

Efficiency of utilization of wheat and milk protein in healthy adults and apparent lysine requirements determined by a single-meal [1-¹³C]leucine balance protocol¹⁻⁴

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ABSTRACT

Background: We recently reported better wheat-protein utilization and a higher apparent lysine requirement than would be predicted, because of adaptive mechanisms of lysine conservation. However, such findings may be subject to the feeding protocol of frequent small meals.

Objective: We used a [1-¹³C]leucine balance, large single-meal protocol to estimate the utilization of wheat and the consequent lysine requirements.

Design: Wheat and milk utilization were compared in 5 adults infused for 9 h with L-[1-¹³C]leucine, in the postabsorptive (0–3 h) and postprandial (3–9 h) states after ingestion of a single meal of either milk (30.4 kJ/kg; 32% of energy as protein) or a mixture of wheat gluten and whole wheat (29.2 kJ; 26.7% of energy as protein). Premeal nitrogen balance was predicted from [1-¹³C]leucine oxidation and postmeal balance predicted from cumulative increased leucine oxidation, enabling evaluation of the metabolic demand for protein, the efficiency of postprandial protein utilization (PPU), and the requirements for wheat protein and lysine.

Results: Mean (\pm SD) PPU was 0.61 ± 0.03 and 0.93 ± 0.02 for wheat and milk ($P \leq 0.001$), respectively, and the estimated average wheat-protein requirement ($0.6 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}/\text{PPU}$) was $0.98 \pm 0.05 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, indicating a lysine requirement of $18.3 \pm 1.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$.

Conclusions: Measured wheat utilization efficiency at 0.61 was considerably higher than the value predicted from wheat lysine intake and milk protein lysine deposition (ie, 0.222 ± 0.004). These results confirm our previous finding that lysine conservation allows wheat protein to be utilized more efficiently than expected and is consistent with a lysine requirement in fully adapted individuals of $19 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, as indicated by recalculated nitrogen balance data. *Am J Clin Nutr* 2002;76:1326–34.

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

INTRODUCTION

The importance of protein quality has been under intensive debate since the rejection by the FAO/WHO (1) of the previously proposed adult amino acid scoring pattern (2). The determination of lysine requirements for human adults and of the protein quality of lysine-limited proteins, such as wheat, is inherently difficult. Studies that used the indicator oxidation method identified

lysine requirements of 37 mg/kg (3) and 45 mg/kg (4), from which a mean lysine requirement of 43 mg/kg was derived (4). Difficult technical issues need to be resolved in relation to these particular studies (5). Such a requirement implies high prevalence rates of lysine deficiency for UK vegetarians with an estimated intake of 44 mg lysine/kg (6), who are generally perceived as having low morbidity and mortality (7). Young et al (8) suggest a requirement of 30 mg lysine/kg. However, we have grave reservations about the way this pattern was derived (6, 9–11) and about the interpretation of experimental studies that validate it (12). The designs of more recent, direct, 24-h [¹³C]lysine oxidation balance studies (13) that validated 30 mg lysine/kg as the requirement are problematic. For example, the lysine intake from meals was significantly lower than the 24-h test intake studied because a significant mass of the tracer was fed in the postabsorptive state. The results of 24-h [1-¹³C]leucine balance studies of various lysine intakes in Indian subjects, which are also reported as supporting a requirement of 30 mg/kg, are difficult to interpret because of the influence of both leucine and lysine intakes on leucine balance and, in some cases, of weight loss in persons consuming purified amino acid mixture diets (14, 15). Also, the logistic difficulties of conducting these studies make it difficult to study sufficient test intakes to accurately define a requirement. Thus, in the most recent of these types of studies published (15), intakes of 12, 20, 28, and 36 mg lysine/kg resulted in a positive daily leucine balance, although balances were significantly higher at lysine intakes of 28 and 36 mg/kg than at intakes of 12 and 20 mg/kg. This finding might be interpreted as indicating a value between

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³ Dedicated to the memory of Peter Reeds and Bernard Beaufre, each of whom made enormous contributions to our understanding of protein and amino acid requirements and directly participated in the development of the conceptual basis of the work described in this article.

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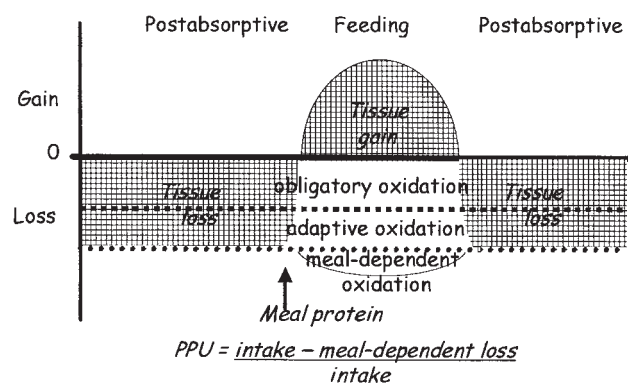


FIGURE 1. Model assumed for determination of protein utilization during a single meal. Dietary protein provides for both obligatory and adaptive metabolic demands that occur throughout the diurnal cycle. Leucine oxidation in the postabsorptive state is assumed to trace overall amino acid oxidation and nitrogen excretion derived from tissue protein loss. Meal protein is utilized to provide for obligatory and adaptive demands and for tissue gain associated with repletion of postabsorptive losses. Any increased leucine oxidation with feeding (meal-dependent oxidation measured as cumulative increased leucine oxidation) represents the inefficiency of meal protein utilization. Postprandial protein utilization (PPU) was calculated from cumulative increased oxidation and nitrogen intake after conversion of utilized leucine into nitrogen to account for differences in meal and tissue leucine-nitrogen ratios.

20 and 28 mg lysine \cdot kg⁻¹ \cdot d⁻¹. The finding of a positive daily leucine balance at all intakes, which in previous studies was interpreted as indicating dietary adequacy, implies methodologic uncertainties with 24-h [¹³C]leucine balances comparable with those associated with the nitrogen balance method, which led to the rejection of adult nitrogen balance data by the FAO/WHO (1). In fact, reasonably robust nitrogen balance data exist for lysine (16). These data were reanalyzed (6) assuming a value of 5 mg for unmeasured integumental losses, similar to the value of 4.7 mg predicted from the data of Calloway et al (17), indicating an apparent lysine requirement of 19 mg \cdot kg⁻¹ \cdot d⁻¹. Rand and Young (18) also reanalyzed these data, assuming higher values for unmeasured integumental losses (8 mg N \cdot kg⁻¹ \cdot d⁻¹). They identified a requirement of 30 mg \cdot kg⁻¹ \cdot d⁻¹, even though they were unable to resolve an asymptotic analysis of the pooled data. This suggests that actual integumental losses were less.

We recently reported studies of wheat-protein utilization using a novel [¹³C]leucine balance protocol (19) involving half-hourly feeding of small meals (20–23). From measurements of [¹³C]leucine oxidation and balance, we predicted nitrogen balance enabling calculation of the metabolic demand for protein, the efficiency of postprandial protein utilization (PPU), and the apparent requirements for wheat protein and lysine. Wheat utilization was better than would be expected from theoretical considerations, confirming adaptive mechanisms of lysine conservation and allowing wheat to be utilized more efficiently than would be expected (6, 20). The lysine requirement calculated from wheat utilization was lower than most previous recent estimates but was similar to our recalculated (6) nitrogen balance data reported by Jones et al (16). However, frequent small meals might be used more efficiently than larger meals. For this reason, we used a [¹³C]leucine balance protocol adapted to enable study of protein utilization from a single large meal.

SUBJECTS AND METHODS

Experimental design and metabolic model

The use of a leucine balance protocol to evaluate the metabolic demand for protein, the efficiency of PPU, and the apparent protein requirement (metabolic demand/PPU) was extensively discussed elsewhere (19–23). However, this is our first deployment of a non-steady state, large, single-meal [¹³C]leucine balance protocol. The assumed model is shown in **Figure 1**. Daily balance is assumed to occur within a cycle of postprandial net protein gain and postabsorptive net protein loss that occurs with an increasing amplitude of gains and losses with an increasing habitual protein intake (24). The adaptive metabolic-demand model of protein requirements involves metabolic consumption of amino acids by both obligatory and adaptive pathways occurring throughout the 24 h cycle, which are supplied from tissue protein in the postabsorptive state and from food in the postprandial state (6, 12, 20). Meal protein is assumed to be completely utilized to provide for metabolic demands as judged by a return to premeal baseline leucine oxidation during the 6-h post meal period. Preliminary studies were conducted to identify the minimum period required for this, which was identified as being 5 h after the meal. On this basis, we chose a 3-h premeal, 6-h postmeal protocol.

Leucine oxidation measured in the premeal postabsorptive phase allows the prediction of total metabolic demands. Leucine oxidation measured in the postmeal phase allows the prediction of milk- and wheat-protein utilization from the extent of any increase in leucine oxidation over the premeal rate. The actual protein utilization, expressed in terms of the efficiency of nitrogen utilization (PPU_{nitrogen}), is calculated from nitrogen intake and the nitrogen equivalent of leucine utilized for the metabolic demand and deposition in tissue protein, ie, leucine intake minus the cumulative excess of leucine oxidation (ie, postmeal – premeal rate). From the values obtained with milk protein, lysine utilization can also be calculated so that, on the assumption that lysine limits wheat utilization, a predicted value for PPU_{nitrogen} can be calculated that can be compared with the measured value for wheat PPU_{nitrogen} calculated from the leucine oxidation after the wheat meal. The apparent protein requirements for milk and wheat are then calculated as metabolic demand/PPU_{nitrogen} and a requirement for lysine is calculated from the estimated average requirement for wheat protein (0.6/PPU_{nitrogen}), assuming it to be the limiting amino acid of the wheat protein in the meal.

Subjects

Five healthy subjects (4 men and 1 woman) were studied on 2 occasions. The time between studies was 6 mo on average, but no changes in the diets or body weights of the subjects occurred during this period. The subjects were aged 33.2 \pm 12.8 y, weighed 66.5 \pm 9.4 kg, and had a body mass index (in kg/m²) of 22.6 \pm 2.4. Before the studies began, there had been no restrictions on activities of daily living; however, the subjects were encouraged to regulate their eating patterns (ie, 12 h of feeding and 12 h of fasting beginning at 0900) over the week before the study began. The study was approved by the University of Surrey ethical committee, and all subjects gave their informed consent after the nature of the protocol had been fully explained to them.

Infusion protocol

A schematic representation of the protocol is shown in **Figure 2**. The subjects were asked to complete their last meal at 2100 at

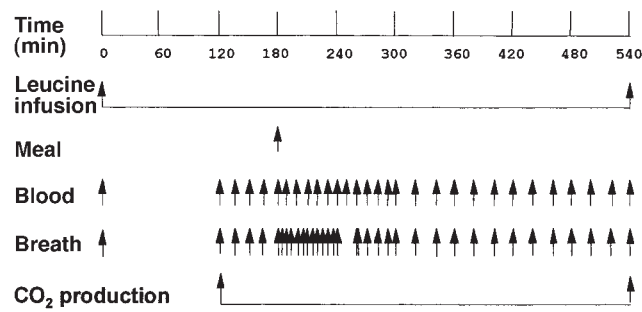


FIGURE 2. Study protocol. The infusion started after an overnight fast, and sampling times within each period are shown for blood, breath, and carbon dioxide production. The single meal, given as either a milk drink or a wheat-gluten pancake and the milk drink, was given at the times shown.

home and to report to the metabolic ward at 0730. At this time, intravenous L-[1-¹³C]leucine was infused as described previously (21, 24, 25) after baseline blood and expired breath samples (in duplicate) were collected and priming doses of NaH¹³CO₂ (0.2 mg/kg) and L-[1-¹³C]leucine (99% ¹³C; MassTrace, Boston) were administered. The wheat studies were performed after the milk studies. After a review of the isotope need, it was decided that the tracer-infusion rate could be halved from 1 to 0.5 mg · kg⁻¹ · h⁻¹ without sacrificing measurement precision. Thus, the priming doses of L-[1-¹³C]leucine were either 0.5 or 1 mg/kg and were followed immediately by a continuous infusion of L-[1-¹³C]leucine (either 0.5 or mg · kg⁻¹ · h⁻¹) for 9 h, starting, in most cases at 0800 (11 h postabsorptive).

A cannula for blood sampling was inserted into a superficial vein on the arm not receiving the infusion. Blood samples were taken before administration of the tracer to determine basal enrichment. Blood samples were then taken every 15 min during the third hour, because it was predicted that the isotopic plateau would have been reached by this time. After ingestion of the test meal at 3 h, blood was sampled every 10 min for 2 h and then every 20 min for the remaining 4 h. Blood (≈4 mL) was collected at each time point and placed in lithium heparin-containing tubes, which were centrifuged immediately at 500 × g for 10 min at 4 °C. The plasma was separated and dispensed equally into 2 tubes, which were stored at -20 °C.

Breath samples were collected for the analysis of expired ¹³CO₂. Baseline samples were taken before the infusion started and then every 15 min during the third hour. After the meal was ingested at 3 h, breath samples were collected every 10 min for 2 h and then every 20 min for the remaining 4 h. Expired breath samples were collected in a urine collection bag with a one-way valve and tap and were then transferred, with the use of a 20-mL syringe, to a 20-mL evacuated tube (Vacutainer; Becton Dickinson, Franklin Lakes, NJ).

Carbon dioxide production rates were measured for 1 h during the postabsorptive period and continuously during the 6 h after the meal with the use of an indirect calorimeter with a ventilated tent system.

The meal was designed to provide 50% of the UK average daily protein intake and a relatively high protein-energy value. The meal contained 0.5 g protein/kg and provided 30 kJ/kg, ie, a protein-energy ratio of 30%. The fat content of the meal was kept as low as possible to maximize gastric emptying. Water was freely

TABLE 1
Meal composition by protein type¹

	Milk	Wheat
Total energy (kJ/kg)	30.4 ± 2.8	29.2 ± 2.1
Protein (% of energy)	32.3 ± 4.5	26.7 ± 4.1 ²
Carbohydrate (% of energy)	65.7 ± 8.7	72.3 ± 6.4
Fat (% of energy)	2.0 ± 0.3	1.0 ± 0.1
Nitrogen (mg/kg)	90.2 ± 4.2	78.5 ± 9.7 ²
Leucine (mg/kg)	64.2 ± 3.0	39.1 ± 4.8 ²
Lysine (mg/kg)	55.8 ± 2.6	9.5 ± 1.2 ²

¹ $\bar{x} \pm 1$ SD; n = 5.

²Significantly different from milk, P ≤ 0.05.

available throughout the study. Samples of the meals were taken and stored frozen before measurement of the leucine and nitrogen contents.

The milk-protein meal consisted of fresh skim milk and dissolved potato dextrose with low natural ¹³C enrichment. The meal was consumed within 5–10 min. The wheat-protein meal consisted of wheat gluten (Tunnel Refineries, Greenwich, United Kingdom), plain flour (Tesco London), and low naturally ¹³C-enriched potato dextrose (Avebe, Veendam, Holland). Wheat gluten was used to provide a high protein content in a small volume. It was fed as a dry fried pancake made from a dough prepared from an equal weight of gluten and plain flour with water and was served hot, accompanied by a drink containing dissolved potato starch with a small amount of sugar-free orange beverage. The meal was consumed within 10–15 min. Prior feeding studies were performed to measure the extent to which background ¹³CO₂ enrichment was influenced by either meal. Samples of each diet were analyzed for total nitrogen by Kjeldahl analysis. The macronutrient content of the 2 meals and protein intakes as leucine, nitrogen, and lysine are shown in **Table 1**.

Sample analysis

Blood glucose was measured with a standard, automated, hexokinase technique. Plasma insulin was measured with a double-antibody, polyethylene glycol, dual-incubation assay with [¹²⁵I]insulin, and with a monoclonal antiinsulin (donkey anti-guinea pig) antiserum as the second antiserum. The concentration of urea nitrogen in the urine and plasma was measured with a Cobas Fara II Autoanalyser (Roche Products Ltd, Diagnostics Division, Welwyn Garden City, United Kingdom) with the use of proprietary reagent kits. The ¹³C enrichment of leucine, ketoisocaproate (KIC), and expired breath samples were measured by mass spectrometry as described previously (21).

Calculations

Leucine oxidation (O) was calculated, per minute, as follows:

$$O = F^{13}\text{CO}_2 / (R \times \text{MPE}_p) \times 100 \quad (1)$$

where $F^{13}\text{CO}_2$ is the ¹³CO₂ excretion rate (in mol · kg⁻¹ · h⁻¹), R is the fraction that accounts for ¹³CO₂ recovery during the study, and MPE_p is the ¹³C enrichment of plasma [¹³C]KIC (mol% excess), at the premeal plateau or at each postmeal sampling time (21, 26).

Cumulative leucine oxidation (μmol/kg) for the 6 h after the meal (O_{cum}) was calculated as follows:

$$O_{\text{cum}} = \Sigma(F^{13}\text{CO}_2 / (R \times \text{MPE}_p) \times 100 \times (t_2 - t_1)) \quad (2)$$

where t_1 and t_2 are the times at the start of each 10- or 20-min post-meal period. Bicarbonate recovery was assumed to gradually increase in the fed state from a fasted value of 0.76 (27) to a maximum value of 0.91 (27), with a time course similar to that for the increase in the carbon dioxide production rate: ie, on average, recovery was assumed to be 0.76, 0.76, 0.79, 0.81, 0.84, 0.88, and 0.91 at 180, 190, 200, 210, 220, 230, and 240 min, respectively. The maximum recovery rate was assumed to be maintained throughout the remainder of the study because bicarbonate recovery increases with time, and the carbon dioxide production rate remained elevated throughout most of the study.

Leucine utilization was calculated, assuming that it was complete by 6 h after the meal:

$$\text{Leucine utilization} = \text{leucine intake} - \text{cumulative excess leucine oxidation} \quad (3)$$

The efficiency of protein utilization, expressed in terms of nitrogen, $\text{PPU}_{\text{nitrogen}}$, is defined as

$$\text{PPU}_{\text{nitrogen}} = \text{nitrogen utilization/nitrogen intake} \quad (4)$$

The conversion of leucine utilization to nitrogen utilization (19, 23) involves an assumed leucine-nitrogen ratio for tissue protein, as discussed below, and is necessary because of the differences between the leucine content of dietary and tissue proteins. Nitrogen intake was corrected for digestibilities, which were assumed to be 93% for wheat (28) and 100% for milk. No adjustment of meal leucine intake for tracer intake was made because tracer oxidation was assumed to be constant throughout the entire 9-h infusion; only excess leucine oxidation was used in the calculation.

Conversion factors for leucine-nitrogen and lysine-nitrogen ratios in meals and tissue protein and the metabolic demand

Leucine intakes were calculated from the measured nitrogen content of the milk and gluten meals and from the leucine-nitrogen ratios calculated from the reported ratio of leucine to total amino acids in bovine milk (29), manufacturer's data for gluten (90% of the protein meal), and food-table values for whole wheat (10% of the protein) (30). Calculation of the leucine-nitrogen ratios assumed that reported values for aspartate and glutamate were derived from mixtures of their amides and the dicarboxylic amino acids in which the amides amounted to 10% and 40%, respectively, for aspartate and glutamate, as indicated by the amino acid sequences of the principal milk proteins in the gene sequence database (P Reeds, personal communication, 2002), with the same distribution for dicarboxylic acid and amide in wheat gluten. On this basis, the ratios of total amino acids to nitrogen were 7.31 and 7.45 for milk and wheat, respectively, and the leucine and lysine contents were 712 and 618 mg/g nitrogen for milk and 497 and 121 mg/g nitrogen for the wheat meal, respectively. These values represent amino acids after peptide hydrolysis (ie, more than the equivalent weight of protein because of the added water of hydrolysis) and are thus larger than the usually quoted values that convert nitrogen to protein, ie, that based on the mean ratios of peptide residue to nitrogen of 6.35 and 6.47 for milk and wheat, respectively. In calculating the lysine requirement from the average wheat-protein requirement, a value of 18.7 mg lysine/g wheat protein was used, ie, 121/6.47.

The leucine utilization of the milk and wheat protein was converted to nitrogen utilization by using a leucine-nitrogen conversion factor for tissue protein of 625 mg/g nitrogen, assuming values of 85.5 mg leucine/g protein and 7.31 for the ratio of total amino acid to nitrogen

in tissue protein, the same as in milk protein, with a lysine-leucine ratio of 0.94. In fact, as is apparent in Figure 1, utilized nitrogen provides for not only tissue gain (repletion of postabsorptive protein losses) but also for the oxidative component of obligatory and adaptive metabolic demands, $\approx 40\%$ of the nitrogen utilization in the milk study, which may have a different leucine-nitrogen ratio. However, only the leucine-nitrogen ratio of the tissue protein gain can be predicted, ie, the weighted mean value for the labile protein mobilized and replaced during the diurnal cycle. This value is probably greater than the whole-body value, ie, 74 mg leucine/g protein (29), because this includes collagen at only 32 mg leucine/g protein, which is unlikely to be mobilized (as indicated from a nitrogen balance calculated from this whole-body value considerably in excess of the intake). The composition of the muscle contractile proteins is equivalent to 91 mg leucine/g protein (31), and the mean values for muscle and liver are 91 and 80 mg leucine/g protein (32). Previously (19), we used a beef muscle value of 82 mg leucine/g protein (30), but a better value can be derived from a 50:50 mixture of liver and muscle, which both participate (33), ie, 85.5 mg leucine/g protein. Lysine utilization was calculated as leucine utilization \times 0.94 the lysine-leucine ratio of tissue protein identified as above.

Requirement for wheat protein and lysine and the predicted PPU of wheat protein

The apparent requirement (AR) for milk and wheat protein was calculated as follows:

$$\text{AR} = \text{metabolic demand}/\text{PPU}_{\text{nitrogen}} \quad (5)$$

where metabolic demand = $24 \times$ hourly postabsorptive leucine balance (leucine tracer intake - leucine oxidation), expressed as protein calculated as discussed above.

The estimated average requirement (EAR) for wheat protein and lysine was calculated as

$$\text{EAR}_{\text{wheat}} (\text{g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}) = 0.6/\text{PPU}_{\text{nitrogen,wheat}} \quad (6)$$

where 0.6 is the currently accepted average requirement for high-quality protein (2).

The EAR for lysine was calculated as shown:

$$\text{EAR for lysine (mg/kg)} = \text{EAR}_{\text{wheat}} \times 18.7 \quad (7)$$

where 18.7mg/g is the lysine content of wheat protein, assuming that lysine limits wheat protein quality.

The predicted PPU of wheat protein was calculated as follows:

$$\text{Predicted PPU} = \frac{\text{wheat lysine intake (mg/g nitrogen)}/\text{tissue protein lysine deposition from milk (mg/g nitrogen milk intake)}}{\quad} \quad (8)$$

The predicted PPU is likely to be higher than the observed PPU to the extent that utilization is improved by 1) a decrease in the lysine oxidation component of the obligatory and adaptive metabolic demands in response to a low intake and 2) recycling of lysine from the free pool to allow, in effect, supplementation of dietary lysine. The adjustment of lysine intake and deposition for nitrogen intake is made to account for any minor difference in the size of the milk and wheat meals.

The data obtained in these studies allow non-steady state analysis of leucine kinetics in terms of protein synthesis and proteolysis with the use of various modeling approaches. These data, however, do not add to the nutritional issue of estimating wheat protein utilization. It will be reported elsewhere.

TABLE 2
Leucine and derived nitrogen utilization by protein type¹

	Milk	Wheat
Digestible nitrogen intake (mg/kg) ²	90.2 ± 4.2	73.0 ± 9.0 ³
Digestible leucine intake (mg/kg)	64.2 ± 3.0	36.3 ± 4.5 ³
Digestible lysine intake (mg/kg)	55.8 ± 2.6	8.8 ± 1.1 ³
Leucine oxidation		
Postabsorptive (μmol · kg ⁻¹ · min ⁻¹)	0.45 ± 0.13	0.44 ± 0.09
Cumulative postmeal (mg · kg ⁻¹ · 6 h ⁻¹)	34.0 ± 5.5	29.2 ± 4.3
Cumulative postmeal excess (mg · kg ⁻¹ · 6 h ⁻¹)	11.9 ± 1.1	8.3 ± 1.6 ³
Leucine utilization		
Intake - postmeal excess oxidation (mg · kg ⁻¹ · 6 h ⁻¹)	52.3 ± 2.7	27.8 ± 4.1 ³
Nitrogen utilization (mg · kg ⁻¹ · 6 h ⁻¹)	83.7 ± 4.3	44.9 ± 6.5 ³
Lysine utilization (mg · kg ⁻¹ · 6 h ⁻¹) ⁴	49.2 ± 2.3	26.4 ± 3.8 ³
PPU _{nitrogen} ⁵	0.93 ± 0.02	0.61 ± 0.03 ³
Predicted PPU _{nitrogen} ⁶	—	0.222 ± 0.004

¹ $\bar{x} \pm 1$ SD; $n = 5$. PPU, postprandial protein utilization.

²Assumes digestibility values of 1 and 0.93 for milk and wheat, respectively.

³Significantly different from milk, $P \leq 0.05$.

⁴Assumes a lysine-nitrogen ratio in tissue protein of 0.94.

⁵Fractional efficiency of nitrogen utilization (utilization/intake).

⁶Predicted from observed lysine deposition from the milk and lysine contents of wheat.

Statistics

The results are expressed as means ± 1 SD. The influence of protein source on the time course of the postprandial responses was examined by analysis of variance (ANOVA) with repeated measures and post hoc testing at individual time points with Tukey's honestly significant difference test. The influence of dietary protein source on the various variables was examined with simple t tests. We used STATISTICA for WINDOWS (StatSoft, Tulsa, OK) for the statistical analysis. A P value ≤ 0.05 was assumed to indicate significance.

RESULTS

Leucine, lysine, and nitrogen intakes

The meal composition and the gross nitrogen, leucine, and lysine intakes (per kg) during the study are shown in Table 1, and digestible leucine, lysine, and nitrogen intakes are shown in **Table 2**. Intakes include leucine tracer at 0.06 and 0.10 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for wheat and milk, respectively. Although isonitrogenous meals were planned, the measured nitrogen value of the wheat gluten was slightly lower than expected; therefore, actual nitrogen intakes from wheat were 20% lower. Because of the much lower leucine content of wheat than of milk (497 and 712 mg/g N, respectively), leucine intakes from the wheat meals were only 50% of that from milk. The markedly lower lysine content of wheat than of milk (121 and 618 mg/g N, respectively) means that the lysine intakes from wheat were only 20% of those from milk.

Hormonal and biochemical responses

Plasma insulin rose immediately after each protein source was fed, reaching a maximum at 60 min after the meal in each case; the concentrations returned to baseline 4 h after the meal, and no

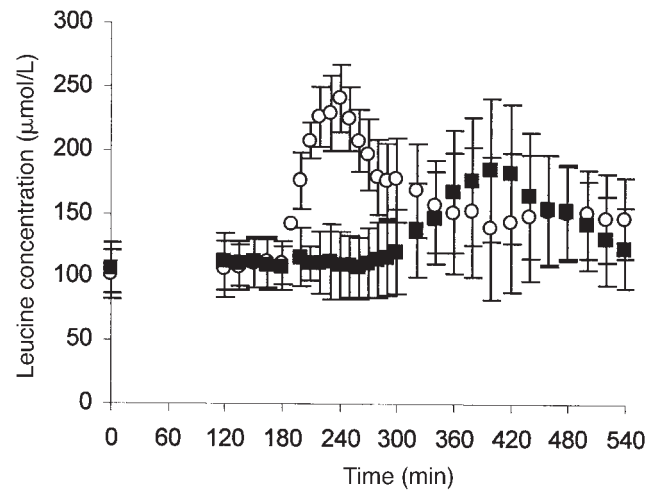


FIGURE 3. Mean (± 1 SD) plasma leucine concentrations during the last 60 min of the postabsorptive period and at the times shown after a meal of either milk (\circ) or wheat (\blacksquare) given at 180 min. $n = 5$. ANOVA: $P = 0.185$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions.

significant differences in the responses between milk and wheat were observed. For plasma glucose, the time course of the response was not significantly different from that of insulin, no significant differences between the 2 meals were observed, and values returned to baseline by 420 min (4 h postmeal).

The responses of plasma leucine to the 2 meals differed, and significant treatment \times time interactions were observed: $P = 0.185$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions (**Figure 3**). After the milk-protein meal, leucine concentrations increased immediately to a peak after 1 h; the concentrations subsequently fell but remained elevated above the premeal value throughout the 6-h postmeal period. After the wheat-protein meal, the leucine concentration peak occurred much later (220 min postmeal), returning to baseline by 6 h postmeal ($P \geq 0.05$ compared with premeal baseline values at 520 and 540 min).

Leucine oxidation and balance

Before each meal there was a plateau enrichment of [¹³C]KIC as evidenced by the lack of a significant difference between means (ANOVA: $P = 0.25$ and 0.961 for milk and wheat, respectively, between 120 and 180 min) and no significant correlations with time ($P = 0.14$ and 0.84 for milk and wheat, respectively, between 120 and 180 min). The enrichment of KIC after the milk meal decreased and subsequently, but slowly, returned toward the premeal value (**Figure 4**). After the wheat meal, the enrichment of KIC did not change initially, decreasing only after 2 h (300 min) to a minimum at 3 h and returning to baseline by 6 h (540 min). There was also a plateau for ¹³CO₂ enrichment, judged as above, and enrichment increased after the meals. The increase was marked for milk, reaching a maximum at ≈ 2 h postmeal (300 min); enrichment decreased after that, more slowly for wheat, reaching a maximum at ≈ 4 h after the meal (420 min). The carbon dioxide production rate also increased after the meals, reaching a peak earlier after the milk meal and returning to baseline but again reaching a peak much later after the wheat meal and remaining elevated for the 6 h postmeal. Leucine oxidation after the 2 meals displayed a pattern not significantly different from the

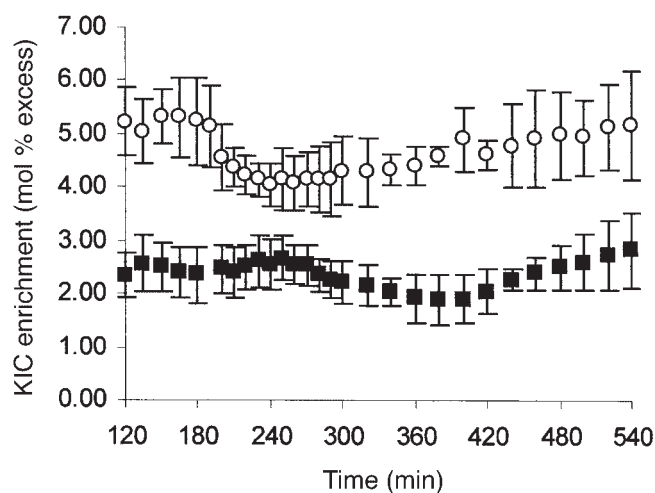


FIGURE 4. Mean (± 1 SD) ^{13}C enrichment of plasma α ketoisocaproate (KIC) during the last 60 min of the postabsorptive period and at the times shown after a meal of either milk (○) or wheat (■) given at 180 min. $n = 5$. Plateau enrichment was achieved, as judged by the lack of significant difference between means (ANOVA: $P = 0.25$ and 0.961 for milk and wheat, respectively, between 120 and 180 min) and the lack of significant correlations with time ($P = 0.14$ and 0.84 for milk and wheat, respectively, between 120 and 180 min).

changes in plasma leucine concentrations (Figure 5). After the milk meal, the rate increased rapidly, peaking much earlier than after the wheat meal and falling slowly back to baseline by 460 min ($P \geq 0.05$ compared with premeal baseline). After the wheat meal, a delayed increase in oxidation was observed, such that the peak rate was not reached until 3 h postmeal; nevertheless, oxidation returned to premeal baseline by 480 min ($P \geq 0.05$ compared with premeal baseline). Significant diet \times time interactions were observed: $P = 0.108$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions.

Calculation of the cumulative leucine oxidation as a percentage of intake indicated that $18.5 \pm 1.6\%$ and $22.9 \pm 3.7\%$ of the meal leucine was oxidized after the milk and wheat meals, respectively (Figure 6). Significant diet \times time interactions were

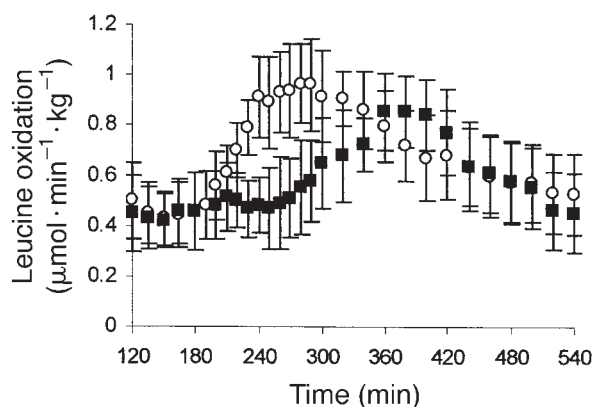


FIGURE 5. Mean (± 1 SD) leucine oxidation during the last 60 min of the postabsorptive period and at the times shown after a meal of either milk (○) or wheat (■) given at 180 min. $n = 5$. ANOVA: $P = 0.108$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions.

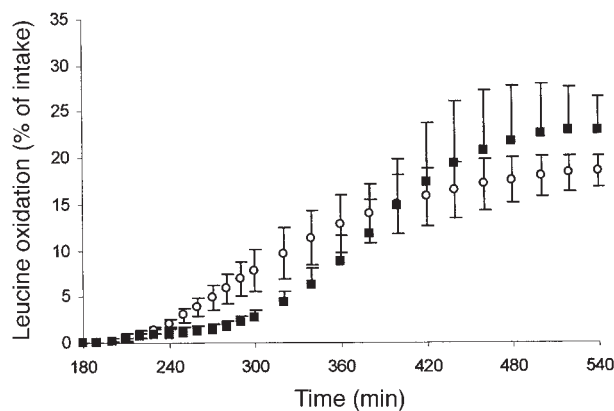


FIGURE 6. Mean (± 1 SD) cumulative postprandial increases in leucine oxidation after a meal of either milk (○) or wheat (■) given at 180 min. $n = 5$. ANOVA: $P = 0.684$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions.

observed: $P = 0.684$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions.

Plasma urea concentrations did not change significantly after the milk meal, consistent with a very efficient overall utilization, but did increase significantly 3 h after the wheat meal from 4.32 ± 1.36 to 5.17 ± 1.19 mmol/L (mean values for all subjects measured between 0–3 and 3–6 h postmeal). Plasma urea concentrations normalized to individual premeal values are shown in Figure 7. Significant diet \times time interactions were observed ($P = 0.0888$ for diet, $P < 0.00001$ for time, and $P < 0.0001$ for diet \times time interactions), and values for wheat were significantly greater than those for milk from 380 min (200 min postmeal), consistent with a less efficient utilization for wheat.

Metabolic demand, PPU, and apparent protein requirement

The digestible intakes, rates of leucine oxidation, and derived rates of leucine, nitrogen, and lysine balances and PPU are shown in Table 2. The rates of leucine oxidation and balance were not

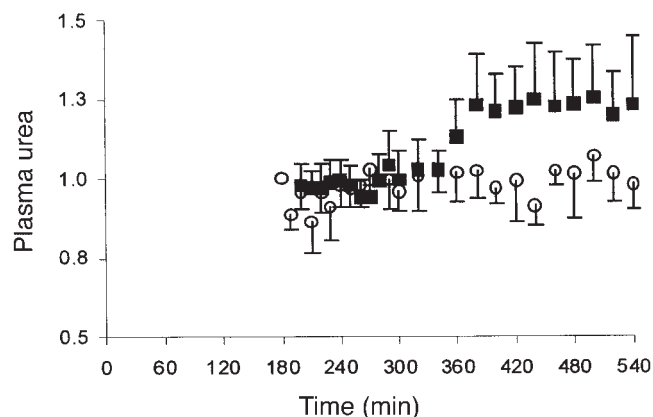


FIGURE 7. Mean (± 1 SD) plasma urea concentrations expressed as a multiple of the premeal values at the times shown after a meal of either milk (○) or wheat (■) given at 180 min. $n = 5$. ANOVA: $P = 0.089$ for diet, $P < 0.0001$ for time, and $P < 0.0001$ for diet \times time interactions. Values for wheat were significantly greater than those for milk from 380 min (200 min postmeal) on, $P \leq 0.05$.

TABLE 3
Metabolic demand and wheat protein and lysine requirements¹

	Wheat	Milk
Postabsorptive loss (mg leucine · kg ⁻¹ · 6 h ⁻¹) ²	2.75 ± 0.90	3.0 ± 0.64
Metabolic demand (g protein · kg ⁻¹ · d ⁻¹) ³	0.81 ± 0.16	
Apparent protein requirement (g · kg ⁻¹ · d ⁻¹) ⁴	1.31 ± 0.23	0.87 ± 0.17 ⁵
EAR for wheat protein (g · kg ⁻¹ · d ⁻¹) ⁶	0.98 ± 0.05	—
EAR for lysine (mg · kg ⁻¹ · d ⁻¹) ⁷	18.3 ± 1.0	—

¹ $\bar{x} \pm$ SD; $n = 5$. EAR, estimated average requirement; PPU, postprandial protein utilization.

²Leucine oxidation – tracer intake.

³Calculated from postabsorptive leucine losses scaled to 24 h (mean values obtained for each subject in both the milk and wheat studies; $n = 10$), assuming that leucine oxidation represents an equivalent loss of tissue protein nitrogen at 4.77 mg leucine/g nitrogen and that the total amino acid–nitrogen conversion factor is 7.31 (see text).

⁴Apparent dietary requirement of either milk or wheat protein for daily balance, calculated as the metabolic demand/PPU_{nitrogen} values in Table 2.

⁵Significantly different from wheat, $P \leq 0.05$.

⁶Calculated from the currently accepted EAR (0.6 g protein · kg⁻¹ · d⁻¹)/PPU_{nitrogen} for wheat in Table 2.

⁷Calculated from the lysine content of the EAR for wheat protein at 18.7 mg/g protein.

significantly different before each of the 2 meals. After the meals, absolute values of cumulative excess leucine oxidation were not significantly different between meals, as opposed to a greater oxidation with the wheat-protein meal than with the milk-protein meal as a proportion of the dose (Figure 6). Leucine utilization (ie, intake – postmeal excess oxidation) and calculated nitrogen and lysine utilization were much greater after the milk meals. PPU_{nitrogen}, calculated from nitrogen utilization as a fraction of nitrogen intake, was greater ($P \leq 0.001$) with the milk meal (0.93 ± 0.02) than with the wheat meal (0.61 ± 0.03). 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 (lysine intake/lysine deposition from milk) was 0.222 ± 0.004.

The metabolic demand and wheat protein and lysine requirements are shown in **Table 3**. The metabolic demand calculated from premeal leucine balance (oxidation – tracer intake) was not significantly different between the 2 studies (0.77 ± 0.25 and 0.84 ± 0.18 g protein · kg⁻¹ · d⁻¹ with the milk and wheat meals, respectively); therefore, a mean value of 0.81 ± 0.16 was used. Calculations with the use of the mean values for metabolic demand and PPU_{nitrogen} for each protein source resulted in apparent protein requirements of 0.87 ± 0.17 and 1.31 ± 0.23 g · kg⁻¹ · d⁻¹ for the milk and wheat meals, respectively. The estimated average protein requirement for wheat (0.6/PPU_{nitrogen}) was 0.98 ± 0.05 g · kg⁻¹ · d⁻¹, indicating a lysine requirement of 18.3 ± 1.0 mg protein · kg⁻¹ · d⁻¹ on the basis of the lysine concentration in our wheat meals of 18.7 mg lysine/g protein.

DISCUSSION

In the present study, we assumed that both protein meals were utilized in the 6-h postmeal period. In fact, in each case, the data indicate that digestion and absorption were delayed. After the milk meal, despite plasma glucose and insulin responses that suggested complete absorption within 3 h and rapid postmeal increases in leucine oxidation and concentrations, oxidation fell slowly and

leucine concentrations did not return to premeal baseline values by 6 h. This suggests a 2-stage digestion and absorption. Whey proteins are rapidly digested and absorbed (34) and together with milk sugars and added starch would mediate the rapid increases in insulin, glucose, and leucine. In contrast, bovine casein precipitates at an acidic pH, forming a clot in the stomach (34); therefore, in our study, there could have been delayed absorption of some of the casein fraction. However, leucine oxidation was only slightly (9%) and not significantly elevated above baseline at 6 h ($P \geq 0.05$ at and after 460 min), resulting in a very small error in calculated PPU values. Even if leucine oxidation took an additional 3 h to return to baseline, this would result in an error in the estimation of the efficiency of milk-protein utilization of <2%. With the wheat meal, leucine oxidation peaked > 3 h after the meal, which, together with the pattern of the other metabolic responses, indicated a delayed absorption of wheat protein, possibly a feature of the dry-fried wheat gluten pancake. Nevertheless, both leucine concentration and oxidation did return to baseline values by 6 h postmeal, with no significant change in the cumulative increased oxidation during the last 60 min. This suggests that absorption was complete.

A second assumption relates to the nature of the utilized dietary protein. We assume that meal leucine is utilized within the body for tissue protein deposition with an assumed leucine–tissue nitrogen ratio to convert leucine balance to nitrogen balance as discussed above. In our previous low-protein, high-protein, steady state, small, frequent-meal protocol, leucine balance was measured at a time when only net protein deposition was occurring. Thus, the leucine–nitrogen ratio in tissue protein would entirely account for the actual leucine–nitrogen ratio of leucine utilization. However, as shown in Figure 1, the meal protein provided for both the obligatory and adaptive oxidative losses (≈42% of utilization at the premeal rate) and net protein deposition (≈58% of utilization). Although a leucine–nitrogen factor can be identified to convert net leucine deposition into nitrogen, the composition of the oxidative losses component in the fed state is unknown, and no such factor can be calculated. Thus, conversion of utilized leucine (ie, intake – excess oxidation) to utilized nitrogen will be uncertain, although the leucine–nitrogen ratio of tissue protein will be the major determinant. Furthermore, leucine may be a major part of the adaptive component of the oxidative loss component of the metabolic demand, given the dietary sensitivity of the branched-chain dehydrogenase enzyme (26). Nevertheless a slightly lower true leucine–nitrogen ratio than we assumed may account for the slightly lower PPU values (0.93 and 0.61) than in our previous study (1.00 and 0.67; 19).

A potential error in PPU_{nitrogen} relates to whether leucine gain involves unbalanced expansion of the free rather than the protein-bound leucine pool. After the wheat meal, the leucine concentration returned to baseline at 6 h, so no errors were involved. However, after the milk-protein meal, the leucine pool size was greater at the end of the test period. Because this amounted to only ≈3.7% of the meal leucine, with leucine oxidation returned to baseline, it might be assumed that this slight expansion of the free amino acid pool represents amino acids that would be available to meet the metabolic demand as either net protein deposition or any other metabolic fate. Alternatively, the slight elevation in concentration might represent surplus leucine in milk protein, still to be oxidized at a slightly elevated rate, that was not detectable. Importantly, in either case, no appreciable error was likely.

Finally, our design objective of isonitrogenous meals was not achieved. The nitrogen content of the wheat meal was 81% that of

the milk meal. It might be assumed, therefore, that wheat-protein utilization was better at this lower intake, simply on the assumption of an improving efficiency of utilization with decreasing protein intakes, as indicated by previous nitrogen balance studies. However, our own studies failed to detect any influence of meal nitrogen content on the efficiency of milk-protein utilization (35).

As in our previous study (19), the efficiency of wheat utilization was much higher than would be predicted if wheat supplied all of the lysine needs for the required net protein deposition (ie, that observed in the same subjects with milk protein): ie, utilization of 26.4 mg lysine/kg in the 6 h after the meal, which only supplied 8.8 mg lysine/kg with 17.6 mg/kg unaccounted for. Part of this likely reflects the recycling of lysine released by net proteolysis during the postabsorptive phase into net protein deposition during feeding (9, 6, 12) by virtue of the relatively larger free intracellular pool size of lysine than of most other indispensable amino acids (36). The reduction in the free lysine pool in human muscle after feeding protein-free meals (36) is equivalent to 17.5 mg/kg lean tissue per 3 h, which is sufficient to entirely account for the deficit implied by the present studies. Thus, even though lysine oxidation adapts to the level of intake and varies throughout the day in meal-fed rats (37), it is not cleared from the free pool in human muscle as quickly as are leucine and other indispensable amino acids after a protein meal (36). Also, in lysine-deficient rats, a delayed lysine supplement (given 12 h after the remaining protein and amino acids) was utilized as effectively as was lysine given as part of a balanced meal (38). This was in contrast 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. Neither plasma nor intracellular lysine was measured in these experiments, so that the extent to which lysine recycling occurs requires further study. 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 fall, reducing the requirement for lysine for net protein deposition.

A second mechanism that could have accounted for the better than expected utilization is an acute decrease in the adaptive metabolic demand component of lysine oxidation in response to the low-lysine meal. Lysine oxidation represents part of the 28 mg lysine utilized after the milk meal (≈ 12 mg, ie, the lysine oxidation contribution to the postabsorptive loss; Figure 1), so that an acute decrease in this component means that the intake was only having to provide for 16 mg as actual protein deposition, implying a much lower deficit.


A third mechanism involves the possibility of some *de novo* synthesis of lysine by colonic microflora utilizing urea nitrogen (39), as observed in infants (40)—when urea salvage rates are high (41)—and in adults (42). In those studies ^{15}N was transferred from urea or ammonia to systemic lysine, implying *de novo* synthesis of lysine in nutritionally significant amounts (40, 42). Because we would expect relatively low rates of urea salvage in our subjects given their background protein intakes, in this case the *de novo* lysine supply may be less than is maximally possible. However, it may be more important in subjects chronically fed wheat protein.

As discussed previously (19), the implications of these values in the context of the current debate about lysine requirements and protein-quality evaluation in human adults can be examined from many perspectives.

First, nitrogen balance studies of wheat-protein utilization indicate a much lower value for net protein utilization (0.41 of that of

egg protein; 28). However, nitrogen balance assays of protein quality are metabolically complex and differ from slope assays obtained in growing animals. This is because, within an adaptive model of protein homeostasis and requirements, in multilevel feeding trials with subjects adapted to each intake, the metabolic demand will increase with the intake so that the slope of the relation between nitrogen balance and nitrogen intake will markedly underestimate the true efficiency of any protein utilization (3, 35).

Second, the implied lysine requirement of $18.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ reported here and the value of $23.2 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ in our recent small-meal protocol study (19) is only an approximate estimate of the requirement and is likely to be greater than the physiologic minimum. This is because the adaptive metabolic-demand model of protein and amino acid requirements includes several responses to intake, which make prediction of protein utilization and quality difficult. Thus, in response to either a lower protein intake (12, 24) or a lower-quality protein intake (6), a decrease in the adaptive metabolic demand results in a decrease in postabsorptive losses and postprandial repletion, ie, a decrease in the amplitude of the diurnal cycle of protein gains and losses, reducing the metabolic demand for lysine for net protein deposition after a meal. Similarly, a reduction in the length of the postabsorptive period from the 12 h in our studies will also reduce postabsorptive losses and the need for net protein deposition after a meal. On this basis, the requirement of $19 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ indicated by the nitrogen balance data (6, 16) and a higher consequent PDCAA (protein digestibility corrected amino acid) score of wheat (0.74) would appear to be realistic.

In conclusion, the results of our studies show that in subjects adapted to generous protein and lysine intakes, the efficiency of wheat-protein utilization from a single large meal is higher than would be expected from recent reports of lysine requirements and higher than would be expected from theoretical predictions. The results are similar to those from our previous studies in subjects fed frequent small meals and imply that adaptive mechanisms of lysine conservation occur in subjects with typical mixed protein intakes. Our data indicate an average lysine requirement of $18.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, a value consistent with that indicated by the nitrogen balance data and lower than other more recently reported values. 

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