

TABLE 1
Effects of Phthalate Monoesters on the Activity of ACMSD or QPRT^a

	Vehicle	MEP	MBP	MHP	MEHP
Rat ACMSD activity ($\mu\text{mol/h/g}$ liver)	9.3 ± 0.3	10.0 ± 0.4	$7.2 \pm 0.6^*$	$3.9 \pm 0.2^*$	$0.9 \pm 0.3^*$
Mouse ACMSD activity ($\mu\text{mol/h/g}$ kidney)	3.07 ± 0.13	2.88 ± 0.15	$2.63 \pm 0.30^*$	$1.12 \pm 0.34^*$	$0.21 \pm 0.16^*$
Human ACMSD activity ($\mu\text{mol/h/mg}$ protein)	21.5 ± 1.1	22.6 ± 1.3	17.7 ± 1.3	$3.5 \pm 1.0^*$	$1.6 \pm 0.4^*$
Rat QPRT activity ($\mu\text{mol/h/g}$ liver)	1.27 ± 0.03	1.30 ± 0.07	1.29 ± 0.04	1.29 ± 0.05	1.25 ± 0.04
Human QPRT activity ($\mu\text{mol/h/mg}$ protein)	10.7 ± 0.4	11.5 ± 0.3	11.7 ± 0.4	11.1 ± 0.2	11.1 ± 0.5

^aEnzyme activities were measured in the presence or absence of 3 mmol/L phthalate monoester. ACMSD (α -amino- β -carboxymuconate- ϵ -semialdehyde decarboxylase) was assayed with extracts from rat liver, mouse kidney, and COS-7 expressing recombinant human enzyme, and QPRT (quinolinate phosphoribosyltransferase) was assayed with extracts from rat liver and COS-7 expressing human enzyme. Extracts prepared from five animals and five cultures of COS-7 cells were assayed; values are means \pm SEM ($n = 5$). MEP: monoethyl phthalate; MBP: mono-*n*-butyl phthalate.

* $P < 0.05$ versus vehicle.

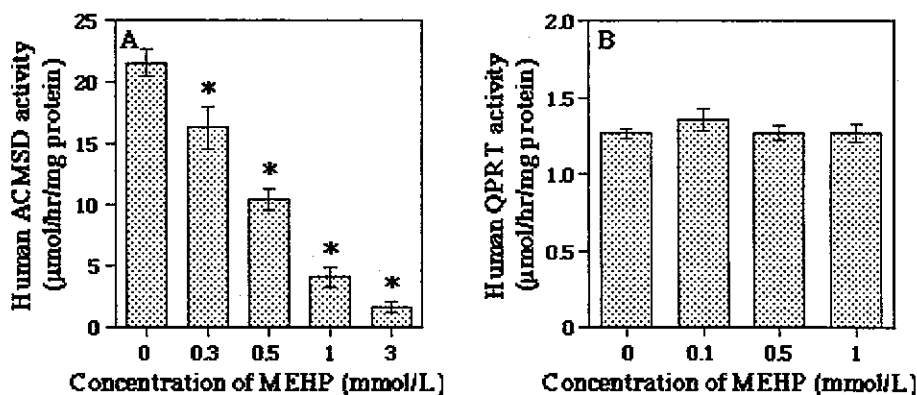


FIG. 4. MEHP inhibits human ACMSD activity in a dose-dependent manner. ACMSD activity is shown as the amount of enzyme that generates μmoles of AMS per hour per milligram of protein. QPRT activity is shown as the amount of enzyme that generates μmoles of nicotinic acid mononucleotide per hour per milligram of protein. Values are means \pm SEM, $n = 5$. * $P < 0.05$ versus the control.

tryptophan-NAD pathway but did not change the excretion of 3-HA, suggesting involvement of ACMSD in this phthalate ester-induced change of the tryptophan metabolism; the cellular concentration of ACMSD protein may be decreased or ACMSD activity may be directly inhibited by these esters. To test the latter possibility, rat, mouse, and human ACMSD activities were measured *in vitro* in the presence and absence of phthalate monoesters such as MEP, MBP, MHP, and MEHP. Extracts from rat livers, mouse kidneys, and COS-7 cells that express human recombinant ACMSD were used as enzyme sources. To show the specificity of the inhibition, QPRT from rat livers and COS-7 cells producing human enzyme were also assayed. Assays with the corresponding diesters could not be performed because the addition of the diesters caused turbidity in the mixture for the enzyme assay. Table 1 shows the results when ACMSD was assayed with and without 3 mmol/L phthalate ester. The high activity of human ACMSD compared with that from rats and mice, is probably due to the high expression of this recombinant human enzyme in COS cells. The same would be true for QPRT. MEP, the monoester with the shortest chain, was not inhibitory whereas low inhibition was found

with MBP. The inhibition became more potent as the length of the side chains in esters became longer; MHP and MEHP severely blocked the ACMSD activity from all sources, and MEHP was the most potent inhibitor. In contrast, neither human nor rat QPRT was affected by any of the phthalate monoesters. Figure 4A shows a dose-dependent inhibition of human ACMSD by MEHP; the presence of MEHP at 0.3 mmol/l caused a significant inhibition, and at 3 mmol/l the activity was inhibited by more than 90%. In agreement with Table 1, MEHP was not inhibitory to human QPRT at any of the concentrations tested (Fig. 4B).

To investigate whether the inhibition of ACMSD by MEHP was reversible or whether MEHP irreversibly inactivated the enzyme, human ACMSD was incubated for 10 min at 25°C with 3 mmol/l MEHP, at which the ACMSD activity would be inhibited by more than 90% (see Fig. 4). The enzyme was then diluted fivefold with 50 mmol/l Tris-HCl buffer, pH 7.6, and the diluted enzyme preparation was subjected to the enzyme assay. The final concentration of MEHP in the assay mixture was 0.06 mmol/l at which the inhibition of the enzyme should be negligible if the inhibition is reversible. The activity of the

diluted ACMSD was found to be comparable to that of the control enzyme diluted without treatment with MEHP (data not shown), indicating that the inhibition is reversible. A similar result was obtained using rat ACMSD.

DISCUSSION

This study was undertaken to investigate effects of phthalate esters orally given to rats on the tryptophan metabolism. DEHP, which has been most widely used in the formation of plastics, and its primary metabolite, MEHP, caused the most dramatic change in tryptophan metabolism. They markedly enhanced the urinary excretion of QA and its downstream metabolites, while the excretion of 3-HA was unchanged, raising the possibility that ACMSD, a critical enzyme acting at the branching point of tryptophan metabolism, is inhibited by these phthalate esters. In fact, MEHP reversibly inhibited ACMSD from human, mouse, and rat sources in a dose-dependent manner. Some of phthalate esters with different side chains promoted urinary excretion and inhibited ACMSD but their potency was far less than that of DEHP and MEHP. Thus it is very likely that the inhibition of ACMSD by phthalate esters blocks conversion of tryptophan to acetyl CoA and directs tryptophan metabolism to the NAD pathway (see Fig. 1), and thus cellular QA and Nam metabolites are accumulated, resulting in their increased excretion into the urine.

Isenberg *et al.* (2000) explored the metabolism of DEHP given orally to rats. MEHP was the most prominent hepatic metabolite of DEHP, and elevation of the hepatic MEHP concentration was time-dependent and dose-dependent, whereas the levels of DEHP and phthalate were minimal and did not correlate with the dose of DEHP or the time after its administration (Isenberg *et al.*, 2000). Oral administration of phthalate and 2-ethylhexanol, hydrolysis products of DEHP, did not affect the conversion rate of tryptophan to NAD (Fukuwatari *et al.*, 2002b). In agreement with the proposal that phthalate monoesters are produced from the diesters in the intestine before absorption (Lake *et al.*, 1977), these results indicate that MEHP is mainly responsible for the perturbation of tryptophan metabolism.

According to Isenberg *et al.* (2000), hepatic MEHP concentration in rats fed 1000 ppm DEHP for 2 weeks, conditions similar to those used in the present experiments, was 9 $\mu\text{mol/g}$ tissue. *In vitro* inhibition of ACMSD by MEHP was apparent (33%) at 0.3 mmol/l and greater than 90% at 3 mmol/l. These results suggest that the liver in rats fed 1000 ppm DEHP accumulates MEHP at the concentration sufficient to exhibit its inhibitory effect on ACMSD, although all of the MEHP molecules in the liver may not necessarily be available for this inhibition.

Previously we showed that ACMSD activity in the liver extracts from rats fed DEHP was similar to that of control animals (Fukuwatari *et al.*, 2002b). This result is not contradictory to our present finding that ACMSD is inhibited *in vitro* by MEHP. The inhibition is reversible, and therefore, even if

MEHP is accumulated in the liver of rats fed DEHP at concentrations sufficient to block ACMSD, MEHP would be washed out during the preparation of the enzyme; the resulting enzyme preparations would contain MEHP at levels that show little inhibition of ACMSD. Taken together, we conclude that phthalate esters perturb tryptophan metabolism through direct inhibition of ACMSD but not by reducing the ACMSD protein level. The mechanism by which ACMSD is inhibited remains to be examined. The very labile nature of ACMS, the substrate of ACMSD, hampers kinetic studies of this enzyme.

Quinolate is a potential endogenous toxin; it is neurotoxic and has been suspected of being involved in the development of a number of brain diseases (see review by Stone and Darlington, 2002). Although it is believed that the liver is a major site of tryptophan metabolism, expression of ACMSD, as well as its mRNA in the brain and kidney (Fukuoka *et al.*, 2002), suggests that the phthalate ester-induced metabolic alteration occurs in these organs. However, to date, there are no reports that show neurotoxicity of phthalate esters. When DEHP (0–200 mg/kg/day) was given to rats via oral gavage, few adverse effects on neurobehavioral evaluations were found (Moser *et al.*, 2003). DEHP given to mice through the diet to provide levels of 0.01–0.09% did not show detrimental effects on neurobehavioral parameters (Tanaka, 2002). Examination of tissue distribution by the use of the radioactive DEHP did not show significant accumulation of the radioactivity in the brain of rats (Tanaka *et al.*, 1975) and mice during the pre-weaning period (Eriksson and Darnerud, 1985). Quinolate also can be a uremic toxin responsible for anemia with renal failure by reducing the renal production of erythropoietin, a growth factor essential for erythrocyte formation (Pawlak *et al.*, 2003). When DEHP at a high dose (12,500 ppm in the diet) was given to rats, the erythrocyte count, hemoglobin, and hematocrit values were significantly lower than controls, but these effects were not found with a lower dose (2500 ppm; David *et al.*, 2000). Although no data indicating that phthalate esters exhibit adverse effects through accumulation of QA are available, effects of the concurrent intake of a tryptophan-rich diet and phthalate esters are worthy of further investigation. Such a series of misfortunes may contribute to triggering and/or exacerbating various diseases.

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ノ ー ト

魚類における3-ヒドロキシアンスラニル酸オキシゲナーゼ/
アミノカルボキシムコン酸セミアルデヒド脱炭酸酵素活性比
からのトリプトファン-ニコチンアミド変換率の推定

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**Estimated Conversion Efficiency of Tryptophan-Nicotinamide
Using an Activity Ratio of 3-Hydroxyanthranilate 3,4-dioxygenase to
Aminocarboxymuconate-semialdehyde Decarboxylase in Fishes**

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In order to estimate the conversion efficiency of tryptophan-nicotinamide in freshwater fishes, carp and crucian, the liver 3-hydroxyanthranilate 3,4-dioxygenase (3-HAO) and aminocarboxymuconate-semialdehyde decarboxylase (ACMSD) activities were measured. The activity ratios of 3-HAO/ACMSD in carp and crucian were both around 5. These values were extremely low compared with that of rat (650), which can synthesize a significant amount of nicotinamide from tryptophan. The activities of quinolinate phosphoribosyltransferase in carp and crucian were below the limit of detection. From these results, it was suggested that fishes cannot biosynthesize nicotinamide from tryptophan.

Key Words: fish, tryptophan, nicotinamide, 3-hydroxyanthranilate 3,4-dioxygenase, aminocarboxymuconate-semialdehyde decarboxylase

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緒 言

ヒトにおいて、B-群ビタミンの一つであるニコチンアミド(NiA-NH₂)が必須アミノ酸の一つであるトリプトファンから生合成されることはよく知られている。トリプトファン-NiA-NH₂ 転換率は、摂取トリプトファ

ン量と尿中に排泄される NiA-NH₂ 異化代謝産物量との比較から求めるのが、通常の方法である。しかしながら、尿の採取が困難である動物においては、他の方法の考案が必要である。田口ら¹⁾は、家畜類の飼料を考案する上で、トリプトファンからどの程度 NiA-NH₂ が生合成されているかが重要であるという認識から、トリプトファン-NiA-NH₂ 転換経路で鍵中間代謝物質である

キノリン酸の生成量を支配する二つの酵素, すなわち, キノリン酸生成酵素である3-ヒドロキシアンスラニル酸3,4-ジオキシゲナーゼ(3-HAO)とキノリン酸の生成量を抑制する酵素アミノカルボキシムコン酸-セミアルデヒド脱炭酸酵素(ACMSD)の活性比率から, トリプトファン-NiA-NH₂ 転換率を推定する方法を報告した。今回は, その方法を使用して, 琵琶湖の固有種であり, 近年生息数が激減しているニゴロブナの本転換率を推定したので報告する。合わせて, コイについても調べた。

実験方法

生後1年6ヶ月, 体長15~20 cm, 体重30~40 gのニゴロブナ(*Car. auratus grandoculis* Temminck et Schlegel)とコイ(*Cyprinus carpio* Linnaeus)を滋賀県水産試験場から入手した。表1に飼育用飼料の組成を示した。哺乳動物ではトリプトファン-NiA-NH₂ 代謝経路は肝臓のみに局在している。そこで, 魚においても肝臓を用いた。ニゴロブナの肝臓の量は非常に少ないため, 5匹から得られた肝臓をひとまとめにして処理した。コイからは肝臓を得, 5匹から得られたものをひとまとめにして処理した。各々得た臓器は5倍量の50 mM リン酸カリウム緩衝液(pH 7.0)を加え, ガラスステフロンホモゲナイザーで均一化した。ホモジネートは10,5000 × g で20分間遠心分離した後の上清を3-hydroxyanthranilate 3,4-dioxygenase(3-HAO, EC 1.13.11.6)活性, aminocarboxymuconate-semialdehyde decarboxylase(ACMSD, EC 4.1.1.45)活性, およびQuinolinate phosphoribosyltransferase(QPRT, EC 2.4.2.19)測定のための酵素源とした。また, 肝臓と筋肉中の総NiA-NH₂ 含量を測定した。

対照データを得るために, トリプトファンからNiA-NH₂ を効率よく合成できる動物として, ラットを使用した。ラットは雄Wistar系で, ナイアシン欠-20%カ,

ゼイン食(ラットは20%カゼインを含む飼料を与えれば, すでにビタミン体となっているナイアシンを全く含まない飼料を与えても, 正常に生育する²⁾)で飼育した7週齢の肝臓を用いた。酵素源の調製方法は, 魚の場合と同じである。

3-HAO活性はDeckerら³⁾の方法により, ACMSD活性はIchiyamaら⁴⁾の方法により, QPRTはShibataら⁵⁾の方法により測定を行った。

総NiA-NH₂ 含量の測定はShibataら⁶⁾の方法に従って行った。

結果並びに考察

表2にコイとニゴロブナの3-HAO活性, ACMSD活性, および3-HAO/ACMSDの活性比を示した。トリプトファンから効率よくNiA-NH₂ を生合成できるラットの値と比較して, コイもフナも3-HAO活性は顕著な差異は認められなかった。しかしながら, ACMSD活性は, ラットと比較して, 数十倍も高く, その結果, この二つの活性比はラットと比較して, 数十分の一という値であった。以前に報告¹⁾したように, 鳥類においても, 3-HAO活性の種による差異は2~3倍程度であったが, 肝臓におけるACMSD活性(μmol/h/g)は顕著な差異が認められ, ニワトリでは5.5, ハトは9.0, スズメは32.7, カラスは52.2であった。哺乳動物でも, トリプトファンをNiA-NH₂ の前駆体として利用できないネコ⁷⁾では, ACMSD活性が50 μmol/h/g 肝臓と高くこの活性比は4.75と報告されている⁸⁾。

さらに, トリプトファン-NiA-NH₂ 転換経路の鍵物質であるキノリン酸をユニバーサルにナイアシン活性を有する物質に転換する酵素であるQPRT活性を測定したが, コイにおいても, フナにおいても, 検出限界以下であった(表3)。

結論として, コイとフナはNiA-NH₂ の前駆体としてトリプトファンを利用できないと推測された。

さらに, トリプトファンからNiA-NH₂ が合成できないと推定されるコイ・フナと合成できるラットの筋肉中と肝臓中の総NiA-NH₂ 含量を調べた結果(表3), 魚の含量が低かった。このことと, トリプトファン-NiA-NH₂ 生合成経路の強弱との関係についてはさらなる検討を有するが, 興味あることである。

NiA-NH₂ の補酵素であるNAD・NADPは代謝に関わっている全酵素の約20%が必要としている。トリプトファンからの生合成経路は, この補酵素が必要な時

表1. Composition of the Diet for Fishes.

Materials	%
Fish powders	50
Wheat flour	21
Vegetable oil cake	14
Rice bran	9
Food yeast	6

The food contains 43.0% crude protein, 3.0% crude fat, 3.0% crude ash, 2.0% Ca, and 1.5% P.

表2. The Activities of 3-HAO, ACMSD, and the Ratio of 3-HAO/ACMSD in the Livers of Fishes.

	3-HAO ($\mu\text{mol/h/g}$)	ACMSD ($\mu\text{mol/h/g}$)	3-HAO/ACMSD
Carp	324	64	5.1
Crucian	430	69	6.2
Rat	481	0.74	650

The values are the mean of the separate three experiments.

表3. Comparison of the QPRT Activity and the Concentration of Total NiA-NH₂ in Liver and Muscle.

	QPRT ($\mu\text{mol/h/g}$)	Total NiA-NH ₂	
		Liver (nmol/g)	Muscle (nmol/g)
Carp	N.D.	1129	293
Crucian	N.D.	626	188
Rat	0.35	2093	647

The values are the mean of the separate three experiments.

に、必要な量だけ速やかに供給するために存在していると考えている。従って、本経路が作動していない魚の飼育においては、NiA-NH₂の供給を適切に行うことが、養殖において一つの重要なポイントになるものと思われる。

ま と め

コイとフナのトリプトファン-NiA-NH₂転換率を3-HAO/ACMSD活性比から推定した結果、効率良く利用できるラットと比較して、数十分の一程度であった。肝臓においてのみナイアシン活性を示すキノリン酸という中間代謝産物をユニバーサルにナイアシン活性を有する化合物に転換する酵素であるQPRT活性もコイとフナには検出されなかった。以上のことから、魚においては、NiA-NH₂はトリプトファンからきわめて合成されにくいことが、はじめて明らかとなった。

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Effects of dietary di(2-ethylhexyl)phthalate, a putative endocrine disrupter, on enzyme activities involved in the metabolism of tryptophan to niacin in rats

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Abstract

We have reported that the conversion ratio of tryptophan to niacin increased with increasing dietary concentration of di(2-ethylhexyl)phthalate (DEHP); the conversion ratio was about 2.0% in the control rat, which increased by about 30% in the rat fed with 3.0% DEHP diet. In this study, we investigated whether this abnormal increase in the conversion ratio by DEHP occurred through the alteration of the enzyme activities involved in the metabolism of tryptophan to niacin. Rats were fed with a diet containing 0%, 0.1%, 0.5%, or 1.0% DEHP for 21 days. The nine kinds of enzyme activities involved in the biosynthesis and catabolism in the liver and kidney were measured. Based on previous findings that the formation of quinolinic acid and its metabolites significantly increased with DEHP administration, we proposed that the activity of 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase would be inhibited by DEHP intake. However, we found that the activities in the liver and kidney did not decrease in the rat fed with DEHP-containing diet. We discuss the discrepancy between the metabolite results and the enzyme activities.

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Keywords: Phthalate ester; Di(2-ethylhexyl)phthalate; Enzyme activity; Tryptophan metabolism; Niacin; Rat

1. Introduction

Treatment of rats with the plasticizer di(2-ethylhexyl)phthalate (DEHP) increases the liver weight due to both hyperplasia and hypertrophy [1], and the induction of several metabolic enzymes, including those involved in peroxisomal β -oxidation [2]. Ganningm et al. [3] reported that phthalate esters increased protein synthesis and decreased protein breakdown. Nair and Kurup [4] reported that the specific incorporation of amino acids into the proteins of whole liver and hepatic mitochondria was not increased in DEHP-treated animals, but that the half-lives of whole liver proteins and of mitochondria were increased in DEHP-treated animals.

We studied the toxic mechanism of phthalate esters and reported that the administration of large amounts of phtha-

late esters such as di(*n*-butyl)phthalate [5] and DEHP [6] increased the conversion ratio of tryptophan to niacin in rats. We also found that the administration of phthalate esters increased the formation of quinolinic acid, which is a key intermediate in the tryptophan-niacin metabolism. Shin et al. [7] reported that administration of a MF diet (a commercial diet; obtained from Oriental Yeast, Tokyo, Japan) containing 2% DEHP for 2 weeks increased rat liver quinolinate phosphoribosyltransferase (QPRT) activity, decreased 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase (ACMSD) activity, and as a result, increased liver NAD⁺ content. Shin's [7] and our [8] results indicate that phthalate esters disturb the metabolism of tryptophan to niacin through changes in some enzyme activities. In a previous paper in this series [8], we reported that the feeding of DEHP increased the urinary excretions of quinolinic acid and its metabolites in a time-dependent manner, and that the increase in these excretions reached a peak 11 days after the rats started on the DEHP diet [8].

To investigate how DEHP affects enzyme activities of tryptophan to niacin metabolism and the amounts of quino-

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linic acid and its metabolites, we measured all the enzyme activities involved in the tryptophan-niacin metabolism in the liver and kidney, and intermediates of tryptophan to niacin metabolism in the liver and blood. Furthermore, we discuss a putative mechanism by which DEHP disturbs the tryptophan-niacin metabolism.

2. Materials and methods

2.1. Chemicals

Nicotinamide mononucleotide, NAD⁺ and NADP⁺ were purchased from Sigma Chemical Company (St. Louis, MO, USA). Vitamin-free milk casein, sucrose, L-methionine, nicotinamide, tryptophan, and anthranilic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Kynurenine sulfate, N¹-methylnicotinamide (MNA) chloride, xanthurenic acid, kynurenic acid and 3-hydroxyanthranilic acid were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). N¹-methyl-2-pyridone-5-carboxamide (2-Py) and N¹-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick [9] and of Shibata et al. [10], respectively. The mineral and vitamin mixtures were obtained from Oriental Yeast Kogyo, and all other chemicals used being of the highest purity available from commercial sources.

2.2. Animals, diets and treatments

The animal room was maintained at a temperature of around 22 °C and a humidity of about 60% with a 12 h light/12 h dark cycle. Body weight and food intake were measured daily at around 09:00 a.m., and food and water were renewed daily. 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 (6 weeks old) were obtained from Clea Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; Clea Japan). They were then divided into four groups, and fed ad libitum for 21 days (Table 1). On day 21, the rats were decapitated, a 10 µl sample of blood was taken from the carotid artery, and their livers and kidneys were used for measuring the enzyme activities and intermediates involved in the tryptophan-niacin metabolism.

2.3. Analyses

2.3.1. Assay of enzymes

2.3.1.1. Enzyme preparation. The rats were killed by decapitation at about 09:00 h on day 21. Their livers and kidneys were dissected, and a portion of liver and a portion of kidney were immediately homogenized with a Teflon-glass homogenizer in five volumes of cold 50 mM

Table 1
Diet compositions

	Control	0.1% DEHP	0.5% DEHP	1.0% [*] DEHP
Casein	20	20	20	20
L-Methionine	0.2	0.2	0.2	0.2
Gelatinized cornstarch	45.9	45.8	45.5	45.2
Sucrose	22.9	22.9	22.8	22.6
Corn oil	5	5	5	5
Mineral mixture ^a	5	5	5	5
Vitamin mixture ^a (NiA-free)	1	1	1	1
DEHP	0	0.1	0.5	1.0

Each value is expressed as g/100 g of diet.

Male rats of the Wistar strain (6 weeks old) were obtained from Clea Japan. The rats were immediately placed in individual metabolic cages (CT-10; obtained from Clea Japan). They were divided into four groups and then fed freely with one of the above diets for 21 days.

^a The compositions of the mineral and vitamin mixtures are described in Ref. [13].

KH₂PO₄-K₂HPO₄ buffer, pH 7.0. The resulting homogenates were used as enzyme sources. For measuring the activities of 3-hydroxyanthranilic acid dioxygenase and ACMSD, homogenates were centrifuged at 20,000 × g for 20 min, and the resulting supernatant were used as enzyme sources.

2.3.1.2. Tryptophan dioxygenase (TDO) (tryptophan: oxygen 2,3-oxidoreductase (deacylizing), EC 1.13.11.11). The activities of the liver and kidney homogenates were measured by using HPLC. The samples were prepared as follows [11]: each reaction mixture (total volume of 1500 µl) contained 120 µl of 0.5 M KH₂PO₄-K₂HPO₄ buffer (pH 7.0), 740 µl of water, 70 µl of 50 mM L-tryptophan, and 60 µl of enzyme source. The reactions were started by addition of the enzyme source, incubated for 60 min at 37 °C, and stopped by addition of 60 µl of 70% perchloric acid. The kynurenine formed in the deproteinized supernatant was measured by the HPLC method described below (Section 2.3.2.7). The activity of holo-TDO was expressed as nmol kynurenine formed per h per g of wet weight of tissue. The kynurenine is converted to the anthranilic acid by kynureninase, and kynureninase is contained in the enzyme source. On the measurement of TDO activity in this protocol, it is considered the kynurenine is not converted to the anthranilic acid because K_m value of kynureninase (substrate; kynurenine, 2.4 × 10⁻⁴ M) is higher than the condition of this protocol (10⁻⁹ M level) [12].

2.3.1.3. Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3). The activities of the liver and kidney homogenates were measured by using HPLC, and the samples were prepared as follows [11]: each reaction mixture (total volume of 1000 µl) contained 200 µl of 0.5 M Tris-HCl buffer (pH 8.0), 590 µl of water, 10 µl of 50 mM L-kynurenine sulfate, and 200 µl of enzyme source. The

reactions were started by the addition of enzyme source, incubated for 30 min at 37 °C, and stopped by the addition of 500 μ l of 10% trichloroacetic acid. The anthranilic acid formed in the deproteinized supernatant was measured by the HPLC method described below (Section 2.3.2.5). The activity of kynureninase was expressed as nmol anthranilic acid formed per h per g of wet weight of tissue.

2.3.1.4. 3-Hydroxyanthranilate-3,4-dioxygenase (3-HAO) (3-hydroxyanthranilate: oxygen 3,4-oxidoreductase (deacylizing), EC 1.13.11.6). The activities of the supernatants of the liver and kidney homogenates were measured by using a UV-spectrophotometer as follows [11]: each reaction mixture in a cuvette (total volume of 1500 μ l) contained 500 μ l of 0.5 M Tris-acetate buffer (pH 8.0), 940 μ l of water, 50 μ l of 3.3 mM 3-hydroxyanthranilic acid, and 10 μ l of enzyme source. The reactions were started by the addition of enzyme source, and monitored over 30 s at 25 °C (the absorbance increases under these conditions). In order to calculate the enzyme activity, the molar extinction coefficient of the reaction product, 2-amino-3-carboxymuconate-6-semialdehyde (ACMS), (45,000 M⁻¹ cm⁻¹ at 360 nm) was used [13]. The activity of 3-HAO was expressed as μ mol ACMS formed per h per g of wet weight of tissue.

2.3.1.5. 2-Amino-3-carboxymuconate-6-semialdehyde decarboxylase ACMSD (2-amino-3-(3-oxoprop-2-enyl)but-2-enedioate carboxylase, EC 4.1.1.45). The activities of the supernatants of the liver and kidney homogenates were measured by using a UV-spectrophotometer as follows [13]: each reaction mixture in a cuvette (total volume of 1500 μ l) contained 500 μ l of 0.5 M Tris-acetate buffer (pH 8.0), 830 μ l of water, 20 μ l of 3.3 mM 3-hydroxyanthranilic acid, and 50 μ l of partially purified 3-HAO [13]. After the absorbance at 360 nm reached a plateau, 100 μ l of the enzyme source was added to the cuvette in order to start the ACMSD reaction, and the absorbance was then monitored over 5 min at 25 °C (the absorbance decreases under this condition). The ACMSD activity was expressed as μ mol ACMSD disappearance per h per g of wet weight tissue.

2.3.1.6. Quinolinic acid phosphoribosyltransferase (QPRT) (nicotinatenucleotide: pyrophosphate phosphoribosyltransferase (carboxylating), EC 2.4.2.19). The activities of the liver homogenates were measured by using HPLC. The samples were prepared as follows [14]: each reaction mixture (total volume of 500 μ l) contained 50 μ l of 0.5 M KH₂PO₄–K₂HPO₄ buffer (pH 7.0), 50 μ l of 10 mM quinolinic acid, 50 μ l of 10 mM 5-phosphoribosyl-1-pyrophosphate, 10 μ l of 100 mM MgCl₂, 290 μ l of water, and 50 μ l of enzyme source. The reactions were started by addition of the enzyme source, incubated for 60 min at 37 °C, and stopped by immersion of the reaction tubes in a boiling water-bath for 5 min. The nicotinic acid mononu-

cleotide formed in the deproteinized supernatant was measured by HPLC [14]. The activity of QPRT was expressed as nmol nicotinic acid mononucleotide formed per h per g of wet weight of tissue.

2.3.1.7. Nicotinamide mononucleotide adenylyltransferase (NMNAT) (ATP: NMN adenylyltransferase, EC 2.7.7.1). The activities of the liver homogenates were measured as follows [15]: each reaction mixture (total volume of 160 μ l) contained 20 μ l of 0.5 M KH₂PO₄–K₂HPO₄ buffer (pH 7.0), 10 μ l of 50 mM nicotinamide mononucleotide, 20 μ l of 50 mM ATP, 20 μ l of 100 mM MgCl₂, 40 μ l of 1 M nicotinamide, 20 μ l of water, and 30 μ l of enzyme source. The reactions were started by the addition of enzyme source, incubated for 30 min at 37 °C, and stopped by immersion of the reaction tubes in hot water at 90 °C for 1.5 min. The NAD⁺ formed in the deproteinized supernatant was measured by the enzyme cycling method [16]. The activity of NMNAT was expressed as nmol NAD⁺ formed per h per g of wet weight of tissue.

2.3.1.8. NAD⁺ synthetase (deamido-NAD⁺: L-glutamine amido-ligase (AMP-forming), EC 6.3.5.1). The activities of the liver homogenates were measured as follows [11]: each reaction mixture (total volume of 200 μ l) contained 20 μ l of 0.5 M Tris–HCl buffer (pH 8.0), 20 μ l of 10 mM nicotinic acid adenine dinucleotide, 10 μ l of 50 mM ATP, 20 μ l of 200 mM of L-glutamine, 10 μ l of 100 mM MgCl₂, 20 μ l of 560 mM KCl, 40 μ l of 1 M nicotinamide, 30 μ l of water, and 30 μ l of enzyme source. The reactions were started by the addition of enzyme source, incubated for 30 min at 37 °C, and stopped by immersion of the reaction tubes in hot water at 90 °C for 1.5 min. The NAD⁺ formed in the deproteinized supernatant was measured by the enzyme cycling method [16]. The activity of NAD⁺ synthetase was expressed as nmol NAD⁺ formed per h per g of wet weight of tissue.

2.3.1.9. NAD⁺ kinase (ATP: NAD⁺ 2'-phosphotransferase, EC 2.7.1.23). The activities of the liver homogenates were measured as follows: each reaction mixture (total volume of 400 μ l) contained 100 μ l of 1 M Tris–HCl (pH 7.0), 40 μ l of 0.1 M NAD⁺, 40 μ l of 0.13 M ATP, 40 μ l of 0.1 M nicotinamide, 40 μ l of 0.1 M sodium pyruvate, 40 μ l of 0.1 M MgCl₂, 50 μ l of water, and 50 μ l of enzyme source. The reactions were started by addition of the enzyme source, incubated for 30 min at 37 °C, and stopped by immersion of the reaction tubes in 90 °C water for 1.5 min. The NADP⁺ formed in the deproteinized supernatant was measured by the enzyme cycling method [17]. The activity of NAD⁺ kinase was expressed as nmol NADP⁺ formed per h per g of wet weight of tissue.

2.3.1.10. NAD⁺ degrading enzymes. The activities of the liver homogenates were measured as follows: each reaction

mixture (total volume of 500 μ l) contained 50 μ l of 0.5 M KH_2PO_4 – K_2HPO_4 buffer (pH 7.0), 100 μ l of 5 mM NAD^+ , 340 μ l of water, and 10 μ l of enzyme source. The reactions were started by the addition of enzyme source, incubated for 10 min at 37 °C, and stopped by immersion of the reaction tubes in 90 °C water for 1.5 min. The remaining NAD^+ in the deproteinized supernatant was measured by the enzyme cycling method [16]. The activity of NAD^+ degrading enzymes was expressed as nmol NAD^+ disappearance per h per g of wet weight of tissue.

2.3.1.11. Nicotinamide methyltransferase (NMT) (*S*-adenosyl-*L*-methionine: nicotinamide *N*-methyltransferase, EC 2.1.1.1). The activities of the liver homogenates were measured by using HPLC, and samples were prepared as follows [18]: each reaction mixture (total volume of 500 μ l) contained 50 μ l of 0.5 M KH_2PO_4 – K_2HPO_4 buffer (pH 7.0), 5 μ l of 1 M nicotinamide, 50 μ l of 10 mM *S*-adenosylhomomethionine, 295 μ l of water, and 100 μ l of enzyme source. The reactions were started by the addition of enzyme source, incubated for 30 min at 37 °C, and stopped by the addition of 30 μ l of 70% perchloric acid. The MNA formed in the deproteinized supernatant was measured by the HPLC method described below (Section 2.3.2.2) [19]. The activity of NMT was expressed as nmol MNA formed per h per g of wet weight of tissue.

2.3.1.12. 2-Py-forming MNA oxidase (aldehyde: oxygen oxidoreductase, EC 1.2.3.1) and 4-Py-forming MNA oxidase (EC number not determined). The activities of the liver homogenates were measured by using HPLC. The samples were prepared as follows [20]: each reaction mixture (total volume of 100 μ l) contained 20 μ l of 0.5 M KH_2PO_4 – K_2HPO_4 buffer (pH 7.0), 20 μ l of 20 mM MNA, 10 μ l of 0.5 M NH_4Cl , and 50 μ l of enzyme source. The reactions were started by the addition of enzyme source, incubated for 10 min at 37 °C, and stopped by the addition of 400 μ l of 0.75% perchloric acid. The 2-Py and 4-Py formed in the deproteinized supernatant were measured by the HPLC method described below (Section 2.3.2.1) [10]. The activities of 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase were expressed as nmol 2-Py or 4-Py formed per h per g of wet weight of tissue.

2.3.2. Measurement of tryptophan metabolites

2.3.2.1. Nicotinamide, 2-py, and 4-py. The amounts of nicotinamide, 2-Py, and 4-Py were measured simultaneously by the HPLC method developed by Shibata et al. [10]. This method employs a Chemcosorb 7-ODS-L (250 \times 4.6 mm I.D.) column eluted with 10 mM KH_2PO_4 –acetonitrile (96:4, v/v; pH adjusted to 3.0 by the addition of concentrated H_3PO_4) at a flow rate of 1.0 ml/min. The UV detector was set at 260 nm, and the column temperature was 25 °C. Nicotinamide, 2-Py and 4-Py in the deprotei-

nized supernatant were extracted into diethyl ether under saturated K_2CO_3 conditions [10].

2.3.2.2. MNA. MNA was measured by the HPLC method developed by Shibata [19]. MNA in the deproteinized supernatant was reacted with acetophenone in a strong alkali medium at 4 °C in the presence of a large amount of isonicotinamide for 10 min. After reaction, formic acid was added, the mixture was cooled at 4 °C for 15 min, and then heated at 95 °C for 5 min. The reaction product, 1-methyl-7-phenyl-1,5-dihydro-5-oxo-1,6-naphthyridine was analyzed. The method used a Tosoh ODS 80Ts (250 \pm 0.6 mm I.D.) column eluted with a mixture of 1000 ml of 30 mM KH_2PO_4 (pH adjusted to 3.0 by the addition of concentrated H_3PO_4) containing 1 g of heptanesulfonic acid sodium salt and 290 ml of acetonitrile at a flow rate of 1.0 ml/min. The MNA derivative was measured at an excitation wavelength of 382 nm and an emission wavelength of 440 nm, and the column temperature was 40 °C.

2.3.2.3. Kynurenic acid. Kynurenic acid was measured by the HPLC method developed by Shibata [21]. This method employs a Chemcosorb 5-ODS-H (150 \times 4.6 mm I.D.) column eluted with 10 mM sodium acetate-acetic acid buffer (pH 4.5) and acetonitrile (96:5, v/v) at a flow rate of 1.0 ml/min. Kynurenic acid separated under these conditions was reacted with 1 M zinc acetate (used as the post-column reagent) delivered at a flow rate of 1.0 ml/min. Kynurenic acid- Zn^{2+} was measured at an excitation wavelength of 344 nm and an emission wavelength of 398 nm. The column temperature was maintained at 40 °C. The deproteinized supernatant was injected directly into the HPLC system.

2.3.2.4. Xanthurenic acid and 3-hydroxyanthranilic acid. The amounts of xanthurenic acid and 3-hydroxyanthranilic acid were measured simultaneously by the HPLC method developed by Shibata and Onodera [22]. This method employs an STR ODS II (250 \times 4.6 mm I.D.) column eluted with 50 mM KH_2PO_4 (pH adjusted to 3.0 by the addition of concentrated H_3PO_4) containing 3 mg/l of EDTA-2Na and acetonitrile (10:1, v/v) at a flow rate of 1.0 ml/min. Xanthurenic acid was measured at 340 nm by UV detector, and 3-hydroxyanthranilic acid was amperometrically measured at +300 mV vs. Ag/AgCl. The column temperature was 40 °C. The deproteinized supernatant was injected directly into the HPLC system.

2.3.2.5. Anthranilic acid. Anthranilic acid was measured by the HPLC method developed by Shibata and Onodera [23]. This method employs a Tosoh ODS 80Ts (250 \times 4.6 mm I.D.) column eluted with 50 mM KH_2PO_4 (pH adjusted by concentrated phosphoric acid) and acetonitrile (65:35, v/v) at a flow rate of 1.0 ml/min. Anthranilic acid was measured at an excitation wavelength of 340 nm and

an emission wavelength of 410 nm. The column temperature was maintained at 40 °C. The deproteinized supernatant was injected directly into the HPLC systems.

2.3.2.6. Quinolinic acid. Quinolinic acid was measured by the HPLC method developed by Mawatari et al. [24]. This method employs a Unisil Q C18 (250 × 4.6 mm I.D.) column eluted with 35 mM KH₂PO₄ (pH adjusted to 3.8 by 0.2 M citric acid) containing 350 mM H₂O₂ and 0.05 mM tetramethylammonium hydroxide at a flow rate of 0.6 ml/min. The quinolinic acid in the column effluent was irradiated with ultraviolet light to produce fluorescence. This fluorescence was monitored with an excitation wavelength of 326 nm and an emission wavelength of 380 nm. The column temperature was maintained at 30 °C. The deproteinized supernatant was injected directly into the HPLC systems.

2.3.2.7. Kynurenine. The kynurenine content in the liver and blood was measured as follows: for the measurement of kynurenine in liver, the liver was homogenized with 10 volumes of cold 3% perchloric acid, and the resulting homogenate was centrifuged at 10,000 × g for 5 min at 4 °C. The supernatant was injected directly into the HPLC. Kynurenine was separated by reversed-phase chromatography (a Tosoh ODS 80Ts was used as the analytical column)

using a mixture of 20 mM KH₂PO₄ (adjusted to pH 3.7 by addition of H₃PO₄) containing 1-heptanesulfonate sodium salt (1 g); acetonitrile = 10:1 (v/v) as a mobile phase. The flow rate was 1.0 ml/min and the detection wavelength was 360 nm. The column temperature was maintained at 40 °C.

2.3.2.8. NAD⁺+NADH. The amount of NAD (NAD⁺+NADH) was measured by the enzyme cycling method of Shibata and Murata [16].

2.3.2.9. NADP⁺+NADPH. The amount of NADP (NADP⁺+NADPH) was measured by the enzyme cycling method of Shibata and Tanaka [17].

3. Results

3.1. Effect of various DEHP concentrations on the liver enzyme activities involved in the tryptophan-niacin metabolism

A 0.1% and 0.5% amount of DEHP in the diet did not affect the body weight gain, as shown in Table 2. Liver weight was increased with increasing DEHP concentration.

The metabolic pathway of tryptophan to niacin is depicted in Scheme 1. The activities of kynureninase, ACMSD,

Table 2
Effect of dietary DEHP on the enzymes involved in the metabolism of tryptophan to niacin in the rat liver

	Control	0.1% DEHP	0.5% DEHP	1.0% DEHP
Body weight gain (g/21 days)	137.6 ± 9.8	143.8 ± 7.7	138.7 ± 5.7	95.2 ± 6.0*
Liver weight (g)	12.1 ± 1.0	17.6 ± 0.5*	20.9 ± 1.1*	19.3 ± 1.2*
<i>Biosynthesis of NAD⁺ from tryptophan</i>				
TDO ^a	1.28 ± 0.11 (15.3 ± 1.1)	0.76 ± 0.04* (13.4 ± 0.8)	0.51 ± 0.03* (10.7 ± 0.9)	0.51 ± 0.05* (9.7 ± 0.7)
Kyase ^b	0.76 ± 0.03 (9.2 ± 0.4)	0.70 ± 0.06 (12.3 ± 1.1)	0.68 ± 0.01 (14.2 ± 0.3)*	0.84 ± 0.09 (16.2 ± 1.7)*
3-HAO ^c	715 ± 20 (8,644 ± 568)	433 ± 9* (7,618 ± 246)	289 ± 27* (5,937 ± 243)	286 ± 14* (5,550 ± 517)
ACMSD ^d	1.05 ± 0.24 (12.7 ± 2.9)	1.15 ± 0.31 (20.3 ± 5.5)	1.06 ± 0.15 (22.1 ± 3.1)	1.14 ± 0.16 (22.0 ± 3.1)
QPRT ^e	1.27 ± 0.03 (15.4 ± 1.4)	1.35 ± 0.07 (23.9 ± 1.6)*	1.27 ± 0.05 (26.5 ± 2.0)*	1.27 ± 0.06 (24.5 ± 1.7)*
NMN ATase ^f	9.01 ± 0.53 (109 ± 6)	9.05 ± 0.51 (159 ± 9)*	8.59 ± 0.41 (179 ± 9)*	8.76 ± 0.66 (169 ± 13)*
NAD ⁺ synthetase	0.41 ± 0.03 (4.96 ± 0.37)	0.45 ± 0.03 (7.92 ± 0.51)*	0.42 ± 0.03 (8.76 ± 0.54)*	0.39 ± 0.02 (7.53 ± 0.41)*
NAD ⁺ kinase	2.50 ± 0.22 (30.2 ± 1.9)	2.09 ± 0.14 (36.8 ± 2.4)	1.70 ± 0.15* (35.5 ± 4.1)	1.89 ± 0.12* (36.5 ± 3.2)
<i>Degradation of NAD⁺</i>				
NAD ⁺ Dase ^g	30.8 ± 6.8 (372 ± 82)	36.0 ± 7.0 (634 ± 123)	29.1 ± 7.3 (607 ± 151)	31.4 ± 2.9 (607 ± 57)
NMT ^h	0.57 ± 0.07 (6.89 ± 0.81)	0.35 ± 0.03* (6.16 ± 0.48)	0.23 ± 0.05* (4.80 ± 0.96)	0.18 ± 0.01* (3.48 ± 0.23)
MNAO ⁱ (2-Py)	1.85 ± 0.37 (22.4 ± 4.5)	0.71 ± 0.10* (12.5 ± 1.7)	0.30 ± 0.05* (6.3 ± 1.0)	0.18 ± 0.01* (3.5 ± 0.2)
MNAO ⁱ (4-Py)	4.17 ± 0.07 (50.4 ± 0.9)	1.11 ± 0.06* (19.6 ± 1.1)	0.32 ± 0.05* (6.7 ± 1.0)	0.23 ± 0.02* (4.4 ± 0.4)

Values are expressed in mmol/h/g of liver (μmol/h/liver) and presented as means ± S.E. for five rats; values with different subscripts are significantly different from control group.

^a TDO = tryptophan dioxygenase.

^b Kyase = kynureninase.

^c 3-HAO = 3-hydroxyanthranilic acid oxygenase.

^d ACMSD = 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase.

^e QPRT = quinolinate phosphoribosyltransferase.

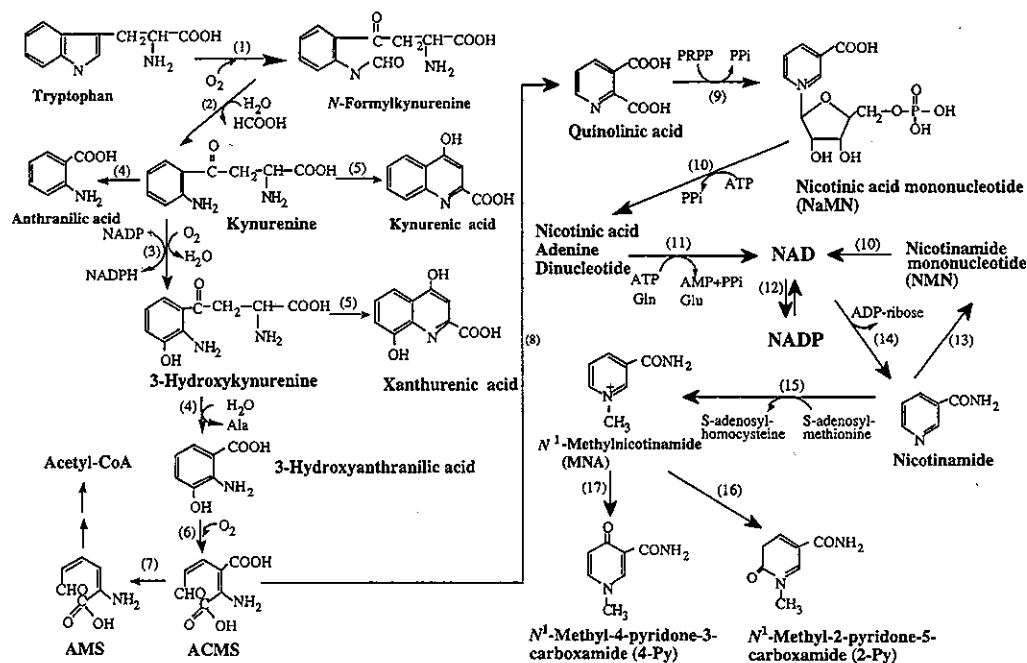
^f NMN ATase = nicotinamide mononucleotide adenylyltransferase.

^g NAD⁺ Dase = NAD⁺ degrading enzymes.

^h NMT = nicotinamide methyltransferase.

ⁱ MNAO = MNA oxidase.

* *P* < 0.05, calculated by a Student–Newman–Keuls multiple comparison test.



Scheme 1. Metabolism of tryptophan to niacin in liver. (1) Tryptophan dioxygenase, (2) formylase, (3) kynurenine 3-hydroxylase, (4) kynureninase, (5) kynurenine aminotransferase, (6) 3-hydroxyanthranilic acid oxygenase, (7) 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase, (8) non-enzymatic reaction, (9) quinolinate phosphoribosyltransferase, (10) nicotinic acid (nicotinamide) mononucleotide adenylyltransferase, (11) NAD⁺ synthetase, (12) NAD⁺ kinase, (13) nicotinamide phosphoribosyltransferase, (14) NAD⁺ degrading enzyme, (15) nicotinamide methyltransferase, (16) 2-Py-forming N¹-methylnicotinamide (MNA) oxidase, (17) 4-Py-forming MNA oxidase.

QPRT, NMN adenylyltransferase, NAD⁺ synthetase, or NAD⁺ kinase were not affected by diets containing DEHP. In contrast, the activities of TDO and 3-HAO were decreased by diets containing DEHP, as shown in Table 2. The decreased activities of these two enzymes usually induce a decreased conversion ratio of tryptophan to niacin, which was not consistent with the previous finding [6,8] that the conversion ratio of tryptophan to niacin increased with feeding of DEHP. Expressing these enzyme activities as per liver, the activities of kynureninase, QPRT, NMN adenylyltransferase and NAD⁺ synthetase were increased by DEHP intake.

As for the catabolic metabolism of NAD⁺, the activities of several NAD⁺ degrading enzymes were not affected by DEHP intake. On the other hand, the activities of nico-

tinamide methyltransferase, 2-Py-forming MNA oxidase, and 4-Py-forming MNA oxidase were significantly decreased by DEHP intake. The changes of these enzyme activities do not relate to the conversion ratio of tryptophan to niacin, and the amounts of these catabolic metabolites must be dependent on precursors derived from tryptophan, such as quinolinic acid and nicotinamide.

3.2. Effect of various DEHP concentrations on the kidney enzyme activities involved in the tryptophan-niacin metabolism

As shown in Table 3, TDO activity was not detected in the kidney. In addition, only a trace amount of kynureninase activity was detected. 3-HAO activity was detected, but it

Table 3
Effect of dietary DEHP on the enzymes involved in the metabolism of tryptophan to niacin in rat kidneys

	Control	0.1% DEHP	0.5% DEHP	1.0% DEHP
Kidney weight (g)	2.27 ± 0.08	2.27 ± 0.10	2.45 ± 0.07	2.08 ± 0.05
TDO	N.D. ^a	N.D.	N.D.	N.D.
Kyase	trace ^b	trace	trace	trace
3-HAO	302 ± 26 (685 ± 59)	364 ± 11 (826 ± 25)	338 ± 16 (828 ± 39)	337 ± 18 (701 ± 37)
ACMSD	10.9 ± 0.9 (24.7 ± 2.1)	12.1 ± 0.5 (27.4 ± 1.2)	13.8 ± 0.7* (33.9 ± 1.6)	15.9 ± 0.7* (33.2 ± 1.4)

Values are expressed in μmol/h/g of kidney (μmol/h/kidney) and presented as means ± S.E. for five rats; values with different subscripts are significantly different from control group.

^a N.D. = not detected.

^b Trace = 0.003–0.004 μmol/h/g of kidney.

* *P* < 0.05, as calculated by a Student–Newman–Keuls multiple comparison test.

was not affected by feeding of DEHP. It has been reported that ACMSD activity in the kidney is about 10-fold higher than in the liver [13]. In the present experiment, the ACMSD activities in the liver and kidney of the control group are consistent with previous findings. This activity increased with increasing the DEHP intake. Higher ACMSD activity generally means lower formation of quinolinic acid. Therefore, this DEHP effect was not consistent with a previous report that lower ACMSD activity increased quinolinic acid formation [6]. The subsequent enzymes in the metabolism depicted are detected in the kidneys [25], but we did not measure these in the present experiment.

3.3. Effect of dietary DEHP on the intermediates involved in the tryptophan to niacin metabolism in the liver

The metabolites in the tryptophan-niacin pathway such as kynurenine, 3-hydroxyanthranilic acid, xanthurenic acid, anthranilic acid, and kynurenic acid were not detected in the liver, as shown in Table 4. Trace amounts of quinolinic acid were detected. Total nicotinamide content, which is the sum of free nicotinamide, NAD, and NADP, in the liver did not affected by feeding of DEHP. MNA, the catabolite of nicotinamide, was detected in the liver, and the MNA content in rats fed with DEHP containing diet was higher than in the control group.

3.4. Effect of dietary DEHP on the intermediates involved in tryptophan to niacin metabolism in the blood

Blood kynurenine concentrations were 3.5 ± 0.4 (nmol/ml of whole blood) in the control group, 3.7 ± 0.4 in the 0.1% DEHP group, 3.4 ± 0.5 in the 0.5% DEHP group and 3.7 ± 0.8 in the 1% DEHP group, respectively. Kynurenine concentration in the blood was not affected by DEHP intake. This means that the enzymes catalyzing the reaction from tryptophan to kynurenine in non-hepatic tissues were

not affected by DEHP. 3-Hydroxyanthranilic acid, xanthurenic acid, anthranilic acid, kynurenic acid, and quinolinic acid were not detected. Blood NAD concentrations were 90.6 ± 4.6 (nmol/ml of whole blood) in the control group, 97.4 ± 4.3 in 0.1% DEHP group, 93.2 ± 5.1 in the 0.5% DEHP group and 91.5 ± 3.4 in the 1% DEHP group, respectively. Also, Blood NADP concentrations were 12.1 ± 0.9 (nmol/ml of whole blood) in the control group, 13.5 ± 0.7 in 0.1% DEHP group, 12.4 ± 0.5 in the 0.5% DEHP group and 11.1 ± 0.9 in the 1% DEHP group, respectively. The concentrations of NAD and NADP were not affected by DEHP intake.

4. Discussion

Our previous papers [6,8] reported a significantly increased conversion ratio of tryptophan to niacin in rats fed with DEHP. The urinary excretion of anthranilic acid, kynurenic acid, xanthurenic acid, and 3-hydroxyanthranilic acid did not increase with DEHP intake, and these results suggest that DEHP does not affect to the tryptophan to 3-hydroxyanthranilic acid metabolism. In contrast, urinary excretion of quinolinic acid and its metabolites increased significantly. These results suggest that DEHP affects quinolinic acid formation, and the possible target enzyme is QPRT and/or ACMSD. The aim of the present experiment is to investigate how DEHP affects enzyme activities of tryptophan to niacin metabolism, and increases quinolinic acid and its metabolites formation. However, we failed to evaluate changes in these two enzyme activities with DEHP intake.

Some reports showed that some drugs, which induce a deterioration of liver function, affected the conversion ratio of tryptophan to niacin. Egashira et al. [26] reported that D-galactosamine-induced liver injury change the tryptophan-niacin metabolism in rat. The urinary excretions of MNA, 2-

Table 4
Effect of dietary DEHP on the intermediates involved in the metabolism of tryptophan to niacin in rat liver

	Control	0.1% DEHP	0.5% DEHP	1.0% DEHP
Kynurenine	N.D. ^a	N.D.	N.D.	N.D.
3-Hydroxyanthranilic acid	N.D.	N.D.	N.D.	N.D.
Xanthurenic acid	N.D.	N.D.	N.D.	N.D.
Anthranilic acid	N.D.	N.D.	N.D.	N.D.
Kynurenic acid	N.D.	N.D.	N.D.	N.D.
Quinolinic acid	Trace ^b	Trace	Trace	Trace
Total Nam ^c (nmol/g of liver) ($\mu\text{mol/liver}$)	1405 ± 72 (17.0 ± 0.8)	1522 ± 237 (20.8 ± 4.2)	1897 ± 255 (39.6 ± 5.3)*	1809 ± 199 (34.9 ± 3.8)*
MNA (nmol/g of liver) (nmol/liver)	28.9 ± 8.2 (349 ± 99)	50.2 ± 3.0 (884 ± 53)*	54.2 ± 10.3 (1130 ± 215)*	54.3 ± 4.4 (1049 ± 85)*
2-Py	N.D.	N.D.	N.D.	N.D.
4-Py	N.D.	N.D.	N.D.	N.D.

Values are expressed in nmol/g of liver and presented as means \pm S.E. for five rats; values with different subscripts are significantly different from control group.

^a N.D. = not detected.

^b Trace = 4–5 nmol/g of liver.

^c Total Nam = the sum of free nicotinamide, NAD and NADP.

* $P < 0.05$, as calculated by a Student–Newman–Keuls multiple comparison test.

Py and 4-Py were higher in the D-galactosamine-injected group than in the control group, and hepatic TDO and ACMSD activity was decreased by the injection of D-galactosamine. We reported that the administration of clofibrate, hypolipidemic drug, increased conversion ratio of tryptophan to niacin in rats [27]. Clofibrate is known as a peroxisomal proliferator, same as DEHP, and the conversion ratio of tryptophan to niacin increased about 10 times higher in the clofibrate group than in the control group. The activities of TDO, ACMSD, 2-Py-forming MNA oxidase and 4-Py-forming MNA oxidase were lower in the clofibrate group than in the control group. The decreased activity of TDO induces a decreased of the conversion ratio of tryptophan to niacin, while the decreased activity of ACMSD induces an increased conversion ratio. Both reports showed that the effects of drugs on the tryptophan- niacin metabolism were consistent with the change of ACMSD activity. In the present study, the TDO activity decreased with DEHP intake, and the other enzyme activities did not change significantly with DEHP in the diet (Table 2). These enzyme activities were not consistent with the increase of tryptophan- niacin metabolism. However, it is possible that DEHP and/or its metabolites could be an inhibitor of some enzyme. Mono-ethylhexylphthalate (MEHP) is a major metabolite of DEHP [28], and phthalic acid is also a metabolite of DEHP [29]. Although phthalic acid is an inhibitor of QPRT [30–32], the administration of phthalic acid did not affect the metabolism of tryptophan to niacin [8]. The inhibitory effect of MEHP on the ACMSD activity *in vitro* was examined by the directly addition of MEHP to rat liver homogenate. As result of this experiment, MEHP inhibited about 90% of rat hepatic ACMSD activity (unpublished data). Although ACMSD activity did not change in rats fed with DEHP, it might be due to the dilution of a putative inhibitor with buffer in the liver homogenate.

The kidney did not contribute biosynthesis of niacin from tryptophan in the control rats, since the activities of TDO and kynureninase were below the detection limit or trace, and ACMSD activity was much higher than in the liver (Table 3). Our results of enzyme activities in the kidney were consistent with previous reports that rabbit kidney contained 3-HAO and ACMSD [33]. Shibata et al. [32] have already reported that QPRT activity was found in the rat kidney. In the present study, the administration of DEHP did not affect kidney weight, and increased the kidney ACMSD activity. The increased ACMSD activity usually decreases the quinolinic acid formation, but the increase of quinolinic acid formation was inconsistent with the change of kidney ACMSD activity [6,8]. Therefore, the kidney enzymes involved in the metabolism of 3-hydroxyanthranilic acid to nicotinamide in rats fed with DEHP did not contribute to the effects of DEHP on the tryptophan- niacin metabolism.

Previous papers from our laboratory [6,8] reported that the sum of nicotinamide and its metabolites was significant-

ly increased by the DEHP diet [6,8]. However, the enzyme activities involved in the catabolic metabolism, such as nicotinamide methyltransferase, 2-Py-forming MNA oxidase, and 4-Py-forming MNA oxidase were all decreased by the DEHP diet, while the enzyme activities of NAD⁺ degrading enzymes were not affected (Table 2). These results indicate that the influx to the catabolic pathway is determined by the amount of precursors but not by the enzyme activities. A previous paper from our laboratory [6] also reported that the excretory pattern of nicotinamide, MNA, 2-Py, and 4-Py was significantly affected by the feeding of the DEHP diet [6]. The urinary excretion of MNA increased with increasing DEHP intake, the excretion of 2-Py was saturated at a level of 0.5% DEHP in the diet, and the excretion of 4-Py was the highest at a level of 0.1% DEHP in the diet [6]. These changes were attributed to a change in the two MNA oxidase activities, but not to a change in nicotinamide methyltransferase.

In the present experiment, we measured the amounts of tryptophan- niacin metabolites in the liver and blood. The major compounds in the liver were the niacin-active compounds such as nicotinamide, NAD and NADP (Table 4). In contrast, major urinary metabolites such as 3-hydroxyanthranilic acid, xanthurenic acid, anthranilic acid, kynurenic acid, quinolinic acid, 2-Py, and 4-Py were all below the detection limit. These results mean that rats immediately excrete these intermediates into urine when they are not metabolized further along the pathway. Therefore, the urinary excretions of these compounds reveal the systemic metabolism of tryptophan- niacin. The effect of DEHP on the amount of nicotinamide in the liver was very low compared to its effect on urinary quinolinic acid and its metabolites. This would mean that the nicotinamide stored in the liver is limited. MNA was detected in the liver and the amount increased in correlation with the DEHP intake. In the blood, similar findings were obtained. These results showed that NAD, and NADP were found in the body, but the other intermediates and the catabolic metabolites were not found in the body.

In summary, the increased urinary excretion of nicotinamide and its catabolic metabolites by the feeding of a DEHP diet is the result of increased quinolinic acid formation. The possible target enzyme is liver ACMSD, but the present methods failed to evaluate the decrease of liver ACMSD activity in rats fed with DEHP. There is a very slight possibility that the increase in quinolinic acid and its metabolites was the result of increased catabolic metabolism due to the DEHP diet, and not due to an increase in the catabolic enzyme activities. As is discussed in a previous paper [8], it is probable that the phthalate monoester and related compounds could be competitive inhibitors of ACMSD. The dilution by liver homogenization should be increased so that the inhibitor was under the effective concentration. In the future, we would like to detect such a water-soluble inhibitor derived from fat-soluble DEHP.

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<Proceeding of JSTRY Meeting>

Establishment of Niacin-deficient Mice

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Summary Although knockout or transgenic mouse would be useful tool for elucidation of the tryptophan-niacin metabolism, mouse for evaluation of niacin nutrition status remains to be established. A niacin deficient animal is difficult to make because niacin is biosynthesized from tryptophan even in mammals. The point making the diet caused to a niacin-deficient animal is to make a diet which tryptophan must be the first-limiting amino acid for mice. We made many kinds of foods. As the result, in the 0.03% tryptophan diets with or without nicotinic acid on the composition of zein supplemented with some amino acids, and sucrose, the body weight, food intake, liver and kidney weights were significantly higher in the nicotinic acid-containing group than in the non-nicotinic acid diet. The NAD and NADP contents in liver and kidney were also significantly higher in the nicotinic acid-containing diet. We succeeded the niacin deficient diet for mice.

Key words: Niacin, Deficiency, Mouse, Tryptophan, Diet

1. Introduction

Recently, techniques for making a knock-out mouse have been developed. In the study on the metabolism of tryptophan (Trp)-niacin, a knock-out mouse would be a useful item for elucidation of the role and regulation. We are now making a quinolinic acid phosphoribosyltransferase (QPRT)-knockout mice because we isolated cDNA encoded QPRT¹⁾. QPRT is the key enzyme in the Trp-niacin metabolism and is catalyzed quinolinic acid (QA) to nicotinic acid

mononucleotide in the presence of 5-phosphoribosyl 1-pyrophosphate²⁾. If this enzyme is defected, niacin cannot be synthesized from Trp and, so, an ideal niacin-deficient mice would be made. However, when the deficient mice are fed with niacin, the animals grow normally because animals contain nicotinic acid and nicotinamide phosphoribosyltransferases²⁾. Nevertheless, the knockout-mouse might manifest abnormal behaviors due to accumulate QA, which is a toxin of neuron³⁾. Thus, the establishment of niacin-deficient mice is needed to compare to the features

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Table 1 Composition of the Diets

	0% Trp diet		0.03% Trp diet		0.07% Trp diet	
	0% NiA	0.006% NiA	0% NiA	0.006% NiA	0% NiA	0.006% NiA
Zein	15	15	15	15	15	15
Glycine	2	2	2	2	2	2
L-Histidine	0.3	0.3	0.3	0.3	0.3	0.3
L-Lysine	1	1	1	1	1	1
L-Methionine	0.3	0.3	0.3	0.3	0.3	0.3
Sucrose	70.4	70.4	70.4	70.4	70.4	70.4
Corn oil	5	5	5	5	5	5
Mineral mixture*1	5	5	5	5	5	5
Vitamin mixture*1 (NiA-free)	1	1	1	1	1	1
Tryptophan	0	0	0.03	0.03	0.07	0.07
Nicotinic acid	0	0.006	0	0.006	0	0.006

*1AIN 93 is used (Reeves, P. G., Components of the AIN-93 diets as improvements in the AIN-76A diet. *J. Nutr.*, 127, 838S-841S, 1997.)

between the QPRT-knockout and the niacin-deficient mice.

Making of a niacin-deficient animal by the feeding of a specific diet is difficult because niacin is biosynthesized from Trp even in mammals. In order to make a niacin-deficient animal, the intake of Trp must be limited. Furthermore, just a limitation of Trp causes a Trp deficiency. The point of making the diet caused to niacin deficient animals is to make a diet, which Trp is the first-limiting amino acid for mice.

2. Materials and Methods

Chemicals

NAD⁺ and NADP⁺ were purchased from Sigma Chemical Company (St. Louis, MO, USA). N¹-methyl-2-pyridone-5-carboxamide (2-Py) and N¹-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick⁹⁾ and of Shibata et al.⁵⁾, respectively. Nicotinamide (Nam) N-oxide was purchased from Aldrich chemical (Milwaukee, WI, USA). N¹-methylnicotinamide (MNA) chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Nicotinic acid (NiA), Nam, QA, zein, sucrose, L-methionine, glycine, L-histidine, and L-lysine were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan), respectively. The mineral

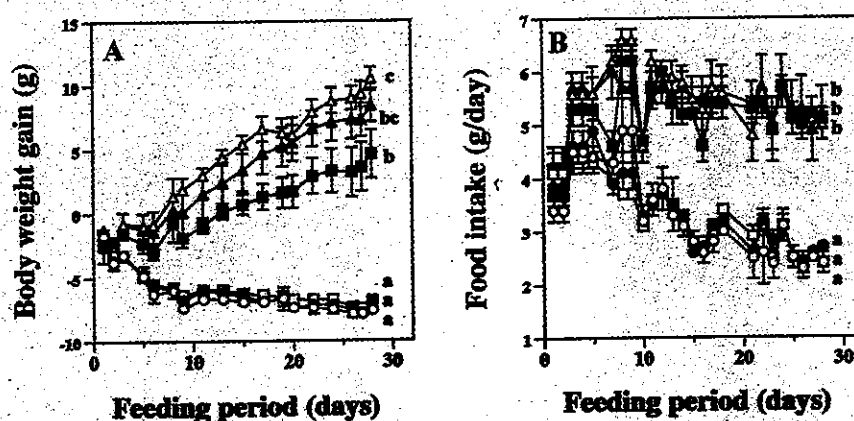


Fig. 1 Effect of Dietary Trp Content in the Presence or Absence of NiA on the Body Weight Gain of Young Mice. ○, 0% Trp and 0% NiA; ●, 0% Trp and 6 mg% NiA; □, 30 mg% Trp and 0% NiA; ■, 30 mg% Trp and 6 mg% NiA; △, 70 mg% Trp and 0 mg% NiA; ▲, 70 mg% Trp and 6 mg% NiA. Values are expressed as means \pm SEM for five mice and a different superscript letter in the last day means significant difference at $p < 0.05$, calculated by Student-Neuman-Keuels multiple comparison test.

and vitamin mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan), all the other chemicals used being of the highest purity available from commercial sources.

Animals

The care and treatment of the experimental animals conformed with The University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. Male mice of the ICR strain (4 weeks old) were obtained from Clea Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CL-0355; Clea Japan). They were then divided into six groups and fed *ad libitum* for 28 days with an NiA-free and Trp-limited diet adding 0, 0.03, and 0.07% Trp with or without 0.006% NiA. The composition of the diets is shown in Table 1.

The room temperature was maintained at around 20°C and about 60% humidity, and a 12-hr light/12-hr dark cycle was maintained. Body weight and food intake were measured daily at around 10:00 a.m.. Urine samples (24-hr; 10:00 a.m.- 10:00 a.m.) at the last day were collected in amber bottles containing 1 ml of 1 M HCl, and were stored at -25°C until needed. The mice were killed by decapitation at around 10:00 a.m. on the last day of the experiment. To measure NAD and NADP, the liver and kidneys of each animal were removed, and a portion (approximately 0.2 g)

was immediately treated as described in the literature⁶.

Analyses

The contents of NAD (NAD⁺ + NADH) and NADP (NADP⁺ + NADPH) were measured by the calorimetric method of Shibata and Murata⁶ and Shibata and Tanaka⁷, respectively. The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the HPLC method of Shibata et al.⁸, while the content of MNA in the urine was measured by the HPLC method of Shibata⁹. The content of Nam N-oxide was measured by the method of Shibata⁹.

3. Results

Creation of niacin deficient mice

In mammals including humans, niacin is biosynthesized from Trp. The efficiency of conversion from Trp to niacin is different from mammals. Shibata¹⁰ has been reported the establishment of niacin-deficient rats. However, we failed to make the establishment of niacin-deficient mice by feeding the niacin-deficient diet for rats. We had been developed a diet for creating niacin deficient mice. Zein was selected for major source of protein because which little contain Trp¹¹ and sucrose was selected as carbohydrate. Sources for lipids, minerals, and vitamins were selected

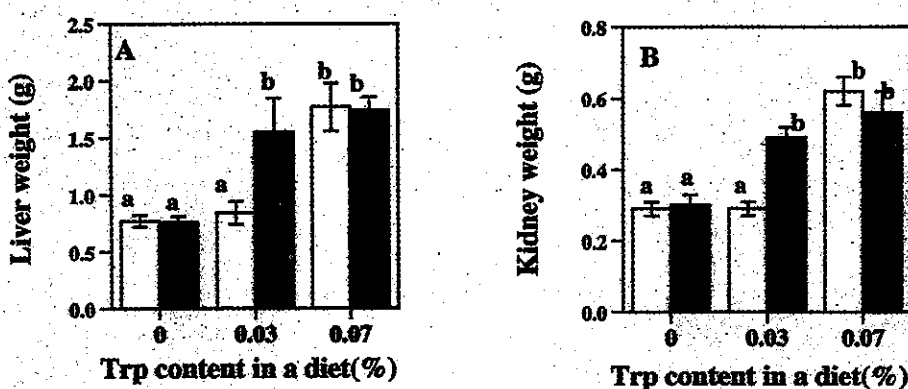


Fig. 2 Effect of Dietary Trp Content in the Presence or Absence of NiA on the Liver and Kidney Weights of Young Mice. Open column is the groups fed on the diets without NiA, on the contrary, closed column is with NiA. A, Liver weight; B, kidney weight. Values are expressed as means \pm SEM for five mice and a different superscript letter in the last day means significant difference at $p < 0.05$, calculated by Student-Neuman-Keuels multiple comparison test.

Table 2 Effects of Dietary Trp Content in the Presence or Absence of NiA on the Urinary Excretion of Nam and Its Metabolites in Mice

	0% Trp diet		0.03% Trp diet		0.07% Trp diet	
	0% NiA	0.006% NiA	0% NiA	0.006% NiA	0% NiA	0.006% NiA
Nam	N.D.	63.0±18.0	N.D.	111.8±13.1	N.D.	142.1±14.0
MNA	N.D.	14.4±1.2	0.24±0.09	21.5±2.6	1.59±0.34	26.7±2.6
2-Py	N.D.	9.0±2.0	N.D.	32.9±3.0	8.75±2.63	50.1±5.4
4-Py	N.D.	5.2±3.4	N.D.	17.2±2.1	4.89±1.11	25.3±2.9
Nam N-oxide	N.D.	40.2±2.4	N.D.	59.8±3.1	N.D.	102.9±3.8
SUM	N.D.	131.8±15.5	0.24±0.09	243.2±18.6	15.2±4.72	347.1±21.2

Values are expressed as nmol/g of diet and means ± SEM for five mice.

conventional ones, but NiA was removed from the vitamin mixtures. The fundamental composition of the diet was consist of 15% zein, 2% glycine, 1% lysine, 0.3% histidine, 0.3% methionine, 70.4% sucrose, 5% corn oil, 5% mineral mixture, 1% vitamin mixture without NiA. This basal diet did not have Trp and NiA. The supplement of suitable amount of NiA to

this diet did not contribute the growth of mice as shown in Fig. 1-A. A similar pattern was observed in the food intake as shown in Fig. 1-B. The addition of 0.03% Trp to the fundamental diet also gave no weight gain, but another supplement of suitable amount of NiA significantly gave the weight gain (Fig. 1-A). In the food intake, another addition of NiA gave an

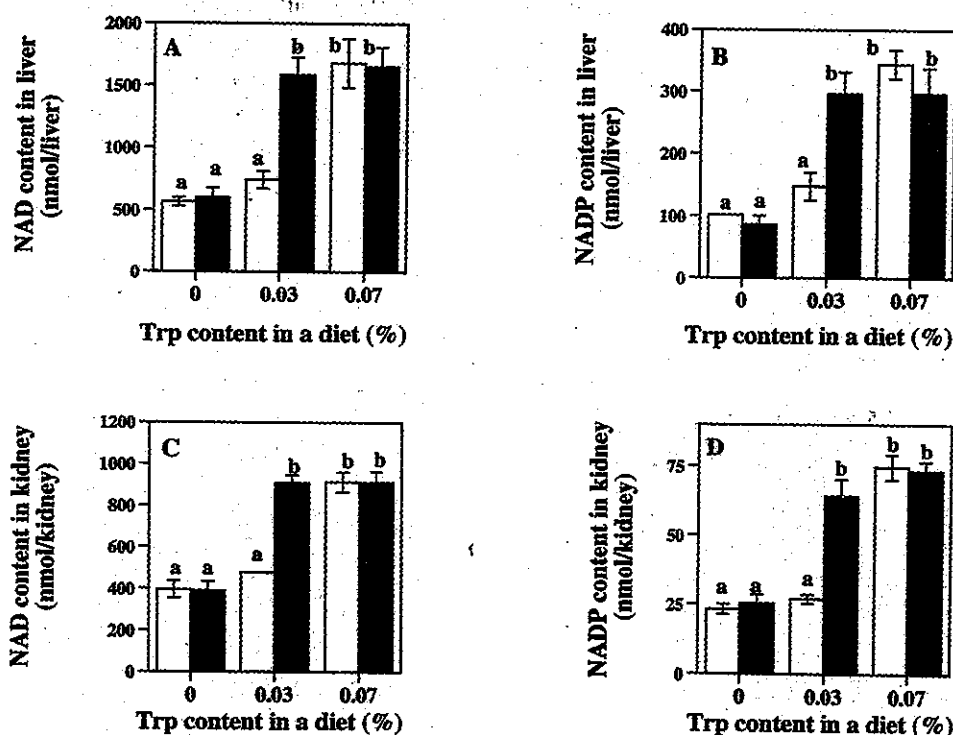


Fig. 3 Effect of Dietary Trp Content in the Presence or Absence of NiA on the Liver and Kidney NAD and NADP Contents of Young Mice.

A, liver NAD content; B, liver NADP content; C, kidney NAD content; D, kidney NADP content. Open column is the groups fed on the diets containing without NiA, on the contrary, closed column is with NiA.

Values are expressed as means ± SEM for five mice and a different superscript letter means significant difference at p < 0.05, calculated by Student-Neuman-Keuels multiple comparison test.

increase in food intake in the 0.03% Trp diet. Namely, the fundamental diet supplemented 0.03% Trp was a niacin deficient diet for mice. The addition of 0.07% Trp to the fundamental diet gave significant growth gain, while another addition of NiA did not give further gain of body weight (Fig. 1-A). In the food intake, another addition of NiA had no increase in food intake (Fig. 1-B). This result means that the addition of 0.07% Trp to the fundamental diet gave a sufficient supply of NiA from the Trp, therefore, another addition of NiA gave no additional body weight gain.

Liver and kidney weight

In the 0.03% Trp diet with or without NiA, liver and kidney weights were significantly higher in the NiA-containing group than in the non-NiA diet as shown in Fig. 2. In the 0% Trp diet, the addition of NiA gave no increase in liver and kidneys weight. The addition of 0.07% Trp to the fundamental diet gave a significant increase in liver and kidneys weight as shown in Fig. 2, but another addition of NiA gave no gain.

NAD and NADP contents in liver and kidney

In the 0.03% Trp diets, NAD and NADP contents in liver and kidney were significantly higher in the NiA-containing diet. In the 0% Trp diet, the addition of NiA gave no increase in NAD and NADP contents in liver and kidney. The addition of 0.07% Trp to the fundamental diet, a significant increase in NAD and NADP contents, but another NiA addition gave no further increase. Therefore, the 0.03% Trp diet was found to be a true niacin-deficient diet.

Urinary excretion of Nam and its catabolic metabolites

The urinary values of the metabolites of niacin are used as indices of the vitamin nutrition. In mice, the catabolic metabolites are MNA, 2-Py, 4-Py, and Nam N-oxide. In the 0% Trp and 0% NiA diet, these urinary excretion were all under detections, while the addition of NiA significantly increased these metabolites as shown in Table 2. In the 0.03% Trp and 0% NiA diet, trace amount of MNA excretion was observed, but the other metabolites were under the detection. The

addition of NiA significantly increased these metabolites. In the 0.07% Trp and 0% NiA diet, urinary excretion of MNA, 2-Py, and 4-Py were observed, but Nam N-oxide and Nam were still under the detection. The addition of NiA also significantly increased these metabolites.

4. Discussion

We succeeded a niacin deficient diet for mice. The composition of the diet is shown in Table 1. The point of this diet is to make the diet that the first limiting amino acid is Trp and the amount should be the minimum requirement for the growth of mice. For protein sources, casein is used in the niacin-deficient diet for rats¹⁰, but, we failed to make a niacin-deficient diet for mice. Then, we selected zein as a protein source because the Trp content of zein is almost zero¹¹. Zein has numbers of limiting amino acids and so, suitable amounts of histidine, lysine, and methionine were supplemented to zein for making the diet that Trp is the first limiting amino acid. Glycine was further added to the protein source because Henderson et al.¹² clarified the essentiality of glycine to produce a niacin-deficiency, but the mechanism is still unknown.

For the dietary carbohydrate sources, when rats were fed with a diet low in protein and Trp, the growth of the rats was different among kinds of carbohydrates¹³. Henderson et al.¹² reported that the superiority of polysaccharides to sucrose was attributed to activation of some microorganisms in small intestine. However, Harper and Katayama¹⁴ reported that the administration of succinylsulfathiazole did not affect the growth of the rats fed on a diet low in protein and Trp containing polysaccharide. Shibata et al.¹⁵ confirmed the effect of kind of dietary carbohydrate on the growth of weaning rats fed with a niacin-free and low-protein diet; when rats were fed with the sucrose diet, the conversion ratio of Trp to niacin was lower than when rats were fed with the cornstarch diet. Although, the mechanism why the intake of sucrose gives lower conversion ratio of Trp to niacin has not been elucidated, we selected sucrose for dietary carbohydrate in the present experiment. The other sources of