What Is Apparent Is Not Always Real: Lessons from Lysine Requirement Studies in Adult Humans

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The importance of obtaining reliable estimations of the quantitative requirements for the indispensable amino acids, especially of lysine, in human protein nutrition has been articulated (1,2). However, there has been a great deal of discussion and also disagreement about the methods used for determining the estimated requirements (3–7). Tracer-based experimental approaches have gained preference over the use of earlier nitrogen balance techniques, which provided the basis for the FAO/WHO (8) and FAO/WHO/United Nations University (UNU)2 (9) international recommendations for amino acid requirements and dietary amino acid requirement patterns. Nevertheless, Millward and co-workers (10) recently criticized earlier tracer-based estimations of the lysine requirement in healthy adults, including those involving the 24-h direct (11,12) and indicator amino acid oxidation (IAAO) methods (13,14) as well as those based on the short-term, fed-state IAAO technique (15,16). Hence, these investigators (10) determined an “apparent requirement” for lysine in subjects adapted to their usual diets. The determination was based on the oxidation of 13C-leucine, using a 13C-leucine tracer model that involved a single meal of wheat gluten protein, over a 6-h postprandial period. At the end of 6 h, leucine oxidation and plasma concentrations had returned to their premeal values, suggesting that the absorption and initial metabolism of the meal protein was complete.

The postprandial utilization (efficiency of utilization; PPU) of the nitrogen (N) intake was calculated as the ratio of the N utilized in protein deposition (measured as the cumulative difference between the postmeal leucine oxidation rate and the premeal oxidation rate, with a conversion to an N retention value based on an assumed body tissue protein leucine:N ratio) and the N intake supplied by the meal. Thus, the N utilized in this case is assumed to be the equivalent of “leucine intake less the meal-dependent leucine oxidation.” Also, assuming that the utilization of wheat gluten is limited by its lysine content, the requirement for lysine was then calculated from the ratio of an assumed average requirement for good quality protein [0.6 g/(kg · d)] and the PPU, with the lysine content of the largely wheat gluten protein meal being taken to be 18.7 mg/g wheat protein. On the basis of this approach, Millward et al. (10) concluded that the daily lysine requirement for healthy subjects, adapted to their usual and presumably adequate diet, is 18.3 mg/(kg · d). This interpretation of the data raises two critical issues: 1) the derivation of this so-called “apparent” requirement value; and 2) whether it is appropriate for the authors to conclude that their study “indicates an average lysine requirement of 18.3 mg lysine per day . . . .” In doing so, they link this estimate to the amino acid requirement values in United Nations reports (8,9), used for the evaluation of food protein quality, diet adequacy and for the planning of food supplies and diets.

We examine here the assumptions and approach used by Millward et al. (10) and show how difficult it is to draw, from their model, any firm conclusions about the physiological requirements for lysine in healthy adults. Thus, with respect to the first issue above, there are problems with both the reasoning and the specific assumptions made by Millward et al. (10) in applying their PPU approach. It is impossible to estimate exactly how much of the leucine oxidized during the fed period is due specifically to exogenous and endogenous sources of leucine. Therefore, it is not possible to measure precisely the PPU nitrogen for the N present in the test meal. Thus, for example, the assumption might be made that essentially all of the leucine oxidized in the fed state is derived from the exogenous leucine (wheat protein). In this case, a recalculated PPU would be 0.2, and the lysine “requirement” would be ~58 mg/(kg · d). However, this is an unlikely metabolic condition because wheat gluten N is retained with a higher efficiency (17) than this calculation would imply. Hence, it is likely that the “apparent lysine requirement” as determined by the present PPU approach would be less than this relatively high, upper estimate.

Millward and co-workers (10) recognized that the rate of endogenous protein breakdown and, in turn, of endogenous leucine oxidation declines with ingestion of a protein-containing meal. We reviewed our data (unpublished) generated on leucine kinetics in well-nourished, Indian subjects given a protein intake of 1 g/(kg · d), with differing levels of leucine intake [50 and 107 mg/(kg · d); n = 60] and with all other indispensable amino acids given in adequate amounts. Our analysis indicates a 29% reduction in protein breakdown with the feeding of small frequent meals. Studies by other investigators, using the same kinetic model and tracer, have reported similar results. Melville et al. (18) observed a 65% reduction in protein breakdown postprandially in subjects receiving 1.5 g protein/(kg · d). Motil et al. (19,20) reported 29 and 49% decreases in postprandial protein breakdown in adults consum-

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2 Abbreviations: EAR, estimated average requirement; IAAO, indicator amino acid balance; IAAO, indicator amino acid oxidation; PPU, postprandial protein utilization; UNU, United Nations University.
ing 0.6 and 1.3 g protein/kg·d, respectively. In another short-term leucine kinetic study of normal Indian adults receiving 1.1 g protein/kg·d, the postprandial protein breakdown was reduced by 36% (21). In children fed ~4 g protein/kg·d, the postprandial protein breakdown was reduced by 34% (22). Hence, a reasonable assumption is that there is a 35% reduction in protein breakdown with ingestion of a complete meal. If so, then this would lead to a higher calculated value for the postmeal-dependent excess leucine oxidation [by ~7 mg/(kg·6 h)] and, thus, a lower PPU for wheat nitrogen, or ~0.45, according to our calculation. This recalculated PPU, along with an estimated average requirement (EAR) for protein of 0.6 g/(kg·d) would then give a requirement for wheat protein of 1.32 g/(kg·d), and an apparent requirement for lysine of ~25 mg/(kg·d). In this calculation, the intake of tracer leucine [3 mg/(kg·d)] is ignored, as was also done by Millward et al. (10). Further support for this recalculation comes from a study of postprandial milk protein oxidation by Gaudichon et al. (23), who found that 15–20% of the protein-based amino acid intake was oxidized, leading to a PPU nitrogen for milk protein of 0.85. This compares with the higher estimation of the PPU nitrogen for milk of 0.93 made by Millward et al. (10) and in part probably indicates the approximate error involved in estimating the meal-derived N utilization by this method.

A further assumption made by Millward et al. (10) is that the bicarbonate recovery factor was constant for the last 5 h of the fed state. This factor was based on data, reported in an abstract, from an hourly, small meal feeding experiment (24), rather than from an experiment that mimicked the single large meal protocol used by Millward et al. (10). Direct experiments at MIT on the recovery of bicarbonate using a large meal feeding paradigm have shown that the recovery of bicarbonate increases from ~75% in the fasted state to 90% immediately after the meal begins, and then declines gradually such that it is near premeal level ~3 h after beginning the meal (25). If this reduction in the recovery of bicarbonate also applies to the study by Millward et al. (10), this would tend to raise the estimate of lysine requirement; a reduction in the mean recovery of bicarbonate over 6 h, from the assumed value used of 0.9 to that of 0.85 or 0.8, would lead to an increase in the apparent requirement for lysine of ~2–3 mg/(kg·d). Similarly, if the tracer given during the postabsorptive state was oxidized rather than retained, this would result in a further increase in the estimated rate of meal-dependent oxidation and thus the estimate of requirement. These and a number of other problems related to 13C isotopic measurements of amino acid oxidation rates were reviewed by Millward (26) but they were not critically considered in the evaluation of their recent experiment (10). However, we (27) recently determined, in the case of a Gauvain tracer given during the postabsorptive period, that the tracer is not oxidized and is thus presumably retained in the free amino acid pool with its later utilization for protein synthesis or oxidation when a mixture of meal-derived amino acids is absorbed.

The EAR for protein used in the calculations by Millward et al. (10) for estimating the lysine requirement is 0.6 protein/kg·d. However, a recent analysis of a large body of published N balance data leads to an EAR of 0.66 g/kg for good quality protein (28). If this new figure is used to estimate the lysine requirement in the example above, it would further increase the apparent lysine requirement value (Table 1), although only slightly.

The use of a bolus feeding pattern, in contrast to a small meal feeding pattern, appears to result in somewhat lower leucine oxidation rates, and the amount of leucine oxidized at each subsequent meal during the day may not be the same (25). This effect of meal pattern on the rate of leucine oxidation seems to be independent of the prevailing leucine intake (29). Again, this response of leucine oxidation to meal pattern may be relevant to the interpretation of short-term studies in which a 5-h postmeal leucine oxidation rate is the primary outcome variable such as with the Millward model (10). Assuming a 16% higher rate of leucine oxidation as might occur with the multiple meal feeding pattern (25), recalculation as detailed above would generate a lysine requirement estimate of 30–35 mg/(kg·d), depending on whether the leucine tracer intake was included in the total leucine intake. Thus, the conclusion drawn by Millward et al. (10), that the value of 18.3 mg lysine/(kg·d) is an average apparent requirement is based on a set of questionable assumptions. As argued above and depending on the assumptions, the value might well be close to 30 mg lysine/(kg·d) (Table 1). Millward et al. (10) criticized our 24-h IAAO and indicator amino acid balance (IAAB) studies of the lysine requirement (14) because of the estimated positive leucine balance values obtained. It might be pointed out, however, that an analysis of the pattern of the 12-h fed state and the 24-h leucine oxidation rate, as well as the daily absolute leucine balance, with changes in lysine intake all gave similar requirement estimates. Furthermore, in contrast to the positive IAAB (13C-leucine balances) obtained in our earlier studies, which we consider resulted from the high leucine intake given to subjects in that study (14), a recent experiment from our laboratories (30) using the same approach but with leucine intakes that were closer to requirement levels, provided a similar lysine requirement estimate of 30 mg/(kg·d), while simultaneously showing that the daily leucine balances were at equilibrium when lysine intakes were at or above the requirement level of lysine intake. The point about the value of the use of the pattern of change in oxidation for estimating the requirement by the indicator amino acid oxidation approach is also underscored by the recent short-term IAAO study by Kringsnynos et al. (31). These investigators showed that although the absolute rate of oxidation of the 13C-phenylalanine indicator was higher when it is given orally compared with the intravenous rate, the estimated lysine requirement was virtually identical.

Finally, although our recalculation of the data of Millward et al. (1) provide an “apparent” lysine requirement value that

| Table 1 |
| Leucine and derived N utilization for wheat protein: a recalculation¹ |
| - Digestible N intake, mg/kg | 73.0 |
| - Digestible leucine intake, mg/kg | 36.3 |
| - Leucine oxidation | 13.5 |
| - Postabsorptive, mg/(kg·6 h) | 30.9 |
| - Cumulative postmeal, mg/(kg·6 h) | 17.4 |
| - Cumulative postmeal excess, mg/(kg·6 h) | 18.9 |
| - Nitrogen utilization, mg N/(kg·6 h) | 30.2 |
| - PPU nitrogen | 0.41 |
| - Lysine requirement, mg/(kg·d) | 29.8 |

¹ Recalculated from Table 2 in Millward et al. (10) assuming the following: 35% reduction in endogenous postprandial leucine oxidation; change in 13C-bicarbonate recovery from 0.9 to 0.8 (postmeal); estimated average requirement (EAR) for protein of 0.66 mg/(kg·d) (see text). PPU, postprandial utilization.
is similar to the lysine requirement value obtained from our tracer studies and close to requirement estimates derived by the Toronto group (15,16) it should not be concluded that the PPU model, as applied in this investigation by Millward et al. (10), is necessarily suitable for measuring the physiologic requirement for lysine in healthy adults. We believe that the experiment, more specifically, provided an approximate estimate of the lysine retention following a wheat gluten-containing meal when given to healthy subjects who were fully adapted to their usual and generous intakes of both protein and lysine. The relationship between this lysine retention figure and a physiologic lysine requirement value, which we would define as the lowest continuing intake of lysine that is sufficient to maintain body lysine equilibrium, was not determined in that study nor by these investigators in their earlier, similar experiment (32). Indeed, we (30) were critical of the approach used in the latter study (32) as well, in which the PPU of the N intake was calculated as the slope of the line relating N balance (determined from 15N-leucine trs) (10,13) to N intake. However, the postprandial expansion of the free leucine pool was unaccounted for when calculating the retention of protein-bound leucine (balance). This, in turn, would have resulted in an overestimate of the efficiency of N utilization from wheat protein and an underestimate of the apparent requirement for lysine. Additionally, it is to be appreciated that the Millward model is based on a single level of protein/lysine intake with extrapolation of the findings to an “apparent” requirement intake level. It would be highly desirable to validate such an extrapolation by conducting studies at multiple intake levels of leucine.

In our opinion, these short-term protocols based on the metabolic demand and PPU concept (10,32) illustrate more importantly, the ability of tissues to recycle during the prandial period the lysine released into the free leucine pool via proteolysis during the postprandial/fasting phase of amino acid metabolism. Additionally, they imply that this recycling can occur with a high degree of efficiency when a single meal deficient in lysine is consumed. It would be of considerable interest to assess the quantitative rates of leucine oxidation and utilization as in the present experiment but in subjects who were adapted to different and lower lysine intakes before the tracer study. The longer-term, steady-state, nutritional significance of a so-called “apparent” lysine requirement value of 18.3 mg/(kg · d) is not evident. In our view, the PPU model, without further validation and as applied in this (10) and an earlier study (32) in which the “apparent” lysine requirement was estimated to be 22 mg/(kg · d), does not provide a satisfactory basis for nor a suitable alternative to the 24-h IAAO and IAAB methods (14,30,33) for establishing an adult amino acid requirement.

In our view, the PPU model, as applied in this investigation by Millward et al. (10,32) and the Toronto group (31,34), directed toward the development of new advances in methods of assessing dietary amino advances in methods of assessing dietary amino acid requirements for adult humans. J. Nutr. 125: 2907–2915.


