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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.

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 phthalate 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, N^1-methylnicotinamide (MNA) chloride, xanthurenic acid, kynurenic acid and 3-hydroxyanthranilic acid were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). N^1-methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick [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.

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].

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 KH_2PO_4–K_2HPO_4 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 (decyclizing), 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 KH_2PO_4–K_2HPO_4 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 μl 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 (decyclizing), 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 μl) contained 500 μl of 0.5 M Tris-acetate buffer (pH 8.0), 940 μl of water, 50 μl of 3.3 mM 3-hydroxyanthranilic acid, and 10 μl 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 μmol 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 μl) contained 500 μl of 0.5 M Tris-acetate buffer (pH 8.0), 830 μl of water, 20 μl of 3.3 mM 3-hydroxyanthranilic acid, and 50 μl of partially purified 3-HAO [13]. After the absorbance at 360 nm reached a plateau, 100 μl 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 μmol ACMD disappearance per h per g of wet weight tissue.

2.3.1.6. Quinolinate 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 μl) contained 50 μl of 0.5 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 50 μl of 10 mM quinolinic acid, 50 μl of 10 mM 5-phosphoribosyl-1-pyrophosphate, 10 μl of 100 mM $MgCl_2$, 290 μl of water, and 50 μl 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 mononucleotide 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 μl) contained 20 μl of 0.5 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 10 μl of 50 mM nicotinamide mononucleotide, 20 μl of 50 mM ATP, 20 μl of 100 mM $MgCl_2$, 40 μl of 1 M nicotinamide, 20 μl of water, and 30 μl 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 μl) contained 20 μl of 0.5 M Tris–HCl buffer (pH 8.0), 20 μl of 10 mM nicotinic acid adenine dinucleotide, 10 μl of 50 mM ATP, 20 μl of 200 mM of L-glutamine, 10 μl of 100 mM $MgCl_2$, 20 μl of 560 mM KCl, 40 μl of 1 M nicotinamide, 30 μl of water, and 30 μl 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 μl) contained 100 μl of 1 M Tris–HCl (pH 7.0), 40 μl of 0.1 M NAD^+, 40 μl of 0.13 M ATP, 40 μl of 0.1 M nicotinamide, 40 μl of 0.1 M sodium pyruvate, 40 μl of 0.1 M $MgCl_2$, 50 μl of water, and 50 μl 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 μl) contained 50 μl of 0.5 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 100 μl of 5 mM NAD^+, 340 μl of water, and 10 μl 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 μl) contained 50 μl of 0.5 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 5 μl of 1 M nicotinamide, 50 μl of 10 mM *S*-adenosylhomomethionine, 295 μl of water, and 100 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 30 μl 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 was prepared as follows [20]: each reaction mixture (total volume of 100 μl) contained 20 μl of 0.5 M KH_2PO_4–K_2HPO_4 buffer (pH 7.0), 20 μl of 20 mM MNA, 10 μl of 0.5 M NH_4Cl, and 50 μl 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 μl 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 × 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 deproteinized 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 add, 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 ± 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 × 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 xanthrenic 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 × 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 × 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 KH_2PO_4 (pH adjusted to 3.8 by 0.2 M citric acid) containing 350 mM H_2O_2 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 KH_2PO_4 (adjusted to pH 3.7 by addition of H_3PO_4) 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.

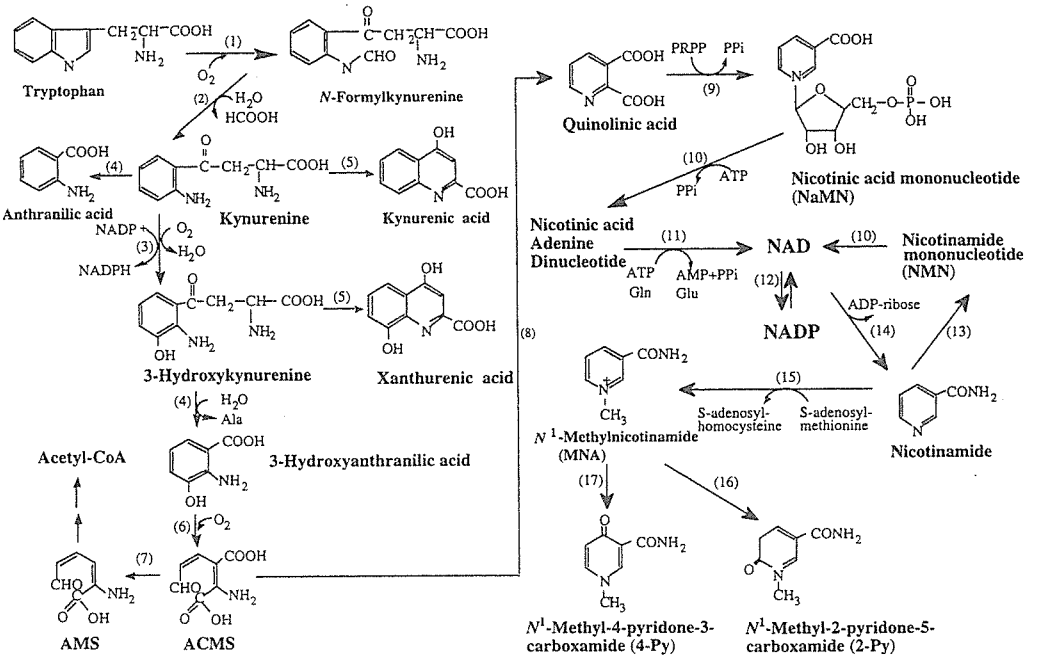
The metabolic pathway of tryptophan to niacin is depicted in Scheme 1. The activities of kynureninase, ACMSD,

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*
Biosynthesis of NAD^+ from tryptophan				
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)
Degradation of NAD^+				
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 N^1-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 nicotinamide 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.
Xanthruenic 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) (μmol/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 ± 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 significantly 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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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 posporibosyltransferase (QPRT)-knockout mice because we isolated cDNA encoded QPRT[1]. 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-pyrophosphate[2]. 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 phosphoribosyltransferases[2]. Nevertheless, the knockout-mouse might manifest abnormal behaviors due to accumulate QA, which is a toxin of neuron[3]. 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 Colowick[4] 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

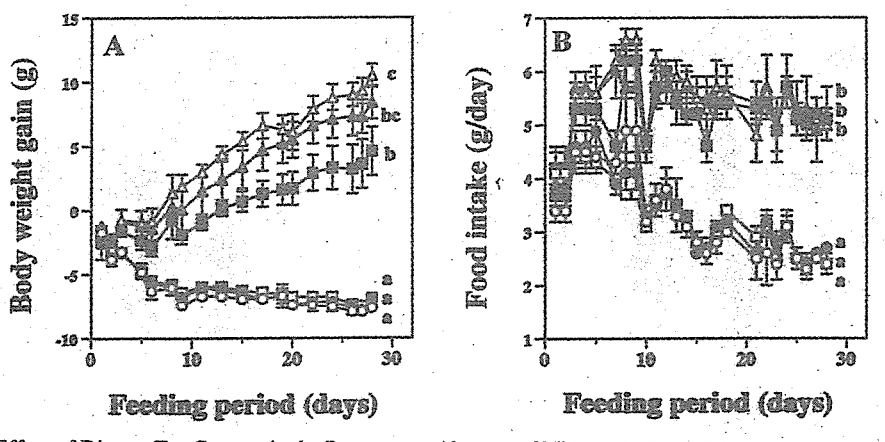

Fig. 1 Effect of Dietary Trp Content in the Presence or Absence of NiA on the Body Weight Gain of Young Mice.
○, 0% Trp and 0% NiA; ●, 0% Trp and 6 mg% NiA; □, 30 mg% Trp and 0% NiA; ■, 30 mg% Trp and 6 mg% NiA; △, 70 mg% Trp and 0 mg% NiA; ▲, 70 mg% Trp and 6 mg% NiA.
Values are expressed as means ± 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 20oC 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℃ 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 literature[6].

Analyses

The contents of NAD (NAD^+ + NADH) and NADP ($^{NADP+}$ + NADPH) were measured by the calorimetric method of Shibata and Murata[6] and Shibata and Tanaka[7], respectively. The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the HPLC method of Shibata et al.[5], while the content of MNA in the urine was measured by the HPLC method of Shibata[8]. The content of Nam N-oxide was measured by the method of Shibata[9].

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. Shibata[10] 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 Trp[11] and sucrose was selected as carbohydrate. Sources for lipids, minerals, and vitamins were selected

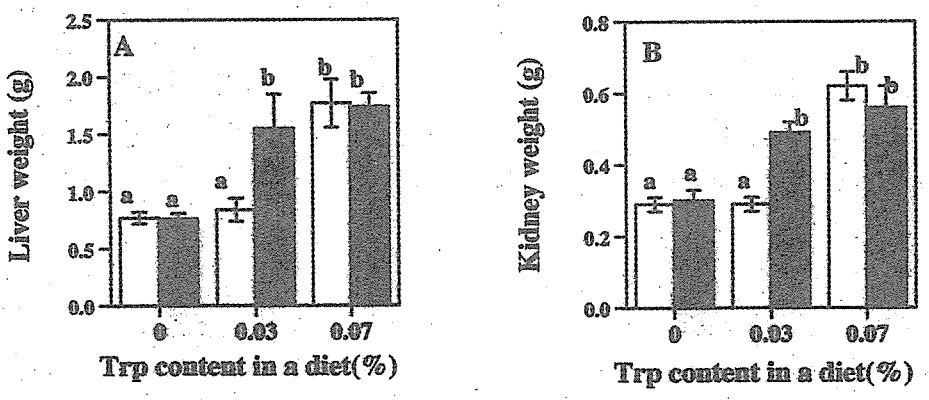

Fig. 2 Effect of Dietary Trp Content in the Presence or Absence of NiA on the Liver and Kidney Weights of Young Mice. Open column is the groups fed on the diets without NiA, on the contrary, closed column is with NiA. A, Liver weight; B, kidney weight. Values are expressed as means ± SEM for five mice 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

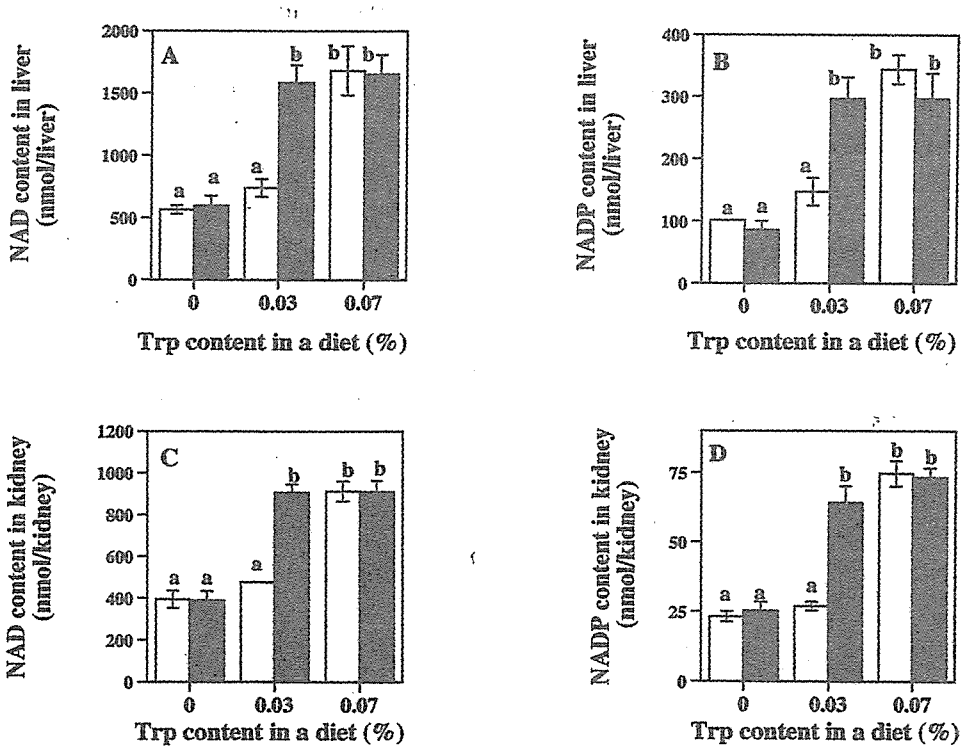

Fig. 3 Effect of Dietary Trp Content in the Presence or Absence of NiA on the Liver and Kidney NAD and NADP Contents of Young Mice.

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 rats[10], 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 zero[11]. 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 carbohydrates[13]. 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 Katayama[14] 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

the diet were not special. In the present experiment, the minimum requirement of Trp in the growing mice was found around 0.03g/100 g of diet in the presence of a suitable amount of NiA.

From the comparison of the body weight gain, food intake, liver and kidney weights, NAD and NADP contents in live and kidney, and the urinary excretion of Nam and its metabolites between the groups fed with the diets of 0.03% Trp-0% NiA and 0.03% Trp-0.006% NiA, the diet containing 0.03% Trp-0% NiA diet (the composition is shown in Table 1) was an excellent diet for producing a niacin-deficiency for mice. Therefore, we get a useful item for comparisons of behaviors and metabolic disturbances between the QPRT-knockout mice and niacin-deficient mice.

Acknowledgment

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〈トリプトファン研究会記録〉

ナイアシン欠乏マウスの作成

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要旨　近年、特定の遺伝子を欠損させた動物を作製する技術が開発され、その動物を解析することにより様々な病態との関係を明らかにすることが可能となってきた。Trp-ナイアシン代謝研究においても、今後はナイアシン合成経路に関与する遺伝子を欠損させたノックアウトマウスを使う研究が期待され、当研究室でもキノリン酸代謝に関与するQuinolinate phosphoribosyltransferase（QPRT）遺伝子ノックアウトマウスの作製を進めている。その際、QPRT遺伝子ノックアウトマウスのナイアシン欠乏状態を野生型のマウスと比較することが必要となる。ところが、ナイアシンはTrpから生合成できるため、ナイアシン欠乏状態にするためにはTrp量をうまく制限した特殊な組成の飼料を開発する必要がある。我々は、このマウス用ナイアシン欠乏飼料の作成に成功したので報告する。Trpをほとんど含まないツェインにTrpが第一制限アミノ酸となるように他のアミノ酸を適量添加し、糖質源をすべてショ糖とした。これに種々の含量のTrpを加え、各々の飼料にニコチン酸を添加して、ニコチン酸の添加効果が認められた時の飼料をナイアシン欠乏飼料とした。Trp含量0.03%において、NiA添加群はNiA非添加群と比して、体重、肝・腎重量が顕著に増加し、肝・腎中のNAD量も有意に増加した。

キーワード：ナイアシン、欠乏、マウス、トリプトファン、飼料

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〈トリプトファン研究会記録〉

運動とトリプトファン代謝　ー血中動態からの推測ー

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内藤 純子[1)]、中上　寧[1)]、長村 洋一[1)]

要旨　トリプトファンの主要な代謝経路であるキヌレニン経路の代謝産物にはキヌレン酸、アントラニル酸、NADなど重要な生理活性を有するものが多い。我々はこれまでにキヌレニン経路の中間代謝産物であるキヌレニンの血中濃度が、運動により変化することを報告した。また、その変化は負荷強度に依存しているように思われる。しかしながら、被検者の運動習慣あるいは主観的な負荷強度の違いにより、血中キヌレニン濃度は増加する場合や減少する場合がある。これはトリプトファン代謝の大部分を占めるキヌレニン経路の酵素活性が運動の影響を受けることを意味する。すなわち、末梢のトリプトファン代謝系が運動パフォーマンスや運動による爽快感や疲労感などに関与する可能性が示唆される。これらの生理現象が、運動へのエネルギー代謝のみに関与しているのか、あるいは知覚神経系を介した中枢神経系にも関与するのかについて、我々は現在研究を続けている。

キーワード：運動、トリプトファン代謝系、キヌレニン経路

Ⅰ. 緒言

運動に際して発生する疲労には、中枢疲労と末梢疲労があるとされている。中枢疲労の主な要因は、運動により血中に増加した遊離トリプトファンの脳内への取り込みによって生じた余剰のセロトニンであるとNewsholmeらによって提唱されている[1)]。Davisらは運動で発生する中枢疲労は、トリプトファンとトランスポーターを競合する分岐鎖アミノ酸および血中遊離脂肪酸を抑制する糖分を摂取することによりその発生を遅延できることを示した[2,3)]。一方の末梢疲労は筋疲労であり、これはグリコーゲンなどエネルギー源の消耗であるとされている[4)]。しかしながら、脳内トリプトファンの大多数はキヌレニン経路により代謝されること、また、トリプトファンはキヌレニン経路を介して解糖系やニコチンアミド代謝系へ代謝されることから、我々は運動によって発生する疲労の一部は少なくともトリプトファンのキヌレニン経路の代謝産物に由来すると考えている。すなわち、トリプトファンはキヌレニン経路を介してキヌレニン、キヌレン酸、3-ヒドロキシキヌレニン、アントラニル酸、キノリン酸などのほか解糖系、ニコチンアミド代謝系へと代謝され神経伝達物質やエネルギー賦活源あるいはフリーラジカルの産生源などになる。運動を行うと、これらキヌレニン経路の代謝産物の増加あるいは減少により中枢疲労あるいは末梢疲労を増減させるのではないかと考えている。これらのことから、

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我々はこれまでにキヌレニン経路の初期の中間代謝産物であるキヌレニンの運動前後での血中動態について調べ[5,6]、適度な運動の指標となる可能性について検討してきた[5,7]。

運動中にはトリプトファンは主にトリプトファン2,3-ジオキシゲナーゼ（EC 1.13.11.11: TDO）およびインドールアミン2,3-ジオキシゲナーゼ（EC 1.13.11.42: IDO）によってN-ホルミルキヌレニンへと分解される。また、継続する運動習慣はキヌレニン経路を活性化し、血中のトリプトファンおよびNAD濃度を高める[6,8]。この論文では、運動による血液中のトリプトファン、キヌレニン、3-ヒドロキシキヌレニンおよびNADの変化を調べ、そこから運動負荷時のトリプトファン代謝について考察した。

Ⅱ. 被検者と方法

1) 被検者

被検者は運動習慣のあるグループと運動習慣の無いグループとした。全ての被検者には予め実験の内容を説明し、インフォームドコンセントを得た。

2) 採血

採血は、運動開始の1時間前と運動終了直後に行った。採血後直ちに氷中に置き、血液凝固後直ちに遠心分離して血清を採取した。NADは、採血直後に全血20μlを専用の緩衝液で十分撹拌した後、90℃で2分間加熱処理した[9]。

3) 測定法

トリプトファン：血清トリプトファンは、日立L-8500高速アミノ酸分析計（日立；東京）で測定した。

キヌレニン：血清キヌレニンはSaitoらの方法で行い、ODCカラムを用いたHPLC法で測定した[10]。

3-ヒドロキシキヌレニン：血清3-ヒドロキシキヌレニンはNaitoらの方法で行い、ODS-Aカラムを用いたHPLC法で測定した[11]。

NAD：NADは色素法を用いて測定した[9]。

POMS試験：POMS検査は、気分の変化を評価する質問紙法としてMcNairらによって開発された方法であり、緊張－不安（tention-anxiety）、抑鬱－落ち込み（depression-dejection）、怒り－敵意（anger-hostility）、活気（vigor）、疲労（fatigue）および混乱（confusion）の6項目の気分尺度を測定できる[12]。結果の解釈は緊張-不安、抑鬱-落ち込み、怒り-敵意、疲労および混乱ではスコアは低い方が良く、活気ではスコアは高い方が良いとされる[12]。この研究ではPOMS試験を運動直前および運動終了直後に行った。

4) 統計学的処理

走行前後の有意差検定にはStudent paired t 検定を用い、危険率5％以下を統計学的有意とした。不良標本の棄却にはThompsonの棄却検定を用いた。また、結果の表示は平均値±標準偏差

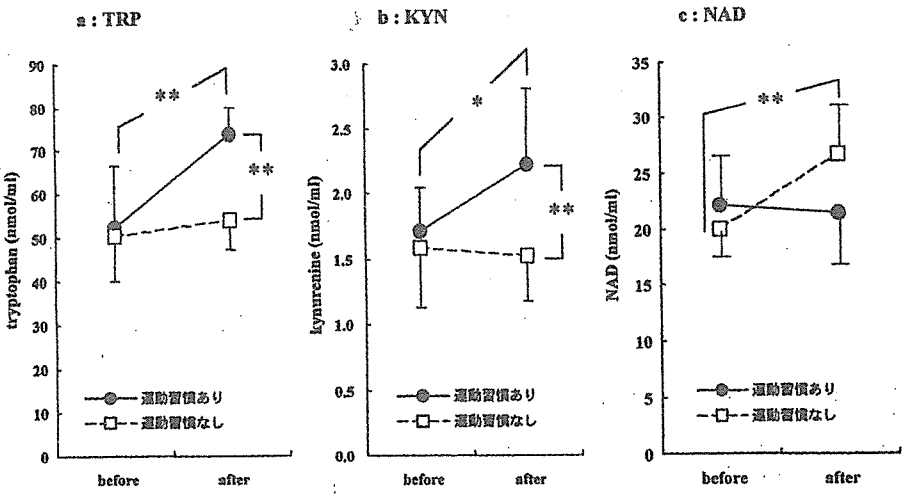

図1　運動習慣を持つ被検者と持たない被検者でのハーフマラソン前後のトリプトファン（a: TRP）、キヌレニン（b: KYN）、NAD（c: NAD）の比較。**: $p<0.01$、*: $p<0.05$

で表した。

Ⅲ. 結果

前述の各条件下におけるトリプトファンおよびその代謝産物の変動は次のようであった。

1) トリプトファン

運動習慣のある人では、血中トリプトファン濃度は、1時間以上の有酸素運動によって増加したが、運動習慣の無い人では増加しなかった（図1a)。また、安静時の血中トリプトファン濃度は、運動習慣のある人と運動習慣の無い人で差はなかった（図2a)。

2) キヌレニン

運動習慣のある人では、血中キヌレニン濃度は、1時間以上の有酸素運動によって増加したが、運動習慣の無い人では増加しなかった（図1b)。また、有酸素運動の前と後で、トリプトファンとキヌレニンはそれぞれ高い相関があった（図3)。また、安静時の血中キヌレニン濃度は、運動習慣のある人では運動習慣の無い人より有意に高かった（$p<0.01$)。しかし、アスリートでは低値であった（図2b)

3) 3-ヒドロキシキヌレニン

3-ヒドロキシキヌレニンは、運動習慣のある人では運動の前後でほとんど変化はなかった（図4)。また、運動の前にはキヌレニンと3-ヒドロキシキヌレニンの間に相関があったが、運動の後にはキヌレニンと3-ヒドロキシキヌレニンの間に相関はなく、運動による血中キヌレニ

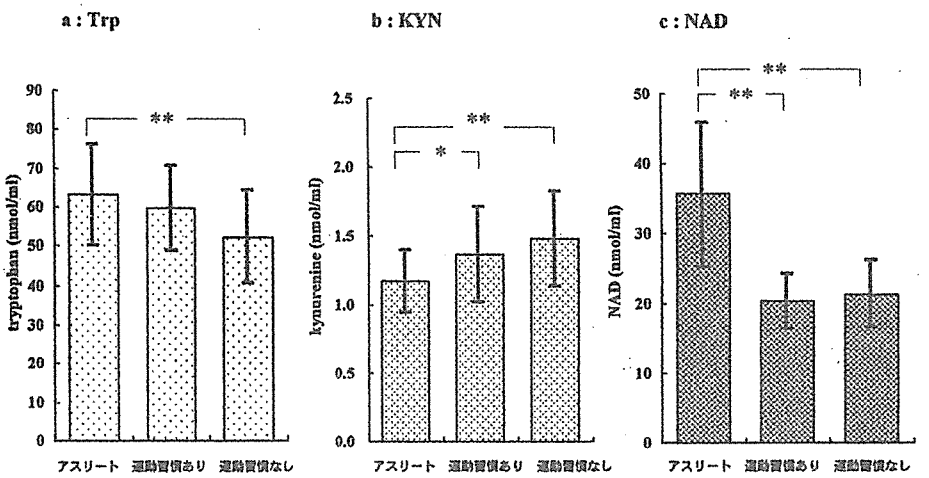

図2　運動習慣を持つ被検者、持たない被検者およびアスリートでの安静時のトリプトファン（a: Trp)、キヌレニン（b: KYN)、NAD（c: NAD）の比較。**: $p<0.01$、*: $p<0.05$

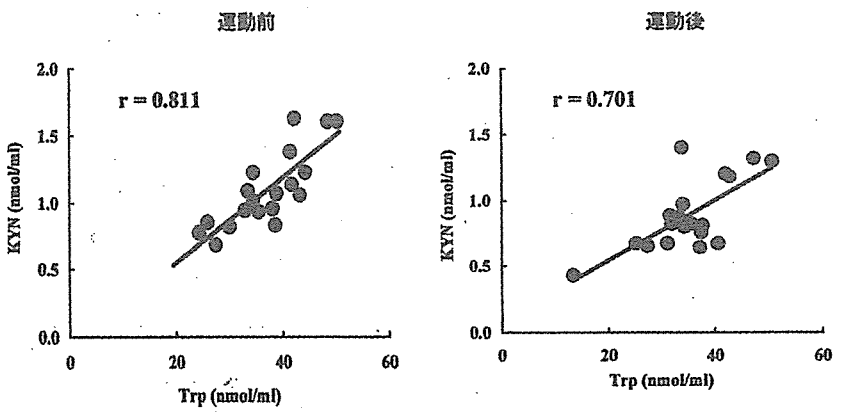

図3　運動前後のトリプトファンとキヌレニンの血中濃度の相関

ンレベルの変化に依存しない可能性が示された（図５）。

4) NAD

運動習慣のある人では、血中NAD濃度は、１時間以上の有酸素運動によって増加しなかったが、運動習慣の無い人では増加した（図１c）。また、安静時の血中濃度は運動習慣のある人は運動習慣の無い人より有意に高かった（$p<0.01$）（図２c）。

最後にトリプトファン代謝と精神活動状態を調べるためにPOMSテストのスコアとの関連性を調べてみた。その結果、POMSスコアと血中キヌレニン濃度の間に相関が認められた。活気とキヌレニン濃度の間には正の相関があった。また、その他の５項目との間には負の相関があった（表１）。

Ⅳ. 考察

今回は運動の前後におけるトリプトファンおよびその代謝産物の変動と疲労感や活気など精神活動の関係を調べた。その結果、運動中に血中トリプトファンはキヌレニンに代謝されるが、運動習慣の有無によりその動態に差があることが示された。その代謝の中心となる酵素はIDOと考えられる[13]。すなわち、IDO活性は運動中に発生するNOやIFNγなどのサイトカインによって誘導され、トリプトファンをキヌレニンに代謝する[10]。被検者の中にはキヌレニン濃度が低下する人もあったが、その原因は基質となるトリプトファン量が減少したためと考えられた。キヌレニンはキヌレニン3-モノオキシゲナーゼ（EC 1.14.13.9）によって3-ヒドロキシキヌレニンへ代謝される。3-ヒドロキシキヌレニンは強いフリーラジカルの産生源であり、運動によっ

表１　運動前後でのPOMSスコアと血清キヌレニンの相関

(n=22)

	before exercise	after exercise
tention-anxiety	−0.410*	−0.383
depression-dejection	−0.492*	−0.272
anger-hostility	−0.429*	−0.156
vigor ※	0.437*	0.573*
fatigue	−0.506*	−0.541*
confusion	−0.569*	−0.596*

**：相関係数が有意（$p<0.05$）であった。

※：活気は正の相関が認められた。

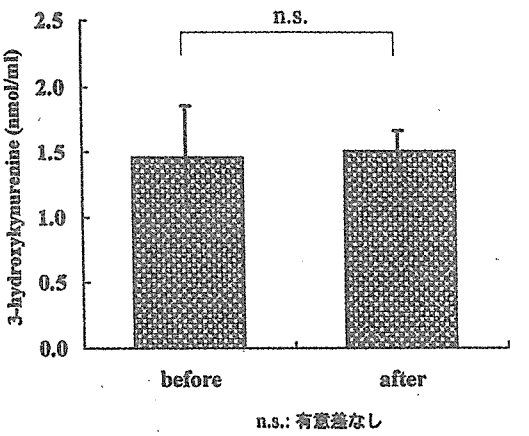

図４　運動前後での血清３-ヒドロキシキヌレニンの変化

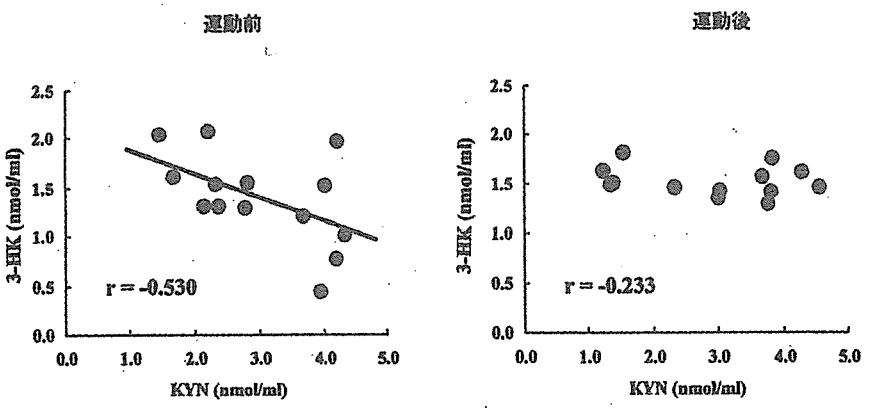

図５　運動前後でのキヌレニンと３-ヒドロキシキヌレニンの関係の変化