

# Human adult amino acid requirements: [1-<sup>13</sup>C]leucine balance evaluation of the efficiency of utilization and apparent requirements for wheat protein and lysine compared with those for milk protein in healthy adults<sup>1-3</sup>

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## ABSTRACT

**Background:** There is considerable debate about the human lysine requirement and the consequent nutritional value of wheat protein.

**Objective:** We used a novel [1-<sup>13</sup>C]leucine balance protocol to examine whether adaptive mechanisms to conserve lysine allow wheat to be utilized more efficiently than expected according to current estimates of lysine requirements and wheat utilization.

**Design:** Wheat and milk proteins were compared in 6 adults infused for 9 h with L-[1-<sup>13</sup>C]leucine in the postabsorptive state (0–3 h), who were fed half-hourly with low-protein (2% of energy, 3–6 h) and isoenergetic higher-protein (12–13% of energy, 6–9 h) meals providing maintenance energy intakes. From acute measurements of [1-<sup>13</sup>C]leucine balance, we predicted nitrogen balance, the metabolic demand for protein, the efficiency of postprandial protein utilization (PPU), and the requirements for wheat protein and lysine.

**Results:** Leucine balance was higher after the milk than after the wheat feeding because of the greater inhibition of proteolysis by milk. PPU, calculated as the ratio of  $\Delta$ nitrogen balance to  $\Delta$ nitrogen intake between the low-protein and higher-protein periods, was  $0.68 \pm 0.06$  for wheat and  $1.00 \pm 0.09$  for milk ( $P \leq 0.001$ ). The estimated average wheat protein requirement (0.6/PPU) was  $0.89 \pm 0.08 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ , indicating a lysine requirement of  $23.2 \pm 2.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ . The measured PPU for wheat,  $0.68 \pm 0.06$ , was higher than the value calculated from wheat lysine intake and milk protein lysine deposition,  $0.26 \pm 0.02$ , and higher than predicted by most published estimates of lysine requirements, apart from a value of 19 mg/kg indicated by nitrogen balance studies.

**Conclusions:** The results show that adaptive mechanisms of lysine conservation allow wheat protein to be utilized more efficiently than expected. *Am J Clin Nutr* 2000;72:112–21.

**KEY WORDS** Healthy adults, protein quality, protein turnover, stable isotopes, nitrogen balance, amino acid requirements, wheat protein, dairy protein, milk, lysine, leucine

## INTRODUCTION

Currently, there is great interest in the magnitude of the nutritional requirement for lysine. This is because the importance of protein quality in human nutrition has been under intensive debate after the rejection in 1991 by the Food and Agriculture Organization of the World Health Organization (FAO/WHO; 1) of the adult amino

acid-scoring pattern proposed previously by FAO/WHO/United Nations University (UNU) (2) for use in the assessment of protein quality. Because cereal proteins differ most from animal proteins in terms of lysine content, with the lysine content of wheat protein only one-third of that in meat or milk, the nutritional value of cereal proteins, the major world protein source, has become the focus of concern (3, 4). The FAO/WHO was unable to identify an unequivocally acceptable alternative set of amino acid requirement values and consequent scoring pattern for older children and adults. However, in an attempt to resolve this issue, several reports have been published that provide new data relating to adult amino acid requirements (5–7). We have argued that the evaluation of protein quality in humans is a complex issue because the requirements for lysine and other indispensable amino acids (IAAs) include an adaptive component that is likely to vary according to the magnitude and nature of the habitual protein intake (8–10). We have also suggested that there are mechanisms that allow some recycling of lysine and threonine during the daily losses and gains that occur with the fasting-feeding cycle in adults in overall balance, which would result in better-than-predicted utilization of proteins such as that in wheat and a lower requirement for wheat than would be predicted (10, 11). We report here [1-<sup>13</sup>C]leucine balance studies designed to examine this hypothesis by evaluating the utilization of wheat compared with milk protein in the context of an adaptive model of protein homeostasis. The experimental approach also allows calculation of an estimated average requirement for lysine.

## SUBJECTS AND METHODS

### Experimental design

Our evaluation of the metabolic demand (MD) for protein, the efficiency of postprandial protein utilization (PPU), and the

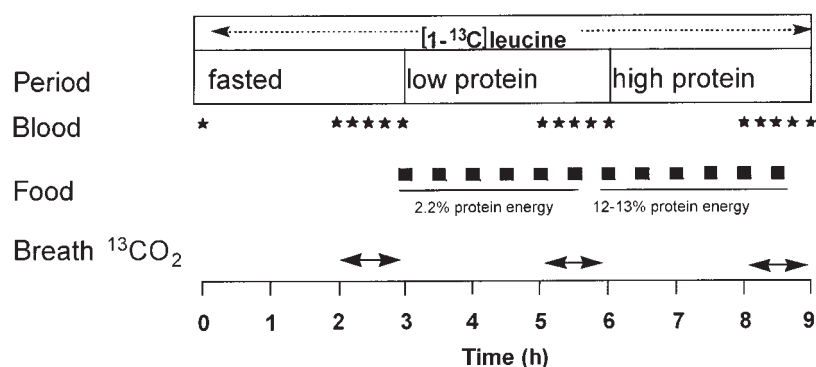
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**FIGURE 1.** Protocol for the study. Infusion started after an overnight fast and sampling times within each period are shown for blood, breath, and carbon dioxide production. Meals, either the milk or the bread slices and the drink, were given at the times shown.

apparent protein requirement involved a leucine balance protocol that was discussed extensively elsewhere (12–15). A steady state  $[1-^{13}\text{C}]$ leucine balance protocol with 3 phases was used: 1) a postabsorptive (PA) phase; 2) a low-protein-meal (LP) phase with frequent, small meals; and 3) an isoenergetic higher-protein-meal (HP) phase, also with frequent, small meals. The protein source under examination was provided in the LP and HP phases. The leucine balance measured in each phase allowed the MD for protein to be determined from the postabsorptive losses, PPU to be calculated from the slope of the leucine intake–balance curve, and the apparent protein requirement to be calculated as MD/PPU. As discussed below, the model was developed further here in terms of calculating 1) true PPU independent of the leucine content of the test proteins, 2) a predicted PPU, and 3) a requirement for the limiting amino acid of the protein of interest.

### Subjects

Six subjects, 4 men and 2 women in good general health, were studied on 2 occasions. The time between studies varied from 3 to 12 mo but no significant changes in diet or body weight occurred during this period. The subjects were aged  $32.0 \pm 11.4$  y, weighed  $62.9 \pm 8.4$  kg, and had a mean body mass index (BMI;  $\text{kg}/\text{m}^2$ ) of  $21.5 \pm 2.2$ . Before the studies, no restriction was placed on activities of daily living but subjects were encouraged to regulate their eating pattern to 12 h of feeding and 12 h of fasting for the week before study, starting at 0900 daily. The study was approved by the University of Surrey ethical committee and all subjects gave informed consent after the nature of the protocol had been fully explained to them.

Because the metabolic studies required feeding in amounts that represented standardized maintenance energy intake and habitual protein intakes, estimates of these values were needed. The resting metabolic rate (RMR) was measured with a ventilated-hood indirect calorimeter. Habitual dietary protein intake was calculated from three 24-h measurements of total urinary nitrogen excretion on the assumption that subjects were in nitrogen equilibrium—ie, total nitrogen loss was the same as nitrogen intake. Total nitrogen excretion was estimated from measured urinary and estimated fecal and surface nitrogen losses on the assumption that the diet was 95% digestible (1), the obligatory fecal nitrogen loss was 12 mg N/kg, and the surface nitrogen loss was 8 mg N/kg (2).

Timed, 24-h urine collections were taken from all subjects in preweighed plastic, lidded containers with 5 mL of 11 mol

HCl/L as a preservative; a sample of the urine was refrigerated ( $4^\circ\text{C}$ ) until analyzed for nitrogen by using a semiautomated Kjeldahl method (Tecator Kjeltac Auto 1030 Analyzer; Tecator, Hoganas, Sweden). Mean protein intake estimated in this way was  $1.19 \pm 0.07$   $\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ .

### Infusions

The volunteers were asked to complete their last meal at home by 2100 and to report to the metabolic ward at 0700–0730. Intravenous cannulas were inserted into superficial veins of both arms or hands, one allowing continuous infusion of the tracer, the other allowing repeated blood sampling. The blood for sampling was arterialized by placing the hand in a heated chamber. After collection of baseline blood and expired breath samples (in duplicate), priming doses of  $\text{NaH}^{13}\text{CO}_2$  (0.2 mg/kg) and L- $[1-^{13}\text{C}]$ leucine (99%  $^{13}\text{C}$ ; Masstrace, Boston) were given. The wheat studies were performed after the milk studies, and when we reviewed the need for isotope, we decided that the tracer infusion rate could be halved from 1 to 0.5  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  without sacrificing precision. Thus, priming doses of L- $[1-^{13}\text{C}]$ leucine were either 0.5 or 1 mg/kg. These were followed immediately by a continuous infusion of L- $[1-^{13}\text{C}]$ leucine (either 0.5 or 1  $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) for 9 h, starting in most cases at 0800 (11 h postabsorptive).

The experimental protocol is shown in **Figure 1**. Blood and expired breath samples (in duplicate) were collected every 15 min throughout the last hour of each 3-h phase. Concentrations of glucose, KIC, leucine, and lysine (wheat studies only) and plasma  $\alpha$ -ketoisocaproate (KIC)  $^{13}\text{C}$  enrichment were measured at the beginning and end of each 3 h, insulin was measured at 2 and 3 h of each 3-h period. Total carbon dioxide production rates were measured for  $\geq 60$  min during each phase with a ventilated-hood indirect calorimeter.

### Meals

The milk and wheat feedings used in these studies were isoenergetic, as were the LP and HP feedings during each individual study. Subjects were fed every 30 min to maintain a metabolic steady state during each 3-h feeding phase. The milk feedings were formulated from a carbohydrate source naturally low in  $^{13}\text{C}$  (potato dextrose; Avebe, Veendam, Holland), double cream, and either full-cream milk (LP diet) or skim milk (HP diet). The wheat feedings were made from stone-ground, soy-free,

whole-meal wheat bread (Baker's Choice, St Michael, United Kingdom), margarine (Sainsbury's, London), and the potato dextrose and were served as crust-free bread slices with margarine and a drink of the potato dextrose dissolved in water flavored with some sugar-free orange soda (Tesco's Finest, Tesco, London). Because the baseline  $^{13}\text{C}$  enrichment of the bicarbonate pool may be affected by the abundance of  $^{13}\text{C}$  in the meal, measurements were made of  $^{13}\text{CO}_2$  enrichment of the breath in subjects fed without any L-[ $^{13}\text{C}$ ]leucine infusion. For both wheat and milk, the  $^{13}\text{CO}_2$  enrichment of the breath was not significantly different from baseline.

The LP and HP diets were individually formulated for each subject to be isoenergetic but to vary between subjects to provide overall energy intakes at an hourly intake equivalent to one-twelfth (ie, 50% in total during the 6-h feeding) of the subject's estimated daily maintenance energy requirements, calculated as  $1.4 \times$  predicted RMR for each subject (14). Mean energy intakes were  $77.3 \pm 5.8$  kJ/kg per 6-h infusion. The protein intakes in the HP phase were calculated to provide an hourly intake, that, if consumed for 12 h would provide the subjects' habitual protein intake ( $1.19 \pm 0.06$  g  $\cdot$  kg $^{-1} \cdot$  d $^{-1}$ ) with the LP intake equivalent to  $0.2$  g  $\cdot$  kg $^{-1} \cdot$  d $^{-1}$ . The macronutrient contents of the milk and wheat meals were similar with carbohydrate fixed at 60% of energy and with dietary fat and protein exchanged isoenergetically in the LP and HP formulations so that protein accounted for 2–3% and 12–14% of the energy in the LP and HP formulations, respectively. The macronutrient contents of the meals are shown in Table 1. Samples of each diet were analyzed for total nitrogen by Kjeldahl analysis. The leucine-nitrogen ratios of the milk and wheat proteins were determined by gas chromatography–mass spectrometry (GC-MS) analysis of an acid-hydrolyzed milk sample (for leucine) and Kjeldahl analysis (for nitrogen). The results of these analyses indicated the leucine contents of the milk and wheat diets to be 638 and 421 mg leucine/g N, respectively.

### Sample analysis

Blood glucose was measured by using a standard automated hexokinase technique and plasma insulin was measured by using a double-antibody, dual-incubation polyethylene glycol assay with [ $^{125}\text{I}$ ]insulin and a monoclonal anti-insulin (donkey anti-guinea pig) antiserum as the second antiserum.

Plasma KIC was derivatized to its trimethylsilyl derivative with ketovaleric acid (10 ng) acting as an internal standard. The  $^{13}\text{C}$  enrichment of KIC was measured on an MD800 (Fisons Instruments, VG Scientific, East Grinstead, United Kingdom) GC-MS analyzer in selective ion recording mode with monitoring at mass-to-charge ratios ( $m/z$ ) of 232.1 and 233.1 under electron impact ionization conditions. Plasma leucine was derivatized to its *tert*-butyldimethylsilyl derivative with monitoring at  $m/z$  302.2 and 303.2. All values were corrected for the respective calibration curves.

Expired breath  $^{13}\text{CO}_2$  enrichment was measured in samples collected before the tracer infusion and at every 15 min during the last hour of each 3-h phase. These were collected in a 2-L latex bag and were immediately transferred into 20-mL Vacutainers (Becton Dickinson Vacutainer Systems Europe, Crowley, Oxford, United Kingdom). The  $^{13}\text{C}$  enrichment in the expired carbon dioxide was measured with a dual-inlet, double collector isotope ratio mass spectrometer (Finnigan Delta S; Finnigan MAT, Bremen, Germany) and all measurements were made within 24 h of gas collection.

**TABLE 1**

Total energy and macronutrient contents of wheat and milk meals<sup>1</sup>

Macronutrient and meal type	Wheat	Milk
Total energy (kJ/kg)		
LP	6.5 $\pm$ 0.5	6.4 $\pm$ 0.4
HP	6.4 $\pm$ 0.5	6.3 $\pm$ 0.6
Protein (% of energy)		
LP	2.1 $\pm$ 0.1	2.2 $\pm$ 0.1
HP	11.9 $\pm$ 0.4	13.2 $\pm$ 0.6
Carbohydrate (% of energy)		
LP	61.6 $\pm$ 3.9	60.2 $\pm$ 4.8
HP	61.5 $\pm$ 4.2	60.1 $\pm$ 3.9
Fat (% of energy)		
LP	36.3 $\pm$ 2.7	37.6 $\pm$ 2.9
HP	26.6 $\pm$ 2.9	26.7 $\pm$ 1.8

<sup>1</sup> $\bar{x} \pm$  SD;  $n = 6$ .

### Calculations

#### Leucine kinetics

Leucine kinetics were calculated from the KIC plateau enrichment by using a 2-pool model assuming an isotopic and metabolic steady state (16). This was established from inspection of the slope of the regression of the plasma KIC enrichment and concentration against time in the 5 blood samples taken during the last hour of each 3-h period. In each case, the slope of the line was not different from zero and values were calculated from the mean of the 5 points.

The flux ( $Q$ ) of the amino acid was determined from tracer dilution as follows:

$$Q = i (MPE_i/MPE_p) \quad (1)$$

where  $i$  is the tracer infusion rate ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ),  $MPE_i$  the  $^{13}\text{C}$  enrichment of the infused tracer, and  $MPE_p$  is the  $^{13}\text{C}$  enrichment of plasma [ $^{13}\text{C}$ ]KIC at plateau. Leucine oxidation was calculated from the excretion rate of  $^{13}\text{CO}_2$  and the  $^{13}\text{C}$  enrichment of plasma KIC as follows:

$$O = F^{13}\text{CO}_2/(R \times MPE_p) \times 100 \quad (2)$$

where  $F^{13}\text{CO}_2$  is the  $^{13}\text{CO}_2$  excretion rate in  $\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  and  $R$  is the fraction that accounts for  $^{13}\text{CO}_2$  recovery during the study. Previous measurements of  $R$  during  $4 \times 4$  h fast-and-feed studies (17) produced values of 0.76 (fasted) and 0.91 (fed); we used these values here.

The relation between leucine flux and individual components of protein turnover during the steady state is represented by the equation

$$Q = D + I + i = S + O \quad (3)$$

where  $D$  is endogenous appearance (proteolysis),  $I$  is dietary leucine intake,  $S$  is nonoxidative disappearance (protein synthesis), and  $O$  is leucine oxidation, all values in  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ . Thus,

$$D = Q - (I + i) \quad (4)$$

and

$$S = Q - O \quad (5)$$

Leucine balance ( $B$ ) was calculated as intake ( $I + i$ ) minus oxidation ( $O$ ); all values are expressed as  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ .

### Leucine balance and metabolic demands

Leucine balance (digestible leucine intake – leucine oxidation) is assumed to indicate protein-bound leucine balance. The metabolic model views daily balance as a cycle of postprandial net protein gain and postabsorptive net protein loss (12, 18, 19). The MD for protein in subjects in overall balance involves metabolic consumption of amino acids by both obligatory and adaptive pathways throughout the day and is met by tissue protein in the postabsorptive state and by food in the postprandial state (11). Daily MD is calculated as  $24 \times$  hourly postabsorptive loss (14, 15), assuming a tissue protein leucine content of 3.93 mmol leucine/g N (18). This is the same as the sum of net protein gain and endogenous and adaptive protein oxidation in the postprandial state in subjects in overall balance (11).

### Efficiency of protein utilization

PPU can be calculated from the slope of the leucine balance–intake relation but there are 2 issues that influence how this is done. First, the changes in balance between the PA and LP phases and between the LP and HP phases reflect physiologically different processes. The PA-LP transition involves a reduction in postabsorptive loss, in part through a reduction in amino acid oxidation, and reflects the sensitivity of proteolysis to insulin. The slope of this phase can exceed unity. The LP-HP transition involves mainly amino acid–mediated net protein deposition. PPU can be calculated as either  $PPU_{\text{meal}}$  from  $\Delta\text{balance PA-HP}$ , reflecting the combined influence of energy (PA-LP) and protein (LP-HP), or as  $PPU_{\text{protein}}$  from  $\Delta\text{balance LP-HP}$ , reflecting only amino acid supply.  $PPU_{\text{meal}}$  is more appropriate for comparisons between subjects with similar high-quality protein intakes, such as young and elderly (14, 15) subjects, between whom differences may exist in insulin sensitivity that could influence the PA-LP transition.  $PPU_{\text{protein}}$  is only influenced by protein intake and is more appropriate for between-subject comparisons of protein quality, such as in this study. This latter value will be used here.

Second, although PPU calculated from leucine balance does allow comparisons of relative rates of protein utilization of different subject groups fed the same protein, as we have reported (14, 15), PPU calculated in this way may differ from the true value for protein utilization. This leucine utilization value ( $\Delta\text{balance}/\Delta\text{intake}$ ) does not predict protein or nitrogen utilization when the ratio of leucine to nitrogen content of balance (tissue protein) differs from the ratio of leucine to nitrogen content of the intake. This is the case with milk, which is relatively leucine rich (4.89  $\mu\text{mol}$  leucine/mg N) compared with tissue protein (3.93  $\mu\text{mol}$  leucine/mg N) (18), and with wheat, which is relatively leucine poor (3.21  $\mu\text{mol}$  leucine/mg N). Thus, when all milk protein is deposited, some excess leucine remains to either expand the free pool or be oxidized, so milk leucine utilization (leucine balance/leucine intake) will underestimate milk protein utilization (milk protein balance/protein intake). For wheat, the opposite is the case, ie, all of the leucine could be utilized with no increased oxidation when protein utilization is <100%.

Thus, protein utilization in these studies is more accurately calculated from protein or nitrogen balance. This can be done because the nitrogen intake is known and nitrogen deposition can be predicted from leucine deposition assuming only 1) that leucine balance (intake – oxidation) represents leucine in tissue protein and 2) a value for the leucine nitrogen content of tissue

protein. Although this value is not known with certainty, the composition of tissue proteins in the main organs (eg, liver and muscle) does not differ significantly in terms of the leucine-nitrogen ratio, so that a reasonable estimate can be made. Thus, changes in nitrogen balance are calculated from measured nitrogen intake and deposition predicted from leucine balance. Thus,  $PPU_{\text{nitrogen}}$  is calculated as  $\Delta\text{nitrogen balance}/\Delta\text{nitrogen intake}$  for the LP-HP transition. This is the appropriate and best estimate that can be made of PPU in these studies and is certainly more accurate than PPU based only on leucine balance.

### Predicted PPU of wheat protein

The magnitude of the PPU of wheat protein allows examination of the extent to which wheat protein utilization is limited by amino acid content or whether adaptive recycling of lysine can occur from the free pool. Thus, lysine deposition from the milk protein can be calculated from leucine balance and the leucine-lysine ratio of tissue protein. The lysine content of the wheat meal as a fraction of the lysine deposited from the milk meals should indicate the theoretical PPU of wheat. Ideally, in such a comparison, nitrogen intakes should be exactly the same in each case but to allow for small differences, as in the current studies, lysine intake and deposition were adjusted for nitrogen intake. Thus, the value is calculated as follows:

$$\begin{aligned} \text{Predicted PPU} &= \Delta\text{wheat lysine intake (LP-HP)} \\ &\quad (\mu\text{mol} \cdot \text{g N}^{-1} \cdot \text{h}^{-1}) \\ &\quad / \Delta\text{tissue protein lysine} \\ &\quad \text{deposition from milk (LP-HP)} \\ &\quad (\mu\text{mol} \cdot \text{g N milk intake}^{-1} \cdot \text{h}^{-1}) \end{aligned} \quad (6)$$

with the lysine intakes and deposition rates calculated by assuming leucine-lysine ratios in wheat and tissue protein to be 0.320 and 1.00, respectively (20).

### Requirements for wheat protein and lysine

PPU together with the MD estimated as described above enable an apparent protein requirement to be calculated as  $\text{MD}/PPU_{\text{nitrogen}}$ . This is an estimate of the requirements for the protein source under the specific nutritional conditions of the study, ie, during consumption of frequent small meals in subjects at rest. The  $PPU_{\text{nitrogen}}$  for wheat also allows calculation of an estimated average requirement for both wheat protein and lysine. The currently accepted estimated average requirement (EAR) value for a high-quality protein is 0.6  $\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  (2). It can be assumed that this would be met by 0.6 g milk protein. Thus, the wheat protein necessary to meet this requirement is indicated by the relative efficiency of utilization of wheat compared with milk in our studies, ie,  $\text{EAR of wheat} = 0.6 \times \text{PPU of wheat}/\text{PPU of milk}$ . Because it is generally accepted that lysine limits wheat utilization, the lysine requirement indicated by our studies can be calculated as the lysine content of the EAR of wheat.

### Statistics

Values are expressed as means  $\pm$  SDs. All data involving repeated measurements during the 3 phases of the infusion during the 2 diets were analyzed by a two-way fixed-effects multivariate analysis of variance (MANOVA), with diet and period analyzed as within-subject, repeated measures both separately and together to identify interactions between them. For those measures for which there was a significant interaction, post hoc testing of the individual means was performed with the Tukey test. Those measures with diet as the

**TABLE 2**

Comparison of the biochemical responses to intermittent feeding of wheat or milk meals fed as low (LP) or high (HP) amounts of protein compared with the postabsorptive (PA) state<sup>1</sup>

Biochemical measure and period	Wheat	Milk
Glucose (mmol/L)		
PA	4.5 ± 0.6	4.0 ± 0.4
LP	5.4 ± 0.1	5.1 ± 1.1
HP	5.4 ± 0.3	5.0 ± 0.9
Insulin (pmol/L)		
PA	28 ± 6	24 ± 7
LP	215 ± 63	231 ± 123
HP	183 ± 54	236 ± 86
Leucine (μmol/L)		
PA	90 ± 18	89 ± 13
LP	59 ± 11	63 ± 13
HP	91 ± 18	96 ± 27
KIC (μmol/L)		
PA	72 ± 3	86 ± 16
LP	42 ± 4	64 ± 17
HP	56 ± 12	70 ± 21
Lysine (μmol/L)		
PA	121 ± 41	—
LP	83 ± 13	—
HP	80 ± 15	—
Carbon dioxide production (mL/min)		
PA	188 ± 19	171 ± 38
LP	226 ± 15	210 ± 31
HP	229 ± 18	208 ± 31

<sup>1</sup> $\bar{x} \pm SD$ ;  $n = 6$ . Measurements made during the third hour of the PA period or after feeding of frequent, small LP or HP meals. There were significant effects of period for all biochemical measures,  $P < 0.05$ . KIC,  $\alpha$ -ketoisocaproate.

only variable were analyzed by paired  $t$  test. We used STATISTICA for WINDOWS (StatSoft, Tulsa, OK) for the statistical analysis.

## RESULTS

### Hormonal, biochemical, and physiologic responses

No differences in postabsorptive glucose or insulin concentrations were detected between the milk and wheat studies. These are shown in **Table 2** as the mean values of 2 measurements made at the beginning and end of the final hour of each phase. Increases occurred in both glucose and insulin concentrations during the LP phase and the higher concentrations were maintained throughout feeding with no significant differences between the LP and HP phases. There were no differences between the glucose or insulin responses to the milk and wheat meals.

The measurements of leucine and KIC concentrations indicated that a metabolic steady state was achieved at the end of each 3-h feeding phase because there were no changes in concentrations during the final hour. There were no significant differences between the milk and wheat studies in the postabsorptive concentrations of either leucine or KIC. Thus, for each protein source, leucine concentrations fell when the LP meals were fed and increased after the HP meals were fed to mean values similar to baseline concentrations. The responses of the KIC concentrations were also similar for each protein source, ie, falling after the LP meals and increasing after the

HP meals. Lysine concentrations were measured only in the wheat studies. Concentrations fell after the meals but were not influenced by the amount of protein fed. Carbon dioxide production increased after the meals feeding but was not influenced by the amount of protein fed.

### Leucine intake, oxidation, and turnover

Leucine intakes (**Table 3**) are shown as digestible leucine intake, assuming digestibility to be 93% for wheat (21) and 100% for milk. Intakes include tracer at concentrations shown by PA values. Although the diets for each phase were designed to be isonitrogenous, the measured nitrogen value of the wheat was slightly lower than expected so that wheat nitrogen intakes were 16% lower than those for milk (**Table 4**). Because of the lower leucine-nitrogen ratio of wheat than for milk (3.2 and 4.9 μmol leucine/g N), leucine intakes from the wheat meals were only 50% of that from milk. There was a plateau enrichment of [<sup>13</sup>C]KIC and carbon dioxide observed during the last hour of each feeding phase in all subjects (**Figure 2**). PA leucine oxidation rates were lower for the wheat than the milk studies (**Table 3**). However, because the tracer contributes to the oxidation rate, the lower amount of tracer with the wheat accounted for the lower oxidation rate. Net oxidation and leucine balance did not differ significantly between wheat and milk. There was a significant effect of period and diet on leucine oxidation, explained by the higher rates in the milk series at each period and by the higher oxidation rate in the milk HP period than in the LP and PA periods.

Leucine balance (**Table 3**) became less negative with LP feeding and became positive with HP feeding. However, the magnitude of the positive balance after the HP-milk meals was considerably greater than that after the wheat meals. In the PA state, there were no differences in the flux or derived rates of protein synthesis and proteolysis (**Table 3**). The response of the flux to feeding did not differ between the milk and wheat diets, falling after the LP meals and increasing after the HP meals.

Similarly, the pattern of response of protein synthesis was not influenced by diet but was influenced by period with rates falling with the LP meals and increasing with the HP meals. For proteolysis, rates fell with feeding and although there was no simple influence of diet on the pattern of this response, there was a significant diet-period interaction. This was due to a greater inhibition of proteolysis by milk than by wheat in the HP period. Thus, the higher balance achieved after the HP-milk meals was a result of the higher leucine intake and the greater inhibition of proteolysis with the HP-milk meals than with the LP-milk meals.

### Efficiency of protein utilization

As indicated above, the measured nitrogen value of the wheat was slightly lower than expected so that wheat nitrogen intakes were 16% lower than those from milk (**Table 4**). Nitrogen balance was calculated from leucine balance so that it varied as leucine balance, ie, it became less negative with LP feeding and became positive with HP feeding, with the milk meals inducing a higher nitrogen balance than the wheat meals.

PPU<sub>leucine</sub>, the fraction of the leucine intake deposited, calculated from LP and HP leucine balance data, was not significantly different from the 2 protein sources. However, PPU<sub>nitrogen</sub>, the fraction of the nitrogen intake deposited, calculated from LP and HP nitrogen balance data, was lower for wheat than for milk ( $P \leq 0.001$ ). Thus, milk was utilized perfectly in these studies.

TABLE 3

Leucine intake, oxidation, balance, and turnover by period and protein type<sup>1</sup>

	Wheat	Milk	P value		
			Period	Diet	Period × diet
	<i>μmol·kg<sup>-1</sup>·h<sup>-1</sup></i>				
Leucine intake <sup>2</sup>					
PA	3.7 ± 0.1 <sup>a</sup>	7.1 ± 0.1 <sup>a</sup>	<0.05	<0.05	<0.05
LP	10.3 ± 0.5 <sup>b</sup>	20.1 ± 0.1 <sup>d</sup>			
HP	42.8 ± 3.7 <sup>c</sup>	83.0 ± 4.8 <sup>e</sup>			
Leucine oxidation					
PA	20.0 ± 4.5 <sup>a,b,d</sup>	28.6 ± 2.9 <sup>b,c,d</sup>	<0.05	<0.05	<0.05
LP	14.9 ± 1.5 <sup>a,b</sup>	25.9 ± 4.1 <sup>a,c,d</sup>			
HP	19.6 ± 2.7 <sup>a,b,d</sup>	38.1 ± 4.9 <sup>e</sup>			
Leucine balance					
PA	-16.3 ± 4.6 <sup>a</sup>	-21.5 ± 2.9 <sup>a</sup>	<0.05	<0.05	<0.05
LP	-4.6 ± 1.6 <sup>b</sup>	-5.7 ± 4.1 <sup>b</sup>			
HP	23.2 ± 5.2 <sup>c</sup>	44.9 ± 3.9 <sup>d</sup>			
Flux					
PA	135 ± 22	129 ± 16	<0.05	NS	NS
LP	112 ± 13	118 ± 16			
HP	140 ± 13	151 ± 21			
Synthesis					
PA	115 ± 21	100 ± 12	<0.05	NS	NS
LP	97 ± 13	92 ± 13			
HP	120 ± 13	113 ± 17			
Proteolysis					
PA	131 ± 22 <sup>a,d</sup>	122 ± 15 <sup>a,b,d</sup>	<0.05	NS	<0.05
LP	101 ± 12 <sup>b,c,d</sup>	97 ± 16 <sup>b,c</sup>			
HP	97 ± 11 <sup>b,c</sup>	68 ± 18 <sup>e</sup>			

<sup>1</sup> $\bar{x} \pm$  SD;  $n = 6$ . Measurements were made during the third hour of the postabsorptive (PA) period or after feeding frequent, small low-protein (LP) or high-protein (HP) meals. Values with different superscript letters are significantly different,  $P < 0.05$ .

<sup>2</sup>Digestible intakes.

The markedly lower lysine content of wheat compared with milk (leucine-lysine molar ratios of 0.32 and 0.72 for wheat and milk, respectively; 20) means that the lysine intakes from wheat were only 20% of those from milk. Assuming that wheat utilization is limited by lysine, the predicted value for wheat protein utilization in these subjects under the conditions of the experimental protocol, calculated from the lysine content of the wheat meal as a fraction of the lysine deposited from the milk meals, was  $0.26 \pm 0.02$ .

#### Metabolic demand and apparent protein requirement

MD, indicated by the magnitude of postabsorptive losses scaled to 24 h, was measured twice in each subject and because mean values on each occasion did not differ significantly, MD was calculated for each subject from the mean of the 2 values. The mean of this value (ie,  $n = 12$ ) is shown in Table 5. Individual mean values for MD ( $n = 2$ ) and values for  $PPU_{\text{nitrogen}}$  were used to calculate apparent protein requirement, MD/PPU, for each protein source. On this basis, the apparent protein requirement for wheat was 42% higher than that for milk. This is the requirement observed in subjects habituated to protein intakes of  $\approx 1.2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  and having an MD as measured here. Also shown in Table 5 is the EAR for wheat calculated from the current EAR for high-quality protein of  $0.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  (2) and the PPU for wheat as  $0.6/PPU_{\text{nitrogen}}$ . The lysine requirement is also calculated as the lysine content of the EAR for wheat assuming that lysine limits wheat protein quality.

#### DISCUSSION

Our intention in these studies was 1) to measure the extent and mechanisms of protein utilization of wheat compared with those of milk, 2) to test the hypothesis that mechanisms exist to allow adaptive conservation of lysine and improve wheat protein utilization compared with a predicted value, and 3) to estimate the lysine requirement of adults adapted to typical protein intakes of omnivores in the United Kingdom (22).

As described previously (13, 19, 23), milk protein utilization was affected by insulin-mediated (LP meals) and amino acid-mediated (HP meals) inhibition of proteolysis and stimulation of protein synthesis (HP meals). The lower utilization of wheat protein was explained by the lack of any amino acid-mediated inhibition of proteolysis. This presumably occurred because of the lower amounts of IAAs (eg, lysine, leucine, threonine, and tryptophan) in wheat than in milk because previous reports also indicated that amino acid mixtures mediated a decreasing inhibition of proteolysis as the relative amounts of IAAs decreased (24). Thus, inhibition of proteolysis and protein deposition are dependent on specific IAAs rather than on overall amounts of IAAs; and this may well be a characteristic of dietary protein that determines protein quality. Hepatic proteolysis is responsive to the multiphasic action of 7 amino acids (including leucine, glutamine, and tyrosine), part of which involves receptor-mediated inhibition of autophagy by leucine (25). Stimulation of protein synthesis by the 2 proteins was not significantly different, suggesting that this anabolic response is less suscepti-

**TABLE 4**  
Efficiency of wheat and milk protein utilization by period<sup>1</sup>

	Wheat	Milk	P value		
			Period	Diet	Period × diet
Nitrogen intake (mg N·kg <sup>-1</sup> ·h <sup>-1</sup> ) <sup>2</sup>					
PA	0.05 ± 0.00 <sup>a</sup>	0.1 ± 0.00 <sup>a</sup>			
LP	2.1 ± 0.1 <sup>b</sup>	2.8 ± 0.0 <sup>b</sup>	<0.05	<0.05	<0.05
HP	12.3 ± 1.1 <sup>c</sup>	15.6 ± 1.0 <sup>d</sup>			
Nitrogen balance (mg N·kg <sup>-1</sup> ·h <sup>-1</sup> )					
PA	-4.1 ± 1.2 <sup>a</sup>	-5.5 ± 0.7 <sup>a</sup>	<0.05	<0.05	<0.05
LP	-1.2 ± 0.4 <sup>b</sup>	-1.5 ± 1.0 <sup>b</sup>			
HP	5.9 ± 1.3 <sup>c</sup>	11.4 ± 1.0 <sup>d</sup>			
PPU <sup>3</sup> <sub>leucine</sub>	0.85 ± 0.05	0.81 ± 0.07	—	NS	—
PPU <sup>4</sup> <sub>nitrogen</sub>	0.68 ± 0.06	1.00 ± 0.09	—	<0.05	—
Lysine intake (μmol·kg <sup>-1</sup> ·h <sup>-1</sup> )					
LP	2.1 ± 0.2 <sup>a</sup>	10.4 ± 0.0 <sup>c</sup>	<0.05	<0.05	<0.05
HP	12.5 ± 1.2 <sup>b</sup>	60.7 ± 3.9 <sup>d</sup>			
Lysine balance (μmol·kg <sup>-1</sup> ·h <sup>-1</sup> )					
LP	-4.6 ± 1.6 <sup>a</sup>	-5.7 ± 4.1 <sup>a</sup>	<0.05	<0.05	<0.05
HP	23.2 ± 5.2 <sup>b</sup>	44.9 ± 3.9 <sup>c</sup>			
Predicted PPU <sup>5</sup> <sub>nitrogen</sub>	0.26 ± 0.02	—	—	—	—

<sup>1</sup> $\bar{x} \pm$  SD;  $n = 6$ . Measurements were made during the third hour of the postabsorptive (PA) period or after feeding frequent, small low-protein (LP) or high-protein (HP) meals. Values with different superscript letters are significantly different,  $P < 0.05$ .

<sup>2</sup>Digestible intakes.

<sup>3</sup>Postprandial protein utilization, fractional efficiency of leucine utilization.

<sup>4</sup>Fractional efficiency of nitrogen utilization.

<sup>5</sup>Predicted from observed lysine deposition from milk and lysine content of wheat.

ble to dietary amino acid composition. Thus, the amino acid composition of a protein may influence its utilization by having a regulatory effect on proteolysis as well as by limiting the amount of substrate for protein synthesis.

One potential error in the calculation of PPU<sub>nitrogen</sub> relates to whether leucine gain involves expansion of the free rather than protein-bound leucine pool. Leucine concentrations were higher after HP than LP meals but did not differ from the PA state (Table 2). We interpret this as indicating that the HP meals replenished the free amino acid pool after its depletion during the LP meals, such increases being part of nutritional demands.

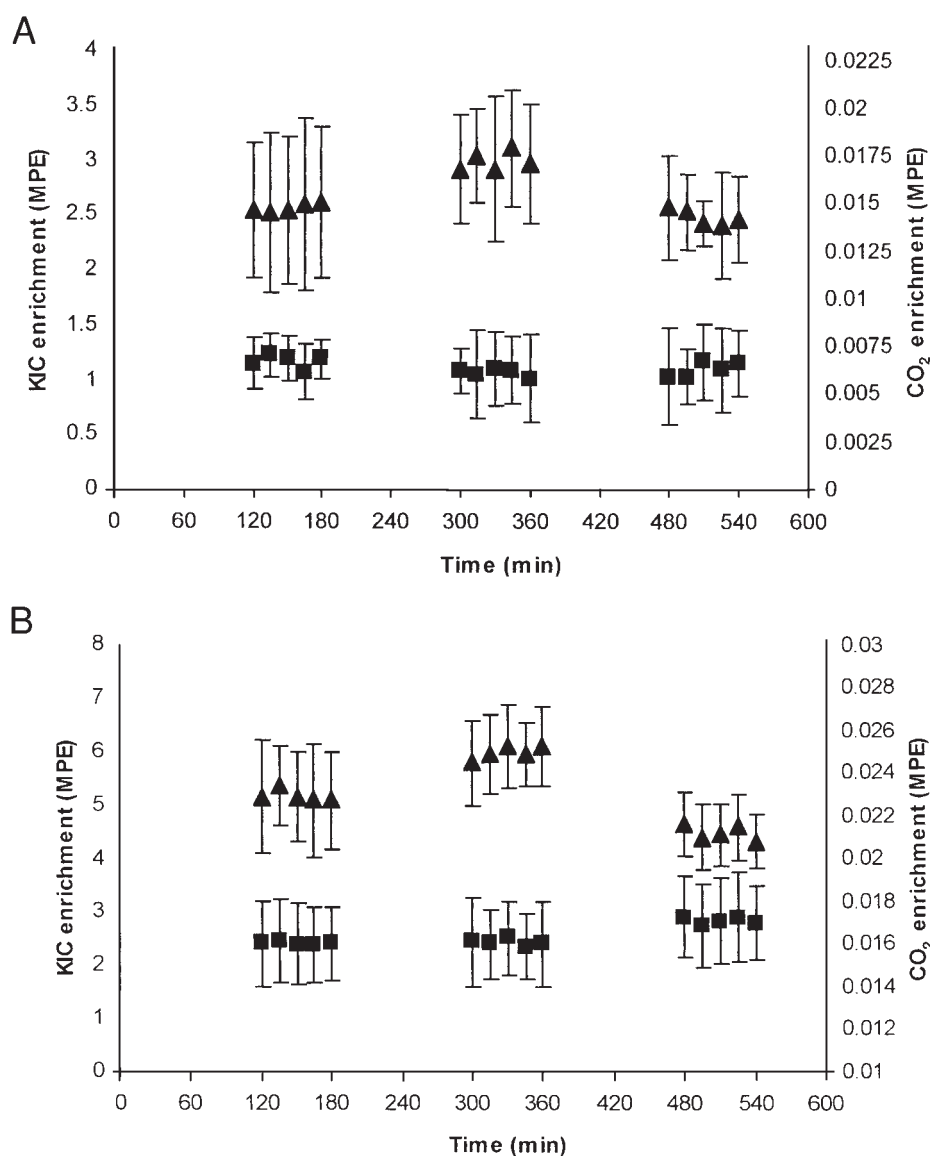
The absolute magnitude of PPU<sub>nitrogen</sub> depends on the value of the leucine-nitrogen ratio of tissue protein deposited, and the true value of this is unknown. In this and other studies (14, 18), we used a leucine-nitrogen ratio in tissue protein of 3.93 μmol/mg N, which is based on a value for the composition of bovine muscle (20). Use of a lower value would increase the value of the PPU and lower the lysine requirement and vice versa. The fact that our value results in a PPU<sub>nitrogen</sub> value of 1 for milk means that a lower leucine-nitrogen ratio value would result in an unrealistically higher value (>1) for PPU<sub>nitrogen</sub> and a lower lysine requirement. Thus, our main concern is whether we underestimated the true leucine-nitrogen ratio of human tissues and overestimated PPU<sub>nitrogen</sub>. There are only limited data in the leucine-nitrogen ratio of human tissues but Block and Weiss (26) report values ranging from 3.4 to 4.5 μmol/mg N for muscle and from 2.29 to 3.4 μmol/mg N for liver. The median of the values for muscle and liver combined is 3.4 μmol/mg N for human tissues and 4.2 μmol/mg N for beef. Use of these 2 values would result in PPU<sub>nitrogen</sub> values for milk of 1.16 and 0.94, respectively. On this basis, we believe that our data are probably accurate, and in any case, errors would not influence the relative values of milk and wheat protein reported here unless different tissue proteins

were deposited after milk than after wheat consumption. This possibility has been suggested (27) but seems unlikely. We have confidence, therefore, in our PPU<sub>nitrogen</sub> values of 0.68 for wheat and 1.0 for milk.

A PPU<sub>nitrogen</sub> of 0.68 for wheat is much higher than would be predicted if wheat had to supply all of the lysine needs for the required net protein deposition (ie, that observed in the same subjects with milk protein). The difference between predicted and observed utilization was considerable. A change in balance at the LP-HP transition of 27.8 μmol lysine·kg<sup>-1</sup>·h<sup>-1</sup> was observed with a change in intake of 10 μmol lysine·kg<sup>-1</sup>·h<sup>-1</sup>. Thus, ≈18 μmol·kg<sup>-1</sup>·h<sup>-1</sup> was unaccounted for: 8 mg during the 3-h HP feeding and 32 mg during the day.

One explanation of this better-than-expected utilization of wheat protein is that the larger free intracellular pool size of lysine compared with that of most other IAAs (28) enables recycling of lysine released by net proteolysis during the PA phase into net protein deposition during feeding (8, 10–12). Indeed, the reduction in the free lysine pool in human muscle after protein-free meals is equivalent to 120 μmol·kg lean tissue<sup>-1</sup>·3 h<sup>-1</sup> (28). This is more than enough to account for the deficit of 18 μmol·kg<sup>-1</sup>·h<sup>-1</sup> implied by our observed versus theoretical wheat PPU<sub>nitrogen</sub> value. The amount needed for the 12-h feeding cycle is equal to less than half (43%) of the free pool. The larger free pool of lysine reflects a higher  $K_m$  for lysine oxidation compared with many other IAAs (8). Thus, even though lysine oxidation does adapt to the level of intake and varies throughout the day in meal-fed rats (29), it is not cleared from the free pool (in human muscle) as quickly as are leucine and other IAAs after a protein meal (28). Also, in rats fed a lysine-deficient mixture of protein and amino acids, a delayed lysine supplement (given 12 h after the other amino acids) was utilized as effectively as was lysine given within a balanced meal (30). This was in con-





**FIGURE 2.** <sup>13</sup>C enrichment of plasma α-ketoisocaproate (KIC; ▲) and <sup>13</sup>CO<sub>2</sub> (■) during L-[1-<sup>13</sup>C]leucine infusions with small, frequent meals of wheat (A) and milk (B).

trast with tryptophan, which is only utilized when it is given within a balanced meal, presumably because it is rapidly oxidized if it cannot be used for net protein synthesis.

Thus, our results are consistent with the lysine requirement being lower than might be predicted due to recycling. Clearly, repeated feeding of the low-lysine diet would lower the free lysine concentration, limiting the amount available for recruitment into protein deposition. However, in our model, lysine oxidation and the amplitude of diurnal cycling would decrease, reducing the requirement for lysine for net protein deposition.

In addition, some de novo synthesis of lysine by colonic microflora utilizing urea nitrogen could contribute to reducing the lysine requirement (31). We showed in infants that when urea salvage rates are high (32), <sup>15</sup>N is transferred from urea to systemic lysine, implying de novo synthesis of lysine in nutritionally significant amounts (33). However, we would expect relatively low rates of urea salvage in our subjects, given their

**TABLE 5**

Metabolic demand and wheat protein and lysine requirements<sup>1</sup>

	Wheat	Milk
Metabolic demand (g protein·kg <sup>-1</sup> ·d <sup>-1</sup> ) <sup>2</sup>	0.73 ± 0.18	
Apparent protein requirement (g·kg <sup>-1</sup> ·d <sup>-1</sup> ) <sup>3</sup>	1.07 ± 0.20	0.75 ± 0.18 <sup>4</sup>
EAR for wheat protein (g·kg <sup>-1</sup> ·d <sup>-1</sup> ) <sup>5</sup>	0.89 ± 0.08	—
EAR for lysine (mg·kg <sup>-1</sup> ·d <sup>-1</sup> ) <sup>6</sup>	23.2 ± 2.0	—

<sup>1</sup> $\bar{x} \pm SD$ ;  $n = 6$ .

<sup>2</sup>Calculated from postabsorptive leucine losses scaled to 24 h; mean of values obtained for each subject with both milk and wheat ( $n = 12$ ).

<sup>3</sup>Calculated from metabolic demand and the fractional efficiency of postprandial nitrogen utilization (PPU<sub>nitrogen</sub>).

<sup>4</sup>Significantly different from wheat,  $P < 0.05$ .

<sup>5</sup>Estimated average requirement for wheat protein (EAR) calculated from the currently accepted EAR of 0.6 g protein·kg<sup>-1</sup>·d<sup>-1</sup> and PPU<sub>nitrogen</sub> for wheat protein.

<sup>6</sup>Calculated from the lysine content of the EAR for wheat protein.

**TABLE 6**

Comparisons of present postprandial protein utilization (PPU) value for wheat with previous net protein utilization (NPU) or calculated protein digestibility–corrected amino acid (PDCAA) scores for wheat

Scoring pattern	Lysine requirement	NPU, PDCAA score, or PPU <sup>f</sup>	Reference
<i>mg/g protein</i>	<i>mg·kg<sup>-1</sup>·d<sup>-1</sup></i>		
—	—	0.41 (0.27 true) <sup>2</sup>	21
50	30	0.49 <sup>3</sup>	34
—	<28	<0.50 <sup>3</sup>	7
—	43	0.33 <sup>3</sup>	6
—	19	0.74 <sup>3</sup>	4, 35
—	23	0.68 <sup>4</sup>	Current value

<sup>1</sup>PDCAA score calculated as the ratio of digestible wheat lysine content (0.94·26 mg lysine/g protein)/ lysine content of scoring pattern (mg lysine requirement·kg<sup>-1</sup>·d<sup>-1</sup>)/0.6 g protein).

<sup>2</sup>NPU measured directly.

<sup>3</sup>PDCAA score.


<sup>4</sup>PPU as reported currently.

protein intakes, so that de novo lysine supply may be of only minor importance. The implications of these values in the context of the current debate about lysine requirements and evaluation of protein quality in human adults can be examined from several such values, as indicated in **Table 6**.

A PPU of 0.68 for wheat protein utilization is higher than the previously derived net protein utilization value (0.41 for that of egg protein) from nitrogen balance studies (21). However, nitrogen balance assays of protein quality, not withstanding any discussion about methodologic difficulties (11, 36, 37), are metabolically complex and differ from slope assays obtained in growing animals. This is because, within our adaptive model of protein homeostasis and requirements in multilevel feeding trials with subjects adapted to each intake, the MD will increase with the intake so that the slope of the nitrogen balance–nitrogen intake relation will markedly underestimate the true efficiency of protein utilization (12).

The amino acid score of wheat calculated from a digestibility of 0.94 and a suggested lysine requirement of 30 mg/kg, 50 mg lysine/g protein (34), is 0.49. However, we have grave reservations about the way this pattern was derived (9–11, 38), and a recent report from Young's group is not inconsistent with a lysine requirement <28 mg·kg<sup>-1</sup>·d<sup>-1</sup> (7). A score of 0.35 can be derived from the mean lysine requirement of 43 mg/kg reported by the Toronto group (5, 6). There are difficult technical issues that need to be resolved in relation to these particular studies (11). Indeed, the safe requirement value derived from the Toronto study (63 mg·kg<sup>-1</sup>·d<sup>-1</sup>) would, if correct, imply that the UK vegetarian population, who have an average intake of 44 mg lysine·kg<sup>-1</sup>·d<sup>-1</sup> (4), would exhibit high prevalence rates of deficiency. On the other hand, our PPU of 0.68 is less than the value of 0.74, which would be calculated from a lysine requirement of 19 mg·kg<sup>-1</sup>·d<sup>-1</sup> indicated by nitrogen balance data (35) adjusted for miscellaneous losses and recalculated on the basis of body weight (4). However, as argued elsewhere (11), within our adaptive model of protein homeostasis, the MD for lysine will fall in response to either a lower protein intake or lower protein quality. On this basis, the lower requirement value of 19 mg·kg<sup>-1</sup>·d<sup>-1</sup> and the higher protein digestibility–corrected amino acid score for wheat of 0.74, indicated by nitrogen balance data (4, 35), would appear to be realistic.

In conclusion, our studies showed that in subjects adapted to generous protein and lysine intakes, the efficiency of wheat protein utilization is higher than would be expected from recent

reports of lysine requirements and higher than would be expected from theoretical predictions. This implies that there are adaptive mechanisms of lysine conservation in subjects with typical mixed protein intakes. The data we report indicate an average lysine requirement of 23 mg·kg<sup>-1</sup>·d<sup>-1</sup> and are consistent with a lysine requirement in adapted individuals of 19 mg·kg<sup>-1</sup>·d<sup>-1</sup>. 

## REFERENCES

1. FAO/WHO. Protein quality evaluation. Rome: FAO, 1991. (FAO Food and Nutrition paper 51.)
2. FAO/WHO/UNU. Energy and protein requirements. World Health Organ Tech Rep Ser 1985;724.
3. Young VR, El-Khoury AE. Human amino acid requirements: a re-evaluation. *Food Nutr Bull* 1996;17:191–203.
4. Millward DJ. The nutritional value of plant based diets in relation to human amino acid and protein requirements. *Proc Nutr Soc* 1999;58:1–12.
5. Zello GA, Pencharz PB, Ball RO. Dietary lysine requirement of young adult males determined by oxidation of L-[1-<sup>13</sup>C]phenylalanine. *Am J Physiol* 1993;264:E677–85.
6. Duncan AM, Ball RO, Pencharz PB. Lysine requirement of adult males is not affected by decreasing dietary protein. *Am J Clin Nutr* 1996;64:718–25.
7. Kurpad AV, El-Khoury AE, Beaumier L, et al. An initial assessment, using 24-h [<sup>13</sup>C]leucine kinetics, of the lysine requirement of healthy adult Indian subjects. *Am J Clin Nutr* 1998;67:58–66.
8. Millward DJ, Rivers J. The nutritional role of indispensable amino acids and the metabolic basis for their requirements. *Eur J Clin Nutr* 1988;42:367–93.
9. Millward DJ. Can we define indispensable amino acid requirements and assess protein quality in adults? *J Nutr* 1994;124:1509–16.
10. Millward DJ. Human amino acid requirements. *J Nutr* 1997;127:1842–6.
11. Millward DJ. Metabolic demands for amino acids and the human dietary requirement: Millward and Rivers (1988) revisited. *J Nutr* 1998;128:2563S–76S.
12. Millward DJ, Pacy PJ. Postprandial protein utilisation and protein quality assessment in man. *Clin Sci* 1995;88:597–606.
13. Gibson NR, Fereday A, Cox M, Halliday D, Pacy PJ, Millward DJ. Influences of dietary energy and protein on leucine kinetics during feeding in healthy adults. *Am J Physiol* 1996;33:282–91.
14. Fereday A, Gibson NR, Cox M, Pacy PJ, Millward DJ. Protein requirements and ageing: metabolic demand and efficiency of utilisation. *Br J Nutr* 1997;77:685–702.
15. Millward DJ, Fereday A, Gibson NR, Pacy PJ. Aging, protein requirements, and protein turnover. *Am J Clin Nutr* 1997;66:774–86.

16. Waterlow JC, Garlick PJ, Millward DJ. Protein turnover in mammalian tissues and in the whole body. Amsterdam: Elsevier North-Holland Biomedical Press, 1978.
17. Wenham D, Pacy P, Price GM, Millward DJ, Halliday D. Bicarbonate recovery during feeding and fasting. *Proc Nutr Soc* 1991;50:47A (abstr).
18. Price GM, Halliday D, Pacy PJ, Quevedo MR, Millward DJ. Nitrogen homeostasis in man: influence on the amplitude of diurnal cycling of body nitrogen. *Clin Sci* 1994;86:91-102.
19. Pacy PJ, Price GM, Halliday D, Quevedo MR, Millward DJ. N homeostasis in man: 2. The diurnal responses of protein synthesis, degradation and amino acid oxidation to diets with increasing protein intakes. *Clin Sci* 1994;86:103-18.
20. Paul AA, Southgate DAT, Russell J. First supplement to McCance and Widdowson's *The Composition of Foods*. London: MAFF/MRC Her Majesty's Stationery Office, 1980.
21. Young VR, Fajardo L, Murray E, Rand WM, Scrimshaw NS. Protein requirements of man: comparative nitrogen balance response within the submaintenance-to-maintenance range of intakes of wheat and beef proteins. *J Nutr* 1975;105:534-44.
22. Jackson AA, Margetts BM. Protein intakes in the adult population of the UK. *Int J Food Sci Nutr* 1993;44:95-104.
23. Fereday A, Gibson NR, Cox M, Pacy PJ, Millward DJ. Variation in amino acid mediated, insulin activated inhibition of proteolysis determines the efficiency of protein utilisation. *Clin Sci* 1998;95:725-33.
24. Marchini JS, Cortiella J, Hiramatsu T, Chapman TE, Young VR. Requirements for indispensable amino acids in adult humans: longer-term amino acid kinetic study with support for the adequacy of the Massachusetts Institute of Technology amino acid requirement pattern. *Am J Clin Nutr* 1993;58:670-83.
25. Miotto G, Venerando R, Marin O, Siliprandi N, Mortimore GE. Inhibition of macroautophagy and proteolysis in the isolated rat hepatocyte by a nontransportable derivative of the multiple antigen peptide Leu8-Lys4-Lys2-Lys-beta Ala. *J Biol Chem* 1994;269:25348-53.
26. Block RJ, Weiss KW. *Amino acid handbook*. Springfield, IL: Charles C Thomas, 1956.
27. Fuller MF, Garlick PJ. Human amino acid requirements: can the controversy be resolved? *Annu Rev Nutr* 1994;14:217-41.
28. Bergstrom J, Furst P, Vinnars E. Effect of a test meal with and without protein on muscle and plasma free amino acids. *Clin Sci* 1990;79:331-7.
29. Blemings KP, Crenshaw TD, Benevenga NJ. Mitochondrial lysine uptake limits hepatic lysine oxidation in rats fed diets containing 5, 20 or 60% casein. *J Nutr* 1998;128:2427-34.
30. Yang SP, Tilton KS, Ryland LL. Utilization of a delayed lysine or tryptophan supplement for protein repletion of rats. *J Nutr* 1968;94:178-84.
31. Fuller MF, Reeds PJ. Nitrogen cycling in the gut. *Annu Rev Nutr* 1998;18:385-411.
32. Badaloo A, Boyne M, Reid M, et al. Dietary protein, growth and urea kinetics in severely malnourished children and during recovery. *J Nutr* 1999;129:969-79.
33. Millward DJ, Forrester T, Ah-Sing E, et al. The transfer of <sup>15</sup>N from urea to lysine in the human infant. *Br J Nutr* 2000;83:505-12.
34. Young VR, Bier DM, Pellett PL. A theoretical basis for increasing current estimates of the amino acid requirements in adult man, with experimental support. *Am J Clin Nutr* 1989;50:80-92.
35. Jones EM, Bauman CA, Reynolds MS. Nitrogen balances in women maintained on various levels of lysine. *J Nutr* 1956;60:549-59.
36. Hegsted DM. Balance studies. *J Nutr* 1976;106:307-11.
37. Young VR. Nutritional balance studies: indicators of human requirements or adaptive mechanisms. *J Nutr* 1986;116:700-3.
38. Millward J. Amino acid requirements in adult man. *Am J Clin Nutr* 1990;51:492-6.

