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Influence of high dietary threonine on growth and amino acids in blood and tissues of rats

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Summary. Diets containing 8 or 15% protein from casein plus limiting amino acids, 25% fat and adequate levels of other nutrients for rat growth were supplemented with 0, 0.5, 1.0, 2.0 or 4% of excess L-threonine. Addition of up to 1% excess threonine had little effect on weight gains or food intakes of weanling rats, but addition of 2 and 4% threonine caused a drastic reduction in weight gains or food intakes (up to 41%); the adverse effect being more severe in rats fed lower protein diets. Addition of graded levels of excess threonine resulted in (5 to 47-fold and 4 to 20-fold) increase in concentration of free threonine in rat plasma and brain, respectively. Addition of excess threonine also caused up to 5-fold increase in plasma level of 3-methylhistidine, suggesting increased muscle protein breakdown.

Keywords: Amino acids – Excess dietary threonine – Rat growth – Tissue amino acids

Introduction

Feeding of excess amounts of indispensable amino acids (IAA) individually in a low protein diet results in depression of food intake and growth of rats (Peng et al., 1973). The relative toxicities of amino acids have been compared by measuring growth response of weanling rats fed 5% of individual amino acid in a low protein diet containing 6% casein. Based on growth depression, threonine was found to be one of the least toxic IAA (Peng et al., 1973). In a 2-wk study, addition of 5% DL-threonine (which is about 4 times the requirement) to a casein-corn diet (about 10% protein) resulted in 27% growth depression of rats but a 14-fold increase in plasma threonine (Sauberlich, 1961). Similarly, the addition of 5% DL-threonine to a 6% casein diet resulted in about a 7-fold increase in plasma threonine and about a 2-fold increase in brain threonine (Peng et al., 1973). Addition of 5% of L-threonine to a 10% casein diet caused a 27% reduction in

food intake and a growth depression of 37% over a period of 3-wk in rats (Muramatsu et al., 1971).

Plasma threonine concentrations have also been observed to increase with increasing threonine intake in human infants, especially premature infants fed formulas containing various amounts of threonine (Kashyap et al., 1987; Rigo and Senterre, 1980). Plasma and urinary threonine concentrations were higher in term infants fed whey-predominant formulas than in those fed casein-predominant formulas or human milk (Järvenpää et al., 1982). Whey-predominant formulas have been reported to contain higher levels of threonine than casein-predominant formulas and human milk (980, 720 and 470 mg/L, respectively) (Rassin et al., 1977, 1988). Plasma concentrations of threonine were about twice as high in premature infants fed whey-predominant formulas than in those fed casein-predominant formulas (Kashyap et al., 1987). Similarly, plasma concentrations of threonine were more than doubled in term infants fed a whey hydrolysate formula compared to those fed human milk (Rigo et al., 1989). High plasma threonine in infants fed the high threonine formulas are of potential concern because cerebrospinal fluid levels increase with increasing plasma concentrations and because of the extensive neurologic development occurring during early infancy (Growdon et al., 1991).

A recent review on the safety of amino acids including threonine as dietary supplements recommended testing in both animals and humans (FASEB, 1992). Since no effort was made to ensure adequacy of control diets in all IAA for rat growth in previous studies (Muramatsu et al., 1971; Peng et al., 1973; Sauberlich, 1961), the relative adverse effects of high dietary threonine (and other amino acids) on rat growth and tissue amino acids may have been underestimated. The purpose of this study was to obtain more information on the potential adverse effects of feeding graded levels (0.5, 1.0, 2.0 and 4.0%) of excess L-threonine in diets otherwise adequate in all nutrients. The influence of high dietary threonine on rat growth, levels of threonine in blood, liver and brain, and the activity of hepatic threonine dehydrogenase was studied.

Materials and methods

Two basal diets (i.e. basal A and basal B) containing 8 and 15% protein (N × 6.25, respectively) from casein were formulated (Table 1). In the formulation of the basal diets, every effort was made to simulate the composition of infant formulas. Both the basal diets contained 25% fat (a 50 : 50 blend of soybean oil : coconut oil) and adequate levels of vitamins and minerals for rat growth. Both the basal diets were supplemented with deficient amino acids to meet all the amino acid requirements for rat growth. Levels of supplemental sulfur amino acids were adjusted to achieve a cysteine/methionine ratio of 0.8, to simulate the ratio in whey-predominant infant formulas. Each basal diet was supplemented with graded levels (0.5, 1.0, 2.0 and 4.0%) of L-threonine. Within each basal, diets were made isonitrogenous by the addition of L-glutamic acid. All the diets were isocaloric and (by calculation) contained 523 kcal/100 g diet; supplying 8–14% calories from protein, 48% calories from fat, and 39–44% calories from carbohydrates. Most milk-based infant formulas contain similar amounts of total calories (about 520 kcal/100 g dry matter), and of calories from protein (10–12%), from fat (48–50%), and from carbohydrates (about 40%).

The ten experimental diets (the two basal and the eight threonine supplemented diets) were fed to male weanling (50 ± 3 g) Sprague-Dawley rats for a period of 2 weeks preceded

Table 1. Composition of experimental diets

Ingredient	Basal A[1]	Basal B[1]
	g/kg diet	
Casein[2]	88.96	166.80
Soybean oil[3]	125.00	125.00
Coconut oil[4]	125.00	125.00
AIN-76 Mineral mixture[5]	44.10	44.10
AIN-76A Vitamin mixture[6]	12.60	12.60
Choline bitartarate[4]	2.00	2.00
Cellulose[7]	50.00	50.00
Amino acid mixture[8,9]	30.36	13.10
L-glutamic acid	49.41	49.40
Cornstarch[10]	472.57	412.00

[1] Each basal diet was supplemented with four graded levels of L-threonine (0.5, 1.0, 2.0 and 4.0%). On analysis, the basal A- and the basal B-based diets contained 12.91–13.62 and 18.04–18.42% protein (N × 6.25), respectively. [2] Animal Nutrition Research Council Reference protein, ICN, St. Laurent, Quebec, Canada. [3] ICN, St. Laurent, Quebec, Canada. [4] Sigma Chemical CO, St. Louis, MO. [5] American Institute of Nutrition (1977). [6] American Institute of Nutrition (1980). [7] Alphacel, Teklad Test Diets, Madison, WI. [8] For Basal A, amino acid mixture provided the following L-amino acids (g/kg diet): Arg, 5.10; His, 0.68; Ile, 2.42; Leu, 1.99; Lys·HCl, 3.39; Met, 2.10; Cysteine·HCl·H_2O, 4.62; Phe, 1.55; Thr, 3.05; Trp, 2.00; Val, 2.62. All amino acids were purchased from Sigma Chemical Company. [9] For Basal B, amino acid mixture provided the following L-amino acids (g/kg diet): Arg, 2.00; Met, 2.25; Cysteine·HCl·H_2O, 6.85; Thr, 0.50; Trp, 0.50. [10] Canada Starch Co., Toronto, Ontario, Canada.

by an adaptation period of 2 days. Each diet was fed to 10 individually caged rats. The Health and Welfare Canada's guide for the care and the use of laboratory animals was followed and the protocol was approved by the Animal Care Committee of the Department.

Rats were housed in stainless steel screen-bottom cages (permitting free droppings of excreta) in a temperature (24–25°C) and humidity (40–50%) controlled facility with 12-h light-dark cycle. Highly absorbent paper was placed under the cages to catch spilled food and to minimize contamination of feces with urine. Rats were given free access to food and water for two weeks and weekly weight grains and food consumption were recorded.

After 2 weeks of testing, animals were exsanguinated after anesthesia with 3% halothane in oxygen for the collection of blood, liver and brain samples.

Blood was drawn from the abdominal aorta at the illiosacral junction, and collected in heparinized tubes. The plasma samples were obtained by centrifuging (model ICE CENTRA-7R, 831a rotor, International Equipment Co, Needham, MA) blood at 3000 × g for 15 min.

The activity of threonine dehydrogenase (EC 1.1.1.103) in fresh livers of rats was determined by the method of Bird and Nunn (1983) by using a Varian 100 UV visible spectrophotometer.

The liver and brain samples were freeze-dried before amino acid analysis. The plasma and the dried liver and brain samples were deproteinized with acetonitrile and analyzed for free amino acid concentrations by using precolumn phenylisothiocyanate derivatization and liquid chromatography (Sarwar and Botting, 1990).

Data for weight gain, food intake, liver weight, brain weight, plasma amino acids, liver amino acids, brain amino acids, and liver threonine dehydrogenase activity were analyzed by one way ANOVA and Tukey's HSD test using the Statistical System for personal computers (SAS, 1985).

Results

Dietary treatments had significant ($P < 0.05$) effects on weight gain, food intake, liver weight and brain weight (Table 2). In the case of basal diet A, the addition of 2.0 and 4.0% threonine caused about 24 and 41% reduction in weight gains or food intakes, respectively (Table 2). Addition of the lower levels (0.5 and 1.0%) of threonine had no effects on these parameters. In the case of basal diet B, only the addition of 4% threonine caused significant reductions in weight gains (19%) and food intakes (29%). Addition of threonine had no effect on gain/food ratios of basal A diets but tended to exert a beneficial effect on the ratios of basal B diets (Table 2). Within each basal group, the addition of threonine had no significant effect on liver weight expressed as g/2-wk or g/100 g body weight (Table 2). Addition of threonine tended to reduce brain weights (g/2-wk), especially in the case of basal A diets (Table 2). However, when expressed as g/100 g body weight, the brain weights tended to increase with increasing levels of supplemental threonine.

The experimental diets had significant effects on levels of threonine in plasma, liver and brain (Table 3). In the case of both the basal diets, the addition of increasing levels of threonine caused marked elevations in plasma threonine (Table 3). The increases in plasma threonine ranged from 5-to 40-fold in the case

Table 2. Growth and organ weights of rats fed the experimental diets

Diet	Weight gain[1] g/2-wk	Food intake[1] g/2-wk	Gain/food ratio	Liver weight[1] g/2-wk	Liver weight[1] g/100 g	Brain weight[1] g/2-wk	Brain weight[1] g/100 g
Basal A	$90^{a,b}$	$140^{a,b}$	0.64	$6.07^{a,b}$	$4.28^{a,b}$	1.68^{a}	$1.19^{c,d}$
plus 0.5% Thr	$88^{b,c}$	$136^{a,b}$	0.65	$6.00^{a,b}$	$4.55^{a,b}$	$1.65^{a,b}$	1.16^{d}
plus 1.0% Thr	$84^{b,c}$	$122^{b,c}$	0.69	6.45^{a}	$4.78^{a,b}$	$1.63^{a,b}$	$1.21^{c,d}$
plus 2.0% Thr	69^{d}	$106^{c,d}$	0.65	$5.79^{a,b}$	4.84^{a}	$1.64^{a,b}$	1.37^{b}
plus 4.0% Thr	53^{e}	83^{e}	0.64	5.03^{b}	4.86^{a}	1.56^{b}	1.52^{a}
Basal B	$95^{a,b}$	$136^{a,b}$	0.70	6.57^{a}	4.23^{b}	1.67^{a}	1.14^{d}
plus 0.5% Thr	$96^{a,b}$	$138^{a,b}$	0.69	6.60^{a}	$4.45^{a,b}$	1.66^{a}	1.11^{d}
plus 1.0% Thr	$96^{a,b}$	$132^{a,b}$	0.73	6.55^{a}	$4.47^{a,b}$	1.67^{a}	1.15^{d}
plus 2.0% Thr	$92^{a,b}$	$123^{b,c}$	0.75	6.89^{a}	$4.83^{a,b}$	1.67^{a}	$1.18^{c,d}$
plus 4.0% Thr	$77^{c,d}$	$97^{d,e}$	0.79	$5.89^{a,b}$	$4.61^{a,b}$	$1.64^{a,b}$	$1.29^{b,c}$
Pooled SEM	9	14	—	0.83	0.41	0.06	0.08

[1] Values are means (n = 10). Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

Table 3. Levels of free threonine in plasma, liver and brain of rats fed the experimental diets[1]

Diet	Plasma Thr μmol/100 mL	Liver Thr nmol/mg dried	Brain Thr nmol/mg dried
Basal A	48[g]	3[g]	4[f]
plus 0.5% Thr	407[e]	12[f]	22[d]
plus 1.0% Thr	774[d]	21[e]	37[c]
plus 2.0% Thr	1311[b]	48[c]	59[b]
plus 4.0% Thr	1508[a]	72[a]	65[a]
Basal B	32[g]	1[g]	3[f]
plus 0.5% Thr	158[f]	5[g]	13[e]
plus 1.0% Thr	392[e]	11[f]	22[d]
plus 2.0% Thr	867[c]	30[d]	39[c]
plus 4.0% Thr	1274[b]	55[b]	61[b]
Pooled SEM	21	1	1

[1] Values are means (n = 10). Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

Table 4. Levels of some free IAA in plasma of rats fed the experimental diets[1]

Diet	Arg	His	Leu	Lys	Met	Trp
	μmol/100 mL					
Basal A	18[a]	4[d,e]	6[c]	48[a,b]	6[c]	8[c,d]
plus 0.5% Thr	15[a,b]	4[d,e]	5[c]	38[d,e]	6[c]	9[c,d]
plus 1.0% Thr	14[a,b]	3[e]	5[c]	36[e]	6[c]	9[c,d]
plus 2.0% Thr	14[a,b]	5[d]	5[c]	36[e]	6[c]	13[b]
plus 4.0% Thr	8[c]	10[a,b]	5[c]	38[d,e]	7[b,c]	15[a]
Basal B	13[a,b]	9[b,c]	11[a]	52[a]	7[b,c]	7[d]
plus 0.5% Thr	15[a,b]	8[c]	10[a]	52[a]	7[b,c]	8[c,d]
plus 1.0% Thr	15[a,b]	9[b,c]	10[a]	46[b,c]	8[a,b]	9[c,d]
plus 2.0% Thr	16[a,b]	9[b,c]	9[a,b]	40[d,e]	8[a,b]	9[c,d]
plus 4.0% Thr	11[b,c]	10[a,b]	8[b]	44[c,d]	9[a]	9[c,d]
Pooled SEM	2	1	2	3	1	1

[1] Values are means (n = 10). Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

of basal A, and from 10- to 47-fold in the case of basal B. The addition of increasing levels of excess threonine also resulted in marked elevations in the levels of liver threonine (up to 55-fold) and brain threonine (up to 20-fold) (Table 3).

The dietary treatments had no significant effects on the plasma levels (μmol/100 mL, mean ± pooled SEM) of isoleucine (8–10 ± 2), phenylalanine (5–6 ± 2) and valine (16–19 ± 3). In general, the levels of plasma histidine, leucine and methionine were higher in rats fed basal B containing diets compared to the levels in those fed basal A containing diets (Table 4). Within each basal

group, the levels of plasma arginine and lysine tended to decrease while the levels of histidine and tryptophan tended to rise with increasing levels of added dietary threonine.

Dietary treatments had no significant effects on the plasma levels (μmol/100 mL, mean $\pm$ pooled SEM) of aspartic acid (3–4 $\pm$ 1), glutamic acid (16–19 $\pm$ 2), tyrosine (8–11 $\pm$ 2) and ornithine (11–16 $\pm$ 3). Within each basal group, the plasma levels of asparagine, serine, glutamine, glycine, alanine, 3-methylhistidine, proline and α-aminobutyric acid tended to rise with increments of added dietary threonine (Table 5).

Table 5. Levels of some free dispensable amino acids in plasma of rats fed the experimental diets[1]

Diet	Asn	Ser	Gln	Gly	Ala	$3CH_3 \cdot$ His	Pro	α-ABA
	μmol/100 mL							
Basal A	7^{d}	39^{f}	131^{d}	30^{e}	90^{b}	2^{d}	32^{c}	2^{d}
plus 0.5% Thr	$8^{c,d}$	$51^{d,e}$	$136^{c,d}$	$31^{d,e}$	94^{b}	7^{b}	32^{c}	$3^{c,d}$
plus 1.0% Thr	$8^{c,d}$	$54^{c,d}$	$153^{a,b}$	37^{d}	92^{b}	7^{b}	32^{c}	$4^{b,c}$
plus 2.0% Thr	10^{b}	79^{b}	170^{a}	66^{b}	94^{b}	11^{a}	$41^{a,b}$	5^{b}
plus 4.0% Thr	10^{b}	92^{a}	176^{a}	94^{a}	116^{a}	11^{a}	46^{a}	6^{b}
Basal	$8^{c,d}$	34^{f}	121^{d}	30^{e}	$75^{b,c}$	2^{d}	$35^{b,c}$	2^{d}
plus 0.5% Thr	7^{d}	34^{f}	120^{d}	30^{e}	73^{c}	4^{c}	33^{c}	2^{d}
plus 1.0% Thr	$9^{b,c}$	48^{e}	129^{d}	38^{d}	$77^{b,c}$	8^{b}	$37^{b,c}$	$3^{c,d}$
plus 2.0% Thr	10^{b}	57^{c}	$148^{b,c}$	49^{c}	$80^{b,c}$	10^{a}	$42^{a,b}$	5^{b}
plus 4.0% Thr	12^{a}	78^{b}	$166^{a,b}$	86^{a}	$83^{b,c}$	10^{a}	45^{a}	8^{a}
Pooled SEM	1	2	7	4	9	1	2	1

[1] Values are means (n = 10). Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

Table 6. Activity of threonine dehydrogenase in livers of rats fed the experimental diets

Diet	Enzyme activity[1] μmol/min per g of liver
Basal A	0.60
plus 0.5% Thr	0.57
plus 1.0% Thr	0.56
plus 2.0% Thr	0.59
plus 4.0% Thr	0.58
Basal B	0.58
plus 0.5% Thr	0.64
plus 1.0% Thr	0.58
plus 2.0% Thr	0.59
plus 4.0% Thr	0.64
Pooled SEM	0.05

[1] means (n = 10).

Dietary treatments had no significant effects on the levels of free amino acids (other than threonine) in liver and brain (data not shown). Similarly, the experimental diets had no significant effect on the activity of hepatic threonine dehydrogenase (Table 6).

Discussion

The basal A and basal B diets were formulated to contain 8 and 15% protein from casein, respectively. The basal diets were supplemented with deficient IAA for rat growth (Table 1). Moreover, basal A or basal B diets were made isonitrogenous by the addition of glutamic acid necessitated by the addition of graded levels of threonine. Therefore, on analysis, the basal A and basal B diets were found to contain 12.91–13.62 and 18.04–18.42% protein (N × 6.25), respectively. Since both the basal diets were formulated to meet all the nutrient requirements (including IAA) for rat growth, the weight gain, food intake, liver weight and brain weight of rats fed basal A and basal B diets were similar (Table 2).

The reduction in food intake and the resultant growth depression caused by the addition of high levels of excess threonine (2 and 4% in the case of basal A diet and 4% in the case of basal B diet) (Table 2) were more severe compared to those reported previously for comparable addition of excess threonine (Muramatsu et al., 1971; Sauberlich, 1961). Similarly, the effects of the additional excess threonine on elevations in plasma and brain threonine were more marked in the present study (Table 3) compared to those reported in previous studies (Peng et al., 1973; Sauberlich, 1961). This comparison would suggest that the adverse effects of excess threonine on rat growth, food intake and tissue threonine were underestimated in the literature reports. This may have been caused by the use of a low protein control diet, not meeting all the amino acid requirements of rats, in these studies (Muramatsu et al., 1971; Peng et al., 1973; Sauberlich, 1961). Addition of 0.5, 1.0, 2.0 or 4.0% of L-threonine to a diet containing 20% protein (corn-soybean meal) did not depress weight gain and food intake in weanling pigs (Edmonds and Baker, 1987). A comparison of our data concerning the adverse effects of excess threonine on rat growth and food consumption (Table 2) with that of Edmonds and Baker (1987) would suggest a species difference in tolerance to excess dietary threonine, with rats being more sensitive than pigs.

Plasma, liver or brain threonine was found to have significant ($P < 0.05$) positive correlations ($r = 0.80–0.82$) with dietary threonine intake (dietary threonine X food intake) in the present study. Liver or brain threonine was also highly correlated with plasma threonine ($r = 0.95–0.99$) in this study. Similar association between dietary threonine (%) or dietary threonine intake and plasma threonine was reported in pigs (Edmonds and Baker, 1987) and in low birthweight infants (Rigo and Senterre, 1980). Plasma and tissue (liver and/or muscle) threonine concentrations were also markedly influenced by dietary threonine in rats (Kang-Lee and Harper, 1978; Yamashita and Ashida, 1971). Yamashita and Ashida (1971) have demonstrated that the catabolic breakdown of [U-^{14}C]-threonine to CO_2 in rats fed a high threonine diet did not increase

as much as that of [U-^{14}C]-lysine in rats fed a high lysine diet. Relative changes in the levels of free threonine in blood and muscle in relation to threonine intake were reported to be greater than those of free lysine in these tissues in relation to lysine intake. A large intake of threonine resulted in greatly elevated tissue threonine concentrations. It was suggested that the degree of the metabolic breakdown of threonine was lower than that of lysine and that threonine may not be conserved as efficiently as other amino acids (Kang-Lee and Harper, 1978; Yamashita and Ashida, 1971). It was further speculated that a possible homeostatic mechanism exists in rats which maintains a constant body level of lysine and that a similar mechanism to maintain threonine levels is absent; consequently, tissue threonine levels fluctuate with dietary threonine intake (Yamashita and Ashida, 1971).

In mammals, three enzymes catalyzing the degradation of L-threonine are known, namely threonine dehydrogenase, threonine dehydratase (EC 4.2.1.16) and threonine aldolase (EC 4.1.2.5) (Bird and Nunn, 1983). The relative importance of each enzyme catalyzing the degradation of threonine has been studied in rat liver (Bird and Nunn, 1983). In normally-fed rats, the activities of hepatic threonine dehydrogenase, threonine dehydratase and threonine aldolase were reported to be 87, 10 and 3% of whole liver threonine catabolic activity, respectively. Similarly, in fed pigs, most of threonine oxidation (76–80%) occurred through threonine dehydrogenase (Ballevre et al., 1990). In gluconeogenic states, however, threonine dehydratase was reported to assume the dominant role in rat hepatic threonine catabolism (Bird and Nunn, 1983).

In the present study, the activity of liver threonine dehydrogenase was not affected by the addition of excess threonine in normally-fed rats (Table 6). Similar observations about the lack of induction of threonine dehydrogenase and/or threonine dehydratase activities by excess threonine intake have been previously made in chickens (Davis and Austic, 1980) and rats (Kang-Lee and Harper, 1978). The developmental pattern of hepatic threonine dehydratase has been investigated in growing rats (Grogan et al., 1988). Activity was found to be low during most of the postnatal period and peaked (3-fold increase) during the late suckling period (3rd postnatal week). The activity, however, decreased drastically by 30 days of age. Threonine dehydratase activity in the liver of adult female rats was slightly higher than the activity observed at 30 days of age. The adult value was, however, significantly lower than that at 20 days of age. Threonine dosing did not affect threonine dehydratase activity at any age (Grogan et al., 1988).

If a similar pattern of enzyme development and lack of induction of enzyme activity by excess threonine intake is present in the human infant, it may explain the increased threonine in plasma of infants fed high-threonine formulas (Rigo and Senterre, 1980). Regardless of the ability to metabolize threonine, higher plasma threonine concentrations of infants fed whey-predominant formulas compared to those fed human milk, reflect excessive threonine intake (Kashyap et al., 1987). The higher plasma threonine concentrations have not been generally associated with adverse clinical outcome. Some low birthweight infants fed a whey-predominant formula were, however, reported to have plasma threonine concentrations which were as high as that observed in a child with convulsions

and growth retardation associated with threoniemia (Kashyap et al., 1987; Reddi, 1978). Adequacy of threonine intake in low birthweight infants has not been properly addressed and deserves further investigation (Kashyap et al., 1987).

Addition of increasing levels of excess dietary threonine resulted in increased levels of several dispensable amino acids, especially glycine and serine (Table 5) due to the close relationship of the metabolism of L-threonine, L-serine and glycine. Similar elevations in the levels of plasma serine and glycine associated with excess dietary threonine were observed in weanling pigs (Edmonds and Baker, 1987). Addition of graded levels of excess threonine also resulted in increased levels of plasma 3-methylhistidine (Table 5), suggesting increased muscle protein breakdown. Since 3-methylhistidine excretion in urine is considered to be a more reliable indicator of muscle protein breakdown in rat or man (Munro and Young, 1978), further experiments are planned to study the effects of excess dietary threonine on urinary excretion of 3-methylhistidine.

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