Effect of Supplementing Methionine and Threonine to a Non-protein Diet on the Induction of Enzymes by Polychlorinated Biphenyls in Rats[†]

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The *N*-sparing action of methionine and threonine in rats fed with a non-protein diet seems to indicate that methionine and threonine are the endogenously most-limiting amino acids. The effect of a dietary supplementation of methionine and threonine to a non-protein diet on enzyme induction by polychlorinated biphenyls (PCB) was investigated. Liver microsomal drug-metabolizing enzymes were further induced by the methionine and threonine supplement to the non-protein diet in PCB-receiving rats. Although feeding with PCB did not increase the urinary ascorbic acid excretion in rats of the non-protein diet group, the supplementation of methionine and threonine to the non-protein diet markedly increased this excretion. From these observations, dietary methionine and threonine might promote the synthesis of drug-metabolizing enzymes and the enzymes related to ascorbic acid biosynthesis in PCB-receiving rats.

Yoshida and Moritoki¹⁾ have reported that the supplementation of methionine and threonine to a non-protein diet reduced the body weight loss and urinary urea excretion in rats. They also reported that dietary methionine and threonine improved the hepatic polysome profile²⁾ and reduced the level of free amino acids in plasma and tissues.³⁾ From these observations, methionine and threonine have been considered to be the endogenously most-limiting amino acids in rats fed with a non-protein diet, and these amino acids might promote reutilization of the endogenous amino acids that are generated from protein turnover.

The administration of polychlorinated biphenyls (PCB) induced drug-metabolizing enzymes and increased urinary ascorbic acid excretion in rats.⁴⁾ These metabolic changes were shown to be influenced by the dietary protein level and quality.^{5,6)} On the other hand, exposure to xenobiotics induced NADPH-generating enzymes.^{7,8)}

We assumed that if methionine and threo-

nine are the endogenously most-limiting amino acids in rats fed with a non-protein diet, a supplementation of methionine and threonine would further increase these PCB-induced metabolic changes. We performed this study to confirm the assumption, and also to discover whether or not dietary PCB would induce NADPH-generating enzymes.

Materials and Methods

Animals and diets. Male rats of the Wistar strain (Japan SLC Co., Hamamatsu, Japan) weighing about 100 g were housed individually and fed with a commercial stock diet (Labo MF Stock, Nihon Nosan Kogyo Co., Yokohama, Japan) for 3 days, and subsequently fed with a 25% casein-sucrose diet for 3 to 5 days. Then the experimental diets were fed for 10 days. The basal non-protein diet contained 3.5% mineral mixture,⁹⁾ 1% vitamin mixture,¹⁰⁾ 0.2% choline chloride and 2% corn oil. Sucrose was used as a source of carbohydrate to make up to 100%, and 0.3% of L-methionine and L-threonine were supplemented to the non-protein diet. The supplemented amount of PCB (Aroclor 1248, Mitsubishi Monsanto Co., Tokyo, Japan)¹¹⁾ was 0.03%. Twenty rats were divided into four groups.

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The rats in groups 1 and 3 were fed with a non-protein diet, and methionine and threonine were added to the diets for groups 2 and 4. The rats in groups 3 and 4 were fed with PCB. To avoid the contamination between the diet and urine, the diets were mixed with the same weight of 3% agar solution to make agar-gel diets. Food and tap water were supplied *ad libitum*. The room temperature was kept at 22°C, with a 12-hour cycle of light (0800–2000 hr) and dark. Urine was collected in 0.1 N HCl on days 9 and 10 of feeding and used for the determination of urea and ascorbic acid. After 10 days of the experimental period, the rats were sacrificed by decapitation (0840–0940). The livers were quickly removed, and aliquots were used to measure the activities of drug-metabolizing enzymes and NADPH-generating enzymes.

Analytical methods. The livers were homogenized in 4 volumes of 1.15% KCl–0.01 M phosphate buffer (pH 7.4) with a Potter–Elvehjem homogenizer, and then centrifuged at $10,000 \times g$ for 15 min. The activities of aminopyrine *N*-demethylase and aniline hydroxylase, and the cytochrome P450 content were measured as described previously.¹²⁾ Using the postmitochondrial fraction, the 7-ethoxycoumarin *O*-deethylase activity was measured by a modification to the method of Aitio,¹³⁾ isocitric acid, isocitrate dehydrogenase and MnCl_2 being omitted from the reaction mixture, and 5 μmol of glucose-6-phosphate being added. Other portions of the liver were homogenized in 3 volumes of 0.25M sucrose with the Potter–Elvehjem homogenizer and centrifuged at $105,000 \times g$ for 1 hr. The cytosol fraction was stored at -80°C until the activities of glucose-6-phosphate dehydrogenase (EC 1.1.1.49),¹⁴⁾ 6-phosphogluconate dehydrogenase (EC 1.1.1.43)¹⁴⁾ and malic enzyme (EC 1.1.1.40)¹⁵⁾ were assayed at 30°C . Blood was clotted at room temperature and serum was obtained, serum ascorbic acid being measured according to the

method of Okamura.¹⁶⁾ Minced carcass, with the gastrointestinal contents removed, and liver were homogenized in 5% metaphosphoric acid and filtered. Ascorbic acid in the tissues and urine was determined by the 2,4-dinitrophenylhydrazine method, 1 or 2 drops of 0.2% 2,6-dichloroindophenol sodium being added to one ml of a sample. If the reddish color remained for 1 min, 1 ml of 1% SnCl_2 in 5% metaphosphoric acid and then 0.5 ml of 2% 2,4-dinitrophenylhydrazine in 9 N H_2SO_4 were added, and the reaction mixture was incubated at 37°C for 3 hr. The reaction mixture was then placed in an ice-cooled water bath, 2.5 ml of 85% H_2SO_4 was added and, after leaving at room temperature for 30 min, the extinction at 540 nm was measured. Urinary urea was measured according to the urease–indophenol method.¹⁷⁾

Statistical analysis. All results presented are mean values $\pm$ the standard error of the mean ($n=5$). A statistical evaluation of the mean was carried out by a two-way analysis of variance (ANOVA) and Student's *t*-test.¹⁸⁾

Results

Feeding with the non-protein diet caused a body weight loss, and this reduction was partially prevented by supplementing with methionine and threonine (Table I). Methionine and threonine reduced the urinary urea excretion in rats fed with the non-protein diet. Relative liver weights were heavier in the PCB-receiving groups, and this effect was more apparent when methionine and threonine were supplemented to the non-protein diet ($p < 0.05$).

Table I. EFFECT OF SUPPLEMENTING METHIONINE AND THREONINE ON THE BODY WEIGHT CHANGE, LIVER WEIGHT AND URINARY UREA EXCRETION IN RATS FED WITH A NON-PROTEIN DIET WITH OR WITHOUT PCB

Group	Initial body weight (g)	Body weight change (g/10 days)	Liver weight (g/100 g of body wt.)	Urinary urea days 9 and 10 (mg/100 g of body wt./day)
1. Non-protein	120 $\pm$ 2	–18.7 $\pm$ 1.1	4.55 $\pm$ 0.19	18.6 $\pm$ 3.0
2. 1+Met, Thr	121 $\pm$ 1	–12.9 $\pm$ 1.4	4.14 $\pm$ 0.17	7.0 $\pm$ 3.1
3. 1+PCB	121 $\pm$ 1	–19.4 $\pm$ 1.1	5.45 $\pm$ 0.29	20.6 $\pm$ 0.7
4. 3+Met, Thr	121 $\pm$ 1	–14.8 $\pm$ 1.9	6.37 $\pm$ 0.23*	5.6 $\pm$ 0.7
ANOVA				
Met, Thr	—	<0.01	NS	<0.01
PCB	—	NS	<0.01	NS
Interaction	—	NS	<0.01	NS

* Significantly different from the values for the rats fed with the non-protein diet containing PCB at $p < 0.05$ by using Student's *t*-test. NS, not significant.

Table II. EFFECT OF SUPPLEMENTING METHIONINE AND THREONINE ON THE CONTENT OF CYTOCHROME P450 AND THE ACTIVITIES OF AMINOPYRINE *N*-DEMETHYLASE, 7-ETHOXYCOUMARIN *O*-DEETHYLASE AND ANILINE HYDROXYLASE IN RATS FED WITH A NON-PROTEIN DIET WITH OR WITHOUT PCB

Group	Cytochrome P450 (nmoles/liver/ 100 g of body wt.)	Aminopyrine <i>N</i> -demethylase	7-Ethoxycoumarin <i>O</i> -deethylase (units/liver/100 g of body wt.)	Aniline hydroxylase
1. Non-protein	29 ± 2	91 ± 12	45 ± 8	20 ± 3
2. 1 + Met, Thr	26 ± 3	151 ± 14	73 ± 9	26 ± 3
3. 1 + PCB	216 ± 31	398 ± 61	311 ± 32	142 ± 9
4. 3 + Met, Thr	390 ± 46***	1064 ± 54***	606 ± 77***	80 ± 11***
ANOVA				
Met, Thr	<0.01	<0.01	<0.01	<0.01
PCB	<0.01	<0.01	<0.01	<0.01
Interaction	<0.01	<0.01	<0.01	<0.01

One unit is one nmol of formaldehyde, 7-hydroxyethoxycoumarin and *p*-aminophenol formed per min for aminopyrine *N*-demethylase, 7-ethoxycoumarin *O*-deethylase and aniline hydroxylase, respectively. *** Significantly different from the values for the rats fed with the non-protein diet containing PCB at $p < 0.001$ by using Student's *t*-test.

Table III. EFFECT OF SUPPLEMENTING METHIONINE AND THREONINE ON THE LEVEL OF ASCORBIC ACID IN THE URINE, SERUM, LIVER AND CARCASS OF RATS FED WITH A NON-PROTEIN DIET WITH OR WITHOUT PCB

Group	Urinary ascorbic acid (mg/100 g of body wt./day)	Serum ascorbid acid (mg/dl)	Liver ascorbic acid (μg/g)	Carcass ascorbic acid (μg/g)
1. Non-protein	0.09 ± 0.02	0.27 ± 0.02	158 ± 11	43 ± 2
2. 1 + Met, Thr	0.08 ± 0.02	0.42 ± 0.02	231 ± 10	58 ± 2
3. 1 + PCB	0.16 ± 0.01	0.68 ± 0.15	424 ± 31	76 ± 3
4. 3 + Met, Thr	4.48 ± 0.43***	1.76 ± 0.16***	653 ± 5***	100 ± 1
ANOVA				
Met, Thr	<0.01	<0.01	<0.01	<0.01
PCB	<0.01	<0.01	<0.01	<0.01
Interaction	<0.01	<0.01	<0.01	NS

The carcass did not contain serum and liver. *** Significantly different from the values for the rats fed with the non-protein diet containing PCB at $p < 0.001$ by using Student's *t*-test. NS, not significant.

The liver microsomal cytochrome P450 content was increased by the administration of PCB, and methionine and threonine further increased ($p < 0.001$) this content (Table II). Enzyme activities are expressed as units per total liver in 100 g of body weight, because they may be meaningful for considering the physiological effect on the whole body. However, similar effects were also observed even if the activities are expressed per unit liver weight (data not shown). The activities of aminopyrine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase were elevated by the

methionine and threonine supplementation, and these effects were most pronounced in the PCB-receiving rats ($p < 0.001$). The induced activity of aniline hydroxylase, which was observed in the PCB-supplemented non-protein group, was significantly suppressed by the methionine and threonine supplement ($p < 0.001$).

PCB administration did not increase the urinary ascorbic acid excretion in the rats fed with the non-protein diet (Table III). Methionine and threonine supplementation markedly increased the urinary ascorbic acid excretion

Table IV. EFFECT OF SUPPLEMENTING METHIONINE AND THREONINE ON THE ACTIVITIES OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE, PHOSPHOGLUCONATE DEHYDROGENASE AND MALIC ENZYME IN RATS FED WITH A NON-PROTEIN DIET WITH OR WITHOUT PCB

Group	Glucose-6-phosphate dehydrogenase	6-Phosphogluconate dehydrogenase (units/liver/100 g of body wt.)	Malic enzyme
1. Non-protein	7.0±0.9	12±1	16±1
2. 1+Met, Thr	6.9±0.9	17±1	20±2
3. 1+PCB	17±3	26±2	27±3
4. 3+Met, Thr	11±1	38±3	34±3
ANOVA			
Met, Thr	NS	<0.01	<0.05
PCB	<0.01	<0.01	<0.01
Interaction	NS	NS	NS

One unit is one nmol of NADPH formed per min. NS, not significant.

in the PCB-receiving rats ($p < 0.001$). PCB elevated the level of serum, liver and carcass ascorbic acid, and further elevation was observed in the methionine and threonine, supplemented group. The correlation between urinary ascorbic acid excretion and the concentration of serum ascorbic acid in the PCB-treated groups was highly significant ($R = 0.85$, $p < 0.01$). From this correlation, we found that the threshold of the serum ascorbic acid concentration for increasing the urinary excretion of ascorbic acid was about 0.5 mg/dl.

Dietary PCB induced the activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme (Table IV).

Discussion

PCB is a potent inducer of liver microsomal drug-metabolizing enzymes, and commercial preparations exhibit two typical induction modes, the phenobarbital- and 3-methylcholanthrene-inducible types.^{19,20} We found a further significant ($p < 0.001$) increment in the content of cytochrome P-450 and induction with the activities of aminopyrine *N*-demethylase and 7-ethoxycoumarin *O*-deethylase in the methionine and threonine-supplemented group receiving PCB. These observations agree well with those in the previous report; the methionine and threonine supplement im-

proved the hepatic polysome pattern.²¹ Methionine and threonine might thus promote protein synthesis. Unexpectedly, methionine and threonine significantly ($p < 0.001$) suppressed the PCB-induced activity of aniline hydroxylase. Methionine alone could also reduce this enzyme activity to the same extent (data not shown). Kato *et al.*^{6,21} have reported that the addition of methionine to a PCB-containing soy protein diet caused no changes in aniline hydroxylase activity. The reduction in aniline hydroxylase due to a methionine and threonine intake may be a specific phenomenon for rats consuming a non-protein diet. Reutilization of the endogenous amino acids generated from protein turnover for each protein synthesis might be changed by the addition of methionine and threonine, although the mechanism for suppressing the activity of aniline hydroxylase due to the methionine and threonine intake remains to be elucidated. The unique action of methionine and threonine has also been reported for tyrosine aminotransferase.¹⁾

Urinary ascorbic acid excretion was increased by the administration of PCB,⁴⁾ and ascorbic acid was required to induce drug-metabolizing enzymes in rats unable to synthesize ascorbic acid.²²⁾ It has been also shown that more sulfur-containing amino acids were required to increase the urinary ascorbic acid excretion than for maximum growth.^{21,24)} Horio and Yoshida²³⁾ have reported that the

administration of PCB increased the biosynthesis of ascorbic acid and accelerated the ascorbic acid turnover in rats. Surprisingly, PCB did not increase the urinary ascorbic acid excretion in rats of the non-protein diet group; however, even in this case, an elevated level of ascorbic acid in the tissues and serum was observed (Table III). This was probably due to the deficiency of amino acids for inducing the ascorbic acid synthesizing enzymes. It is well known that urinary ascorbic acid excretion is influenced by the dietary protein level.⁵⁾ It is noteworthy that methionine and threonine elevated the urinary ascorbic acid excretion just like the protein diet. We conclude that the addition of methionine and threonine would enhance ascorbic acid biosynthesis and, if the serum ascorbic acid level exceeded the threshold of 0.5 mg/100 ml, urinary ascorbic acid excretion would increase and ascorbic acid turnover would be accelerated.

Phenobarbital⁷⁾ and 3-methylcholanthrene⁸⁾ have been reported to be inducers of NADPH-generating enzymes. The activities of drug-metabolizing enzymes⁴⁻⁶⁾ and 3-hydroxy-3-methylglutaryl coenzyme A reductase (EC 1.1.1.34),^{25,26)} the rate-limiting enzyme for cholesterol synthesis, were elevated by the addition of PCB, and these two systems would require NADPH as a cofactor. The elevated activities of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and malic enzyme in the PCB-receiving rats (Table IV) explains the elevated demands of NADPH for the drug-metabolizing system and cholesterol synthesis.

We postulate that methionine and threonine would be the endogenously most-limiting amino acids when rats are fed a non-protein diet; however, the mechanism for the unique nutritional characteristics of methionine and threonine should be further investigated.

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