摂取量：遊離型のパントテン酸摂取量（μmol/日）	86.20
増大したパントテン酸排泄量（μmol/日）＝*データ2*(81.60)－*データ1*(26.47)	55.13
1 mol の遊離型パントテン酸を摂取した時に尿中に排泄されるパントテン酸量（mol/日）＝55.13（尿中パントテン酸排泄量，μmol/日)/86.20（遊離型パントテン酸摂取量，μmol/日）	0.64
生体利用率（%)＝(0.60/0.64)×100	94.50

るので，ここで紹介した方法では生体利用率を計算することはできない。

7.3　日本人の食事摂取基準（2005年版）で採用された生体利用率

食事性ビタミンB_6が75%，食事性葉酸が50%，食事性ビタミンB_{12}が50%という数値が採用された。なお，他のビタミンについては，生体利用率は考慮されていない。示されたデータの出所は，いずれも，日本人を被験者としたものではない。今後，日本人を被験者として，幅広い年齢階層で，代表的な日本食メニュー中の水溶性ビタミンの生体利用率を明らかにしていけば，より質の高い栄養計画をたてることができる。

健常者が寿命の限界まで健康を維持し続けるには，毎日毎日，「日本人の食事摂取基準」[1,2]に記載されている栄養素量を摂れば良い。しかし，先に記載したように，食事摂取基準の数値と食品成分表の数値は直接比較することはできない。食品成分表は食品化学的な立場から，一方，食事摂取基準は生命化学的な立場から数値が決められているからである。水溶性ビタミンに関しては，ビタミンB_6，ビタミンB_{12}，葉酸が生体利用率を考慮にいれて策定されていることは述べた。したがって，これら三つのビタミンに関しては，食品成分表と食事摂取基準との数値は，直接比較可能である。他の五つの水溶性ビタミンは生体利用率を考慮した策定にはなっておらず，栄養計画をたてにくい。食品成分表から計算したビタミン量のみで，食事摂取基準で示された量を摂っていると計画しても，評価はできない。生体側がどれだけ利用しているのかわからないからである。そのためにも代表的な食事中の水溶性ビタミンの生体利用率を求める研究が必要である。

8　摂取した水溶性ビタミンの体内運命

食べた水溶性ビタミンがすべて生体内で利用されるわけではない。水溶性ビタミンはビタミンCとB群ビタミンに分類できる。ビタミンCは食品中でも高分子と比較的ゆるく結合しており，ビタミン体（アスコルビン酸）と活性型（アスコルビン酸として機能を発揮する）は同じである。生体利用率は100%と考えてもよい。

一方，B群ビタミンはビタミン体そのものでは生体内で機能を発揮することはできないので，活性型に変換されて，酵素反応を補助する生体分子である補酵素として存在している。そのため，まず消化管腔内で消化が必要である。酵素タンパク質と結合しているため，タンパク質分解酵素が関与する。タンパク質が加水分解されると，補酵素が遊離状態となる。次に補酵素が消化されて，遊離状態のビタミン体となる。そして，各々の特異的な輸送担体によって，血液中に輸送される。肝臓に取り込まれたビタミンの一部は肝臓のために使用されるが，多くは，備蓄される。そのため，肝臓はB群ビタミン含量が他の組織に比べてきわめて高く，遊離型のビタミン体としても検出される。備蓄された肝臓中のビタミンは，必要に応じて（要求する組織からなんらかの信号がでているのであろうが不明）再度血液中に放出される。必要な組織は，細胞膜の輸送担体数を増

加させるなどして，積極的にビタミンを細胞内に取り込むものと考えられる。取り込まれたビタミンは補酵素型に合成される。そして，補酵素は酵素タンパク質と結合して，はじめて機能を発揮する。機能を終えた酵素は細胞内で加水分解され，補酵素が自由になれる。すると，補酵素が加水分解され，遊離型のビタミンとなる。遊離型のビタミンの供給系が正常に機能している場合は，尿中にビタミンそのものあるいはその異化代謝産物が排泄される。

9　現在の栄養評価方法

栄養計画をたてる方法は，今までの栄養学で行われている食品化学的な方法で良い。

現在の栄養評価は，多くの場合調査対象者に，食べたものの種類・重量を記載してもらい，その記録から，食品成分表を用いて栄養素の摂取量を計算し，その量を食事摂取基準と比較することで，評価を行っている。食べたものを利用する生命側の情報を考慮せずに栄養評価がなされている。もっと深刻な問題がある。食事調査が難しい。いい加減になりやすい。ある報告によると，肥満者は食べた量よりも20％減の報告をするものが多いとか，日本ではやせに分類される人でも20％減の報告をするという。現在よく用いられている食べたものを記載する方法よりも，習慣的に摂取する食品が聞き取れるような質問票もできているので，より精度の高い食事調査が可能となることが期待できる。しかし，あくまでも受け手である人のことを考慮しない評価となることには違いない。

10　新しい栄養評価方法の提案～尿中の水溶性ビタミン量で水溶性ビタミンの栄養評価～

尿中の水溶性ビタミン量から，水溶性ビタミンの栄養評価が可能である。

生体側が要求する必要量を満たすことができない日々が続くと，生体側は危急の防御策として，いったん役割を終えて尿中に排泄されるべき水溶性ビタミンを再利用するように代謝系を切り替える。健常者から非健常者への境界に入らないための抵抗期間である。その結果，尿中への水溶性ビタミンあるいはその異化代謝産物量が低下しはじめる。この時点では，体内の水溶性ビタミン量は，正常範囲内に維持されているので，欠乏症が現れることはなく，まだ，健常者である。尿中への排泄量を極限まで減らしても，体内の水溶性ビタミンの必要量をまかなえなくなると，はじめて血液中の水溶性ビタミン量が低下してくる。健常者ではなくなる。栄養評価は健常者に対しての評価である。

ここで，提案する尿中の水溶性ビタミン量で水溶性ビタミンの栄養評価，つまり，尿中のビタミンあるいはその異化代謝産物量の正常範囲の値をあらかじめ明らかにし，その値と比較することで，水溶性ビタミンの栄養評価をしようとするものである。この方法では，食べたものを調べる必要はない。評価がよければ，現在の食生活を続ければ良い。評価が悪い水溶性ビタミンがあれば，栄養士に栄養計画をしてもらい，1 週間程度，栄養計画にしたがった食事をした後，再度尿中の水溶性ビタミン量を測定すればよい。1 回の栄養指導で尿中の水溶性ビタミンの値が，所定の範囲内にはいれば終了。定期的に尿中の値を測定すればよい。1 回の指導で範囲内に入らなければ，再度栄養士に栄養計画を依頼し，採尿し分析する，ということを繰り返せばよい。

11　どこで尿中のビタミン量が測定してもらえるのか

営業で尿中のビタミンを専門的に測定している企業はない。依頼すれば，測定できる技術を有している企業もある。

大学等の研究室では，我々の研究室がある。

尿中の水溶性ビタミン量を測定することで，水溶性ビタミンの栄養評価を行う。当たり前のようであるが，今までになかったビタミンの新しい利用方法である。

（平成 17 年 12 月 9 日受理）

文　献

1）日本人の栄養所要量－食事摂取基準－検討会編，“日本人の栄養所要量－食事摂取基準－検討会報告書，日本人の食事摂取基準（2005 年版）”，(2004，厚生労働省；http://www.mhlw.go.jp/houdou/2004/11/h1122-2.html)

2）第一出版編集部編，“厚生労働省策定，日本人の食事摂取基準（2005 年版）”，(2005，第一出版).

ノート

高齢者の血液中 NAD および NADP 含量

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Blood NAD and NADP Levels in the Elderly

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We measured the NAD and NADP levels in blood from elderly subjects to obtain data for comparisons with levels in young adults. Our data indicated that the levels of niacin coenzymes in the elderly born in 1927 were almost in the same range as those of young adults. From this, we concluded that the special considerations for calculation of the niacin requirements for the elderly were unnecessary.
Key words: NAD, NADP, niacin, blood, elderly subjects.

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緒　言

2004 年 10 月に厚生労働省は新しい「日本人の食事摂取基準 (2005 年版)」を答申した[1].

しかし，高齢者に対する食事摂取基準の策定は，その根拠となる高齢者を対象とした研究が少ないことから，科学的裏付けが乏しい.

高齢者のナイアシン必要量に関する研究は，柴田ら[2]の報告がある．この報告をもとに，「第六次改定日本人の栄養所要量－食事摂取基準－」[3]も今回の「日本人の食事摂取基準 (2005 年版)」[1]も，高齢者と若年成人間でナイシシン代謝上差異はみられなかったとされているが，ナイアシン代謝産物の尿中排泄量[2]から導かれたものであり，血液中のナイアシンは測定されていない．そこで，本研究では，高齢者の血液中の NAD および NADP 含量を測定した.

実験方法

本研究は 1998 年から開始され 2008 年に終了する予定のコホート研究「高齢者の口腔保健と全身的な健康状態の関係についての総合研究 (新潟市高齢者コホート調査：男 300 名，女 300 名) の追加研究として実施した．コホートの詳細は既に報告されている[4]．なお，本研究は，新潟大学医歯学総合研究科の倫理委員会の承認を得て実施された.

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(1) 被験者

昭和 2 年 (1927 年) 生まれの高齢者 (男女) を対象に新潟市で 6 月に実施されている「新潟市高齢者コホート調査」の検診受診者 2001 年 (男性 236 名，女性 200 名) のうち 61 名 (男性 34 名，女性 27 名) および 2003 年 (男性 215 名，女性 191 名) のうち 125 名 (男性 69 名，女性 56 名) を対象とした．

(2) NAD (NAD^++NADH) と NADP ($NADP^+$+NADPH) の分析方法

Shibata らの方法によった[5)6)7)]．すなわち，上腕部より採取した直後の全血液を 20 μl 取り出し，直ちに 100 mM ニコチンアミドを含む 50 mM KH_2PO_4-K_2HPO_4 緩衝液 (pH 6.0) 400 μl が入っているミクロチューブ中に加えてよく混合し，90℃で 1.5 分間加熱処理した後，冷却後，遠心上清を血中 NAD および NADP 含量の測定試料とした．

血中 NAD 含量の測定は，測定試料 (遠心上清) 10 μl，2.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H tetrazolium bromide (MTT) 溶液 10 μl，1 mg/ml phenazine methosulfate (PMS) 溶液 80 μl，100 mM ニコチンアミドと 500 mM エタノールを含む 65 mM グリシルグリシン-NaOH 緩衝液 (pH7.4) 150 μl，75 IU/ml アルコール脱水素酵素溶液 50 μl をよく混和し，37℃で 20 分間加温した後，570 nm における吸光度を測定した．

血中 NADP 含量の測定は，測定試料 (遠心上清) 20 μl，2.5mg/ml MTT 溶液 10 μl，1 mg/ml PMS 溶液 80 μl，150 mM ニコチンアミドを含む 150 mM グリシルグリシン-NaOH 緩衝液 (pH 7.4) 80 μl，10 mM グルコース 6-リン酸溶液 60 μl，2 IU/ml グルコース 6-リン酸脱水素酵素溶液 50 μl をよく混和し，37℃で 20 分間加温した後，570 nm における吸光度を測定した．なお，2001 年は NADP·NAD を測定し，2003 年は NAD のみを測定した．

(3) 統計処理

NAD，NADP，NAD/NADP の数値は，すべて平均値 ± 標準偏差 (SD) であらわした．2001 年の調査における男女差の有意差検定は，生データを対数変換した後，Student の t-test により危険率 5% にて有意性を判定した．また，2001 年の NAD 値と 2003 年の NAD 値の比較，および高齢者と若年成人間の比較も同様に行った．なお，検定は，統計ソフト Instat (version 2.0：GraphPad，San Diego, CA, USA) を使用しておこなった．

結　果

2001 年における 73，74 歳の高齢者 61 名の全血 1 ml 当たりの NAD 含量の度数分布図を Fig. 1-A に示した．値は 41.3 ± 15.9 nmol/ml であった．また Fig. 1-B に全血 1 ml 当たりの NADP 含量の度数分布図を示した．値は 12.4 ± 1.8 nmol/ml であった．さらに，この時の NAD/NADP 比を Fig. 1-C に示した．この比率の値は 3.4 ± 1.4 であった．この 2001 年の調査において，男女差を調べたが，NAD，NADP，および NAD/NADP 比において，いずれも男女差は認められなかった．

2003 年における 75，76 歳の高齢者 125 名の全血 1 ml 当たりの NAD 含量の度数分布図を Fig. 2 に示した．値は 43.2 ± 7.6 nmol/ml であった．

なお，2001 年の NAD の値と 2003 年の値との間に有意な差異は認められなかった．

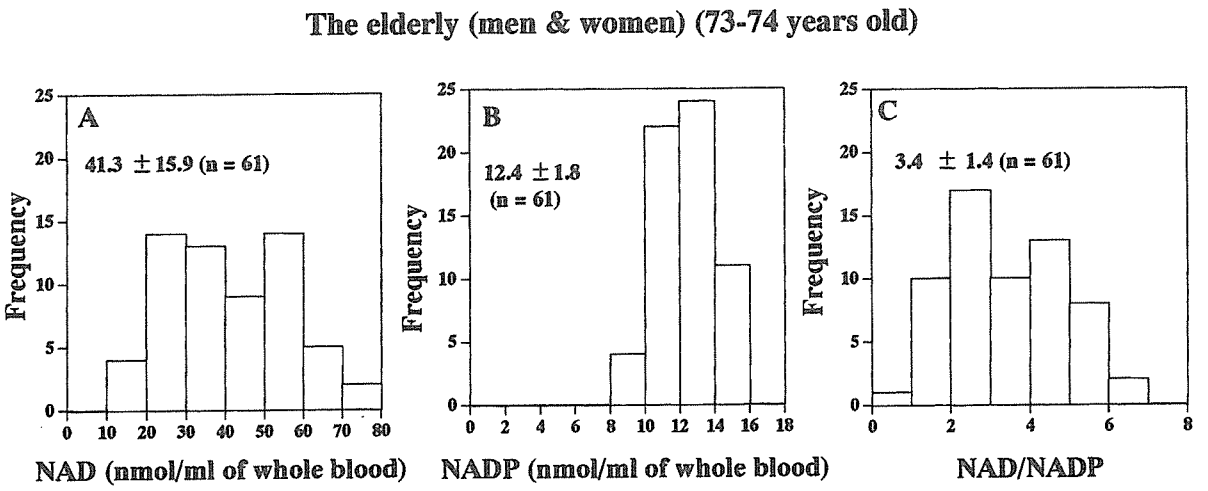

Fig. 1. Histograms of Blood NAD (A) and NADP (B) Levels, and the Ratio of NAD/NADP (C) in the Elderly (men & women) (73-74 years olds).

考　察

本研究の対象者は自立した生活を送っている高齢者であるが，その多くは内科あるいは整形外科などに通院し投薬を受けている．しかし，同じコホートを対象に実施した秤量法の食事調査[8]ではエネルギー2250 ± 400 kcal/日，ナイアシン20.3 ± 6.9mg/日（平均値±標準偏差）摂取しており，平均的にはナイアシン摂取量/エネルギー比は9.1 ± 2.3mg/1000kcalとなっていた．その結果を反映して，全血中のNADは41.3 ± 15.9 nmol/mlおよび43.2 ± 7.6 nmol/mlという値を維持しているものと考えた．

20歳前後の若年成人の値はNADが35 nmol/ml程度[9]，NADPが10 nmol/ml程度[10]であった．血液中のNAD値とNADP値は，若年成人と高齢者間で有意差は認められなかったが，平均値では高齢者の方が高い値を示した．一方，NADP/NAD比は若年成人と変わらず，同じであった．このことより，血液中のナイアシン補酵素レベルが高齢者において低い値を示すことはないことが明らかとなった．高齢者において特異な点は，NAD値の分布が非常に広いということであった．この点を解決するには，食事調査や生体活動状態あるいは，投与薬などを調べる必要性がある．

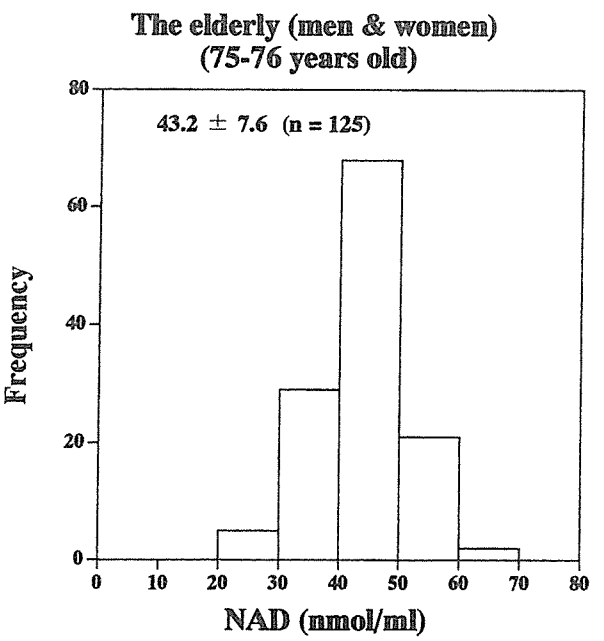

Fig. 2. Histogram of Blood NAD Level in the elderly (men & women) (75-76 years olds).

結　論

今回得られた値と以前に報告した尿中の値[2]から判断すると，高齢者のナイアシン必要量を若年成人の食事摂取基準の推奨量である5.8 mgナイアシン当量/1,000 kcalよりも高い値とした方が良いという理由は見当たらない．

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（平成17.5.6受付）

文　献

1) 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－検討会報告書，2004年10月
2) 柴田克己，真田宏夫，湯山駿介，鈴木健（1994）ナイアシン代謝産物排泄量からみた高齢者におけるナイアシン栄養の評価．ビタミン **68**, 365-372
3) 厚生省，第六次改定日本人の栄養所要量－食事摂取基準－，第六次改定日本人の栄養所要量策定検討会報告書1999年6月
4) Yamaga T, Yoshihara A, Ando Y, Yoshitake Y, Kimura Y, Shimada M, Nishimuta M, Miyazaki H (2002) Relationship between dental occlusion and physical fitness in an elderly population. *J Gerontology: MEDICAL SCIENCES* **57A**, M616-M620
5) Shibata K, Murata K (1986) Blood NAD as an index of niacin nutrition. *Nutr Int* **2**, 177-181
6) Shibata K, Tanaka K (1986) Simple measurement of blood NADP and Blood levels of NAD and NADP in humans. *Agric Biol Chem* **50**, 2941-2942
7) 柴田克己，福渡努，土居美紀，緒方進，下岡令美，田口寛（2001）マイクロプレートリーダーを利用したNADおよびNADPの微量定量方法．ビタミン **75**, 455-462
8) Watanabe R, Hanamori K, Kadoya H, Nishimuta M, Miyazaki H (2004) Nutritional intakes in community-dwelling older Japanese adults: High intakes of energy and protein based on high consumption of fish, vegetables and fruits provide sufficient micronutrients. *J Nutr Sci Vitaminol* **50**, 184-195
9) Shibata K (1987) Blood pyridine nucleotide levels reflect niacin equivalent intake in humans. *J Clin Biochem Nutr* **3**, 37-45
10) 柴田克己，岩井和夫（1990）NADP定量方法の改良と女子学生の血液中のNADP値の訂正．ビタミン **64**, 193-196

栄養表示基準の改訂について

本誌 79 巻 (9) で平成 17 年度から 21 年度に使用される新しい食事摂取基準が策定されたことを紹介した[1]．これは，平成 16 年 10 月 25 日に「日本人の栄養所要量－食事摂取基準－策定検討会（座長：田中平三）においてとりまとめられたものであり，http://www.mhlw.go.jp/houdou/2004/11/h1122-2.html で概要を知ることができる．市販本もある[2]．

表 1．栄養機能食品の規格基準の上限値

Zn	UL －（平均摂取量＋ 2SD）．30 mg － 15 mg = 15 mg
Ca	UL (2300 mg) －目安量の最大値 (1100 mg) ＞医薬部外品最大分量 (600 mg) であるので，医薬部外品最大分量を上限値とする．600 mg
Fe	UL (50 mg) －推奨量の最大値 (13.5 mg) ＞医薬部外品最大分量 (10 mg) であるので，医薬部外品最大分量を上限値とする．10 mg
Cu	UL －（摂取量に関する報告の最大値）．10 mg － 3.6 mg = 6.4 mg → 6 mg
Mg	食物由来 Mg の過剰摂取による好ましくない影響は報告されていない．サプリメント・医薬品などの Mg を多量に摂取した場合に限られる．したがって，通常の食品以外からの摂取量として，5 mg/kg 体重．この値から 300 mg．
ニコチンアミド	UL (300 mg) －推奨量の最大値 (16 mgNE) ＞医薬部外品最大分量であるので，低い方の医薬部外品最大分量を上限値とする．60 mg
ニコチン酸	UL (100 mg) －推奨量の最大値 (16 mgNE) ＞医薬部外品最大分量 (60 mg) であるので，低い医薬部外品最大分量を上限値とする．60 mg
葉酸	UL (1000 μg) －推奨量の最大値 (240 μg) ＞医薬部外品最大分量 (200 μg) であるので，低い方の医薬部外品最大分量を上限値とする．200 μg
ビタミン B_6	UL (60 mg) －推奨量の最大値 (1.5 mg) ＞医薬部外品最大分量 (10 mg) であるので，低い方の医薬部外品最大分量を上限値とする．10 mg
ビタミン A	UL (3000 μg) －推奨量の最大値 (750 μg) ＞医薬部外品最大分量 (600 μg) であるので，低い方の医薬部外品最大分量を上限値とする．600 μg
ビタミン D	UL (50 μg) －目安量の最大値 (5 μg) ＞医薬部外品最大分量 (5 μg) であるので，低い方の医薬部外品最大分量を上限値とする．5 μg
ビタミン E	UL (800 mg) －推奨量の最大値 (10 mg) ＞医薬部外品最大分量 (150 mg) であるので，低い方の医薬部外品最大分量を上限値とする．150 mg
パントテン酸	UL が策定されていないが，医薬部外品最大分量があるので，医薬部外品最大分量を上限値とする．30 mg
ビオチン	UL が策定されていないが，医薬部外品最大分量があるので，医薬部外品最大分量を上限値とする．500 μg
ビタミン B_1	UL が策定されていないが，医薬部外品最大分量があるので，医薬部外品最大分量を上限値とする．25 mg
ビタミン B_2	UL が策定されていないが，医薬部外品最大分量があるので，医薬部外品最大分量を上限値とする．12 mg
ビタミン B_{12}	UL が策定されていないが，医薬部外品最大分量があるので，医薬部外品最大分量を上限値とする．60 μg
ビタミン C	UL が策定されていないが，医薬部外品最大分量があるので，医薬部外品最大分量を上限値とする．1,000 mg

*UL = 上限量

今回は，食事摂取基準の改定に伴い，変更されたものがあるので紹介する．それは，「栄養表示基準」である．これは，平成15年4月24日厚生労働省告示第百七十六号で定められたもので，平成17年7月1日厚生労働省告示第三百十号で改訂されたものが最新である．

食事摂取基準で策定された数値の表[2]は同じ年齢区分・性別でも複数の数値が示されているため，食品の栄養素表示に使用しにくい．そこで，栄養素等表示基準値(NRV)(Nutrient Reference Value)が定められている．栄養素等表示基準値は，国民(集団)の栄養計画を手助けするための簡易食事摂取基準と考えることができ，いわゆる一般向けの簡易食事摂取基準とも考えることができる．

もう少し，具体的に示すと，「日本人の食事摂取基準(2005年版)」の各年齢区分の推定平均必要量(estimated average requirement = EAR)あるいは目安量(adequate intake = AI)に，人口比・性比により加重平均し，値を丸めて，算出したものである．

NRV = ｛栄養素AのEAR(男性6～7歳)×(男性6～7歳の人口)
＋栄養素AのEAR(男性8～9歳)×(男性8～9歳の人口)
・
・
＋栄養素AのEAR(男性70歳以上)×(男性70歳以上の人口)
＋栄養素AのEAR(女性6～7歳)×(女性6～7歳の人口)
＋栄養素AのEAR(女性8～9歳)×(女性8～9歳の人口)
・
・
＋栄養素AのEAR(女性70歳以上)×(女性70歳以上の人口)｝×(1/総人口)

NRVの利用目的は，

①栄養素が含まれている旨の表示(100g当たり，タンパク質はNRVの10%以上，食物繊維・ビタミン・ミネラルはNRVの15%以上)

②栄養素が多く含まれている旨の表示(100g当たり，タンパク質はNRVの20%以上，食物繊維・ビタミン・ミネラルはNRVの30%以上)

③栄養素を含まない旨の表示(100g当たり，脂質は0.5g，飽和脂肪酸は0.1g，コレステロールは5mg，糖質は0.5g，ナトリウムは5mg，エネルギーは5kcal以上では含まない旨を表示できない)

④栄養素が低減されている旨の表示(100g当たり，脂質は3g，飽和脂肪酸は1.5g，コレステロールは20mg，糖質は5g，ナトリウムは120mg，エネルギーは40kcalに低減された量が数値に満たない場合は，「低減された」と表示できない)

表2．栄養素等表示基準値(NRV)の算出根拠の値

エネルギー(推定エネルギー必要量)	身体活動レベルⅡの値
タンパク質	PFC(Protein:Fat:Carbohydrate)比の15%(タンパク質は推定平均必要量ではなく，適正エネルギー比率を採用した)
脂質	PFC比の25%
炭水化物	PFC比の60%
食物繊維	目標量
ビタミン B_1	推定平均必要量
ビタミン B_2	推定平均必要量
ナイアシン	推定平均必要量
ビタミン B_6	推定平均必要量
葉酸	推定平均必要量
ビタミン B_{12}	推定平均必要量
ビタミンC	推定平均必要量
ビタミンA	推定平均必要量
ビオチン	目安量
パントテン酸	目安量
ビタミンE	目安量
ビタミンD	目安量
ビタミンK	目安量
Mg	推定平均必要量
Fe	推定平均必要量
Cu	推定平均必要量
Zn	推定平均必要量
Ca	目安量

⑤栄養機能食品の規格基準の下限値と上限値の算出根拠(下限値はNRVの30%．上限値は表1を参照)

⑥栄養素の必要量の概数の何％が摂取できるかを示すことで，国民の栄養計画の補助とする

である．

表3. 6歳以上のエネルギーと主要栄養素，微量栄養素の NRV（1日当たりの必要量の概数）

エネルギーと主要栄養素

エネルギー	2100 kcal
タンパク質	75 g
脂質	55 g
炭水化物	320 g
食物繊維	20 g

エネルギーと主要栄養素

ビタミン B_1	チアミン塩酸塩	1.0 mg	
ビタミン B_2	リボフラビン	1.1 mg	
ナイアシン	ナイアシン当量	11 mg	
ビタミン B_6	ピリドキシン	1.0 mg	生体利用率 75% として
葉酸	プテロイルモノグルタミン酸	200 μg	生体利用率 50% として
ビタミン B_{12}	シアノコバラミン	2.0 μg	生体利用率 50% として
ビタミン C	アスコルビン酸	80 mg	
ビタミン A	レチノール当量	450 μg	
ビオチン	ビオチン	45 μg	
パントテン酸	パントテン酸	5.5 mg	
ビタミン E	α-トコフェロール	8 mg	
ビタミン D	エルゴカルシフェロールとコレカルシフェロールの合量	5 μg	
ビタミン K	フィロキノンとメナキノン-4 の合計量	70 μg	

注意事項：遊離型のビタミンを添加した場合，生体利用率を考慮する必要あり

ミネラル

Ca	700 mg	25% 程度の吸収率として
Mg	250 mg	40% 程度の吸収率として
Fe	7.5 mg	15% 程度の吸収率として
Cu	0.6 mg	40% 程度の吸収率として
Zn	7 mg	30% 程度の吸収率として

問題点：吸収率を高めた場合，吸収率の問題を考慮する必要あり

栄養素等表示基準値の算出根拠の値を表2に，また表3に6歳以上のエネルギーと主要栄養素，微量栄養素の NRV（必要量の概数）を示した．

「栄養素バランスが良い，悪い」という言葉がよく使用されるが，「栄養素バランス」の基本を示した簡易表が表3である．

（滋賀県立大学人間文化学部生活文化学科食生活専攻
柴田　克己）

文　献

1) 柴田克己，岡野登志夫（2005）新しい食事摂取基準「日本人の食事摂取基準（2005 年版）」が策定された．ビタミン **79**, 461-462
2) 第一出版編集部（2005）厚生労働省策定　日本人の食事摂取基準（2005 年版），第一出版，東京

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

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

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

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

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

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

MATERIALS AND METHODS

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

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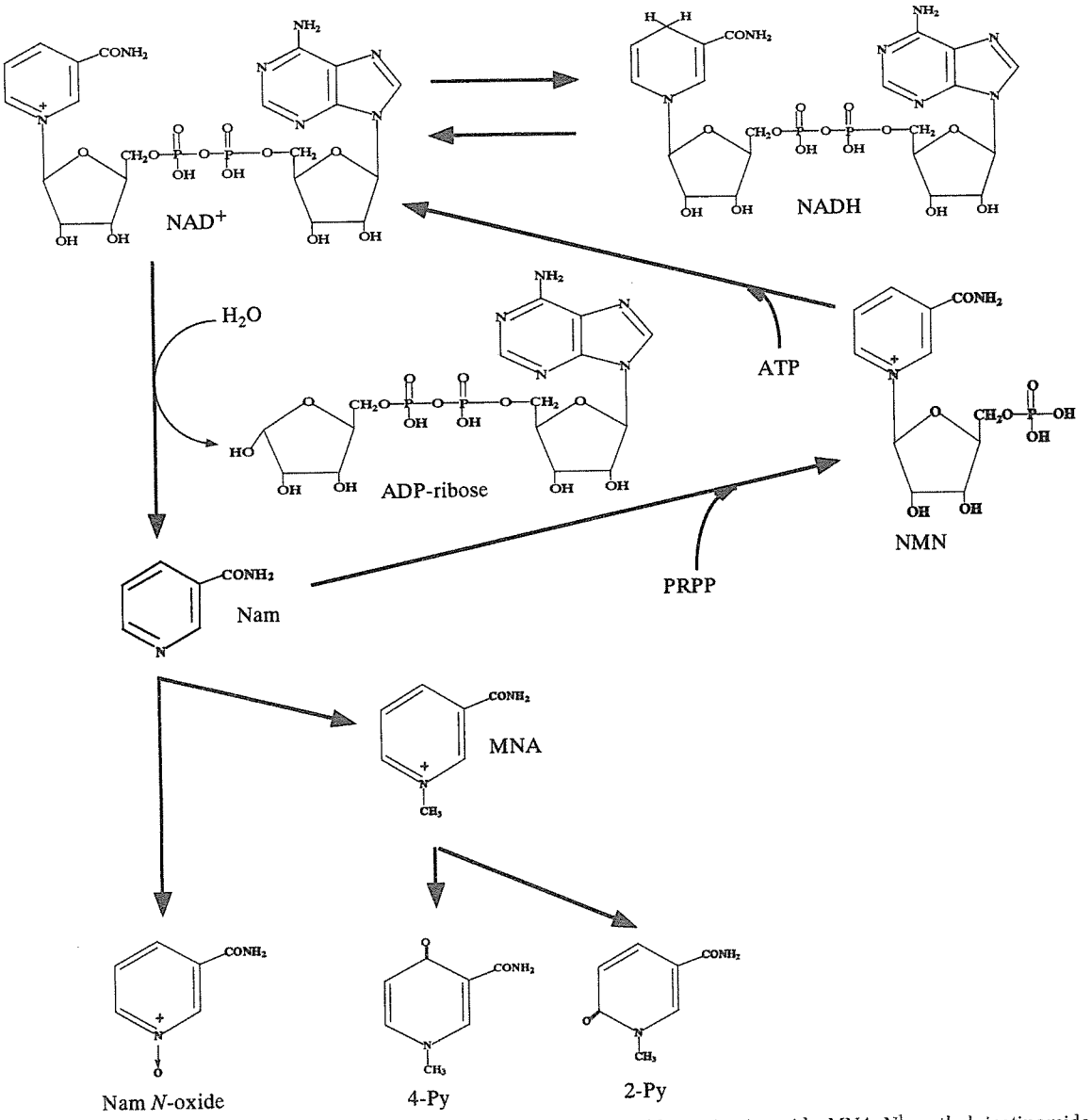

Fig. 1. Proposed metabolic fate of the intraperitoneally injected NADH. Nam, nicotinamide; MNA, N^1-methylnicotinamide; Nam *N*-oxide, nicotinamide *N*-oxide; 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-Py, N^1-methyl-4-pyridone-3-carboxamide; NMN, nicotinamide mononucleotide; PRPP, 5-phosphoribosyl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

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

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

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

tory Animals.

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

Table 1. Composition of the diets.

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

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

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

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

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

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

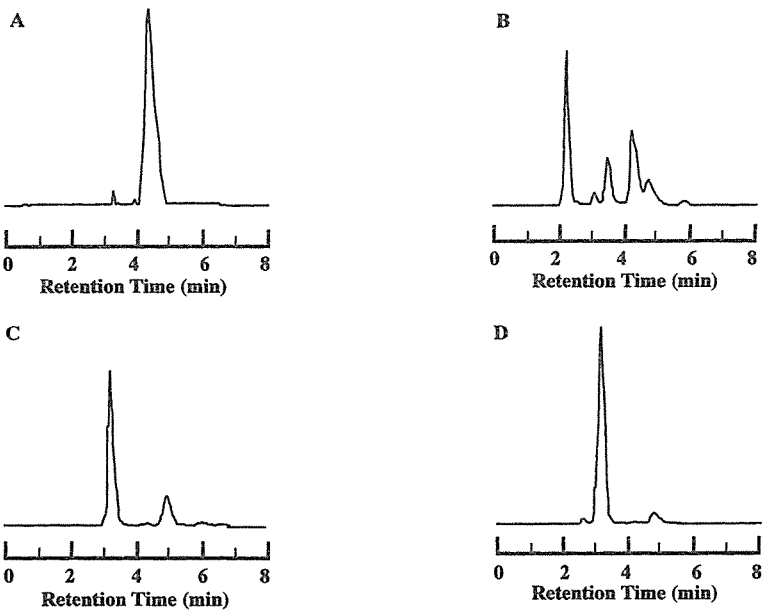

Fig. 2. Time-dependent changes in the HPLC chromatogram of NADH dissolved in 0.1 mol/L HCl. A: NADH was dissolved in H_2O at 0.1 mg/mL and immediately injected into the HPLC. B–D: NADH was dissolved in 0.1 M HCl at 0.1 mg/mL at 25°C, and then injected into the HPLC at (B) 1 min after the dissolution,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

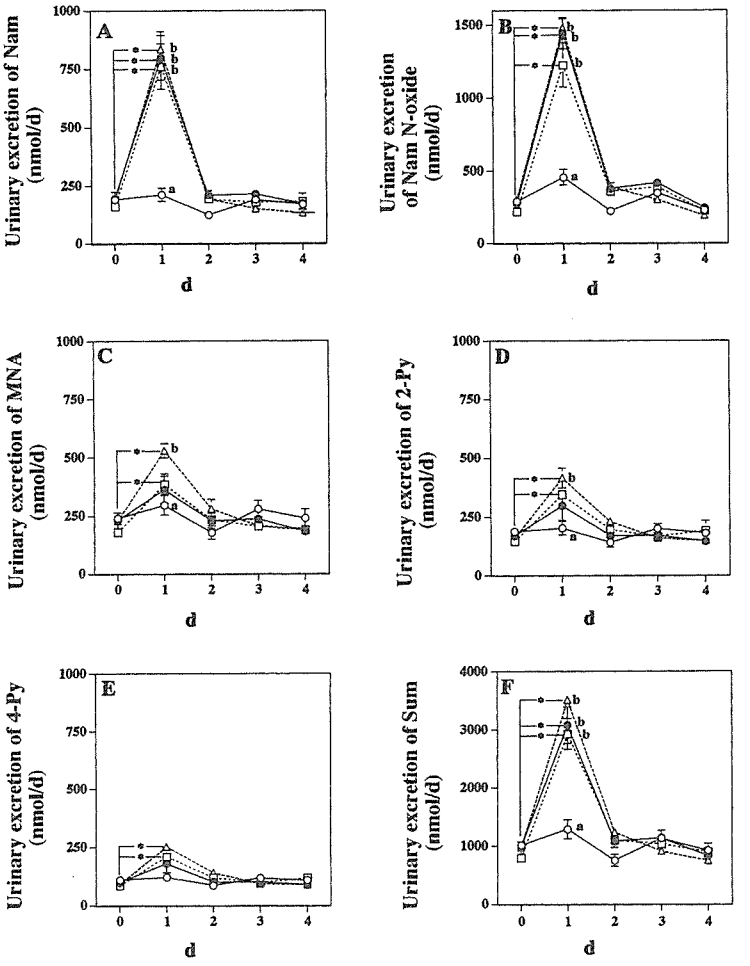

Fig. 3. Effects of intraperitoneal injection of Nam, NAD^+ or NADH on the urinary excretion of nicotinamide and its metabolites in mice. On day 1 at 09:00, 5 μmol/mouse of Nam (●), NAD^+ (□) or NADH (△) dissolved in 0.1 mL of sterile saline was intraperitoneally injected into mice. As a control (○),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

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

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

RESULTS

Changes of NADH and NAD^+ in acidic solution

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

Body weight and food intake

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

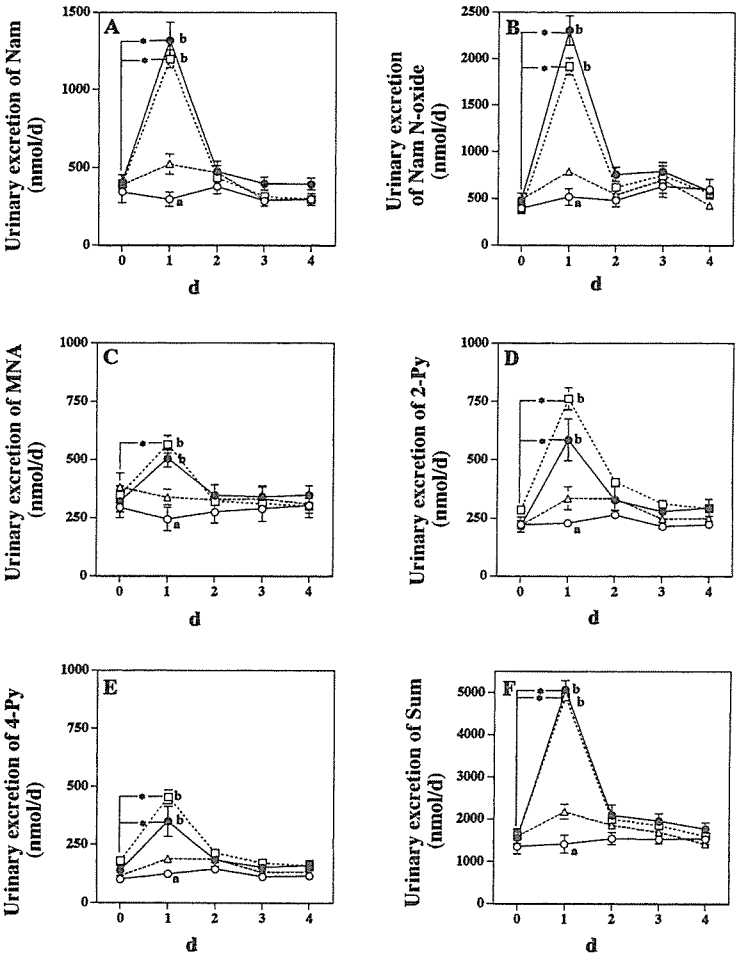

Fig. 4. Effects of oral administration of Nam, NAD^+ or NADH on the urinary excretion of nicotinamide and its metabolites in mice. On day 1 at 09:00, 5 μmol/mouse of Nam (●), NAD^+ (□) or NADH (△) dissolved in 0.1 mL of sterile saline was orally administered to mice. As a control (○), 0.1 mL of sterile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

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

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

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

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

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

DISCUSSION

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

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

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

Acknowledgments

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REFERENCES

1) Gross CJ, Henderson LM. 1983. Digestion and absorption of NAD by the small intestine of the rat. *J Nutr* **113**: 412–420.
2) Henderson LM, Gross CJ. 1979. Transport of niacin and niacinamide in perfused rat intestine. *J Nutr* **109**: 646–653.
3) Shibata K, Kakehi H, Matsuo H. 1990. Niacin catabolism in rodents. *J Nutr Sci Vitaminol* **36**: 87–98.
4) Lowry OH, Passonneau JV, Rock MA. 1961. The stability of pyridone nucleotides. *J Biol Chem* **236**: 2756–2759.
5) Birkmayer JG, Vrecko C, Volc D, Birkmayer W. 1993. Nicotinamide adenine dinucleotide (NADH)—a new therapeutic approach to Parkinson's disease. Comparison of oral and parenteral application. *Acta Neurol Scand Suppl* **146**: 32–35.
6) Birkmayer GJ, Birkmayer W. 1989. Stimulation of endogenous L-dopa biosynthesis—a new principle for the therapy of Parkinson's disease. The clinical effect of nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotidephosphate (NADPH). *Acta Neuro Scan* **126**: 183–187.
7) Swerdlow RH. 1998. Is NADH effective in the treatment of Parkinson's disease? *Drug Aging* **13**: 263–268.
8) Vrecko K, Storge D, Birkmayer JG, Moller R, Tafeit E, Horejsi R, Reibnegger G. 1997. NADH stimulates endogenous dopamine biosynthesis by enhancing the recycling of tetrahydrobiopterin in rat phaeochromocytoma cell. *Biochim Biophys Acta* **1361**: 59–65.
9) Kazula A, Nowak JZ, Iuvone PM. 1993. Regulation of melatonin and dopamine biosynthesis in chick retina: the role of GABA. *Vis Neurosci* **10**: 621–629.
10) Birkmayer GD, Kay GG, Vurre E. 2002. Stabilized NADH (ENADA) improves jet lag-induced cognitive performance deficit. *Wien Med Wochenschr* **152**: 450–454.
11) Santaella ML, Font I, Disdier OM. 2004. Comparison of oral nicotinamide adenine dinucleotide (NADH) versus conventional therapy for chronic fatigue syndrome. *PR Health Sci J* **23**: 89–93.
12) Forsyth LM, Preuss HG, MacDowell AL, Chiazze L Jr, Birkmayer GD, Bellanti JA. 1999. Therapeutic effects of oral NADH on the symptoms of patients with chronic fatigue syndrome. *Ann Allergy Asthma Immunol* **82**: 185–191.
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) Shibata K. 1987. Ultramicro-determination of N^1-methylnicotinamide in urine by high-performance liquid chromatography. *Vitamins* **61**: 599–604 (in Japanese).
16) Shibata K. 1989. High-performance liquid chromatographic measurement of nicotinamide *N*-oxide in urine after extracting with chloroform. *Agric Biol Chem* **53**: 1329–1331.
17) Demarin V, Podobnik SS, Storga-Tomic D, Kay G. 2004. Treatment of Alzheimer's disease with stabilized oral nicotinamide adenine dinucleotide: a randomized, double-blind study. *Drugs Exp Clin Res* **30**: 27–33.
18) Birkmayer W, Birkmayer GJ, Vrecko K, Mlekusch W, Paletta B, Ott E. 1989. The coenzyme nicotinamide adenine dinucleotide (NADH) improves the disability of Parkinsonian patients. *J Neural Transm Park Dis Dement Sect* **1**: 297–302.
19) Bushehri N, Jarrell ST, Lieberman S, Mirdamadi-Zonozi N, Birkmayer G, Preuss HG. 1998. Oral reduced B-nicotinamide adenine dinucleotide (NADH) affects blood pressure, lipid peroxidation, and lipid profile in hypertensive rats (SHR). *Geriatr Nephrol Urol* **8**: 95–100.
20) Rex A, Hentschke MP, Fink H. 2002. Bioavailability of reduced nicotinamide-adenine-dinucleotide (NADH) in the central nervous system of the anaesthetized rat measured by laser-induced fluorescence spectroscopy. *Pharmacol Toxicol* **90**: 220–225.
21) Birkmayer JG, Nadlinger K. 2002. Safety of stabilized, orally absorbable, reduced nicotinamide adenine dinucleotide (NADH): a 26-week oral tablet administration of ENADA/NADH for chronic toxicity study in rats. *Drugs Exp Clin Res* **28**: 185–192.
22) Birkmayer JG, Nadlinger KF, Hallstrom S. 2004. On the safety of reduced nicotinamide adenine dinucleotide (NADH). *J Environ Pathol Toxicol Oncol* **23**: 179–194.
23) Rainer M, Kraxberger E, Haushofer M, Mucke HA, Jellinger K A. 2000. No evidence for cognitive improvement from oral nicotinamide adenine dinucleotide (NADH) in dementia. *J Neural Transm* **107**: 1475–1481.

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

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

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

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

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

Materials and Methods

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

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

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Table 1. Composition of the Diets

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

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

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

Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

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

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

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

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

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

Results

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

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

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

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

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

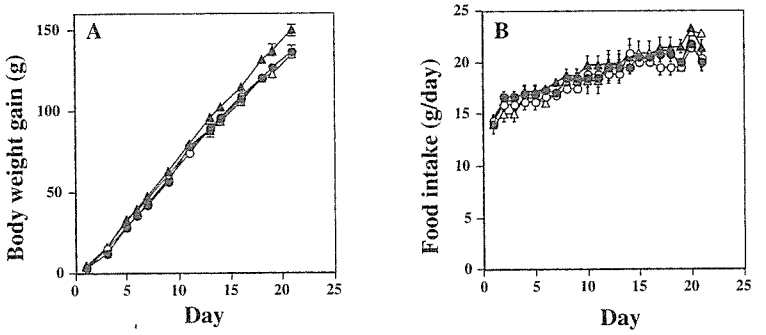

Fig. 1. Effect of DEHP on the Body Weight Gain (A) and Food Intake (B) of Rats Fed with the Gluten or Casein Diet.
Young rats of the 6 weeks olds were fed on respective diet for 21 days. Each point is the mean ± SEM for 5 rats. ●, Gluten diet; ○, gluten diet + 0.5% DEHP; ▲, casein diet; △, casein diet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

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

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

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

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

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

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

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

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

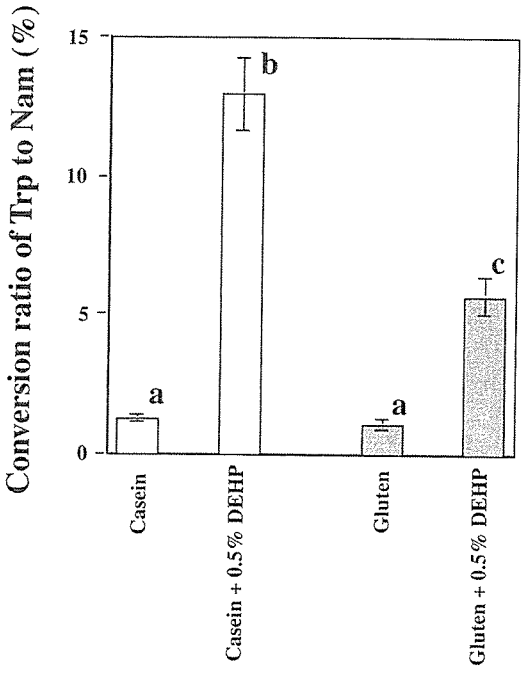

Fig. 2. Effect of DEHP on the Conversion Ratio of Trp to Nam in Rats Fed with the Gluten or Casein Diet.

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

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

Discussion

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

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

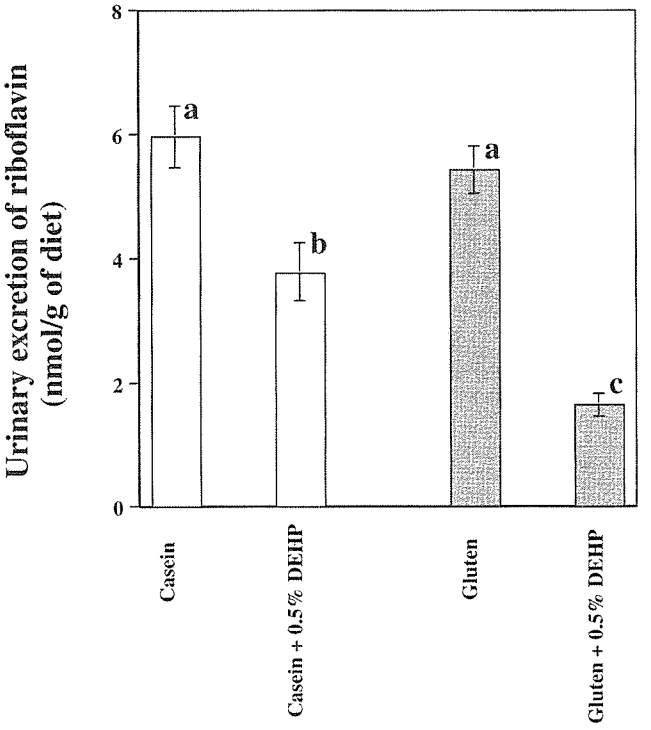

Fig. 3. Effect of DEHP on the Urinary Excretion of Riboflavin in Rats Fed with the Gluten or Casein Diet.
Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column,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

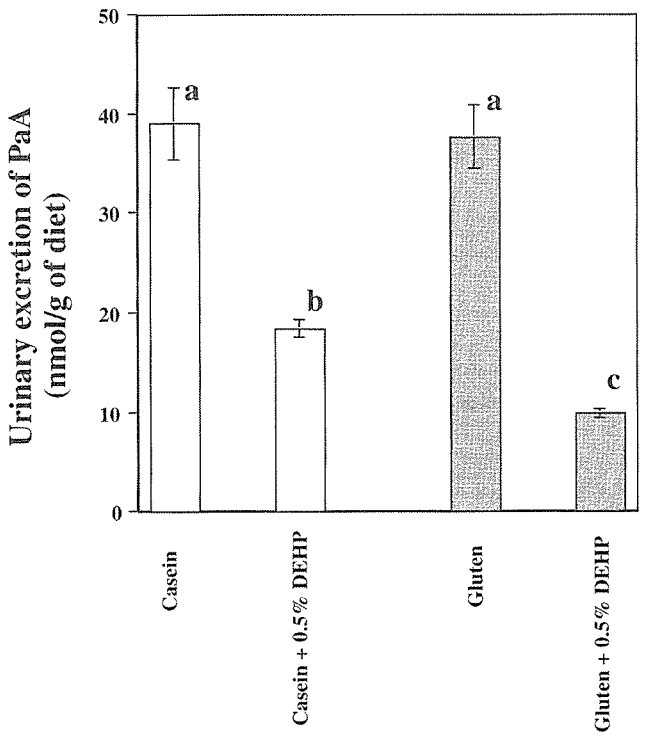

Fig. 4. Effect of DEHP on the Urinary Excretion of Pantothenic Acid (PaA) in Rats Fed with the Gluten or Casein Diet.
Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled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