Evaluation of Protein Requirements Using the Indicator Amino Acid Oxidation Method

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Summary The indicator amino acid oxidation (IAAO) method is a novel method for determining protein requirements. Recently, the protein requirement of healthy young men was reevaluated using this method, and the currently recommended protein requirement based on nitrogen balance study was found to be deficient. Similarly, with respect to experimental animals, the protein concentration used widely in the experimental diets was assumed to be deficient. However, only a few studies have tested the IAAO method in experimental animals. In particular, there are no studies on the protein requirement of adult rats measured using this method. Therefore, we applied the IAAO method to adult rats, to determine their casein protein requirement. Male Wistar/ST rats (15–18 wk old, housed in lighting (lights on from 23:00 to 11:00) conditions) were provided with the test diet including graded casein (5, 7, 9, 13, 17, 21 and 25%) every 2 h from 11:00 to 17:00. Tracer administration of 13C-phenylalanine was performed hourly from 14:00 to 17:00. Breath 13CO2 was measured every 30 min after the first tracer administration. There were significant differences between the 13CO2 concentration of the 5% and 17% casein groups at 17:00 and 18:00 (p < 0.05). The mean casein protein requirement and recommended dietary allowance (RDA) were estimated to be 5.2 g/kg BW/d and 7.0 g/kg BW/d using the mixed-effect change point regression model, respectively. Our results indicated that the recommended casein value may be slightly deficient to satisfy the protein metabolic demand of some adult rats.

Key Words IAAO method, metabolic demand, stable isotope, casein, adult rat

The indicator amino acid oxidation (IAAO) method was developed as a novel method for determining the protein or essential amino acid requirements of humans (1–3), and is widely applied to the elderly, young adults, children, pregnant women, and athletes in the clinic (2, 4–7). Since the IAAO method does not require a dietary adaptation period, it is considered to determine the protein requirement more precisely and easily than nitrogen balance estimations (8).

The mean protein requirements and population-safe intakes proposed by the Food and Nutrition Board based on nitrogen balance studies are 0.66 g/kg BW/d and 0.80 g/kg BW/d, respectively (9). Whereas, the protein requirement and recommended dietary allowance (RDA) for healthy young men, were estimated at 0.93 g/kg BW/d and 1.2 g/kg BW/d, respectively using the IAAO method (2), suggesting that the currently recommended protein requirement is under-estimated. Therefore, the protein requirements determined using the nitrogen balance estimations should be reassessed.

As for experimental animals including rats, the protein requirements is also determined by the nitrogen balance studies (10). Considering the results of human study regarding protein requirements using the IAAO method, it may be assumed that the RDA for the experimental animals is also lower than the actual requirements. Therefore, a reevaluation of protein requirements for experimental animals, especially rodents which are used widely as model animals for nutritional studies, using the IAAO method is needed.

However, only a few studies have applied the IAAO method to evaluate the protein requirements of experimental animals (11–13). In particular, there are no studies on the protein requirement of adult rats using the IAAO method. It was reported that the protein requirements for adult rats are different from that for young rats, as the protein metabolism changes with aging (14). Therefore, in this study; we determined the protein requirement and RDA of adult rats using the IAAO study.

MATERIALS AND METHODS

Animals. This study was approved by the Animal Research Committee of Morinaga Milk Industry (17-011, 17-050). Male Wistar/ST rats (11–12 wk old) were purchased from Japan SLC, Inc., Hamamatsu, Japan. The rats were housed in individual mesh cages under controlled temperature (22 ± 2°C) and lighting (lights on from 23:00 to 11:00) conditions. They were adapted to the laboratory environment for at least 1 wk before starting the experiment. The rats were given free access to water and the AIN-93M diet (Oriental Yeast Co., Ltd., Tokyo, Japan). The food intake was recorded for each
rat for 3 d before the first day of the experiment. The total daily intake was calculated as the average of the 3-d intake. After adaptation, 15–18-wk-old rats were used for the experiment.

**Experimental diets.** The compositions of the experimental casein diets based on AIN-93M are shown in Table 1. The diets are described as casein contents (%). L-Phenylalanine (Phe) and L-tyrosine (Tyr) were added to the diets to achieve equal content of indicator amino acid (L-Phe) and its derivative (L-Tyr) in all diets as previously reported (11, 12). The levels of cornstarch were modified in accordance with the level of protein to keep at an identical energy level (3.6 kcal/g).

**Breath measurement.**

Experimental design: During each experiment day, the rats received one of the seven levels of the experimental casein diets (5, 7, 9, 13, 17, 21, and 25%). The breath measurement experiments were performed seven times to calculate the breakpoint from the breath 13CO2 concentrations of the seven casein diets for each rat (n=8, 15–18 wk old).

The rats were fasted from 18:00 and the breath measurement experiments were performed at 11:00 the next day as starting time of night period. The experimental protocol is depicted in Fig. 1. Blood and tissue samples were collected in the sample collection experiment.

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**Table 1.** Composition of experimental diets.

| Protein content | 5% diet | 7% diet | 9% diet | 13% diet | 17% diet | 21% diet | 25% diet |
|-----------------|---------|---------|---------|----------|----------|----------|----------|
| Casein2,4       | 2,3     | 50      | 70      | 90       | 130      | 170      | 210      | 250      |
| Cornstarch2     | 557.5   | 537.5   | 517.5   | 477.5    | 437.5    | 397.5    | 357.5    |
| AIN-93M2,4      | 392.5   | 392.5   | 392.5   | 392.5    | 392.5    | 392.5    | 392.5    |
| l-Phenylalanine5 | 9.0     | 8.1     | 7.2     | 5.4      | 3.6      | 1.8      | 0        |
| l-Tyrosine6     | 10.0    | 9.0     | 8.0     | 6.0      | 4.0      | 2.0      | 0        |
| Energy (kcal/g) | 3.6     | 3.6     | 3.6     | 3.6      | 3.6      | 3.6      | 3.6      |

1 Content (weight%) of purified casein protein in experimental diets.
2 Oriental Yeast.
3 Protein: 86.2% (N×6.38). Amino acid (mg/g casein): l-alanine: 2,700, l-arginine: 3,300, l-aspartic acid: 6,300, l-cysteine: 430, l-glutamic acid: 19,000, l-glycine: 1,600, l-histidine: 2,700, l-isoleucine: 4,900, l-leucine: 8,400, l-lysine: 7,100, l-methionine: 2,600, l-phenylalanine: 4,500, l-proline: 10,000, l-serine: 4,600, l-threonine: 3,700, l-tryptophan: 1,100, l-tyrosine: 5,000, l-valine: 6,000, total: 93,930.
4 AIN-93M (Oriental Yeast) except for cornstarch, casein and l-cystine.
5 l-Phenylalanine content was kept constant at 11,250 mg/kg diet in all diets.
6 l-Tyrosine content was kept constant at 12,500 mg/kg diet in all diets.

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**Fig. 1.** Protocol used on each IAAO day. a The experimental diets are shown in Table 1. Each rat was given one-twelfth of the daily intake. b Isotope: L-[1-13C]Phe was administrated orally using a feeding needle. c Sample collection: Baseline breath sample was collected before the L-[1-13C]Phe administration. Nine breath samples were collected every 30 min after the initiation of the isotope protocol. Blood, liver, and gastrocnemius muscle samples were collected at 17:30. Breath samples were collected in the breath measurement experiments. Blood and tissue samples were collected in the sample collection experiment.
chambers were continuously charged with fresh room air through the aspiration tube by a pump. Breath samples were injected to the breath-sampling bags (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) through the aspiration tube. The $^{13}$CO$_2$ concentration in the bags was measured by POCone (Otsuka Electronics Co., Ltd., Tokyo, Japan) as reported previously ([15]).

Experimental design: The stable isotope levels in the plasma and liver and muscle tissues in rats on 5% or 17% casein diets was evaluated as representative protein-insufficient and protein-sufficient cases, respectively.

After the rats were fasted overnight from 18:00, they ($n=8$, 15 wk old) were treated as described in Fig. 1 with the exception of breath collection. The 17% or 5% casein diets were given as the experimental diets ($n=4$/group). Blood and tissue samples were collected at 17:30.

Collection and analysis of blood and tissue samples: Blood samples drawn from the inferior vena cava were collected in tubes with heparin. The plasma samples were stored at $-30^\circ$C until analyses. The liver and gastrocnemius muscle were rapidly removed and snap-frozen in liquid nitrogen and stored at $-80^\circ$C until analysis. Approximately 0.5 g tissue was homogenized in 4.5 mL saline and centrifuged at $1,000 \times g$ for 10 min, and the supernatant was used for LC-MS analysis.

LC-MS analysis was conducted by Sumika Chemical Analysis Service, Ltd. (Osaka, Japan). Briefly, 50 μL plasma samples or the tissue supernatant were deproteinized with 5% sulfosalicylic acid (100 μL) and mixed with the internal standards (Phe-d$_8$: 10,000 ng/mL, Tyr-d$_4$: 100,000 ng/mL for $^{13}$C analysis; Phe-d$_8$: 10,000 ng/mL, Tyr-d$_4$: 100,000 ng/mL for $^{12}$C analysis). The solutions were centrifuged at 15,000 $\times g$ for 5 min and the supernatants were used for LC-MS analysis. Phe and Tyr concentration were analyzed with MS (API5000, AB Sciex Pte. Ltd.) coupled with the UFLC system.

Statistical analyses. Differences were considered significant at $p<0.05$. Student’s t-test was used to analyze differences in characteristics between the 17% casein diet group and 5% casein diet group using the JMP13 software (SAS Institute Inc., Cary, NC). All results are presented as the mean±SD. The breakpoints and the 95% confidence intervals were calculated using R version 3.3.3 (R Foundation for Statistical Computing, Vienna, Austria) by applying a mixed-effect change point-regression model (CPRM) as previously reported ([16]). The mean casein protein requirement was estimated as the breakpoints.

RESULTS

Characteristics of the rats

The average total daily food intake per rat was 24.1±0.9 g/d (calorie, 87.4±3.4 kcal/d; protein, 2.9±0.1 g/d) in the breath measurement experiments. The body weights of the rats used for the 5, 7, 9, 13, 17, 21, and 25% casein diet experiments were 440.2±5.9 g, 486.8±24.1 g, 454.5±20.9 g, 479.1±23.8 g, 478.0±23.5 g, 437.5±21.7 g, and 437.5±27.5 g, respectively.

The average total daily food intake per rat in the sample collection experiment (the 5% and 17% casein diet) were 23.7±1.1 g/d and 23.4±1.0 g/d (calorie, 85.8±4.2 kcal/d and 84.8±3.8 kcal/d; protein, 2.9±0.1 g/d and 2.8±0.1 g/d) and the body weights were 410.4±10.7 g and 409.1±8.4 g, respectively.

Breath $^{13}$CO$_2$ concentration and casein protein requirement

The breath $^{13}$CO$_2$ data is shown in Fig. 2. The casein protein intake (x-axis) was normalized to the body weight on each experimental day. The mean casein protein requirement (breakpoint) and RDA (equivalent to the upper 95% CI) were estimated to be 5.2 g/kg BW/d and 7.0 g/kg BW/d, respectively, using the mixed-effect CPRM. To confirm the steady state of breath $^{13}$CO$_2$ concentration and compare the 5% and 17% casein diet groups as representative protein-insufficient and protein-sufficient cases, the time-course of breath $^{13}$CO$_2$ concentration after i-[1-13C]Phe infusion in the 5% and 17% casein diet groups is shown (Fig. 3). There were significant differences between the $^{13}$CO$_2$ concentration of...
The Phe and Tyr concentration of plasma, liver, and gastrocnemius muscle are shown in Table 2. Significant differences were demonstrated in the liver Phe and in the plasma, liver, and gastrocnemius muscle Tyr between 5% and 17% casein diets (p < 0.05). Significant differences were observed at 17:00 and 18:00.

**DISCUSSION**

To the best of our knowledge, this is the first study to evaluate the protein requirement of adult rats using the IAAO method.

In the present study, the mean casein protein requirement and RDA of adult rats were estimated as 5.2 g/kg BW/d and 7.0 g/kg BW/d, respectively. Converting these results to per feed intake using each rat’s body weight and daily food intake, the mean protein requirement and RDA of adult rats were calculated as 11.6% casein and 15.6% casein, respectively. The protein content (14% purified casein), recommended by the conventional experimental diet (AIN-93M) is over the mean casein protein requirement in this study. Considering the RDA, 14% purified casein may be slightly deficient for some adult rats. Regarding AIN-93M, crystalline l-cystine, which is considered as the limiting amino acid in casein for rats, is supplemented (10). Therefore, the protein content in AIN-93M is thought to be sufficient for adult rats. Further studies are expected to reveal the effect of l-cystine supplementation on casein requirement using...
IAAO methods.

We used the average of breath $^{13}\text{CO}_2$ concentrations at 17:00, 17:30, and 18:00 to calculate the breakpoint in this study. The breath $^{13}\text{CO}_2$ concentrations needed to be at equilibrium for breakpoint calculation. After administration of $^{13}\text{C}$-Phe at 14:00, the breath $^{13}\text{CO}_2$ concentrations increased gradually in both the 17% and 5% casein diet groups. Significant differences in breath $^{13}\text{CO}_2$ concentrations were observed at 17:00 and 18:00 between the 17% and 5% casein diet groups (Fig. 3). The experimental diets were also influenced on the breath $^{13}\text{CO}_2$ concentrations. We confirmed that the breath $^{13}\text{CO}_2$ concentrations achieved at a steady state 2.5–3.0 h after the first feed of the experimental diet and was maintained until the end of the experiment in our preliminary examination without tracer administration (data not shown). Taken together the breath $^{13}\text{CO}_2$ concentrations was considered to be at equilibrium after 17:00.

The Phe and Tyr concentrations in the plasma and tissues was expected to be at the same levels in all experimental diets for breakpoint calculation. Therefore the experimental diets were modified to achieve equal content of Phe and Tyr. The Phe and L-[1-13C]-Phe concentrations in the plasma and gastrocnemius muscle were not affected by the amount of protein intake (the 17% and 5% casein diets). These results indicate that the concentration of the indicator amino acid in the major amino acid pool was similar at the different levels of experimental diets.

In contrast, the Phe concentrations in the liver of the 5% casein group were significantly higher ($p<0.05$) than that of the 17% casein group. Moreover, Tyr and L-[1-13C]-Tyr concentrations in the plasma, liver, and gastrocnemius muscle of the 5% casein group were also higher than that of the 17% casein group.

In this study, Phe and Tyr were added to the experimental diet to achieve equal content of these amino acids in all diets. In general, it is known that casein, called “slow protein,” is absorbed at a slower rate than amino acids (17, 18). The absorption speeds of L-Phe and L-Tyr in the form of casein protein or as crystalline amino acids are different, and this would affect the concentration of these amino acids in the plasma and tissues. Actually, Tyr and Phe concentrations in the amino acid pool of the 5% casein group were higher than that in the 17% casein group, indicating that the absorption speed of amino acids or casein protein would affect the amino acid pool within 30 min after feeding. On the other hand, on the previous study, Phe and Tyr concentrations in the plasma, liver and gastrocnemius muscle was similar even with different levels of casein intake in young rats in spite of adding amino acids to the experimental diets (11). This could be attributed to the differences in protein metabolism between young and adult rats. The protein turnover of adult rats is considered to be slower than that of young rats. While excess Phe is hydroxylated to Tyr in the liver, the intake of Phe in this study might far exceed the rate of hydroxylation in adult rats (19–22). Therefore, it was considered reasonable that the Phe concentrations in the liver and Tyr concentrations in plasma and tissues of the 5% casein group were significantly higher than that in the 17% casein group.

Since it is known that Tyr concentration in rats rarely affect the hydroxylation of Phe, Tyr concentrations in the plasma and tissues is thought to have little effect on breath $^{13}\text{CO}_2$ concentration. Therefore, it is considered possible to evaluate protein requirement from breath $^{13}\text{CO}_2$ concentrations. It may not be necessary to add Tyr to achieve equal Tyr content in the experimental diets (23).

The protein requirements determined in this study have some limitations. Protein requirements is affected by life-style including daily protein intake or physical activity. We used Wistar/ST male rats under conventional breeding conditions in this study. Therefore, this result is considered broadly adapted to nutritional studies under conventional conditions. However, protein requirements are also affected by body weight and body composition.

In this study, the protein requirement was evaluated based on body weight. This evaluation is easily affected by high body weight or high body fat. During the experimental period for determining protein requirements, the average body weight gain was approximately 11.2%. The rats used in the present study may have high body fat since they had a low-activity level and were given free access to the AIN-93M diet. Therefore, it may be more appropriate to evaluate protein requirement based on muscle mass rather than body weight, in particular in obesity cases. In future studies, the effect of body composition on the protein requirement should be evaluated. Therefore, we calculated recommended protein amount (%) in the diets.

Also, we may need to consider sex differences on protein requirements. In human studies, Li et al. reported that the comparison of estimated average requirement values determined by the IAAO method between female and male subjects were not significantly different (24). Whereas, in a long-term nitrogen balance study, positive nitrogen balances were observed at about ovulation while negative balances were observed before the onset of menstruation (25). Therefore, protein requirements in female rats before the onset of menstruation may be different to that of male rats. Further IAAO studies are needed in female rats to confirm the differences in protein requirements during menstruation.

In conclusion, the optimum amount of casein in the diets for adult rats was confirmed using the IAAO method. Our results showed that the recommended casein value would be slightly insufficient to satisfy the protein requirements in some adult rats. Besides, the mean casein protein requirement for adult rats in this study is lower than that in previous studies using young rats (11, 12). These indicate that it is warranted to reassess protein requirement for other animal models using the IAAO method. Since the IAAO method is minimally-invasive and does not require an adaptation period, it may be useful for evaluating protein require-
ments in various models, including exercise model, disease model, or various life-stage models for menopause, infancy, and senescence. Moreover, the protein requirement determined by the IAAO method may reflect the lifestyle (26). The accurate protein requirement of various models should be assessed using the IAAO method in future studies.

Disclosure of state of COI

All authors are employees of Morinaga Milk Industry Co. Ltd.

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