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

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In order to find an alleviation method for the adverse effect of environmental endocrine disrupters, we studied the effects of the putative endocrine disrupter and peroxisome proliferator, di(2-ethylhexyl)phthalate (DEHP), on animal growth and vitamin metabolism. It is known that the effects of chemical compounds such as xenobiotics differ according to the dietary protein source. We compared the effects of dietary DEHP administration on rats fed with a diet containing milk casein or wheat gluten. The increased conversion ratio of tryptophan to nicotinamide by DEHP administration was significantly higher in the casein group than in the gluten group. We also investigated the effects of DEHP on the urinary excretion of other vitamins. DEHP administration resulted in decreased urinary excretion of vitamin B₁, vitamin B₂, and pantothenic acid.

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

Phthalic acid esters, which are known to cause malformation of the mice fetus,¹–³) are used in a variety of industrial applications.¹–³) They are constituents of such diverse products as paint, adhesive, cosmetics and polyvinyl chloride plastic.²,³) These esters are widely distributed throughout the environment and have been detected in animals and humans.⁴) We have already reported that the administration of phthalic acid the esters such as di-n-butylphthalate⁵) and di(2-ethylhexyl)phthalate (DEHP)⁶–¹⁰) disturbed the de novo nicotinamide (Nam) synthesis from tryptophan (Trp). Handler and Dann¹¹) and Shibata and Tanaka¹²) have reported that an intake of excess Nam retarded the growth of young rats. We have proposed that part of the toxicity of phthalic acid esters was attributable to excess Nam formation.⁵,⁶) The conversion ratio of Trp to Nam varies according to the amino acid composition of dietary proteins.¹³) Furthermore, there are some reports that dietary grain proteins alleviated the adverse effect of a toxin¹⁴,¹⁵) and reduced the toxicity with an excessive intake of nutrients¹⁶) compared to dietary milk casein. In the present experiment, we report a comparison of the effects of phthalic acid esters on the vitamin metabolism in rats fed with a casein or gluten diet.

Materials and Methods

Chemicals. Vitamin-free milk casein, wheat gluten, DEHP, sucrose, L-methionine, L-lysine, L-threonine, anthranilic acid, nicotinic acid, thiamin hydrochloride, riboflavin, calcium pantothenate, Nam and quinolinic acid (QA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Kynurenine acid (KA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HA) and N¹-methylnicotinamide (MNA) chloride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N¹-Methyl-2-pyridone-5-carboxamide (2-Py) and N¹-methyl-4-pyridone-3-carboxamide (4-Py) were respectively synthesized by the methods of Pullman and Colowick¹⁷) and Shibata et al.¹⁸) Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized cornstarch, and the mineral (AIN-93-G-MX)¹⁹) and Nam-free vitamin (AIN-93-VX)¹⁹) mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan), all the other chemicals used being of the highest purity available from commercial sources.

Animal and diets. The care and treatment of the experimental animals conformed with The University of
Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

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

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

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

The rats were killed by decapitation at around 10:00 a.m. on the last day of the experiment.

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

The contents of KA,22) XA,23) 3-HA,24) AnA,24) QA,25) thiamin,26) and riboflavin27) in the urine were measured by the HPLC method. The urine content of pantothenic acid was measured by a microbiological method.28)

### Results

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

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

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

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

We have previously reported that a target for the disturbance of Trp metabolism was the reaction of $\alpha$-amino-\(\beta\)-carboxymuconate-\(\epsilon\)-semialdehyde (ACMS) $\rightarrow$ $\alpha$-aminomuconate-\(\epsilon\)-semialdehyde (AMS),10) which resulted in the increased formation of QA. As shown in Table 2, the QA formation was significantly increased by the DEHP intake in the experiments with both the

### Table 1. Composition of the Diets

<table>
<thead>
<tr>
<th></th>
<th>20% Gluten diet (%)</th>
<th>20% Casein diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Test 0.5% DEHP</td>
<td>Control</td>
</tr>
<tr>
<td>Gluten</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Casein</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>t-Lysine</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>t-Threonine</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>t-Methionine</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
<td>46.83</td>
<td>46.33</td>
</tr>
<tr>
<td>Sucrose</td>
<td>22.66</td>
<td>22.66</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>(AIN-93G-MX)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(nicotinic acid-free)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEHP</td>
<td>0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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

\(2\) The amino acid contents (total content was 19,194.6 mg) of 100 g of the diet were 972.6 mg of isoleucine, 1,675.2 mg of leucine, 1,432.0 mg of lysine, 940.4 mg of methionine, 86.40 mg of cysteine, 998.8 mg of tyrosine, 729.4 mg of threonine, 226.8 mg of tryptophan, 1,188.8 mg of valine, 540.4 mg of histidine, 648.4 mg of arginine, 540.4 mg of alanine, 1,243.0 mg of aspartic acid, 3,783.0 mg of glutamic acid, 324.2 mg of glycine, 2,026.4 mg of proline, and 918.6 mg of serine.
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Fig. 1. Effect of DEHP on the Body Weight Gain (A) and Food Intake (B) of Rats Fed with the Gluten or Casein Diet.

Young rats of the 6 weeks olds were fed on respective diet for 21 days. Each point is the mean ± SEM for 5 rats. ●, Gluten diet; ○, gluten diet + 0.5% DEHP; ▲, casein diet; △, casein diet + 0.5% DEHP.

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

<table>
<thead>
<tr>
<th></th>
<th>20% Gluten diet (%)</th>
<th>20% Casein diet (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Test 0.5% DEHP</td>
</tr>
<tr>
<td>Liver weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g/rat)</td>
<td>11.5 ± 0.4a</td>
<td>15.5 ± 0.4b</td>
</tr>
<tr>
<td>(g/100 g b.w.)</td>
<td>4.0 ± 0.1a</td>
<td>5.9 ± 0.2b</td>
</tr>
<tr>
<td>Urinary excretion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol/mol Trp intake)</td>
<td>AnA</td>
<td>KA</td>
</tr>
<tr>
<td></td>
<td>0.49 ± 0.03</td>
<td>5.62 ± 0.31a</td>
</tr>
<tr>
<td></td>
<td>0.57 ± 0.03</td>
<td>7.95 ± 0.90b</td>
</tr>
<tr>
<td></td>
<td>0.34 ± 0.05</td>
<td>0.28 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.26 ± 1.33c</td>
<td>4.12 ± 1.33c</td>
</tr>
<tr>
<td></td>
<td>1.80 ± 0.34a</td>
<td>12.26 ± 1.33c</td>
</tr>
<tr>
<td></td>
<td>1.14 ± 0.24a</td>
<td>12.14 ± 1.29c</td>
</tr>
<tr>
<td></td>
<td>0.57 ± 0.16a</td>
<td>5.49 ± 1.09b</td>
</tr>
<tr>
<td></td>
<td>9.00 ± 1.35a</td>
<td>34.87 ± 4.11b</td>
</tr>
</tbody>
</table>

Each value is expressed as the mean ± SEM (n = 5); a different superscript letter in the same row means significantly different at P < 0.05 as calculated by the Student-Neumann-Keuls multiple-comparison test.

* N.D., not detected

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

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

Treatment of rats with DEHP increases the induction of several metabolic enzymes, including those involved in peroxisomal β-oxidation.179 We therefore compared the effects of DEHP administration on the vitamins involved in the β-oxidation pathway such as riboflavin and pantothenic acid in the rats fed with the diets of gluten and casein.

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

Thiamin is not required in fatty acid metabolism, but is in glucose metabolism. Thus, the effect of DEHP administration on the urinary excretion of thiamin was also investigated. As shown in Fig. 5, the urinary
that dietary grain protein alleviated the adverse effect of DEHP. Shibata and Tanaka\textsuperscript{12} have reported that the intake of Nam was the same and the conversion ratio of Trp to Nam was also the same. However, the administration of DEHP significantly increased the formation of Nam acetate and accentuated the mechanism of QA by inhibiting the ACMSD activity. This increased formation resulted in metabolites beyond QA such as \(8\)-hydroxyriboflavin and \(8\)-hydroxyriboflavin 14,15. We therefore compared the effect of DEHP on the vitamin metabolism involved in energy formation between the casein and gluten diets. In the present experiments, the limiting amino acids were appropriately supplemented to the two diets to maintain an equal growth rate (Table 1). However, the effects of DEHP on the metabolism of Trp to Nam differed between the groups fed with the gluten and casein diets (Fig. 2 and Table 2). The difference in the effect of DEHP would have been due to the reaction of ACMS \(\rightarrow\) AMS. This reaction is catalyzed by ACMSD, although the liver ACMSD activity was almost the same between the groups fed with the gluten and casein diets (data not shown). We have reported that the mono-(2-ethylhexyl) phthalate acid ester was an inhibitor of ACMSD.\textsuperscript{10} It is known that the enzyme activities\textsuperscript{16,29} and gene expression\textsuperscript{30–33} are affected by the kind of dietary protein. Therefore, the enzyme activity catalyzing the reaction of DEHP \(\rightarrow\) mono-(2-ethylhexyl)phthalate and/or its mRNA level might be expected to differ between the gluten and casein diets. It is known that the amino acid score is higher in casein than in gluten. In the present experiment, the limiting amino acids were added to the gluten diet to give the same body weight gain between the two dietary groups (Fig. 1). The effect of DEHP on the metabolism of Trp to Nam (Fig. 2 and Table 2), riboflavin (Fig. 3), pantothenic acid (Fig. 4), and thiamin (Fig. 5) was significantly different between the gluten and casein diets. A decreased urinary excretion of riboflavin, pantothenic acid, and thiamin generally means an increased requirement of these vitamins in the body when the intake of these vitamins is the same. Additionally, increasing the catabolism of these vitamins by enhancing the drug metabolizing system could be considered as the reason for decreased urinary excretion of these vitamins by the DEHP intake. \(7\)-Hydroxyriboflavin and \(8\)-hydroxyriboflavin have been reported as catabolic metabolites of riboflavin,\textsuperscript{27} although we were not able to confirm the peaks that corresponded to \(7\)-hydroxyriboflavin and \(8\)-hydroxyriboflavin in the HPLC data. Hydroxylated compounds of pantothenic acid and thiamin have not been reported, so the main reason for the decreased amounts of these vitamins would be attributable to accentuation of the \(\beta\)-oxidation pathway. In other words, the requirement for riboflavin, pantothenic acid, and thiamin might be increased when \(\beta\)-oxidation pathway is accentuated by the DEHP intake. It could be expected that the necessity for Nam would rise as well and that the urinary excretion of Nam and its metabolites would decrease with accentuation of the \(\beta\)-oxidation pathway when the intake of Nam was the same and the conversion ratio of Trp to Nam was also the same. However, the administration of DEHP significantly increased the formation of QA by inhibiting the ACMSD activity. This increased formation resulted in metabolites beyond QA such as Nam, MNA, 2-Py, and 4-Py. Therefore, the increased urinary excretion of Nam and its metabolites did not

\[\text{Conversion ratio of Trp to Nam (\%)}\]

\[\begin{align*}
\text{Casein} & \quad \text{Casein + 0.5% DEHP} \\
\text{Gluten} & \quad \text{Gluten + 0.5% DEHP}
\end{align*}\]

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

Discussion

We have already reported that the administration of DEHP significantly increased the formation of Nam from Trp by inhibiting the activity of ACMSD\textsuperscript{10} and that feeding a casein diet containing over 1% DEHP retarded the growth of young rats.\textsuperscript{5} Handler and Dann\textsuperscript{11} and Shibata and Tanaka\textsuperscript{12} have reported that the intake of excess Nam retarded the growth of young rats. It is therefore considered that part of the toxicity of phthalic acid esters is attributable to excess Nam formation. In a previous report,\textsuperscript{3}\ we stated that the degree of conversion of Trp to Nam differed according to the dietary casein level; the increased conversion was significantly lower in the group fed with the 10% casein diet than in the group fed with the 20% casein diet when the diets contained a phthalic acid ester. Shibata\textsuperscript{13} has clarified that the conversion ratio varied according to the amino acid composition of dietary proteins. Treating the rats with DEHP increased the induction of several metabolic enzymes, including those involved in peroxisomal \(\beta\)-oxidation.\textsuperscript{29} Furthermore, some reports have revealed that dietary grain protein alleviated the adverse effect of a toxin.\textsuperscript{14,15}
Fig. 3. Effect of DEHP on the Urinary Excretion of Riboflavin in Rats Fed with the Gluten or Casein Diet. Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column, casein diet group; hatched column, gluten diet group.

Fig. 4. Effect of DEHP on the Urinary Excretion of Pantothenic Acid (PaA) in Rats Fed with the Gluten or Casein Diet. Each bar is the mean ± SEM for 5 rats; a different superscript letter means significant difference at $p < 0.05$ as calculated by the Student-Neumann-Keuls multiple-comparison test. Unfilled column, casein diet group; hatched column, gluten diet group.
mean that the DEHP administration resulted in a lower need for Nam, because the conversion ratio of Trp to Nam was significantly increased (Fig. 2). It is likely that the need for Nam was increased by the DEHP administration. Therefore, when the toxicity of DEHP is being discussed, it is necessary to include the increased requirement of such vitamins as thiamin, riboflavin, and pantothenic acid. We therefore recommend when DEHP is being administered that it is necessary to increase the dietary intake of vitamin B₁, vitamin B₂, and pantothenic acid.

Acknowledgments

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