A Simple Evaluation Method for the Quality of Dietary Protein in Rats Using an Indicator Amino Acid Oxidation Technique

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Summary We demonstrated that the indicator amino acid oxidation (IAAO) method could be employed for the evaluation of quality of dietary protein by comparing the protein intakes required to meet metabolic demand in rats fed different proteins. The objective of this study was to validate a simple evaluation method for determining the quality of dietary protein using the IAAO technique. Male Sprague-Dawley rats (5–6 wk old) were fed meals composed of graded protein, using either casein, wheat gluten (WG), soy protein isolate (SPI), or egg white protein (EW), every 3 h from 09:00 to 18:00. Administration of L-[1-13C]phenylalanine was performed hourly from 15:00 to 18:00. The 13CO2 level in breath CO2 was measured at 18:30. The protein intake values required to meet the metabolic demand based on the breath 13CO2 data for the dietary casein, WG, SPI, and EW intake were 18.0, 22.2, 17.5, and 10.1 g/kg BW/d, respectively. The breath 13CO2 concentrations corresponding to the protein intake of 7.5 g/kg BW/d for casein, WG, SPI, and EW were 9.8, 10.9, 10.3, and 8.9 (‰)/100 g BW, respectively. A significant correlation was demonstrated between the protein intake required to meet the metabolic demands and the 13CO2 concentration in the breath for a protein intake of 7.5 g/kg BW/d (r=0.967; p<0.05). These results demonstrated that the protein intake required to meet metabolic demand could be estimated and that the quality of the dietary protein could be evaluated using the 13CO2 concentration in the breath with a protein intake of 7.5 g/kg BW/d.

Key Words protein quality, indicator amino acid oxidation, metabolic demand, rats

Since its adoption by the FAO/WHO in 1991 (1), the protein digestibility-corrected amino acid score (PDCAAS) method has been in use for some 20 y and has proved to be of considerable value in actual practice. This method is based on a comparison of the concentration of the first limiting essential amino acid in the test protein with the concentration of that amino acid in a reference (scoring) pattern. The chemical score is corrected for the true fecal digestibility of the test protein (2). Although the principle of the PDCAAS method was widely accepted after it was announced in the 1991 report (1), a number of critical questions have since arisen related to the PDCAAS approach. Digestibility should be based on the true ileal digestibility of each amino acid, and accordingly, in a report published by the FAO in 2013 (3), a new protein quality measurement method and the digestible indispensable amino acid score (DIAAS) were recommended as a replacement for the PDCAAS. Nevertheless, several limitations of the DIAAS have also been recognized, including the validity of its true ileal digestibility. Therefore, it is clear that there are several aspects related to a valid protein quality evaluation that require further consideration.

The indicator amino acid oxidation (IAAO) technique was developed and used to determine the protein metabolism by measuring 13CO2 in breath as changes in the oxidation of orally administered L-[1-13C]phenylalanine. The IAAO method has been widely used in order to determine amino acid requirements in studies on pigs (4–7), and humans (8–13). In 2007, Humayun et al. (14) applied the IAAO method and conducted a reevaluation study on protein requirements in healthy young men.

In a previous study (15), we evaluated protein requirements and dietary protein quality employing casein and wheat gluten as protein sources in experimental diets in rats, using the IAAO method with L-[1-13C]phenylalanine. The results of that study suggested that the IAAO method could be employed for the evaluation of protein quality by comparing the protein intake required to meet metabolic demands in rats. The present study aimed to validate a simple evaluation method for the quality of dietary protein using the IAAO technique. Specifically, the protein intakes required to meet metabolic demand were determined employing casein, wheat gluten (WG), soy protein isolate (SPI), and egg white protein (EW) as protein sources in experimental diets. Using the IAAO

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method, we investigated the relationships between the protein intake required to meet metabolic demands for each diet and the $^{13}$CO$_2$ concentration in the breath for a certain protein intake, 7.5 g/kg BW/d. The premise of the study was that if the results showed a correlation a certain protein intake, 7.5 g/kg BW/d. The premise of each diet and the $^{13}$CO$_2$ concentration in the breath for protein intake required to meet metabolic demands for the SPI and the EW diets determined using the IAAO method in rats.

The objective of the present study was to confirm that the IAAO method could be employed as a simple evaluation technique could be employed as a simple evaluation method for the quality of dietary protein in rats. The eight rats in each group received, in random order without repeats, one of six levels of the casein (4.3, 8.6, 12.9, 17.2, 21.5, 25.8%) diet, a WG (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet, an SPI (4.0, 7.9, 11.9, 15.8, 19.8, 23.7%) diet, or an EW (4.3, 8.7, 13.0, 17.3, 21.6, 26.0%) diet. The diets were provided every 3 h (09:00–18:00). Each meal represented one-eighth of each rat’s daily intake. Stable isotope: Priming doses of l-[1-$^{13}$C]phenylalanine (●) and NaH$^{13}$CO$_3$ (○) were started with the third meal at 15:00, and the administration of l-[1-$^{13}$C]phenylalanine was performed at 16:00, 17:00, and 18:00. A baseline breath sample was collected before the isotope protocol began, and another breath sample was collected at 18:30 (■).

In the 1991 FAO/WHO report (1), the PDCAAS value was 1.00 for casein, 0.25 for wheat gluten, 0.92 for egg white protein, and 1.00 for egg white protein. In our previous study (15), the mean protein intakes for metabolic demands determined by the IAAO method were 13.1 g/kg BW/d for casein and 18.1 g/kg BW/d for the wheat gluten. These results suggested that the protein intake required to meet metabolic demand decreases with good quality (amino acid scoring pattern) protein intake, and increases with poor quality protein intake. Moreover, this was the first study conducted that employed the IAAO method to determine the protein intakes required to meet metabolic demand using SPI and EW as protein sources in experimental rat diets. The present study investigated whether or not the mean protein intakes required to meet metabolic demand for the SPI and the EW diets determined using the IAAO method in rats would be consistent with the PDCAAS values for these proteins.

The objective of the present study was to confirm that the protein intakes required to meet metabolic demand determined using the IAAO method supported our previous results, and validate whether or not the IAAO technique could be employed as a simple evaluation method for the quality of dietary protein in rats.

**MATERIALS AND METHODS**

**Animals.** This study was performed in accordance with the guidelines for animal experimentation at Kyoto Prefectural University, Japan. Male Sprague-Dawley rats (4 wk old) were purchased from Japan SLC, Inc. (Hama-matsu, Japan). The rats were housed in individual mesh cages under controlled temperature (22 ± 2°C) and lighting (lights on from 20:00 to 08:00) conditions. The rats were given free access to water and a 17.2% casein maintenance diet, and they were allowed to adapt to the laboratory environment for at least 1 wk before starting the experiment. After adaptation, 5–6 wk old rats were used for the experiment. The amount of feed available and any feed not eaten were recorded for each rat for 3 d before the first study day, and the total daily intake for each rat, equivalent to the 24-h dietary intake, was calculated on the basis of the average intake during the previous 3 d.

**Experimental design.** Thirty-two rats were used, divided into four groups, each composed of eight rats. Each of the rats in the casein, WG, SPI, and EW groups consumed, respectively, the casein, the wheat gluten, soy protein isolate, and egg white protein diets at all six levels; the different levels were randomly assigned to the rats. Each IAAO study day was separated by 2 d, and the six IAAO studies were completed within 2 wk.

The 17.2% casein maintenance diet employed for all of the studies was provided for at least 24 h, and even when they were measured for the WG, SPI, and EW diets, the 17.2% casein diet was provided as a maintenance diet before the study day for all of the IAAO studies. Then, the rats were fasted overnight for 13 h from 20:00 on the day before the study day, but they had free access to drinking water. The study protocol for all of the IAAO studies is depicted in Fig. 1. On the study day, the rats were weighed in the morning before feeding. Then, the eight rats in each group received, in random order without repeats, one of six levels of the casein (4.3, 8.6, 12.9, 17.2, 21.5, 25.8%) diet (N×6.38) (16), one of six levels of the WG (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet (N×5.70) (16), one of six levels of the SPI (4.0, 7.9, 11.9, 15.8, 19.8, 23.7%) diet (N×5.71) (16), or one of six levels of the EW (4.3, 8.7, 13.0, 17.3, 21.6, 26.0%) diet (N×6.25) (16) (Table 1). The experimental diet was consumed beginning at 09:00 and continued at 3-h intervals until 18:00 for a total of 4 isonitrogenous, isonitrogenous meals. Each meal accounted for one-eighth of the rat’s total daily intake. The rats were allowed free access to drinking water during the experimental period. The rats were fed the remaining half of the daily ration in the evening. The tracer protocol was started with the third meal at 15:00 to measure the phenyl-
| Casein diet          | g/kg diet | Casein diet          | g/kg diet | Casein diet          | g/kg diet | Casein diet          | g/kg diet | Casein diet          | g/kg diet |
|---------------------|----------|---------------------|----------|---------------------|----------|---------------------|----------|---------------------|----------|
| 4.3%                | 5.0      | 4.3%                | 5.0      | 4.3%                | 5.0      |
| 8.6%                | 10.0     | 8.6%                | 10.0     | 8.6%                | 10.0     |
| 12.9%               | 15.0     | 12.9%               | 15.0     | 12.9%               | 15.0     |
| 17.2%               | 20.0     | 17.2%               | 20.0     | 17.2%               | 20.0     |
| 21.5%               | 25.0     | 21.5%               | 25.0     | 21.5%               | 25.0     |
| 25.8%               | 30.0     | 25.8%               | 30.0     | 25.8%               | 30.0     |
| 7.2%                | 10.0     | 7.2%                | 10.0     | 7.2%                | 10.0     |
| 10.8%               | 15.0     | 10.8%               | 15.0     | 10.8%               | 15.0     |
| 11.9%               | 15.0     | 11.9%               | 15.0     | 11.9%               | 15.0     |
| 15.8%               | 20.0     | 15.8%               | 20.0     | 15.8%               | 20.0     |
| 19.8%               | 25.0     | 19.8%               | 25.0     | 19.8%               | 25.0     |
| 23.7%               | 30.0     | 23.7%               | 30.0     | 23.7%               | 30.0     |
| 4.0%                | 5.0      | 4.0%                | 5.0      | 4.0%                | 5.0      |
| 7.9%                | 10.0     | 7.9%                | 10.0     | 7.9%                | 10.0     |
| 11.9%               | 15.0     | 11.9%               | 15.0     | 11.9%               | 15.0     |
| 15.8%               | 20.0     | 15.8%               | 20.0     | 15.8%               | 20.0     |
| 19.8%               | 25.0     | 19.8%               | 25.0     | 19.8%               | 25.0     |
| 23.7%               | 30.0     | 23.7%               | 30.0     | 23.7%               | 30.0     |
| 4.3%                | 5.0      | 4.3%                | 5.0      | 4.3%                | 5.0      |
| 8.7%                | 10.0     | 8.7%                | 10.0     | 8.7%                | 10.0     |
| 13.0%               | 15.0     | 13.0%               | 15.0     | 13.0%               | 15.0     |
| 17.3%               | 20.0     | 17.3%               | 20.0     | 17.3%               | 20.0     |
| 26.0%               | 30.0     | 26.0%               | 30.0     | 26.0%               | 30.0     |

1. AIN-76 (vitamin mixture (per g mixture)): vitamin A, 4,000 IU; vitamin D3, 100 IU; vitamin E, 5 mg; vitamin K3, 0.005 mg; vitamin B6, 0.6 mg; vitamin B12, 0.7 mg; vitamin B12, 0.001 mg; vitamin B12, 0.002 mg; folic acid, 0.2 mg; calcium pantothenate, 1.6 mg; nicotinic acid, 3 mg; choline chloride, 200 mg; sucrose, 9.68 g.
2. AIN-76 (mineral mixture (g/kg mixture)): calcium phosphate dibasic, 500.0; sodium chloride, 74.0; potassium citrate, 220.0; potassium sulfate, 52.0; magnesium oxide, 24.0; manganese carbonate, 3.5; ferric citrate, 6.0; zinc carbonate, 1.6; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.0066; chromium potassium sulfate, 0.55; sucrose, 118.03.
3. L-Tyrosine content was kept constant at 1,500 mg/kg diet in all diets, except the 25.2% WG diet (14,350 mg/kg diet) and the 26.0% EW diet (15,324 mg/kg diet).
alanine kinetics with the use of L-\[1-\textsuperscript{13}C\]phenylalanine, and continued hourly until 18:00. The rats were placed in the chamber immediately after the oral administration of the \( ^{13}C \) tracer. Breath samples were collected and the \( ^{13}CO_2 \) level in the breath was measured at 18:30. Baseline breath samples were collected before the iso- 

to protocol began at 15:00.

Tracer administration protocol. L-\[1-\textsuperscript{13}C\]Phenylalanine (99 atom% excess; Cambridge Isotope Laboratories, Andover, MA) and NaH\( ^{13}CO_3 \) (99 atom% excess; Cambridge Isotope Laboratories) were used as tracers. Labeled compounds were dissolved in saline and stored at 4°C. Isotopic solutions were prepared and adminis- 
tered in a volume of 2.5 mL/kg BW. The L-\[1-\textsuperscript{13}C\]phe- 

nylalanine was administered together with unlabeled phenylalanine for priming. Tracer administration timing is shown in Fig. 1. Oral priming doses of 0.88 mg/kg BW NaH\( ^{13}CO_3 \) and 7.92 mg/kg BW NaHCO\(_3\) were given with the third meal at 15:00. An oral dosing protocol of 3.3 mg/kg BW L-\[1-\textsuperscript{13}C\]phenylalanine and 29.7 mg/kg BW phenylalanine was commenced simultaneously with the third meal, and the administration of 6.0 mg/kg BW L-\[1-\textsuperscript{13}C\]phenylalanine and 54.0 mg/kg BW phenylalanine was continued hourly until the end of the study.

Experimental diets. The composition and source of the powdery experimental diets are shown in Table 1. Casein, WG, SPI, and EW were the sole source of protein in the casein, WG, SPI, and EW diets, respectively. The compositions of the amino acids in the casein, WG, SPI, and EW are shown in the footnote to Table 1 (16). L-Phenylalanine and L-tyrosine were added to the diets to achieve an equal content of these amino acids in all diets. In the present study, L-phenylalanine (13.5 g/kg diet) and L-tyrosine (15.0 g/kg diet) were consumed in excess of these amino acid requirements for rodents (L-phenylalanine, 8.8 g/kg diet; L-tyrosine, 9.3 g/kg diet) (17) in order to minimize the net hydroxylation of phenylalanine to tyrosine. Due to the varying protein content, each experimental diet was kept at an identical energy level by varying the levels of sugar and starch. Thus, all of the diets had a similar energy level (15.4–15.7 kJ/g).

Collection and analysis of the breath samples. The instruments used for the collection of breath samples from the rats consisted of an acrylic chamber (10.6 L) fitted with a drinker, an aspiration pump (Columbus Instruments, Columbus, OH) and an air flow meter (Columbus Instruments). The chambers were continuously charged with fresh room air through the aspiration tube by a pump. The rats were moved outside the chamber for the administration of the \( ^{13}C \) tracer, and thereafter moved back into the same chamber. Because the chambers filled with expired air, the rats were placed in separate compartments for 30 min before the collection of the breath samples.

Breath samples of 200 mL volume drawn into a 200 mL syringe were injected into breath-sampling bags (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The \( ^{13}CO_2 \) concentration in the expired air was measured by attaching the breath-sampling bags to the sampling joint of an infrared spectrometer (POCone; Otsuka Electronics Co., Ltd., Osaka, Japan). Using the measurement system provided by POCone, the concentra- 
tion of CO\(_2\) in the aspirated air in the breath sampling bags was at least more than 0.5%. Therefore fresh room air was drawn through the system at comparatively low rates of approximately 0.4 L/min, and the \( ^{13}CO_2 \) concentration within the chamber was stabilized at 0.8–1.2%. The \( ^{13}CO_2 \) rate was measured as the \( ^{13}CO_2/^{12}CO_2 \) ratio, and followed by a pulse of mixed gas composed of 5% CO\(_2\), 12% O\(_2\) and 83% N\(_2\) for the control. Isotopic abundances were expressed relative to the international Vienna Pee Dee Belemnite standard (‰) as over the baseline (\( \Delta - \Delta_0 \) value, and further normalized by each rat’s weight.

Statistical analysis. Data analysis was performed using Statcel2 software (OMS Publishing Inc., Tokyo, Japan). Statistical analysis for multiple comparisons was performed using one-way analysis of variance (ANOVA) with repeated measures, followed by a Tukey-Kramer post hoc test. All results were presented as the mean±SE. Values of \( p<0.05 \) were considered statistically significant.

The protein intake required to meet metabolic demand was derived by applying a mixed-effect change-point regression model (CPRM) on the \( ^{13}CO_2 \) concentration in the breath, and the regression oxidation rate of the dietary protein contents (18). The first regression line showed a downward slope and the second line was hori- 

zontal with minimal or no slope. The breakpoint, the protein intake with a plateau in oxidation, was regarded as the protein intake required to meet metabolic demand. The 95% confidence intervals of the breakpoints were calculated using GraphPad Prism 5 software (MDF Co., Ltd., Tokyo, Japan).

Pearson’s correlation coefficient analysis was used to identify the relationship between the protein intake required to meet metabolic demand and the \( ^{13}CO_2 \) concentration in the breath for a protein intake of 7.5 g/kg BW/d.

RESULTS

The rats were given free access to a 17.2% casein diet as a maintenance diet for 3 d before the first study day. The total daily intake for each rat used for the casein, WG, SPI, and EW diets experiments employing the IAAO method were 15.8±0.4 g/d (calorie, 244.9±5.6 kJ/d; protein, 2.7±0.1 g/d), 15.7±0.3 g/d (calorie, 243.4± 4.0 kJ/d; protein, 2.7±0.0 g/d), 15.2±0.4 g/d (calo- 

trie, 235.6±5.7 kJ/d; protein, 2.6±0.1 g/d) and 16.5± 0.6 g/d (calorie, 255.8±9.4 kJ/d; protein, 2.8±0.1 g/d), respectively. No significant differences were shown in the average total daily intakes among the 4 groups during the maintenance diet period (\( p=0.20 \)).

The body weights for the rats used for the 4.3, 8.6
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12.9, 17.2, 21.5 and 25.8% casein diet experiment were 136.6 ± 9.5, 143.9 ± 9.8, 146.9 ± 7.8, 155.8 ± 1.9, 138.1 ± 4.7 and 159.0 ± 9.1 g, respectively (p < 0.24).

The body weight for the rats used for the 7.2, 10.8, 14.4, 18.0, 21.6 and 25.2% WG diet experiments were 152.7 ± 9.5, 165.9 ± 9.2, 153.7 ± 10.8, 151.2 ± 5.8, 162.4 ± 7.0 and 162.8 ± 9.4 g, respectively (p < 0.77).

The body weights for the rats used for the 4.0, 7.9, 11.9, 15.8, 19.8 and 23.7% SPI diet experiments were 133.9 ± 8.7, 126.8 ± 3.8, 136.9 ± 4.8, 141.2 ± 5.4 and 137.9 ± 5.5 g, respectively (p < 0.31).

The body weights for the rats used for the 4.3, 8.7, 13.0, 17.3, 21.6 and 26.0% EW diet experiments were 151.4 ± 11.0, 167.3 ± 4.0, 143.4 ± 4.4, 153.5 ± 11.5, 168.8 ± 3.7 and 143.9 ± 5.0 g, respectively (p < 0.07).

Although the rats gained weight regardless of diet throughout the 2-wk experimental period, no significant differences were shown in the mean body weights among the different levels of protein intake.

Figure 2 shows the mean breakpoints illustrated in the breath 13CO2 data, which were representative of the mean protein intake required to meet metabolic demand. As the protein intake increased, breath 13CO2 decreased steadily until the breakpoints were reached. There was no further decrease in breath 13CO2 with the increase in protein intake. The protein (%) included in the casein, WG, SPI, and EW diets was converted into protein intake (g) per day, and further normalized according to each rat’s body weight. The mean protein intakes required to meet metabolic demands for the casein, WG, SPI, and EW diets were estimated to be 18.0, 22.2, 17.5, and 10.1 g/kg BW/d, respectively.

12.9, 17.2, 21.5 and 25.8% casein diet experiment were 136.6 ± 9.5, 143.9 ± 9.8, 146.9 ± 7.8, 155.8 ± 1.9, 138.1 ± 4.7 and 159.0 ± 9.1 g, respectively (p < 0.24). The body weight for the rats used for the 7.2, 10.8, 14.4, 18.0, 21.6 and 25.2% WG diet experiments were 152.7 ± 9.5, 165.9 ± 9.2, 153.7 ± 10.8, 151.2 ± 5.8, 162.4 ± 7.0 and 162.8 ± 9.4 g, respectively (p < 0.77).

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The body weights for the rats used for the 4.3, 8.7, 13.0, 17.3, 21.6 and 26.0% EW diet experiments were 151.4 ± 11.0, 167.3 ± 4.0, 143.4 ± 4.4, 153.5 ± 11.5, 168.8 ± 3.7 and 143.9 ± 5.0 g, respectively (p < 0.07).

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The intake of 7.5 g/kg BW/d was used to calculate the correlation was
5.69 x – 39.70, r = 0.967; p < 0.05) (Fig. 4).

**DISCUSSION**

In previous studies (15), we showed that the IAAO method could be employed for the evaluation of protein quality by comparing the protein intakes required to meet metabolic demand of rats when the rats were fed different source proteins. The present study aimed to validate a simple evaluation method for the quality of fed different source proteins. The present study aimed to determine the protein intake required to meet metabolic demand of rats when the rats were used to meet metabolic demand of 7.5 g/kg BW/d, suggesting that the protein intake required to meet metabolic demand could be estimated and that the quality of the dietary protein employed could be evaluated by the metabolic demand in the breath with a protein intake of 7.5 g/kg BW/d. The linear regression equation for this correlation was $y = 5.69x - 39.70$, but since this is the first study conducted that employed the IAAO method to show a correlation between the protein intake required to meet metabolic demand and the $^{13}$CO$_2$ concentration in the breath for a certain protein intake, this equation should be considered provisional, and it may be necessary to conduct further studies employing other protein sources.

The protein intake required to meet metabolic demand was derived by applying a CPRM model (18) to the $^{13}$CO$_2$ concentration in the breath. The CPRM model has a break point followed by a zero slope, but for confirmation, our data was also applied to a model without the breakpoint. Akaike information criteria (AIC) (18) made it possible to select the best fit model for the data, as the model minimizing the AIC is regarded as the model with the best predictive capability. A comparison showed that the AIC for CPRM (224.0, 227.1, 221.8, and 195.0 for the casein, WG, SPI, and EW, respectively) was smaller than that for the model without the breakpoint (224.0, 228.5, 223.4, and 197.8 for the casein, WG, SPI, and EW, respectively), and accordingly, we demonstrated that the breakpoint is present in our data.

Although the breath $^{13}$CO$_2$ appeared to differ according to the type of protein at a protein intake lower than that meeting the metabolic demand, that difference was not shown at a protein intake greater than the protein intake required to meet metabolic demand. Therefore, the $^{13}$CO$_2$ concentration in the breath for a protein intake of 7.5 g/kg BW/d was used to calculate the correlation with the protein intake required to meet metabolic demand.

Moehn et al. (19, 20) developed the concept of metabolic availability based on the IAAO technique, in growing pigs, as an alternative to ideal digestibility as a measure of amino acid bioavailability. Metabolic availability reflects the proportion of dietary amino acids used for protein synthesis and includes all amino acid losses that occur during digestion, absorption, and metabolic utilization, including endogenous losses (19). In our previous study (15), we showed that the IAAO method could be employed for the evaluation of a measure of protein bioavailability as the protein quality by comparing the protein intake required to meet metabolic demand. For each rat, it was necessary to repeat the IAAO method 6 or 7 times with varying test protein intakes in order to determine the protein intake required to meet metabolic demand. However, using measurements of the $^{13}$CO$_2$ concentration in the breath for a certain protein intake, such as 7.5 g/kg BW/d, it was only necessary to conduct the procedure once using the IAAO method. The
The differences in the protein intake required to meet metabolic demand among the casein, WG, SPI and EW diets will be a function of the limiting amino acid in the respective protein source. This limiting amino acid will be dependent on both the amino acid profile and the digestibility of the protein. The results for the WG diet conformed to the 1991 FAO/WHO report (1). The PDCAAS value for WG was 0.25, and since the protein intake required to meet metabolic demand will increase with poor quality (amino acid scoring pattern) protein intake, our results validated the concept that the IAAO method could be employed to evaluate the quality of protein in the present study. In the 1991 FAO/WHO report (1), the PDCAAS values for casein and EW were each 1.00. The amino acid score and consequent PDCAAS value are expressed as the maximum value for each individual protein, that is, no greater than 1.0 or 100%. However actual calculated values for the amino acid score are higher than 1.0 or 100% because all indispensable amino acids are present in some proteins at higher concentrations than in the reference scoring pattern. Thus it has been argued that truncation of values higher than 100% does not provide a true representation of the actual protein concerned (2). However, the results determined in the present study using the $^{13}$CO$_2$ concentration in the breath for a protein intake of 7.5 g/kg BW/d obtained with IAAO method varied widely. Thus, it may be possible to employ the IAAO method in order to evaluate the quality of protein in detail and obtain more accurate values for each type of protein, compared with the PDCAAS approach. Incidentally, the DIAAS can produce values below, or in some circumstances, above 100%. Values above 100% should not be truncated, which was conventional for PDCAAS values, except when calculating the DIAAS for protein or amino acid intakes for mixed diets (3). The DIAAS values for casein, WG, and SPI are 146% (3, 21, 22), 34% (3), and 119% (3, 23), respectively, which conformed to the order of the $^{13}$CO$_2$ concentration in the breath with a protein intake of 7.5 g/kg BW/d. Because there is no appropriate data for the ileal digestibility of EW, the DIAAS value for EW has not been calculated.

While it can be asserted that the IAAO method can be used to “assess” protein quality, to date there has been no indication as to how values determined employing IAAO method could be compared to published values based on the PDCAAS or DIAAS methods. For instance, our results indicated that the $^{13}$CO$_2$ concentration in the breath, determined using the IAAO method could be represented as a ratio of the test protein to casein in rats. According to the procedures recommended by the AIN-93G diet for laboratory rodents (a purified 20% casein (≥85% protein)), values are converted to dietary content by assuming a dietary intake of 15 g/rat/d for growing rats, and protein intake is 2.55 g/rat/d for a rat fed 20% casein (85% protein). The protein intake required to meet metabolic demand for casein determined using the IAAO method in the present study was 18.0 g/kg BW/d, or 2.64 g/rat/d (approximately 146.7 g BW for rat fed a casein diet). This value was similar to the value recommended by the AIN, which was developed based on the nitrogen balance method, suggesting that the IAAO method is a robust technique for the determination of protein intake required to meet metabolic demand.

The protein intake required to meet metabolic demand was estimated to be covered by 18.0 and 22.2 g/kg BW/d for casein and WG, respectively. In our previous study (15), the mean protein intake required to meet metabolic demand determined by the IAAO method were 13.1 g/kg BW/d for casein and 18.1 g/kg BW/d for wheat gluten. Both studies demonstrated the same pattern, the protein intake required to meet metabolic demand based on wheat gluten was higher than that based on casein. However, the values determined by the present study differ from those of our previous study. The protein intakes required to meet metabolic demand determined in our previous study were based on data obtained from Wistar/ST rats, which varied from the SD rats used in the present study, and these different types could have different patterns of weight gain and reproduction. This study was completed during the period from 09:00 to 19:00, while the light period for the room was from 08:00 to 20:00 in our previous study (15). The dark period was from 08:00 to 20:00 in the present study. Since rats are active during the dark period, it can be considered that environment employed for the present study made it possible to obtain measurements that were close to the protein intake required to meet metabolic demand for the daily life of the rats. Except for these points, all of the experimental basic protocols were the same as those employed in previous study (15).

In our previous study (15), regardless of the protein intake and the 4.3% or 17.2% casein diets, $^{13}$C enrichment in breath gradually increased until 2.5 h after the start of the administration of the L-$^{13}$C phenylalanine isotope. The plateau breath samples were collected during the isotopic steady state every 30 min during the period from 17:30 to 19:00. During the period from 18:30 to 19:00, when the 4.3% casein diet was employed, the enrichment of breath $^{13}$CO$_2$ was significantly greater than that achieved with the 17.2% casein diet, so the protein intake required to meet metabolic demand was determined by the $^{13}$CO$_2$ in breath at 18:30 in our previous study. In our preliminary examination, breath samples were collected and the $^{13}$CO$_2$ level in breath CO$_2$ measured at 30 min intervals from 15:00 to 19:00 from rats fed all of the different types of protein. Regardless of the type of protein, the stable rate of $^{13}$CO$_2$ production in breath was achieved during the period from 17:30 to 18:30 (data not shown). The quality of the protein employed showed an effect on the breath $^{13}$CO$_2$ enrichment. Therefore, the present study also adopted 18:30 as the time employed to measure the breath $^{13}$CO$_2$. In the previous study (15), at 18:30, we showed that the phenylalanine and L-$^{13}$C phenylalanine concentrations in the plasma, liver and gastroc-
muscle were not affected by the amount of protein intake in the 4.3% or 17.2% casein diets, suggesting that the precursor pool for indicator oxidation did not change in size in response to the test protein intake.

In conclusion, this study suggested that the quality of dietary protein could be estimated by $^{13}$CO$_2$ concentration in the breath with a protein intake of 7.5 g/kg BW/d, validating this simple evaluation method for the quality of dietary protein using the IAAO technique in rats. The $^{13}$CO$_2$ concentration in the breath for a relatively low protein intake may reflect a difference in protein metabolism, and the IAAO method could be applied to the measurement of various metabolic demands as future extensions of this study.

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