Effects of Dietary Threonine Levels on the Threonine-degrading Enzyme Activities and Tissue Threonine Related Amino Acid Concentration in Rats

Reiko WATANABE, Shinobu FUJIMURA*,
Motoni KADOWAKI* and Teru ISHIBASHI*

Niigata Women's College, Niigata-shi 950-0806

* Faculty of Agriculture, Niigata University, Niigata-shi 950-2102

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Abstract In order to compare the responses of plasma amino acid (AA) concentrations of glycine (Gly) and serine (Ser) to the dietary excess threonine (Thr) in rats and chickens, adult male Wistar/ST rats weighing 304.5 ± 5.2 g were fed diets with five graded levels of Thr from 0.32 to 0.67%. The composition of experimental diets was similar to that of the basal diet used for laying hens. Tissue concentrations (TC) of three AA, Thr, Gly and Ser, expressed as $\mu\text{mol/g}$ tissue and the enzyme activities expressed as $\mu\text{mol/g tissue/30 min}$ relating to conversion of Thr to Gly and interconversion of Gly and Ser in homogenates of liver, kidney and duodenum were determined. In the liver, when dietary Thr increased from 0.32 to 0.42%, TC of Thr increased from 0.50 to 0.73 $\mu\text{mol/g}$, and then increased linearly with increasing dietary Thr levels. When dietary Thr levels were higher than 0.42%, TC of Thr in the kidney and duodenum were higher 45 and 75% at 0.67% dietary Thr level compared to those at less than 0.42% dietary Thr level, respectively. TC of Gly in the three tissues remained constant regardless of dietary Thr levels, but those in the kidney were two times higher than those in other two tissues. TC of Ser in the liver tended to increase, however, those in the kidney and duodenum were not affected by dietary Thr levels. In the liver, Thr aldolase activity decreased slightly, and then increased at dietary Thr levels exceeded 0.42%. However, all enzyme activities except Ser-hydroxymethyltransferase in the liver were not affected by dietary Thr levels. In contrast to the data of laying hens reported previously, the activity of liver Ser-hydroxymethyltransferase converting Ser to Gly decreased with increasing dietary Thr levels. These responses of enzyme activities in the rat liver might be one of the reasons for the difference in the responses of TC and plasma concentrations of Gly and Ser to the dietary excess Thr in rats and chickens.

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When a threonine (Thr) excess diet was supplied, the plasma concentration (PC) of Thr increased, that of glycine (Gly) remained constant and that of serine (Ser) decreased in chicks³⁾ and laying hens^{10,19)}. However, the responses of PC of Gly and Ser to changes of dietary Thr levels in rats were unclear. For

the degradation of Thr, Thr dehydratase (T-DHT, EC 4.2.1.16) catalyzing Thr to α -ketobutyric acid and Thr aldolase (T-AL, EC 4.1.2.5) catalyzing Thr to Gly in cytosole, and Thr dehydrogenase (T-DHG, EC 1.1.1.103) catalyzing Thr to 2-aminoacetacetic acid in mitochondria, have been recognized as major

enzymes in the mammalian liver⁷⁾.

In the previous paper²¹⁾, it was reported that T-AL activities responded to changes of dietary Thr levels in laying hens. They remained constant at the range of deficient dietary Thr levels from 0.32 to 0.42%, and then increased significantly with increasing dietary Thr levels in the duodenum. The similar tendencies were observed in the activities of T-AL and Ser hydroxymethyltransferase (S-HMTF, EC 2.1.2.1) catalyzing reversible interconversion of Gly and Ser, in the liver and kidney. However, S-HMTF activities from Gly to Ser was not affected by changes of dietary Thr levels, and resulted in the increment of conversion from Ser to Gly, which might be one of the reasons why the tissue Ser concentration decreased, when dietary Thr levels were higher than its requirement in chickens.

Therefore, the objective of this study was to compare the responses of PC of Gly and Ser to the dietary excess Thr in rats and chickens. The tissue amino acid (AA) concentration and Thr metabolic enzyme activities were investigated in rats fed the diets containing graded levels of Thr and similar AA composition to that for laying hens in the previous report²¹⁾.

Materials and Methods

Animals

Male Wistar/ST rats weighing 280 to 290 g at 9 weeks of age were purchased from a commercial farm (Japan SLC, Inc., Shizuoka), and housed in individual wire mesh cages in an environmentally controlled room ($22 \pm 2^\circ\text{C}$ and 12 h light from 07:00 to 19:00). They were fed an experimental diet containing Thr (0.55%) for 5 days to adapt to the experimental diet. And then, rats with average body weight, 304.5 ± 5.2 g, were divided into five experimental dietary groups with 6 rats each, and given free access to the experimental diets and water for 7 days. Feed intake and body weight were recorded every day during the

experimental period.

Diets

The basal diet consisted mainly of corn and wheat bran, and contained 2.92 kcal (12.2 kJ) ME/g and 12.7% crude protein (CP) as shown in Table 1. This dietary composition was the same as that of the basal diet used for laying hens in the previous report²¹⁾, except for 9.2% of calcium carbonate which was essential for eggshell formation in the layer diet was replaced by agar on an equal weight basis. Dietary AA concentrations were calculated from the analytical data of used feedstuffs except for methionine, cystine and tryptophan, and the concentrations of them were calculated from the table in NRC (1994)¹³⁾. To meet 100% of AA of NRC (1994)¹³⁾ requirements for laying hens, seven essential AA were supplemented. Five diets with graded levels of Thr from 0.32 to 0.67% were prepared by balancing the same amount of glutamic acid. The ratio of essential AA to non-essential AA in the added AA mixture was adjusted to be about 2:1. As shown in Table 2, dietary levels of all AA were higher than those for growth and reproduction of rats by NRC (1978)¹²⁾. The difference of ME and CP caused by balancing Thr by glutamic acid was calculated to be maximumly 0.09 kcal ME/g and 0.05% CP of the diet, respectively.

Table 1. Composition of basal diet
(Unit: %)

Corn grain	74.4
Corn gluten meal (62.0% CP)	3.5
Wheat bran	7.0
Soybean oil	1.0
Choline chloride	0.1
Calcium phosphate tribasic	1.2
Sodium chloride	0.3
Vitamin mineral premix ¹⁾	0.3
Amino acid mixture ²⁾	1.9
Glutamic acid	1.1
Agar	9.2

¹⁾ See Yamamoto and Ishibashi¹⁰⁾.

²⁾ See Table 2.

Table 2. Amino acid composition of amino acid mixture, experimental diet and NRC requirements for rats (1978)⁽¹²⁾

(Unit : %)			
Amino acid	Amino acid mixture	Experimental diet	NRC requirement
Threonine	0 to 0.35	0.32 to 0.67	0.4
Arginine	0.29	0.70	0.3
Histidine	—	0.24	0.2
Isoleucine	0.31	0.65	0.4
Leucine	—	1.16	0.7
Lysine	0.42	0.69	0.4
Methionine	0.19	0.30	0.5
Phenylalanine	0.03	0.47	0.4
Tryptophan	0.06	0.15	0.1
Valine	0.25	0.70	0.5
Glutamic acid	1.10 to 0.75	1.55 to 1.20	—

Tissue preparation

On the 7th day after changing to five graded experimental diets, rats were weighed and killed by decapitation after taking about 0.4 ml of blood samples from the *Vena caudalis* by vein puncture with heparinized syringes. The liver, kidney and duodenum were weighed, and quickly sliced in ice-cold 0.14 M KCl solution containing 0.25% Triton X-100 at 4°C. The tissues were homogenized with a polytron sonicator (Kinematica, Switzerland), and diluted with 5 volumes of ice-cold 0.14 M KCl solution. The tissue homogenates were immediately used for assay as described as follows.

Tissue free AA concentration

A portion of tissue homogenates was deproteinized immediately by addition of 5 fold amount of 3.0% sulfosalicylic acid, and the mixture was stood for at least 30 min at 4°C. The precipitated fraction was removed by centrifugation at $1,800 \times g$ for 15 min, and the supernatant was stored at -25°C until analysis of AA. Approximately 10 μ l of samples were applied to a high performance liquid chromatograph (Shimadzu, LC-10A, Kyoto) equipped with a Shimadzu lithium type ion exchange column at 39°C for the determination of AA according to Fujimura *et al.*⁽⁵⁾.

Enzyme assay of Thr, Gly and Ser conversion

The assay system reported by Davis and Austic⁽²⁾ was modified. The concentrations of each substrate, Thr, Gly or Ser, and each tissue homogenate, pH and reaction time were determined in the same conditions as reported in the previous report used laying hens⁽²¹⁾. The reaction mixture of 1.0 ml of 0.1 M phosphate buffer solution (pH 7.5) and 1.0 ml of homogenate was preincubated for 3 min at 37°C, and then 1.0 ml of each substrate in 0.1 M phosphate buffer solution was added. The final concentrations of substrates were 1.0 μ mol/ml for all tissue homogenates. After 30 min, the reaction was stopped by addition of 1.0 ml of 9.0% sulfosalicylic acid solution, and the mixture was stood for at least 30 min at 4°C. The precipitated fractions were separated by centrifugation, and the supernatants were stored at -25°C until analysis. Amino acid analyses were performed in the same manner as above mentioned. Enzyme activities were expressed as μ mol of Gly or Ser formed per 30 min per gram tissue.

Statistical analysis

Statistical significance was estimated by analysis of variance and Duncan's new multiple range test using the General Linear Model

procedure in SAS^{®17)}. Statements of significance were based on $P < 0.05$ unless otherwise stated.

Results

Body and tissue weights, and feed intake

Table 3 shows the average feed intake, body weight change, and weights of liver, kidney and duodenum of rats fed the diets containing graded levels of Thr. The feed intake ranged from 22.2 to 23.4 g, but no differences were found among them. Body weight changes and tissue weights expressed as grams and percentages of body weight were not affected by changes of dietary Thr levels.

Tissue AA concentrations

When the dietary Thr increased, PC of Thr increased from 53.2 to 450.6 nmol/ml and that of Ser from 101.0 to 149.7 nmol/ml, but that of Gly remained constant (Fig. 1). Tissue concentrations (TC) of AA of rats fed the diets with graded levels of Thr are shown in Table 4. Tissue concentrations of Gly and Ser in the liver, kidney and duodenum did not respond to changes of dietary Thr levels. Tissue concentrations of Thr in all tissues remained constant below 0.42% dietary Thr levels, and then increased linearly with increasing dietary Thr levels. Tissue concentrations of Thr, Gly and Ser in the kidney were higher than those in the duodenum, and the latter were higher than those in the liver. Especially, TC of Gly in the

kidney (7.14 to 7.40) were higher than those in the duodenum (2.76 to 3.18) and liver (2.54 to 3.09 $\mu\text{mol/g}$ tissue). These differences were not narrowed by recalculating as $\mu\text{mol/ml}$ of water in tissue.

Enzyme activities

Enzyme activities expressed as μmol of products per gram tissue per 30 min in the liver, kidney and duodenum are summarized in Table 5. The response of enzymes to changes of dietary Thr levels were different among tissues. All enzyme activities in the kidney

Fig. 1. Plasma concentrations of threonine, glycine and serine of adult rats fed diets with five graded levels of threonine for 7 days. Each value is means for 6 rats. $\square$; Thr, $\diamond$; Gly, $\bullet$; Ser.

Table 3. Feed intake, body weight change and tissue weight of adult rats fed diets with five graded levels of threonine for 7 days

Dietary threonine (%)	0.32	0.37	0.42	0.55	0.67	Pooled SE
Feed intake (g/rat/day)	22.2	23.4	22.7	22.3	22.5	0.6
Body weight change (g/day)	3.45	3.37	3.64	3.48	3.52	0.17
Weight of tissues (g)						
Liver	10.3(3.1)	10.1(3.0)	10.0(3.1)	10.0(3.1)	10.0(3.0)	0.3
Kidney	1.86(0.55)	1.81(0.53)	1.82(0.53)	1.93(0.56)	1.82(0.54)	0.04
Duodenum	0.79(0.20)	0.79(0.20)	0.87(0.22)	0.81(0.21)	0.83(0.21)	0.03

Values are means for 6 rats.

Values in parentheses are percentage to body weight.

Table 4. Concentrations of threonine, glycine and serine in liver, kidney and duodenum of adult rats fed diets with five graded levels of threonine for 7 days

(Unit : $\mu\text{mol/g}$ tissue)

Tissue	Amino acid	Dietary threonine (%)					Pooled SE
		0.32	0.37	0.42	0.55	0.67	
Liver	Thr	0.50 ^c	0.64 ^c	0.73 ^c	1.06 ^b	1.44 ^a	0.04
		(0.50 ^c)	(0.63 ^c)	(0.60 ^c)	(1.06 ^b)	(1.33 ^a)	
	Gly	2.54	2.90	2.81	2.69	3.09	0.10
		(2.51)	(2.61)	(2.34)	(2.42)	(2.36)	
	Ser	1.11	1.14	1.21	1.22	1.31	0.07
		(5.49 ^a)	(5.24 ^a)	(5.55 ^a)	(4.56 ^b)	(4.08 ^c)	
Kidney	Thr	1.41 ^c	1.40 ^c	1.46 ^c	1.84 ^b	2.02 ^a	0.06
		(0.50 ^c)	(0.55 ^c)	(0.54 ^c)	(0.94 ^b)	(1.45 ^a)	
	Gly	7.25	7.14	7.23	7.40	7.20	0.12
		(7.07)	(7.64)	(7.48)	(7.57)	(7.60)	
	Ser	3.13	2.89	2.88	2.81	2.84	0.09
		(3.91 ^a)	(3.94 ^a)	(4.02 ^a)	(3.56 ^{ab})	(3.02 ^b)	
Duodenum	Thr	1.11 ^c	1.18 ^c	1.11 ^c	1.62 ^b	1.95 ^a	0.07
		(0.90 ^b)	(1.02 ^b)	(1.01 ^b)	(1.32 ^{ab})	(1.71 ^a)	
	Gly	2.98	2.94	2.76	2.93	3.18	0.13
		(4.48)	(4.67)	(5.00)	(5.00)	(5.09)	
	Ser	2.23	2.39	2.14	2.25	2.34	0.11
		(3.31 ^{ab})	(3.53 ^{ab})	(3.88 ^a)	(3.35 ^{ab})	(2.96 ^b)	

Values are means for 6 rats.

Values in parentheses are means for 6 laying hens in the previous report²¹.^{a-c} Means in the same row with different superscripts differ significantly ($P < 0.05$).

tended to increase at the dietary Thr levels from 0.32 to 0.37 or 0.42% and then decrease with increasing dietary Thr levels. Adversely those in the duodenum tended to decrease in the dietary Thr levels from 0.32 to 0.42%, and then increase with increasing dietary Thr levels, but the differences observed were not significant. In the liver, the enzyme activities converting Thr to Ser, and Gly to Ser remained constant, but those converting Thr to Gly decreased at the dietary Thr levels from 0.32 to 0.42%, and then increased with increasing dietary Thr levels as those in the kidney. Those converting Ser to Gly decreased linearly from 1.98 at 0.32% dietary Thr level to 1.32 $\mu\text{mol/g}$ tissue/30 min at 0.67% dietary Thr level.

Discussion

Although the body weight gain in laying hens increased curvilinearly with increasing dietary Thr levels²¹, there were no differences in those of rats among the five graded Thr groups (Table 3). Accordingly, no differences of tissue weights were observed in rats fed the diets with different Thr levels. These results indicated that these parameters were unavailable for estimation of Thr requirements of adult rats.

Threonine requirements of growing rats have been reported to be 0.42% from data of PC of Thr¹¹, 0.43% from data of growth assay and protein efficiency ratio¹⁵, and 0.50% from data of growth assay⁹. However, recently, there are no reports on the determination of Thr

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Table 5. Glycine and serine formed from three substrates, threonine, glycine and serine in liver, kidney and duodenum of adult rats fed diets with five graded levels of threonine for 7 days
(Unit : $\mu\text{mol/g}$ tissue/30 min)

Tissue	Substrate	Product	Dietary threonine (%)					Pooled SE
			0.32	0.37	0.42	0.55	0.67	
Liver	Thr	Gly	1.61 ^a (1.56)	1.44 ^{ab} (1.51)	1.27 ^b (1.43)	1.37 ^{ab} (1.42)	1.60 ^a (1.55)	0.06
	Thr	Ser	0.80 (1.12)	0.88 (1.00)	0.79 (0.99)	0.74 (1.12)	0.83 (1.05)	0.04
	Gly	Ser	0.97 (1.01)	0.97 (1.12)	0.90 (1.08)	0.85 (1.04)	0.96 (1.11)	0.04
	Ser	Gly	1.98 ^a (1.50)	1.85 ^a (1.52)	1.49 ^{ab} (1.48)	1.35 ^b (1.65)	1.32 ^b (1.76)	0.07
Kidney	Thr	Gly	3.25 (0.96 ^b)	3.67 (0.86 ^b)	3.74 (0.76 ^b)	3.55 (1.20 ^a)	3.09 (1.38 ^a)	0.12
	Thr	Ser	3.14 (1.39)	3.36 (1.40)	3.40 (1.37)	3.43 (1.46)	2.96 (1.50)	0.14
	Gly	Ser	3.40 (1.41)	3.57 (1.36)	3.30 (1.41)	3.17 (1.40)	2.80 (1.43)	0.12
	Ser	Gly	3.27 (1.26)	3.90 (0.93)	3.66 (0.86)	3.59 (1.10)	3.52 (1.26)	0.15
Duodenum	Thr	Gly	7.00 (3.49 ^b)	6.36 (3.58 ^b)	5.70 (3.59 ^b)	6.10 (4.56 ^a)	6.85 (4.96 ^a)	0.31
	Thr	Ser	7.11 (2.61 ^b)	6.43 (2.57 ^b)	5.13 (2.89 ^b)	5.95 (3.28 ^{ab})	6.92 (3.74 ^a)	0.28
	Gly	Ser	6.97 (2.83)	6.79 (2.90)	5.08 (2.88)	5.76 (2.97)	6.93 (3.24)	0.22
	Ser	Gly	6.81 (3.29 ^b)	6.31 (3.79 ^{ab})	6.00 (3.29 ^b)	6.15 (4.31 ^a)	6.44 (4.54 ^a)	0.30

Values are means for 6 rats.

Values in parentheses are means for 6 laying hens in the previous report²¹.

^{ab} Means in the same row with different superscripts differ significantly ($P < 0.05$).

requirement for adult rats, although Thr requirement for maintenance of rats by NRC (1978) was 0.18%¹². Therefore, the dietary Thr levels used in this study might range from deficiency to excess from the above cited reports^{9,11,12,15}. In laying hens, the Thr requirements determined from data of performance agreed well with those determined from data of plasma Thr concentration, and the latter agreed well with those determined from data of TC of Thr²¹. From the data of TC of liver, kidney and duodenum of young adult rats, the break points were estimated to be 0.42,

0.41 and 0.42%, respectively (Fig. 2).

In Tables 4 and 5, the values obtained in laying hens are presented in parentheses. There were no species differences in TC of Thr and Gly in the liver, those of Gly in the kidney, and those of Thr in the duodenum. However, TC of Thr in the kidney were higher in rats, and TC of Gly in the duodenum were higher in laying hens, and TC of Ser of rats were lower than those of laying hens in all tissues. These species differences might be caused by the difference of metabolic pathway for Thr degradation responding to dietary excess Thr. The

Fig. 2. Break points estimated from threonine concentrations in liver, kidney and duodenum of adult rats fed diets with five graded levels of threonine for 7 days.

Each value is means for 6 rats. □; liver, ◆; kidney, ○; duodenum.

constancy of TC of Gly in tissues might be related in a way that the metabolic aspect of Gly is particularly complex involving conversion to Ser or other AA and synthesis of molecules such as purines and creatine, although its structure is the simplest one among naturally occurring AA⁸⁾.

The reported Thr metabolism is summarized as follows. The conversion of Thr by T-AL (Thr to Gly) is recognized to be the most important in the Thr catabolism in mammals, but the major pathway for the degradation of Thr is due to the action of T-DHT (Thr to α -ketobutyric acid) in the rat liver, and can be induced by a high protein diet^{4,6,9,14)}. T-AL should not be a genuine enzyme in the liver, and the apparent enzyme activity may result from a combined effect of T-DHT and lactate dehydrogenase (or oxo acid-linked NADPH dehydrogenase) in the liver cytosolic extract²⁰⁾. T-AL and S-HMTF, T-DHT and Ser-dehydratase (S-DHT, EC 4.2.1.3), appear to be a single protein, respectively^{17,18)}. T-AL activity varies independently from T-DHT

activity¹⁾. There are various studies for Thr-degrading enzymes and the relating enzymes as S-HMTF and S-DHT of the rat liver. Kang-Lee and Harper⁹⁾ determined S-DHT activity with Ser as a substrate in the liver of rat fed dietary Thr levels from 0.15 to 0.85%, and showed that S-DHT activity tended to decrease with increasing dietary Thr levels. These results agreed with those in the present study.

The enzyme activities in the kidney and duodenum of rats were far higher than those of laying hens. The responses of four enzyme activities in the duodenum and three enzyme activities in the liver agreed well with each other. However, the activities of all enzymes in the kidney differed. The enzyme activities in rats increased and then decreased, while those of laying hens decreased and then increased. In the liver, the activity of S-HMTF converting Ser to Gly decreased in rats, but increased in laying hens. Although the significance of the species differences was unclear, the difference in S-HMTF activities between rats and laying hens might be one of the reasons why PC of Ser of laying hens decreased and that of rats increased when the dietary Thr was higher than the requirement.

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ラットのトレオニン分解酵素活性とトレオニン関連の遊離アミノ酸濃度に対する飼料中のトレオニン含量の影響

渡邊令子・藤村 忍*・門脇基二*・石橋 晃*

県立新潟女子短期大学, 新潟市 950-0806

* 新潟大学農学部, 新潟市 950-2102

トレオニン (Thr) を過剰に添加した飼料を給与したときに, ラットの血漿中の遊離グリシン (Gly), セリン (Ser) 濃度の応答を鶏の場合と比較するため, 雄の Wistar/ST ラット (304.5±5.2 g) に飼料中 Thr 濃度が 0.32~0.67% までの 5 種類の試験飼料を給与した. 試験飼料の組成は産卵鶏の場合の基礎飼料の組成と同一とした. 肝臓, 腎臓および十二指腸の各組織中の Thr, Gly および Ser 濃度は $\mu\text{mol/g}$, また, Thr から Gly への転換, および Gly と Ser の相互転換に関係する酵素活性は, 各組織 1 g 当たり 30 分間の反応で生成される各アミノ酸の $\mu\text{mol/g}$ として測定した. 肝臓の Thr 濃度は, 飼料中 Thr が 0.32~0.42% までは徐々に増加し, その後飼料中 Thr 濃度が高くなるに従って直線的に増加した. 腎臓と十二指腸の Thr 濃度もまた飼料中 Thr 濃度が 0.67% のとき, 0.42% 以下のときに比較し各々 45, 75% 増加した. 3 組織中 Gly 濃度は, 飼料中 Thr 濃度に関わらず一定であったが, 腎臓中 Gly 濃度は他の 2 組織の Gly 濃度に比べ 2 倍以上高かった. 肝臓中 Ser 濃度は, 飼料中 Thr 濃度の増加に対応してわずかに増加する傾向がみられたが, 腎臓や十二指腸の Ser 濃度は飼料中 Thr 濃度に影響されなかった. Thr 分解酵素活性は, 飼料中 Thr 濃度が 0.32~0.42% のとき, 肝臓の Thr-aldolase 活性がわずかに減少し, その後, 飼料中 Thr 濃度が高くなるに従って増加した. 肝臓の Ser-hydroxymethyltransferase 以外の他の酵素活性は飼料中 Thr 濃度に影響されなかった. 肝臓の Ser を Gly に転換する Ser-hydroxymethyltransferase だけが, 産卵鶏の場合とは逆に飼料中の Thr 濃度が高くなるに従って減少した. ラットの肝臓におけるこのような代謝調節は, Thr 過剰飼料に対するラットとニワトリとの組織, および血漿中 Ser 濃度の応答の差の理由の一因であることが示唆された.

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