An Evaluation of Protein Intake for Metabolic Demands and the Quality of Dietary Protein in Rats Using an Indicator Amino Acid Oxidation Method

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Summary Currently, protein requirements are generally determined based on nitrogen balance studies, but there are a variety of limitations associated with this method. The indicator amino acid oxidation (IAAO) method, with a theoretical base that differs widely from the nitrogen balance method, was developed as an alternative method for humans. The objective of the present study was to evaluate protein intakes for metabolic demands and protein quality, using protein itself, in rats employing the IAAO technique with L-[1-13C]phenylalanine. Male Wistar/ST rats (5–6 wk old) received a graded casein (4.3, 8.6, 12.9, 17.2, 21.5, 25.8%), or a wheat gluten (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet, along with L-[1-13C]phenylalanine. An isotopic plateau in breath was achieved 210 min after the start of the 13C ingestion. The protein intakes for metabolic demands were calculated by applying a mixed-effect change-point regression model to breath 13CO2 data, which identified a breakpoint at minimal breath 11CO2 in response to graded protein intake. The 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 wheat gluten, showing a tendency similar to that determined by the nitrogen balance method. These results demonstrated that the IAAO method could be employed to evaluate not only the protein intakes for metabolic demands, but the dietary protein quality in freely living rats, suggesting that this method might be viable in a clinical setting.

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

The nitrogen balance method is normally employed to determine protein requirements, as specified in the 2007 WHO/FAO/UNU (1). However, the limitations of the nitrogen balance method, which can result in considerable error in the prediction of balance (2, 3), have been well described (4–6). In the nitrogen balance method, after the diet has been changed, a period of time is usually allowed for adaptation to be complete during the first 5–7 d (7). Therefore, employing the nitrogen balance method, the metabolic demand for protein cannot be assessed in patients with a widely varying metabolic demand. The indicator amino acid oxidation (IAAO) method was originally employed to study amino acid requirements in pigs (8), and thereafter it has been widely used for studies on pigs (9–11) and humans (12–17). Since the IAAO method does not require prior dietary adaptation (18) to each of the varying protein intake levels, it could be available when an assessment of the metabolic demand for protein is required for post-operative patients or patients with injuries or infections.

In 2007, Humayun et al. (19) applied the IAAO method and conducted a reevaluation study on the protein requirements in healthy young men by feeding the subjects graded protein intake as a crystalline amino acid mixture and measuring changes in the oxidation of orally administered L-[1-13C]phenylalanine. However, no studies have previously been conducted on determining the protein requirement using protein itself in animals or humans employing the IAAO method. Therefore, sufficient evidence has not been gathered showing that the IAAO method is viable for measurements of the protein requirement, and it has not been sufficiently validated in studies employing experimental rats up to the present. We should consider that the mechanism of the assimilation of the amino acid mixture differed from that of the protein. Amino acid mix-
tutes will be absorbed very rapidly, and protein utilization will show a higher efficiency, compared with slow proteins such as casein (20). Incidentally, a previous study by Moehn et al. (21) evaluated the metabolic availability of amino acids in peas, and they indicated the applicability of using IAAO for intact protein sources.

Measurements of the quality and quantity of the dietary protein employed can be used to facilitate adjustments to the diet to ensure that the metabolic demands for protein can be met sufficiently. Poor protein quality increases the protein requirement. In the 1991 FAO/WHO/UNU report (22), the protein digestibility corrected amino acid score (PDCAAS) value for casein is 1.00, compared with 0.25 for wheat gluten. Therefore, the protein requirement calculated for rats fed a wheat gluten diet is higher than that for rats fed a casein diet. In a clinical setting, the adequate quality and quantity of protein or amino acid for each disease might be estimated using the IAAO method.

The objective of the present study was to establish whether or not the IAAO method is viable for determining the metabolic demand for protein and to evaluate protein quality using protein itself, employing casein and wheat gluten as protein sources in experimental diets and using the IAAO method with L-[1-13C]phenylalanine.

**MATERIALS AND METHODS**

**Animals.** This study was performed in accordance with the guidelines for animal experimentation at Kyoto Prefectural University, Japan. Male Wistar/ST rats (4 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 08:00 to 20:00) 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- to 6-wk-old rats (initial BW = 130.1±2.3 g) 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.

**Experiment 1.** The objective of Experiment 1 was to examine the effect of L-[1-13C]phenylalanine administration on breath 13CO2 enrichment, and to evaluate whether the protein metabolism could be measured by the IAAO method in rats consuming different protein level diets. All of the eight rats were included in two IAAO studies, consuming both 4.3% and 17.2% casein diets (N×6.38) (23) with a time period of more than 2 d between the studies. The 17.2% casein maintenance diet employed for all of the studies was provided for at least 24 h. Then, the rats fasted overnight for 13 h from 20:00 on the day before the study day, but 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, they received either 4.3% or 17.2% casein diets (Table 1). The study-day diet was provided in 4 isonitrogenous, isonitrogenous diets, and each meal accounted for one-eighth of the rat’s total daily intake. Specifically, the casein diet was consumed beginning at 09:00 and continued at each 3-h interval until 18:00 for a total of 4 meals. The rats were allowed free access to drinking water during the experiment 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 phenylalanine kinetics with the use of L-[1-13C]phenylalanine, and continued hourly until 18:00. The rats were placed in the chamber immediately after the oral administration of the 13C substrate. Breath samples were collected, and the 13CO2 level in breath CO2 was measured at 30-min intervals from 15:00 to 19:00. Baseline breath samples were collected before the isotope protocol began at 15:00. On a later day, the rats were dissected at 18:30; blood, liver, and gastrocnemius muscle samples were collected for subsequent analysis of amino acid concentration in plasma and tissues.

**Experiment 2.** The protein intake for metabolic demands was measured using the IAAO method for rats fed the casein diets, and also for rats fed diets based on

| Time (h) | 09:00 | 12:00 | 15:00 | 16:00 | 17:00 | 18:00 | 19:00 |
|---------|--------|--------|--------|--------|--------|--------|--------|
| Exp. Diet * | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ | ▲ |
| Stable isotope § | ▪ | ▪ | ▪ | ▪ | ▪ | ▪ | ▪ |
| L-[1-13C]Phe | | | | | | | |
| NaH13CO3 | | | | | | | |
| Samples § | | | | | | | |
| Breath | □ | □ | □ | □ | □ | □ | □ |
| Blood and tissues | | | | | | | |

Fig. 1. The protocols employed for each IAAO study day. *The experimental diet was either a 4.3% or 17.2% casein diet. The diet was provided every 3 h (9:00–18:00). Each meal represented one-eighth of each rat’s daily intake. §Isotope: Priming doses of L-[1-13C]phenylalanine and NaH13CO3 were started with the third meal at 15:00, and the infusion of L-[1-13C]phenylalanine was continued hourly until the end of the study. §Sample collection: Baseline breath sample was collected before the isotope protocol began. Nine breath samples were collected every 30 min after the initiation of the isotope protocol. Samples of blood, liver, and gastrocnemius muscle were collected at 18:30.
wheat gluten instead of casein to determine whether it was important to consider the effects of the source of the protein in the diet. Sixteen rats were used, and even when they were measured for the wheat gluten diets, the 17.2% casein diet was provided as a maintenance diet for the 2 d before the study day for all of the IAAO studies. On the study day, eight rats 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/H11003 6.38) (23); and the other eight rats received one of six levels of the wheat gluten (7.2, 10.8, 14.4, 18.0, 21.6, 25.2%) diet (N/H11003 5.70) (23). Tracer administration protocol. L-[1-13C]Phenylalanine (Cambridge Isotope Laboratories, Andover, MA) and NaH13CO3 (Cambridge Isotope Laboratories) were used as tracers. Labeled compounds were dissolved in saline and stored at 4˚C. Isotopic solutions were prepared and administered in a volume of 2.5 mL/kg BW. Oral priming doses of 0.88 mg/kg BW NaH13CO3 and 7.92 mg/kg BW NaHCO3 were given with the third meal at 15:00. An oral dosing protocol of 3.3 mg/kg BW L-[1-13C]phenylalanine and 29.7 mg/kg BW phenylalanine was commenced simultaneously with the third meal, and administration of 6.0 mg/kg BW L-[1-13C]phenylalanine and 54.0 mg/kg BW phenylalanine

Table 1. Composition of experimental diets.

| Protein | Casein diet | Wheat gluten diet |
|---------|-------------|------------------|
|         | g/kg diet   | g/kg diet        |
| Casein1,2 | 4.3%  | 100 | 150 | 200 | 250 | 300 | 350 |
| Wheat gluten3,4 | 7.2% | 100 | 150 | 200 | 250 | 300 | 350 |
| Cornstarch5 | 12.9% | 100 | 150 | 200 | 250 | 300 | 350 |
| Sucrose6 | 17.2% | 100 | 150 | 200 | 250 | 300 | 350 |
| Soy bean oilb | 21.5% | 100 | 150 | 200 | 250 | 300 | 350 |
| Vitamins1,7 | 25.8% | 100 | 150 | 200 | 250 | 300 | 350 |
| Minerals1,8 | 7.2% | 100 | 150 | 200 | 250 | 300 | 350 |
| Cellulose1 | 10.8% | 100 | 150 | 200 | 250 | 300 | 350 |
| t-Phenylalanine9 | 14.4% | 100 | 150 | 200 | 250 | 300 | 350 |
| t-Tyrosine10 | 18.0% | 100 | 150 | 200 | 250 | 300 | 350 |
| 21.6% | 100 | 150 | 200 | 250 | 300 | 350 |
| 25.2% | 100 | 150 | 200 | 250 | 300 | 350 |
| Energy (kJ/g) | 15.4 | 15.4 | 15.5 | 15.5 | 15.5 | 15.6 | 15.6 |

1 Oriental Yeast Co., Ltd., Japan.
2 Protein, 86.2% (N/H11003 6.38). Amino acid (mg/100 g Casein): L-alanine, 2,700; L-arginine, 3,300; L-aspartic acid, 6,300; L-cysteine, 4,300; 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.
3 Weston Bioproducts Ltd., Queensland, Australia.
4 Protein, 72.0% (N/H11003 5.70). Amino acid (mg/100 g wheat gluten): L-alanine, 2,100; L-arginine, 2,700; L-aspartic acid, 2,700; L-cysteine, 1,600; L-glutamic acid, 29,000; L-glycine, 2,700; L-histidine, 1,800; L-isoleucine, 3,000; L-leucine, 5,400; L-lysine, 1,400; L-methionine, 1,300; L-phenylalanine, 4,100; L-proline, 11,000; L-serine, 3,600; L-threonine, 2,000; L-tryptophan, 780; L-tyrosine, 2,500; L-valine, 3,300; total, 80,980.
5 Nisshin Oillio Ltd., Japan.
6 Wako Pure Chemical Industries, Ltd., Japan.
7 AIN-76TM vitamin mixture (per g mixture): vitamin A, 400 IU; vitamin D3, 100 IU; vitamin E, 5 mg; vitamin K1, 0.005 mg; vitamin B1, 0.6 mg; vitamin B2, 0.6 mg; vitamin B6, 0.7 mg; vitamin B12, 0.001 mg; B1, 0.02 mg; folic acid, 0.2 mg; calcium pantothenate, 1.6 mg; nicotinic acid, 3 mg; choline chloride, 200 mg; sucrose, 0.968 g.
8 AIN-76TM 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.
9 t-Phenylalanine content was kept constant at 13,500 mg/kg diet in all diets, except the 25.2% wheat gluten diet (14,350 mg/kg diet).
10 t-Tyrosine content was kept constant at 15,000 mg/kg diet in all diets.
was performed hourly until the end of the study.

**Experimental diets.** The composition and source of the powdery experimental casein and wheat gluten diets are shown in Table 1. Casein and wheat gluten provided the sole source of protein in the casein and wheat gluten diets, respectively. The compositions of the amino acids in the casein and wheat gluten are shown in the footnote to Table 1 (23). 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) (24), in order to minimize the net hydroxylation of phenylalanine to tyrosine. Each casein diet with varying protein content was kept at an identical energy level by varying the levels of sugar and starch. The oil levels in the wheat gluten diet were decreased because the energy level of wheat gluten is higher than those of casein. Thus, all of the diets had a similar energy level (15.4–15.6 kJ/g).

**Breath sample collection and analysis.** The instruments used for the collection of breath samples in 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 substance, and thereafter moved back into the same chamber. Because the chambers filled with expired air were necessary in order to collect the breath samples, 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., Tokyo, Japan). Using the measurement system provided by POCone, the concentration 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 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 the rest of the mixture was 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, further normalized by each rat’s weight.

**Blood and tissue samples collection and analysis.** Blood samples drawn from the inferior vena cava were collected in tubes with heparin, and plasma was separated from the blood samples by centrifugation at 1.500 × g for 5 min. The plasma was stored at −80°C until it was analyzed. The liver and gastrocnemius muscle were rapidly removed and snap-frozen in liquid nitrogen and stored at −80°C for analysis. Approximately 0.5 g of liver and muscle were homogenized in 4.5 mL of saline, centrifuged at 1,000 × g for 10 min.

A 100 μL plasma sample and the supernatant of liver and muscle obtained as described above were deproteinized with 300 mL ethanol and centrifuged at 1,500 × g for 10 min. A 200 mL sample of the supernatant fluid was cleared of contamination by using a strong cation exchanger (AG 50W-X8, Bio-Rad Laboratories, Hercules, CA), dried under a vacuum, derived to its 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) derivative using the Waters AccQ Fluor Reagent Kit (Waters Corp., Milford, MA) and dried. Then the supernatant fluid was reconstituted in 200 μL of 0.1% formic acid. Phenylalanine and tyrosine concentrations were measured by an HPLC system. The individual amino acids were separated by an Inertsil ODS-3 column (250×4.6 mm, GL Sciences, Tokyo, Japan) with a binary LC gradient (0–60% aqueous acetonitrile containing 0.1% formic acid). The areas under the peaks were integrated using Peak Net 5.1c (Dionex Corp., Osaka, Japan). L-[1-$^{13}$C]Phenylalanine and L-[1-$^{13}$C]tyrosine enrichment in the plasma and tissue samples was analyzed with a MS (LCQ Fleet, Thermo Scientific, Waltham, MA) coupled to the HPLC system. Selected ion chromatograms were obtained by monitoring ions m/z 336 and 337 for L-phenylalanine and L-[1-$^{13}$C]phenylalanine, m/z 352 and 353 for L-tyrosine and L-[1-$^{13}$C]tyrosine, respectively.

**Statistical analysis.** Data analysis was performed using Statcel2 software (Oms Publishing Inc., Tokyo, Japan). All results were presented as the mean±SE. Values of p<0.05 were considered statistically significant. Student’s t test was used to analyze differences between two different groups, such as the protein intake. 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.

The protein intake for metabolic demands was derived by applying a mixed-effect change-point model to breath $^{13}$CO$_2$ data (25), and the regression oxidation rate of the dietary protein contents. The first regression line showed a downward slope and the second line was horizontal with minimal or no slope. The breakpoint, the protein intake with a plateau in oxidation, was regarded as the protein intake for metabolic demand.

**RESULTS**

**Experiment 1**

The rats were given free access to a 17.2% casein diet as a maintenance diet for 3 d before the first study day, and the total daily intake for each rat was 16.5±0.5 g/d (calorie, 255.9±7.8 kJ/d; protein, 2.8±0.1 g/d). The body weights for the rats used for the 4.3% and 17.2% casein diet experiments were 144.1±5.7 g and 143.5±5.0 g, respectively.

Complete data sets of 9 breath samples were obtained.
in only 7 of the rats fed the 17.2% casein diet. One rat did not consume its feed completely at 18:00, which affected the $^{13}$CO$_2$ values thereafter. Regardless of the protein intake and the 4.3% or 17.2% casein diets, breath $^{13}$CO$_2$ enrichment gradually increased after the initiation of the isotope protocol (Fig. 2). The plateau breath samples were collected during the isotopic steady state every 30 min during the period from 16:30 to 19:00 in rats fed the 17.2% casein diet, and from 17:30 to 19:00 in rats fed the 4.3% casein diet. This isotope protocol had been shown to achieve a satisfactory isotopic steady state 2.5 h after the start of L-$[1-^{13}$C]phenylalanine isotope administration. In addition, when the 4.3% casein diet was employed, the enrichment of breath $^{13}$CO$_2$ was greater than that achieved with the 17.2% casein diet, and during the period from 17:30 to 19:00, significant differences were shown between the 4.3% and 17.2% casein diets on breath $^{13}$CO$_2$ enrichment at 18:30 ($p<0.01$) and 19:00 ($p<0.01$).

The amino acid concentrations of plasma, liver and gastrocnemius muscle obtained at 18:30 on the IAAO study day are shown in Table 2. In both phenylalanine and tyrosine, $^{13}$C-amino acid concentrations, $^{12}$C-amino acid concentrations, and the total of these concentrations in the plasma and tissues of rats fed the 4.3% casein diet were similar to those of rats fed the 17.2% casein diet, and there were no significant differences.

**Experiment 2**

The rats were given free access to a 17.2% casein maintenance diet for 3 d before the first study day. The total daily intake for each rat used for the casein and wheat gluten diet experiments employing the IAAO method were 16.7±0.3 g/d (calorie, 258.9±4.3 kJ/d; protein, 2.9±0.1 g/d) and 17.3±0.5 g/d (calorie, 268.2±7.0 kJ/d; protein, 3.0±0.1 g/d), respectively. The body weights for the rats used for the 4.3, 8.6, 12.9, 17.2, 21.5, and 25.8% casein diet experiments were 149.4±5.7, 141.0±9.9, 155.2±10.8, 147.4±3.5, 158.0±2.4, and 184.7±3.1 g, respectively. The body weights for the rats used for the 7.2, 10.8, 14.4, 18.0, 21.6, and 25.2% wheat gluten diet experiments were 130.7±3.5, 148.8± 4.8, 157.1±5.4, 160.8±10.0, 134.4±2.8, and 142.4±3.0 g, respectively.

![Fig. 2. The effect of L-$[1-^{13}$C]phenylalanine infusion on $^{13}$CO$_2$ enrichment of the breath. Values are mean±SE for the 4.3% (n=8 per mean) and 17.2% (n=7 per mean) casein diets. On the study day, the rats received either a 4.3% or 17.2% casein diet every 3 h from 09:00. Each meal represented one-eighth of the rat’s total daily intake. The administrations of L-$[1-^{13}$C]phenylalanine were performed at 15:00, 16:00, 17:00, and 18:00. The establishment of a plateau in the breath samples on the basis of no significant differences among the timed samples was confirmed using repeated-measures ANOVA. The isotopic steady state was confirmed at 16:30–19:00 in rats fed the 17.2% casein diet, and at 17:30–19:00 in rats fed the 4.3% casein diet. *The asterisk marks shown significant differences (p<0.01) between the 4.3% and 17.2% casein diets at 18:30 and 19:00.](https://example.com/fig2.png)

Table 2. The concentrations of phenylalanine and tyrosine in the plasma, liver and gastrocnemius muscle in rats fed 4.3% or 17.2% casein diets.

| Diet          | $^{13}$C-Phe (Plasma nmol/mL) | $^{12}$C-Phe (Plasma nmol/mL) | Total (Plasma nmol/mL) | $^{13}$C-Tyr (Liver nmol/g) | $^{12}$C-Tyr (Liver nmol/g) | Total (Liver nmol/g) | $^{13}$C-Phe (Gastrocnemius muscle nmol/g) | $^{12}$C-Phe (Gastrocnemius muscle nmol/g) | Total (Gastrocnemius muscle nmol/g) |
|---------------|------------------------------|------------------------------|------------------------|-----------------------------|-----------------------------|---------------------|-------------------------------------------|-------------------------------------------|--------------------------------------|
| 4.3% casein   | 13.2±2.9                      | 47.2±4.3                     | 60.4±7.0               | 7.5±2.0                     | 113.0±29.4                  | 120.6±30.7          | 13.0±1.7                                 | 46.6±4.5                                 | 59.6±5.7                             |
| 17.2% casein  | 12.1±2.5                      | 50.8±10.0                    | 62.9±11.8              | 8.5±1.4                     | 119.8±15.2                  | 128.3±16.2          | 11.6±1.9                                 | 48.2±2.5                                 | 59.8±3.6                             |
| 4.3% casein   | 10.6±0.4                      | 40.9±5.1                     | 51.5±4.9               | 7.4±1.3                     | 99.4±32.0                   | 106.9±33.2          | 16.2±1.9                                 | 75.6±12.3                                | 91.9±8.7                             |
| 17.2% casein  | 10.4±2.1                      | 43.1±10.5                    | 53.6±12.3              | 8.2±2.8                     | 92.5±7.5                    | 101.4±9.2           | 30.7±1.1                                 | 84.7±5.8                                 | 115.4±7.5                            |
| 4.3% casein   | 13.0±1.7                      | 46.6±4.5                     | 59.6±5.7               | 8.4±0.8                     | 91.9±8.7                    | 100.3±8.5           | 32.0±1.1                                 | 84.7±5.8                                 | 116.4±7.5                            |
| 17.2% casein  | 11.6±1.9                      | 48.2±2.5                     | 59.8±3.6               | 7.0±1.1                     | 84.7±5.8                    | 101.4±9.2           | 15.2±1.9                                 | 64.2±5.8                                 | 79.4±7.5                             |

Values are shown as mean±SE for the 4.3% (n=5) and 17.2% (n=5) casein diets. Student’s t test was performed to assess the effect of protein intake. No significant differences were demonstrated in the plasma and tissues phenylalanine or tyrosine concentrations between the 4.3% and 17.2% casein diets.

$^{13}$C-Phe, L-$[1-^{13}$C]phenylalanine; $^{12}$C-Phe, L-$[1-^{12}$C]phenylalanine; $^{13}$C-Tyr, L-$[1-^{13}$C]tyrosine; $^{12}$C-Tyr, L-$[1-^{12}$C]tyrosine.
According to procedures recommended by the AIN, the breath 13CO2 data, which were representative of the metabolic demands. The linear regression equation for the estimated protein intake for metabolic demands for the casein diet is as follows: $y=10.73-0.35x$ and $y=6.17$, for the wheat gluten diet is as follows: $y=18.87-0.66x$ and $y=6.92$, for the downward slope of the line and the level part of the line, respectively. The protein (%) included in the casein and wheat gluten diets was converted into protein intake (g) per day, and further normalized according to each rat's body weight. The mean protein intakes for metabolic demands for the casein and wheat gluten diets were estimated to be 13.1 g/kg BW/d and 18.1 g/kg BW/d, respectively.

**Figure 3**

The relationship between the intake of various proteins and the production of 13CO2 from the oxidation of L-[1-13C]phenylalanine when the rats were fed a casein diet (n=8 per mean) or a wheat gluten diet (n=8 per mean). Values are shown as mean±SE. The breakpoint estimates the mean protein intake for metabolic demands. The linear regression equation for the estimated protein intake for metabolic demands for the casein diet is as follows: $y=10.73-0.35x$ and $y=6.17$, for the wheat gluten diet is as follows: $y=18.87-0.66x$ and $y=6.92$, for the downward slope of the line and the level part of the line, respectively. The protein (%) included in the casein and wheat gluten diets was converted into protein intake (g) per day, and further normalized according to each rat’s body weight. The mean protein intakes for metabolic demands for the casein and wheat gluten diets were estimated to be 13.1 g/kg BW/d and 18.1 g/kg BW/d, respectively.

**DISCUSSION**

In the current IAAO study on rats, the protein intake for metabolic demands was estimated to be covered by a 13.1 g/kg BW/d for casein. This result was similar to the value recommended by the AIN-93G diet for laboratory rodents (20% casein (≥85% protein)), which was developed based on nitrogen balance studies. According to procedures recommended by the AIN, values were converted to dietary content by assuming a dietary intake of 15 g/rat/d for growing rats, and also for the rats fed 16.7 ± 0.3 g/rat/d in the present study. This is the first study conducted that employed the IAAO method to determine the protein intake for metabolic demands using protein itself in rats, and the determined the protein intake for metabolic demands should be considered provisional.

Temperature, age, and physical activity influence the energy requirements of rats. It is difficult to estimate the energy requirement for growth due to variations in the composition of weight gain (26–30) and variations in the energetic efficiency of net protein and fat synthesis. However, it has been suggested that rats will generally consume enough food to meet their energy requirements (31, 32). The AIN-93 specifications indicate that a diet containing at least 15.0 kJ/g will meet the energy requirement for maintenance and growth if the rats are allowed free access to food and the diet is not deficient in other nutrients. In the present study, the rats accepted a 15.5 kJ/g diet containing 17.2% casein as a maintenance diet. Furthermore, the rats were given free access to this diet and the 24-h dietary intake was regarded as an individual rat’s energy requirement.

Humayun et al. (19) reevaluated the protein requirement in young men employing the IAAO method, and the protein source of the experimental diet was consumed hourly in small meals consisting of a crystalline amino acid mixture. In the present study, casein and wheat gluten were employed as the protein source, and therefore, as the rats consumed the experimental diet at 3-h intervals, it can be considered that the mechanism of assimilation differed from that of the amino acid mixture. Amino acid mixtures will be absorbed very rapidly, and protein utilization will show a lower efficiency, compared with slow proteins such as casein (20).

The phenylalanine and L-[1-13C]phenylalanine concentrations in the plasma, liver and gastrocnemius 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. After phenylalanine is hydroxylated, conversion to tyrosine takes place, so the tyrosine concentration was also examined. In comparison with the ratio of L-[1-13C]phenylalanine to the total phenylalanine concentration, only a trace of L-[1-13C]tyrosine to the total tyrosine occurred in the plasma and tissues, regardless of the protein intake, suggesting that there was no tyrosine deficiency. In previous studies, the loss of the 13C into the protein-bound tyrosine pool or tyrosine metabolites was minimized by providing a high-tyrosine diet before the study (19).

13CO2 breath tests are normally performed in the presence of a large background of naturally occurring isotope of approximately 1.1% 13C (33). The 13C rate of any unlabeled substrate ingested during a 13CO2 breath test must be considered in order to eliminate artifacts that may reduce the sensitivity of the breath test and produce erroneous results (33). In our preliminary
examination, the stable rate of $^{13}$CO$_2$ production in breath was achieved between 5 h and 6 h and maintained until the end of the study. These results suggested that two meals received every 3 h were required to achieve constant $^{13}$CO$_2$ enrichment, and that the effect of the $^{13}$C infusion could be evaluated correctly after the third meal at 15:00.

Experiment 1 demonstrated a similar pattern and a latter steady state ~2.5 h after the start of the stable isotope protocol (Fig. 2), so breath samples for the measurement of the protein metabolism were collected 210 min after the administration of the stable isotope began. Moreover, the protein intake level, the 4.3% or 17.2% casein diets, had a significant effect on breath $^{13}$CO$_2$ concentration at 18:30, showing that this protocol could detect differences in protein metabolism. These results reflected the supposition that if one indispensable amino acid (limiting) was deficient for protein synthesis, then all other indispensable amino acids (including the indicator amino acid, $[^{13}$C]phenylalanine) would be oxidized. Therefore, when the rats were fed a low protein diet, the 4.3% casein diet, most of amino acids were oxidized, and the $^{13}$CO$_2$ concentration in breath increased. By increasing the protein intake with the 17.2% casein diet, the intake of the limiting amino acid also increased, and the values produced by the IAAO method decreased, reflecting the increasing incorporation into protein.

The mean protein intakes for metabolic demands determined by the IAAO method were 13.1 g/kg BW/d for the casein and 18.1 g/kg BW/d for the wheat gluten. Therefore, the protein intakes for metabolic demands based on wheat gluten was higher than that based on casein. The differences between the casein and wheat gluten 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. These results also conformed with our hypothesis, that the protein requirement will decrease with good quality (amino acid scoring pattern) protein intake, and increase with poor quality protein intake, validating the concept that the IAAO method could be employed to evaluate the quality of protein.

In regard to the measured phenylalanine oxidation, the enrichment of breath $^{13}$CO$_2$ differed between the rats fed the casein and wheat gluten diets. The enrichment of breath $^{13}$CO$_2$ was consistently higher in rats fed the wheat gluten diet, compared with rats fed the casein diet, even at the plateau line with a protein intake more than the metabolic demand for protein. According to intake of protein, specifically, the limiting amino acid, the indicator amino acid is partitioned between incorporation into proteins and oxidation. The quality of the protein also affected the $^{13}$CO$_2$ volume in the breath. Future extensions of this study to other protein sources will be necessary in order to confirm this relationship.

Hegsted (34) suggested the necessity of taking account of adaptation in their nitrogen balance methods, arguing that prior adaptation is required. The IAAO method can be conducted in short time periods because no period of adaptation to each intake is employed (35). Therefore, the IAAO method could be employed to evaluate the metabolic protein demand for all age groups (infants, children, adolescents, adults, and the elderly), as well as for post-operative patients or patients with injuries or infections that have specific metabolic conditions, such as a widely varying metabolic demand. In a clinical setting, the adequate quality and quantity of protein or amino acid for each specific condition could be estimated using the IAAO method.

The results of this study demonstrated that the IAAO method can be employed to evaluate not only the protein intake for metabolic demands, but the dietary protein quality in freely living rats. Further studies are necessary to assess the viability of the IAAO method in a clinical setting.

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