

Next Generation Diet Formulation: True Metabolic Availability of Amino Acids in Diets for Pigs

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■ Abstract

Not all of the dietary amino acid that is included in standardized ileal digestibility is metabolically available for protein synthesis. A rapid method was developed to determine metabolic amino acid availability in feedstuffs for protein synthesis. Using the indicator amino acid oxidation technique, a change in phenylalanine oxidation, which is inversely proportional to protein synthesis, was used to measure the metabolic availability of lysine in feedstuffs compared to that of free lysine, which is fully available. Data regarding several methodological considerations were addressed before this technique was validated, including: adaptation time between test diets, repeatability of measurements, predictability of response to lysine intake, dietary phenylalanine availability and lysine intake sensitivities. In addition, an oral dosing regimen was developed to simplify the facilities required and lower costs further.

This isotope dilution method is suitable for the determination of the metabolic availability of amino acids for protein synthesis in a given feedstuff within 2-3 weeks. This method could also be adapted to predict the availability of other economically important amino acids in feedstuffs (ie threonine, methionine, tryptophan). This new approach is an improvement over the chemical methods which are only available for lysine.

■ Introduction

'Nutrient Requirements of Swine' by NRC (1998) introduced a major improvement over previous methods of expressing amino acid requirements

and amino acid supply in feeds. This improvement adjusted amino acid digestibility in feedstuffs by estimates of endogenous losses, primarily the amino acids that are excreted into the intestine as enzymes, and other proteins and amino acids that are lost via intestinal cell turnover. However, we should now consider the next improvement in defining amino acid requirements and amino acid supply in feeds.

The state of the art for expressing amino acids in feed is standardized ileal digestible amino acids. Expressing amino acid contents of feeds as a total amount neglects all losses of amino acids due to incomplete digestion and absorption and inefficiencies in metabolism. This was the standard until 1988 (NRC 1988), when it was recommended that ileal digestibility be adopted, even though at that time there were only a few feeds for which amino acid digestibility was available. Ileal digestibility, although a significant improvement, was soon found to have a major problem; for many ingredients, formulating on ileal digestibility did not accurately predict pig performance. Endogenous amino acid losses were subsequently found to be the reason for differences among feed ingredients. Although a major step forward conceptually, the current 'standardized ileal digestibility', used by NRC (1998), does not account for all the variation among feedstuffs in amino acid losses and inefficiencies that occur.

There are 2 reasons why standardized ileal digestibility is not, and should not be accepted as, the final answer. One reason is that endogenous losses can be very different among feeds, particularly for those feeds that contain anti-nutrients. This problem was clearly shown by slope-ratio growth and serial slaughter trials where pigs were fed diets formulated to various ileal digestible lysine levels (Batterham, 1992). Comparisons of protein deposition (lean growth) and feed conversion at similar intakes of ileal digestible amino acid, using feedstuffs versus diets with synthetic amino acid, showed that different feedstuffs did not produce the same performance as an equivalent intake of synthetic lysine. This large body of time-consuming, expensive and difficult work by Batterham clearly demonstrates that neither ileal digestibility nor standardized ileal digestibility should be considered the final word in accurately predicting the protein deposition of the pig. Therefore the use of a standardized value for endogenous losses, as employed by NRC (1998), is really not a major improvement until we have data on the endogenous losses for all the feed ingredients that we use. This is an enormous undertaking and how to address this problem is the subject of considerable debate. (DPP 2003)

The second reason to move beyond standardized ileal digestibility is that not all of the amino acid that is digested and absorbed is available for protein synthesis (ie protein deposition by the pig). For example, some lysine that is digested and absorbed is in a chemically complexed form that cannot be used by the pig to make body protein (Carpenter and Booth, 1973). This 'metabolically unavailable' lysine is mainly catabolized and used for energy. For

example, consumption of cottonseed meal has been shown to increase the catabolism of lysine (Ball et al, 1995).

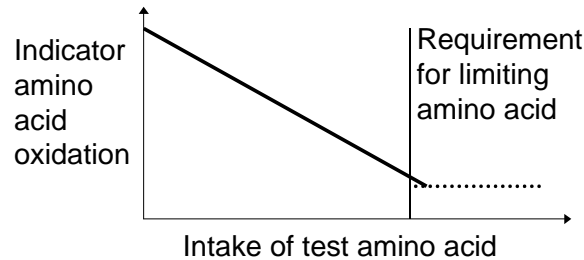
Although “metabolically available amino acid” content of a feedstuff can be determined by slope-ratio growth assays, these assays are very expensive and very time consuming (van Barneveld, 1993). For example, a slope-ratio experiment to determine the metabolically available lysine in a single feedstuff, based on the designs of Batterham (1992), would require 100 pigs, and 8-10 months to complete, at an estimated cost of \$30,000-\$40,000. This information would be out of date by the time it was available because by then most the feedstuff has already been fed commercially. The metabolically available lysine needs to be determined frequently because not only can it vary from batch to batch of canola or soybean meal; it can even vary within a feedstuff when ileal digestibility seems to be the same (Batterham, 1992).

Therefore, there are several reasons why we need to develop a rapid method for determining metabolic amino acid availability for protein synthesis in pig feeds. Such a method would circumvent the need to measure endogenous amino acid losses in all feedstuffs because metabolic availability would include the effects of all losses, regardless of the source or reason. If information on metabolic amino acid availability was available on many feedstuffs, then diets could be formulated much more accurately and more cheaply, relative to performance, and animal performance would be less variable over time. We believe that the indicator amino acid oxidation method can fill this need.

■ Indicator Amino Acid Oxidation

The indicator amino acid oxidation method (IAAO) is based on the fact that, because there is no significant storage of amino acids in the body (Holt et al., 1962), amino acids that are consumed are partitioned between either protein synthesis or oxidation (for reviews see: Zello et al., 1995, Brunton et al., 1998, Bos et al., 2002, Pencharz and Ball, 2003, Bertolo et al., 2004). Briefly, if a dietary amino acid (ie lysine) is limiting protein synthesis, then all other amino acids are in excess and must be oxidized. We have chosen phenylalanine as an indicator of the partitioning of these excess amino acids between oxidation and protein synthesis. Thus, when dietary lysine is deficient, the phenylalanine cannot be incorporated into protein and the excess must be oxidized. As dietary lysine is increased, more phenylalanine is incorporated into protein and less is oxidized. Once lysine is increased above its requirement, then phenylalanine oxidation remains constant because increasing amounts of lysine do not lead to increases in protein synthesis. The dietary lysine level at which phenylalanine oxidation changes from a decreasing pattern to a constant pattern is called the breakpoint and is equivalent to the lysine requirement (**Figure 1**).

Figure 1. Indicator amino acid oxidation response to increasing intake of limiting amino acid



Phenylalanine oxidation reflects protein synthesis, which in turn is dependent on the lysine metabolically available for incorporation into protein. Thus, phenylalanine oxidation reflects 'metabolically available' lysine and does not require any assumptions or require any indirect calculations regarding dietary digestibilities or endogenous losses specific to feedstuffs. Therefore, we can calculate metabolically available lysine from phenylalanine oxidation provided the animal is fed lysine at a level that is deficient, or within the decreasing phase of oxidation. We have tested this hypothesis and validated the technique for use with different feedstuffs of unknown metabolic lysine availability. However, as with all new methods, there were several methodological considerations that needed to be addressed and validated before the final form of the technique was acceptable. The methodological questions included:

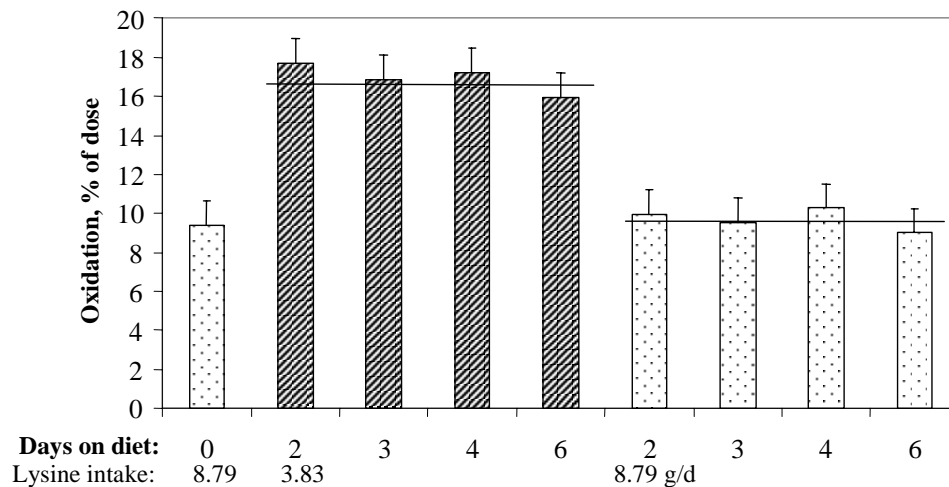
How long do the pigs need to be adapted to the diet before they achieve a steady state in amino acid oxidation and protein synthesis?; How repeatable are the measurements?; What is the metabolic availability of the indicator and is this major problem?; Can we simplify the technique, which has previously used intravenous infusions of labeled indicator, to an oral dosing method?.

■ Adaptation

A time efficient application of the IAAO method to individual growing animals requires knowledge of the time required for changes in protein synthesis to occur in response to changes in the diet. Therefore, we examined the effect of period of adaptation on IAAO (Moehn et al., 2004). We showed that weaner pigs adapted within less than two days to a change in lysine intake (**Figure 2**), regardless of the direction of change in dietary lysine intake. Adaptation may have occurred even earlier, but we did not measure oxidation on the day after

the change in diet. In non-pregnant sows, we determined that a decrease in protein intake caused the phenylalanine oxidation to respond within 18 h, with no further change when the new dietary regimen was maintained for ten days (Moehn et al., 2004). It should be noted that the short adaptation time for the IAAO was demonstrated irrespective of the manipulation of a single amino acid or of all dietary amino acids, except the indicator amino acid which must be held constant. We have previously shown in humans that indicator oxidation was not different after 3, 6 or 9 days of adaptation to a diet deficient or adequate in lysine intake (Zello et al., 1993) and that 2 days adaptation to a fixed diet minimized the variation between individuals (Thorpe et al., 1999). Therefore, the short adaptation time appears to be maintained regardless of the rate of body protein turnover, which differs widely between growing and adult animals or human subjects (Waterlow et al., 1978). The reason for this may be that there is no significant storage of amino acids in the body (Holt et al., 1962) and therefore excess amino acids are rapidly channelled to catabolism. This is in contrast to nitrogen balance which requires a week or more of adaptation to achieve a steady state (Bingham and Cummings, 1985). We have concluded that two days are fully adequate to achieve adaptation when using the IAAO.

Figure 2. Effect of period of adaptation time on phenylalanine oxidation in growing pigs of 20 kg body weight receiving 3 levels of lysine intake. Regression of phenylalanine oxidation on period of adaptation time within lysine intakes was not significantly different ($P > 0.1$) from zero.



■ Repeatability of Measurements

The suitability of a method is dependent on its precision, which can be judged by the repeatability of measurements. The repeatability of the oxidation measurement was derived from the coefficient of variation of the oxidation within pigs and diet level. The CVs of the oxidation measurements in growing pigs (11.2%) and sows (9.25%) were lower than CV estimates for conventional measurements, such as plasma free amino acids, plasma urea and the urinary N to creatinine ratio (Moehn et al., 2004). This result is not surprising when one considers that the oxidation method relies on a single metabolic process (phenylalanine oxidation) rather than the net effect of several complex metabolic processes that are typically involved in nitrogen metabolism. The CV of the apparent and true ileal digestibilities of amino acids was estimated as 7 – 8% (Moughan et al., 1987) in complete feed. However, when using the difference method, the CV appears to be much greater, reaching almost 60% at inadequate inclusion levels (Fan and Sauer, 1994).

■ Metabolic Availability

Under the conditions of these experiments, phenylalanine (PHE) oxidation reflects protein synthesis, which in turn is dependent on the lysine available for incorporation into protein, which we have termed 'metabolically available'. Thus, PHE oxidation reflects only the metabolically available lysine for protein synthesis and does not necessitate any assumptions or any indirect calculations regarding dietary digestibilities or endogenous losses specific to feedstuffs because these are already accounted for. Therefore, we can calculate metabolically available lysine directly from PHE oxidation.

The response of indicator (PHE) oxidation to a diet containing synthetic lysine (which is regarded as 100% available for protein synthesis) versus an intact protein source (where some of the lysine is not available for protein synthesis) is proportional to the metabolic availability of lysine in the feed. We know that this response occurs over long feeding periods when protein synthesis is measured by changes in protein composition of the body (ie the slope ratio assay). However, no one has previously demonstrated that isotope methods could be used to measure lysine availability in feedstuffs based upon this principle.

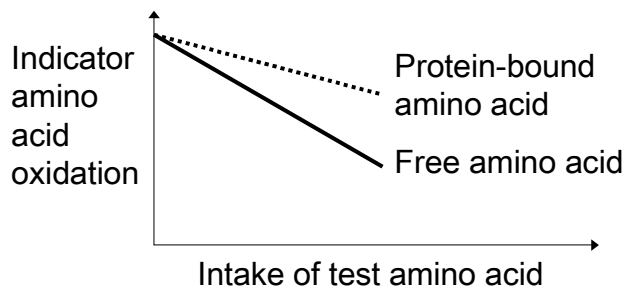
There are three crucial conditions that must be fulfilled for IAAO to be applied to metabolic availability of amino acids. First, the amino acid tested (ie lysine) must be the first limiting amino acid. PHE oxidation, as well as protein synthesis, is driven by the dietary content of the first limiting amino acid. Condition one also implies that all amino acids except lysine are at relative excess and thus not limiting for protein synthesis. Second, the content of PHE

(and of tyrosine) must be maintained constant across all diets employed, including those containing the test feedstuff. This will ensure that change in PHE oxidation is a result of changes in protein synthesis and not due to changes in PHE intake. Third, the response of PHE oxidation to a change in dietary lysine content must be predictable. There are several other conditions that must be met in any experiment to measure amino acid availability, and these continue to hold true for IAAO.

Testing of feedstuffs using IAAO is achieved in a similar fashion as the determination of digestibility by the difference method: A base diet is formulated with a given lysine content and a certain percentage of a lysine-free ingredient (e.g. starch). This lysine-free ingredient can be substituted for feedstuffs to be tested or for free lysine (free amino acid mixture) as the reference. Any change in oxidation relative to the base diet must be a result of the lysine in the feedstuff or in the free amino acid mixture. Therefore, the calculation of the metabolic availability of protein-bound lysine in feedstuffs is as follows (**Figure 3**):

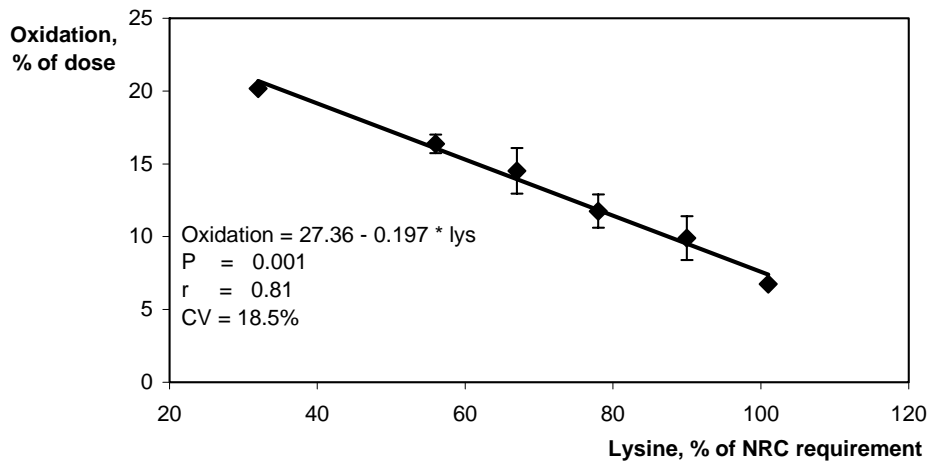
$$\text{Metabolic availability} = \frac{\text{oxidation change per g lysine from test ingredient addition to base diet}}{\text{oxidation change per g free lysine addition to base diet}}$$

Figure 3. Determination of metabolic availability via indicator amino acid oxidation



We have shown that at dietary lysine intakes below the requirement of individual animals, the PHE oxidation decreased linearly with increasing dietary lysine intake (**Figure 4**). PHE oxidation was correlated to the dietary lysine intake with $r = 0.76$ to $r = 0.93$ (all regressions, $p < 0.05$). Furthermore, because the slopes of these regressions were not different between pigs ($P = 0.66$), we were able to combine all 30 observations. The overall correlation between PHE oxidation and dietary lysine intake was $r = 0.81$ and the rate of decline was 0.177 % of PHE oxidation per unit of lysine intake (percent units of NRC recommendation) (SE 0.024, $p = 0.0001$).

Figure 4. Linearity of response of phenylalanine oxidation to a change in lysine intake in growing pigs.



Based on the data for 9 animal studies thus far, we have concluded that limiting the upper intake of the test amino acid lysine at 85% of the requirement, according to NRC (1998), will ensure a linear response in oxidation by growing pigs. This limit is important to maintain because once the requirement of the individual animal is met, then PHE oxidation is no longer linear. This safety margin is approximately two standard deviations below the mean requirement for lysine, which means there is a very low probability that the lysine requirement of an individual animal will be exceeded during the experiment.

■ Oral Isotope Delivery

Amino acid isotope kinetics studies have traditionally been conducted using intravenous infusion in order to be able to accurately quantify the dose delivered into the plasma amino acid pool. We initially developed this technique using surgically installed intravenous catheters in growing pigs in order to prove the concept was feasible and to maintain a constant infusion rate that was as accurate as possible. However, maintaining chronic catheters in fast growing pigs was found to be problematic and expensive and required specialized equipment for infusions. Because of these concerns about the health and welfare of catheterized pigs and in order to facilitate the practical application of this method by others, we developed a less invasive oral isotope delivery model.

In order to validate the oral dosing model, we needed to ensure an accurate, constant intake of isotope by the animal. The pigs were fed their half-daily feed allotment in 8 half-hour aliquots with the isotope mixed with the feed as mash. Typically, all pigs ate their small meals immediately when offered; but even in the few pigs that ignored the occasional feeding, the plateau of dose oxidized was not significantly affected, probably because stomach-emptying and absorption buffers small changes to label appearance in the plasma. Linearity data ($r = 0.81$) were not different from those determined with intravenous dosing and repeatability was improved slightly (CV of 10.0%). Thus, oral administration of isotope in the feed is an equally effective and simpler approach than the previous method of intravenous infusion.

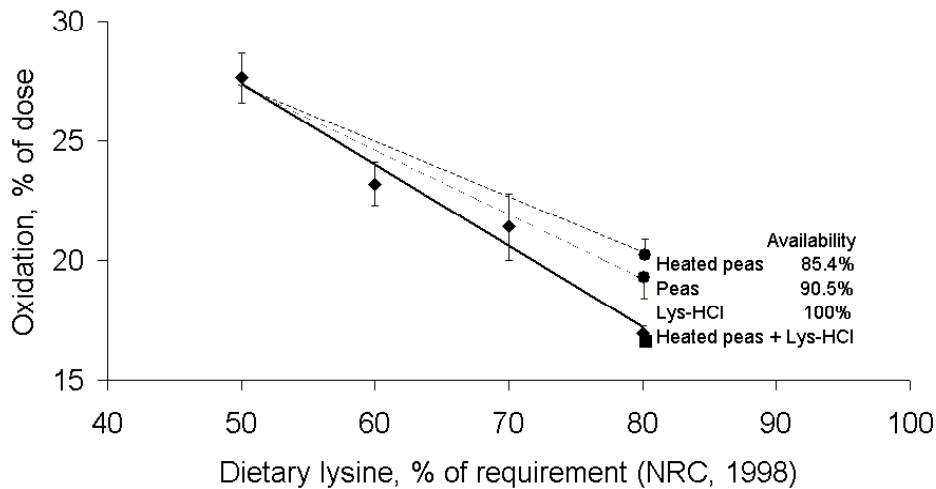
■ Metabolic Availability of Lysine in Test Ingredients

In order to test the concept using practical diets, we first employed peas in a controlled experiment. Peas were chosen as the test ingredient because of the extensive work by van Barneveld et al. (1994a, 1994b, 1994c) to characterize the lysine availability in this feedstuff using several methods. In addition to being a common feedstuff for swine, peas are normally not heat processed, unlike soybean, canola or meat meals. Heat processing is a major cause of decreased metabolic availability of lysine because of the formation of Maillard products, which are digested and absorbed, but are not metabolically available for protein synthesis (Carpenter and Booth, 1973). Therefore, by using peas, we were able to directly test the effects of heating on the metabolic availability of lysine. We hypothesized that the increase in PHE oxidation after heating peas would be reversed by adding free lysine to this diet.

The metabolic lysine availability of all test diets was determined with repeated measurements within pigs to abolish inter-animal variation. First, PHE oxidation was measured when animals received the low-lysine basal diet, supplemented with four levels of free lysine to achieve lysine concentrations corresponding to 50, 60, 70 and 80% of the recommendations of NRC (1998). From these data, we estimated the change in PHE oxidation per g free lysine added to the basal diet. To test the lysine metabolic availability, peas were included in the basal diet to achieve a lysine level of 80% of the requirement. The PHE oxidation using this diet was determined in duplicate for each animal to reduce the variation associated with the measurements. A subsample of the peas was heated according to the procedure described by van Barneveld et al. (1994a), and again the PHE oxidation was determined twice in each animal. Finally, we calculated the lysine in the peas made metabolically unavailable by heating, and supplemented free lysine to the heated pea diet in an amount equivalent to that calculated to achieve 100% availability. Again, PHE oxidation was determined twice in each animal.

We found that lysine in raw peas in this experiment was 90.5% metabolically available, while heating peas reduced the metabolic availability to 85.4% (**Figure 5**). Adding back the lysine lost during heating resulted in an oxidation rate slightly lower than that during feeding of raw peas (i.e. availability of 103%). These data clearly showed that the response in phenylalanine oxidation was entirely due to the changes in the metabolic availability of lysine. Furthermore, the result for the addition of free lysine to the heated pea diet shows that the determination of metabolic availability responds accurately – free lysine has been shown to be 100% available (Susenbeth et al. 2001) – to small dietary inclusions (0.56 g/kg) of lysine HCl. In addition, the metabolic availability of lysine was similar to that determined using intravenous infusion of isotope with the same diets, further validating the use of oral isotope delivery as a simplified technique.

Figure 5. Lysine availability in peas determined using the indicator amino acid oxidation method with oral isotope delivery



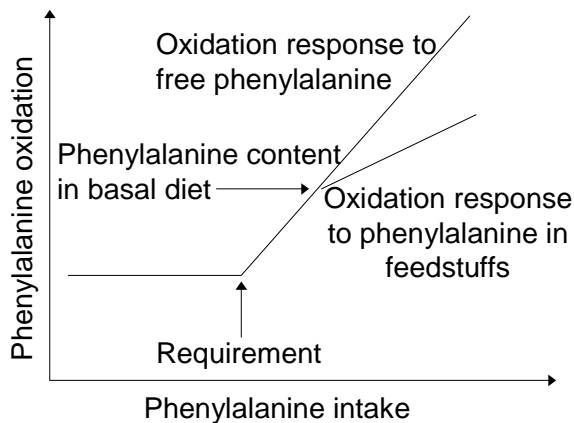
■ Phenylalanine Availability

A critical condition for the application of this technique is that metabolically available phenylalanine must be constant across diets. If PHE intake is not constant among diets then the slope of the line for PHE oxidation will be affected by PHE intake, rather than only by intake of the limiting amino acid. However, when PHE intake is constant and lysine is first limiting, dietary phenylalanine will be oxidized quantitatively in relationship to the change in protein synthesis as a result of intake of the limiting amino acid. In the initial

development of this technique, we made the assumption that true ileal digestibility estimates for phenylalanine published by NRC would be accurate for our purposes. This assumption was considered acceptable to prove our concept because the large difference in lysine availability due to Maillard reactions in heated feedstuffs was considered to be greater than any differences in the availability of phenylalanine, which does not participate in Maillard reactions. However, in order to verify the assumptions we used in early experiments regarding phenylalanine, we used the direct oxidation technique to measure PHE metabolic availability.

Phenylalanine is oxidized quantitatively when supplied in excess of the requirement (**Figure 6**). Therefore, added PHE from feedstuffs, being less available than free phenylalanine, will increase the oxidation rate of PHE to a lesser degree than free PHE. The oxidation change per g added PHE from feedstuffs, divided by that from free phenylalanine, will thus represent the metabolic availability of phenylalanine in the feedstuff. Free phenylalanine or phenylalanine from feedstuffs was added to a base diet providing 120% of the requirement, to obtain diets with 150% of the requirement. By comparing phenylalanine oxidation when provided as free amino acid versus as pea protein, the metabolic availability of phenylalanine in peas was calculated to be 88.5%, which is similar to that estimated by NRC using true ileal digestibility data (87%). This experiment verified our original assumption that NRC estimates for true ileal digestibility of phenylalanine can be used.

Figure 6. Metabolic availability of phenylalanine using the direct oxidation approach.



■ Further Developments for This Technique

The next step to validate this technique is to directly compare batches of feedstuffs using the ileal digestibility technique and our new metabolic availability approach. One limitation of using NRC tables is that batch to batch variability in feedstuffs in amino acid content and availability is significant and prevents an accurate comparison of the methods. In particular, we will compare the heated feedstuffs (ie soybean, canola, cottonseed, meat and bone, blood and feather meals) which tend to be the most variable with respect to lysine digestibility.

Based on the success of the method in pigs, studies are currently underway to develop this methodology for poultry. In addition to its usefulness in determining amino acid requirements of various types of poultry, the method will lend itself well to rapid determination of amino acid availability in feedstuffs. We have already validated the basic IAAO methodology in poultry (Tabiri et al., 2002a, 2002b, Coleman et al., 2003).

This metabolic availability methodology could also be used as a selection tool for plant cultivars to improve amino acid availability. Rather than requiring tonnes of a particular grain for determination of amino acid availability using traditional methods, assays can be conducted rapidly using IAAO and only kilograms of the test feedstuff. This ability offers significant advantages to plant breeders both in terms of number of plant generations required before experiments can be conducted, as well as in development costs. The use of IAAO to determine amino acid availability has already been proven in pigs. Because of the much smaller body size of poultry, even smaller amounts of the test feedstuff will be required, further decreasing costs.

■ Amino Acid Requirements for Individual Animals

Although animal performance is taken into account when calculating amino acid requirements, the resulting values that are used are based on the average performance of a herd of pigs. Differences in individual requirements have been shown in humans (Zello et al., 1993, Pencharz and Ball, 2003), neonatal pigs (House et al., 1998) and growing pigs (Moehn et al., 2001). The main reason that requirements of individual pigs have not been determined to date is the lack of a suitable method. However, the indicator amino acid oxidation method (IAAO) can determine the amino acid requirement of individual pigs. IAAO allows multiple measurements to be performed within the same animal in quick succession, without the animal changing its physiological state during the experiment. Knowledge of the amino acid requirements of individual animals has several important implications: it can be used in advanced feed formulation approaches using probability and stochastic techniques; it can be used to

assess the economic impact of feeding regimens that supply amino acids above or below the average requirement of a herd of pigs; it can be used as a selection criterion to derive a more efficient strain of animal.

Because the development of the metabolic availability method required that we measure many intakes of lysine in several pigs, a corollary of this research was that we also demonstrated that this is a rapid technique to determine lysine requirement of individual pigs. Although amino acid requirements for growing pigs are well established, there are no estimates of the variability of requirements within the population that have been determined by measurements on individual animals. By compiling the data acquired during the metabolic availability studies, we were able to demonstrate that these individual requirements for nine pigs generated a mean lysine requirement of 94% ($\pm 10\%$) of that recommended by NRC for the performance of our pigs (**Figure 3**). This is the first study, to our knowledge, that has directly determined the variation associated with the amino acid requirement of individual pigs. We have also recently concluded an experiments on methionine and threonine requirements in individual pigs between 7 and 18 kg body weight.. The methionine requirement coincided with current recommendations (NRC 1998) at 3.4 g/kg with a variability of $\pm 11.4\%$ (Moehn et al. 2005). The threonine requirement of weaner pigs was 7.1 g/kg, SE 0.1, for semi-synthetic diets and 8.8 g/kg, SE 0.2, for barley based diets (Myrie et al. unpublished). Comparable studies are currently underway in poultry.

The short (<3 weeks) experimental period of the IAAO method allows, for the first time, the generation of an estimate of population variability. This variability estimate allows the accurate calculation of the economic and performance effect of choosing a specified intake above or below requirement on herd performance. For example, the standard curve developed by individual requirement measurements will allow a prediction of the proportion of the herd that will respond to a specified change in lysine intake above or below the population mean requirement. This method is suitable to use with all dietary indispensable amino acids.

■ Conclusions and Implications

We have developed an isotope dilution method which is suitable for determining the amino acid requirement of individual pigs and for determining the metabolic availability of amino acids for protein synthesis in a selection of feed ingredients. This method offers the opportunity to determine the quantity of the amino acid that is metabolically usable by the animals by simple amino acid analysis in feedstuffs and a short experiment (<3 weeks) to determine the metabolic availability in vivo. This approach eliminates the reliance on predicted or assumed values to correct ileal digestibility of lysine for endogenous losses

and unknown corrections for the chemical availability of absorbed lysine. A further advantage of this method is that the amino acid availability can be determined rapidly (within 3 weeks) and much cheaper than the slope ratio growth assay. Additional research is required to characterize additional feedstuffs and determine the metabolic availability of other key amino acids.

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