Review article

Review of advances in metabolic bioavailability of amino acids

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1. Introduction

The animal’s amino acid requirement and the potential of feedstuffs to supply those amino acids in metabolically available form (feedstuff amino acid bioavailability) are intertwined. To fully utilize the knowledge of one, we must also have a complete knowledge of the other. However, methods used to estimate both amino acid requirements and amino acid bioavailability are still a matter of considerable debate in animal and human nutrition research.

Amino acid bioavailability has been defined as the percentage of amino acids digested and absorbed in a form available for utilization for the purpose of maintenance or...
growth of tissue (ARC, 1981; Batterham, 1992; Fan, 1994; Danfaer and Fernández, 1999). Amino acid digestibility, amino acid bioavailability using slope ratio assays, protein digestibility-corrected amino acid score, net postprandial protein utilization and postprandial protein utilization have all been used to estimate protein quality of animal and human food ingredients (Stein et al., 2007, Elango et al., 2009a). Each method has its own unique set of advantages and disadvantages.

In pig nutrition, standardized ileal amino acid digestibility is currently most widely used as an estimate of dietary protein quality. Ileal amino acid digestibility is comparatively easy to determine, and yields results for all amino acids in a reasonably short time frame. However, amino acid digestibility coefficients are highly dependent on an accurate estimate of ileal endogenous amino acid losses (Stein et al., 2005). Numerous factors such as dietary fiber and crude protein content, antinutritional factors, body weight and composition of protein-free diet can all influence the estimate of endogenous amino acid losses (Stein et al., 2007). The reader is directed to Stein et al. (2007) for a detailed description of the terminology and problems with estimation of ileal endogenous amino acid losses. Amino acid digestibility, in its most accurate form (true ileal digestibility), can only measure amino acid disappearance from the gut (Stein et al., 2007) and thus does not account for first-pass metabolism or amino acids absorbed in a chemical complex unsuitable for metabolism. If the degree of first-pass metabolism is different between AA then the measurement of the pattern of AA in the diet, or the residual AA in the digesta, will not reflect the availability of AA to the extra-intestinal tissue (Stoll et al., 1998). Heat processing of feedstuffs can result in AA forming chemical complexes (i.e. lysine and Maillard reaction products) that can be digested and absorbed but cannot be utilized by the animal for protein synthesis (Carpenter and Booth, 1973).

Assays of amino acid bioavailability that measure parameters of animal growth (slope ratio assays) are considered the standard against which other methods of amino acid availability are judged (Lewis and Bayley, 1995). The advantages of slope ratio assays that measure parameters of protein synthesis are that they measure a response that has practical and economic consequences and account for the effect of digestion, absorption and utilization of the amino acids provided by the protein source (Lewis and Bayley, 1995). Therefore, such assays measure the effect of protein quality on the entire metabolism. The importance of this has been shown by Batterham (1992) who demonstrated that heat-damaged protein supported lower protein retention than a control diet despite similar amino acid digestibility. However, practical use of amino acid bioavailability growth assays is limited by time, cost and the limitation of examining only one amino acid at a time (Batterham, 1992).

Recently, the slope ratio assay utilizing the indicator amino acid oxidation technique as the dependent response criterion has been developed to determine the metabolic availability (MA) of amino acids in pigs (Moehn et al., 2005, 2007) and humans (Humayan et al., 2007). This review will demonstrate how the indicator amino acid oxidation (IAAO) technique can 1) address some of the problems associated with the slope ratio assay; 2) be used to determine the metabolic availability (MA) of amino acids in animal feedstuffs; 3) become a valuable measure of amino acid bioavailability; and 4) be applied in practical swine nutrition.

2. Indicator amino acid oxidation concepts and techniques

The IAAO method is based on the principle that amino acids are either utilized for protein synthesis or must be oxidized. Therefore, the change in oxidation of the indicator amino acid is inversely proportional to protein synthesis (Fig. 1). When one essential amino acid (test amino acid) is limiting, all other AA are in excess and must be oxidized (Moehn et al., 2005). As the intake of the limiting amino acid increases, the oxidation of an indicator amino acid decreases linearly until the requirement for the limiting amino acid is reached. Increases of the test amino acid beyond the requirement do not increase protein synthesis further, and there is no further change in oxidation of the indicator amino acid (Fig. 1); indicator amino acid oxidation reaches a plateau.

Ball and Bayley (1986) found that the recovery of radioactivity in liver protein was greatest at the level of dietary protein that minimized 1-14C phenylalanine oxidation. The increase in radioactivity of liver protein indicated that 1-14C phenylalanine was increasingly incorporated into liver protein, hence protein synthesis, until protein synthesis was maximized, represented by a plateau in liver protein radioactivity. Thus the oxidation of the indicator amino acid indicated that the rate of whole body protein synthesis was driven by the limiting amino acid. The breakpoint is defined as the requirement for the test amino acid. The IAAO technique has been extensively developed to determine amino acid requirements and was chosen as the gold standard by the World Health Organization for determination of amino acid requirements of humans (WHO, 2007; Pencharz and Ball, 2003).

Selection of an appropriate indicator AA is an important component of IAAO. Key criteria when selecting an amino acid as indicator are 1) an essential amino acid and 2) the labeled C is irreversibly lost as CO2 during oxidation, preferably during the first steps of amino acid catabolism. Apart from the indicator amino acid of choice, phenylalanine, lysine (Ball and Bayley, 1984a), threonine (Soliman and King, 1969), [14C-methyl]-methionine (Brookes et al., 1972) and leucine (Hsu et al., 2006, Kurpad et al., 1998) have been used as indicator amino acids. The L-[1-13 or 1-14C]-phenylalanine, in the presence of excess tyrosine, has been used most often because of its many advantages: the free pool size is comparatively

![Fig. 1. Inverse relationship between protein synthesis and oxidation of an indicator amino acid. Adapted from Ball and Bayley (1986).](image-url)
small with a quick turn-over rate (Neale and Waterlow, 1974), and the size of the intracellular free phenylalanine pool is tightly regulated (Flaim et al., 1982). Consequently, phenylalanine oxidation was more responsive to dietary changes than lysine or leucine (Neale and Waterlow, 1974). The advantage of the short half-life of the free phenylalanine pool is that it responds rapidly to changes in test amino acid intake. Ball and Bayley (1984b) showed in baby pigs that amino acid requirement could be determined with as little as 4 h of adaptation to the diet. In sows and growing pigs, phenylalanine oxidation responded within 1–2 days of a change in test amino acid intake, and remained constant for up to 10 days after a change in diet (Moehn et al., 2004). Therefore, 2 days of adaptation to a new dietary test amino acid level is deemed sufficient. Recently, Elango et al. (2009b) showed in humans that the adaptation period can be even shorter.

3. IAAO as a measure of metabolic availability of AA in feedstuffs

The change in appearance of $^{13}$C or $^{14}$CO$_2$ in breath reflects the change of whole body protein synthesis. At plateau in oxidation, protein synthesis is maximized and would not be expected to respond to changing supply of test amino acids of differing availability. However, when the intake of the test amino acid is limiting, the change in indicator oxidation reflects the response of whole body protein synthesis to graded levels (or intakes) of the limiting amino acid (Moehn et al., 2005). This slope indicates the change in IAAO per unit change in limiting amino acid. A shallower slope indicates less amino acid per unit intake is available to support protein synthesis. Therefore, the ratios of the slope of the linear response of indicator oxidation can be used to estimate the MA of essential AA in feedstuffs.

Metabolic availability of AA in feedstuffs is defined as the ratio of the indicator oxidation response of the addition of limiting amino acid from test protein source to that of free amino acid (Elango et al., 2009a). Gabert et al. (2001) suggested that this comparison of the slope of the response from a test protein source to the slope of the response to a defined reference protein source represents relative values only. However, the true ileal digestibility of crystalline AA is essentially 100% (Baker, 1992). Thus, the slope of the indicator amino acid oxidation obtained with the crystalline form of the test amino acid represents the maximal unit increase in protein synthesis and is equivalent to 100% metabolic availability of the test amino acid. The oxidation of the indicator amino acid is regressed against the amino acid intake above that provided by the base diet (supplied by free test amino acid) and the protein source (Moehn et al., 2005). The ratio of the slope of the indicator oxidation due to the protein source to the indicator oxidation slope due to the free amino acid gives the MA of the protein source.

The test of the MA concept using the IAAO technique was conducted by Moehn et al. (2005). Addition of free lysine from peas, and even more so from heat-treated peas, to a base diet led to a shallower decrease in oxidation than addition of free lysine (Fig. 2). This indicated a lower availability of lysine in peas and heated peas for protein synthesis. The MA of lysine in peas was calculated to be 88.8% and was similar to previously published estimates of the true ileal digestibility of lysine in peas (NRC, 1998). Heating peas using a protocol similar to that employed by van Barneveld et al. (1994), who used traditional slope ratio assay, reduced the lysine availability further, to 54.8% (Moehn et al., 2005). Similarly, Ball et al. (1995) reported impaired lysine availability in heated cotton seed meal, but found similar oxidation responses for lysine in soybean meal and for free lysine. Therefore, using the IAAO in a slope ratio assay allows determination of the availability of AA in vivo, and is capable, contrary to digestibility studies, to detect reduced protein quality of heat-treated feedstuffs.

4. Diet formulation

There are three important dietary considerations for slope ratio assays: (1) the test amino acid must be first limiting, (2) the response to changes in test amino acid intake must be predictable, preferably linear, and (3) the observed response must not be influenced by other dietary nutrients in the test feed ingredient (Littell et al., 1995, Batterham, 1992). In addition, when applying the IAAO in a slope ratio assay, an isotopic steady state must be achieved.

Batterham (1992) suggested that a basal diet should contain all nutrients in slight excess, except the amino acid of interest. This is relatively simple to accomplish with the use of crystalline AA added alone or in addition to a protein source in the base diet. All AA except the amino acid of interest must be set at greater than 100% of requirement while the maximum intake of the test amino acid should be set at a level at least 2 SD below requirement. Bertolo et al. (2005) estimated the CV for inter-animal variability of lysine requirement was 10%. Restriction of the test amino acid intake to 80% of the mean requirement, therefore, leaves only 2.5% of the animals with a potentially lower requirement. This is important because a linear response to changes in test amino acid intake is critical for calculation of MA. Indicator oxidation was linear as lysine intake increased up to 80%, 2 SD below the mean lysine requirement of growing pigs (Moehn et al., 2005, 2007), Humayan et al. (2007) also noted a linear decrease in $^{13}$C phenylalanine oxidation in adult males over increasing methionine intakes up to the lower 95% confidence interval of the methionine requirement. Numerous other studies measuring different response parameters have shown a linear response to amino acid intakes below requirement: plasma urea nitrogen and nitrogen retention (Coma et al.,

![Fig. 2. Metabolic availability of lysine in peas and heated peas. Adapted from Moehn et al. (2005).](image-url)
Thus, the only factor driving IAAO becomes the level of test and crude protein in both reference and test protein diets, to obtain similar dietary intakes of all essential amino acids. However, with the use of crystalline amino acids, it is possible by the base diet (Moehn et al., 2005).

As discussed previously, the assumption of linearity of the response to graded amino acid intakes holds (Littell et al., 1995). As with all statistical analyses, assumptions required to apply the analysis must hold true and should be tested prior to final data analysis. The assumptions of linearity of the response to graded amino acid intakes and intersection of the regression line are important for calculation of amino acid bioavailability (Littell et al., 1995). As discussed previously, the assumption of linearity of the response to graded amino acid intakes holds true when dietary amino acid intake levels are maintained below 2 SD of the mean amino acid requirement.

To ensure intersection of the regression lines of the reference and test proteins, both the crystalline test amino acid and the test protein source should be added to a semi-synthetic or synthetic basal diet containing the lowest level of test amino acid. The dependent response variable is then regressed against the amino acid intake above that provided by the base diet (Moehn et al., 2005).

Estimates of bioavailability usually demonstrate a high standard error due to the effect of individual variation among test subjects on the slope of the response and the accuracy of the total amino acid analysis of the test protein (Batterham, 1992). The short adaptation time required for the IAAO method (<2 days, Moehn et al., 2004) allows repeated measures in the same animal at all levels of test amino acid intake, thus reducing the effect of individual variation. Total amino acid analysis can vary from 5 to 10% between laboratories (Sarwar et al., 1983; Batterham, 1992). Digestibility experiments require total amino acid analyses to be conducted on the test diets, the protein-free diet, as well as the collected digesta; whereas, only the test diets are analyzed for total amino acid content in MA studies. Additionally, stable and radioactive isotope analyses are measured with great precision and are highly sensitive and thus able to detect smaller differences between treatments than traditional amino acid analysis. The lower sensitivity of amino acid analysis compared to isotopic analysis requires additional numbers of observations to account for differences in the sensitivity of the response variable.

### 6. Advantages of metabolic availability

Although the measurement of amino acid bioavailability by slope ratio assay is regarded as the ‘gold standard’, there are problems with its practical implementation. Conventional response criterion, such as comparative slaughter or N-balance technique, in slope ratio assays requires large number of animals and prolonged periods of time. These problems can be addressed by using the IAAO instead of conventional methods. The short adaptation period to dietary treatments (<2 days, Moehn et al., 2004) allows repeated measurements of indicator oxidation within animals in rapid succession. Therefore, both the number of animals, inter-animal variation and the time frame of an experiment can be reduced drastically. Typically, the MA of an amino acid in the test feed can be tested within 14 days. The IAAO also fulfills the critical requirement that the response criterion used in a slope ratio assay should be a measure of protein retention (Batterham, 1992). This has been shown in numerous studies using the IAAO technique to determine amino acid requirements of pigs (Ball and Bayley, 1984a; Moehn et al. 2005; Levesque et al. 2009), chickens (Ewing et al., 2001) and humans (Elango et al., 2007).

While applying the IAAO in a slope ratio assay is an improvement over conventional techniques, metabolic availability still only provides information for one amino acid at a time. Determination of ileal digestibility yields values for all amino acids simultaneously. However, metabolic availability represents the end result of digestion, absorption and metabolic utilization of amino acids, while digestibility values are only a measure of disappearance in the small intestine. Furthermore, ileal digestibility usually is determined using surgically modified animals, while metabolic availability can be measured in intact pigs. Surgical modification (e.g. T-canal, re-entrant canal, ileal–rectal anastomosis) may disrupt the regular function of the gastro-intestinal tract (Fuller, 1991), and thus yield digestibility values that may deviate from those applicable to intact animals. However, ileal digestibility is the most common measure of protein quality in pig nutrition and the basis for most recommendations for dietary amino acid supply; given its comparatively easy determination, it can be expected that ileal digestibility will be the measure of choice for the foreseeable future. In contrast, Batterham (1992) suggests that amino acid availability values are the most useful for creating amino acid availability reference tables, evaluating the effect of processing conditions,
and as reference values in the development of new techniques. The metabolic availability method becomes the method of choice to determine amino acid (e.g. lysine) availability if losses during and/or after absorption are suspected, e.g. lysine in heat damaged proteins. The metabolic availability method also becomes the method of choice if surgical modification is either unethical, e.g. in humans, or impractical.

7. Practical application of MA

Practical diet formulation relies on the additivity of the estimates of feed amino acid content when creating mixed feed diets. Fan (1994) argued that protein retention is affected by the diet balance of AA, diet level of protein:energy and energy sources, chronological appearance of AA at the tissue level, genotype, and physiological state. Based on this argument, Stein et al. (2007) regarded the metabolic availability estimate as relevant only under the same conditions (dietary, genetic, and physiological state) that the slope ratio study was conducted and deem the availability estimates as not likely additive. However, the factors described by Fan (1994) do not affect the availability estimate by slope ratio if the experiment is properly conducted. Ball and Bayley (1986) showed that at constant phenylalanine intake, the ratio of protein bound to free phenylalanine in the liver was not affected by protein intake ranging from deficient to excess. This lack of influence of dietary protein on the distribution of label between protein bound and non-protein bound fractions suggests that MA estimates would be additive. Additionally, the efficiency of use of protein above maintenance is not affected by pig breed (Kyriazakis and Emmans, 1995). Similar to any digestibility studies, diet formulation and animal selection are used to control potential dietary influences, other than the test amino acid level, on observed responses.

Acknowledging the relationship between amino acid requirement of the animal and feedstuff amino acid bioavailability, it has been suggested that methods used to express both requirement and AA availability should be the same (Lewis and Bayley, 1995; Stein et al., 2007). Advancements in amino acid digestibility methods have improved the relationship between amino acid requirements and the estimation of feedstuffs to meet these requirements; however, amino acid requirements are a reflection of the effect of genetics, physiological state and dietary factors. By definition, amino acid bioavailability insinuates that ingredient characteristics, genetics, physiological state and dietary factors influencing protein retention should be considered. Controlling for the effect of these influences can be accomplished through experimental design but to fully understand how these factors might influence the supply and utilization of AA for protein synthesis appropriate experimental techniques must be utilized. The non-invasive IAAO technique provides a unique opportunity to understand metabolically how the supply of AA for protein synthesis changes with physiological state, ingredient or genetics.

8. Conclusions

The development of the IAAO method can address many of the limitation of previous bioavailability studies using a slope ratio growth assay.

Ideal digestibility will continue to be the most common measure of choice for the foreseeable future. However, the minimally invasive IAAO technique becomes the method of choice when evaluating feedstuffs during sensitive physiological states, when losses during and/or after absorption are expected, when surgical modification is unethical or impractical and when an integration of factors on amino acid utilization is desired.

The development of MA using the IAAO technique also has the potential to ensure that advancements in amino acid bioavailability will keep pace with improvements in knowledge of amino acid requirement.

Conflict of Interest

We have no conflict of interest.

References


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