A New Method for Determining Digestible Reactive Lysine in Foods†

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The study aimed to develop a bioassay for determining digestible reactive lysine in processed feedstuffs. The approach combines the determination of reactive lysine (guanidination method) in diets and digesta with a true ileal lysine digestibility assay. Using optimized reaction conditions, reactive lysine was determined in unheated casein and heated lactose/casein using both guanidination and FDNB methods. Similar values were obtained with both methods (0.53 and 0.32 mmol (g of casein)$^{-1}$ for casein and heated lactose/casein, respectively). Conventional lysine analysis overestimated (0.38 mmol g^{-1}) the reactive lysine in the heated mixture. The true ileal lysine digestibility in casein was 98.8%. The corresponding value in the heated lactose/casein was 70.5%, whereas the true ileal reactive lysine digestibility was 85.9%. The traditional approach to determining the digestible lysine in processed foods appears to considerably underestimate lysine digestibility. The new method, which allows determination of digestible reactive lysine, circumvents some problems inherent in the traditional approach.

Keywords: *Lysine; availability; true digestibility; guanidination; Maillard damage*

INTRODUCTION

Lysine is an essential amino acid and is often the first limiting amino acid in pig and poultry diets. In feedstuffs that have undergone processing or prolonged storage, the ϵ-amino group of lysine can react with other compounds present in feedstuffs to become nutritionally unavailable (Hurrell and Carpenter, 1981). Consequently, numerous assays have been developed to allow determination of the chemically reactive lysine content of foods (Hendriks *et al*., 1994). However, not all of the chemically reactive lysine in heat-treated proteins is absorbed from the small intestine (Schmitz, 1988; Desrosiers *et al*., 1989; Moughan *et al*., 1996). Consequently, methods for determining chemically reactive lysine such as the FDNB method are inappropriate for assessment of available lysine as they incorrectly assume that all of the reactive lysine present in a feedstuff is digested and absorbed. Furthermore, it has been shown that the traditional true ileal amino acid digestibility assay does not always accurately predict the availability of lysine in heat-processed feedstuffs (Batterham, 1992). During the acid hydrolysis step of amino acid analysis, an integral part of the digestibility assay, a portion of the structurally altered nutritionally unavailable lysine derivatives in processed feedstuffs can break down, reverting to lysine and leading to an overestimate of unaltered lysine in diets and digesta samples and therefore inaccuracy in digestibility coefficients.

In this study a new method for determining the ileal digestibility of reactive lysine was developed. The method involves coupling the guanidination reaction, which converts chemically reactive lysine to the acid stable derivative homoarginine, to the traditional true ileal amino acid digestibility assay, whereby the ileal digestibility assay is conducted and the guanidination method is used to determine the reactive lysine content of both the diet being tested and the digesta of animals fed that diet. The true ileal reactive lysine digestibility coefficient can then be calculated and the true digestible reactive lysine (available lysine) can be determined. The new assay has been applied to a heated lactose/casein mixture, which simulated a mildly heated protein source with early Maillard damage.

EXPERIMENTAL PROCEDURES

Materials. 1-Fluoro-1,4-dinitrobenzene (FDNB), dinitrophenyllysine (DNP-lysine), lysozyme, and *O*-methylisourea were obtained from Sigma Chemical Co., St. Louis, MO. Barium hydroxide octahydrate and lactose were obtained from BDH Laboratory Supplies, Poole, England. Lactic casein, skim milk powder, and whey protein concentrate were obtained from the New Zealand Dairy Board, Wellington, New Zealand. Soy protein isolate and concentrate were obtained from Columbit (New Zealand) Ltd., Auckland, New Zealand. Wheat meal, blood meal, meat and bone meal, and soybean meal were obtained from the Feed Processing Unit Massey University, New Zealand, and cottonseed meal was obtained from Cargill Oilseed Ltd., Brisbane, Australia. The enzymatically hydrolyzed casein was obtained from New Zealand Pharmaceuticals Ltd., Palmerston North, New Zealand, and contained peptides no larger than 2000 Da. Centriprep 10 disposable ultrafiltration devices were obtained from Amicon, Inc., Beverly, MA. Laboratory rats were sourced from the Small Animal Production Unit, Massey University, Palmerston North, New Zealand.

FDNB Method. FDNB-reactive lysine was determined according to the method of Carpenter (1960) using the modifications described by Booth (1971). Samples containing approximately 10 mg of reactive lysine (estimated previously using amino acid analysis) were reacted with FDNB in ethanol/$NaHCO_3$ at room temperature for 2 h. The resulting DNP-lysine was liberated from the protein by hydrolysis in 5.8 M HCl for 16 h under reflux conditions. The unreacted FDNB was removed by diethyl ether extraction and the remaining DNP-lysine detected by absorbance at 435 nm.

Preparation of 0.6 M *O*-Methylisourea Solution. A 0.6 M *O*-methylisourea solution was prepared according to a modified procedure based on the methods of Chervenka and Wilcox (1956), Shields *et al*. (1959), Mauron and Bujard (1964), and Kassell and Chow (1966). Four grams of barium hydroxide octahydrate was added to approximately 16 mL of boiling distilled deionized water that had been preboiled for 10 min to remove carbon dioxide. The solution was heated to near boiling and then added to 2 g of *O*-methylisourea (sulfate salt)

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† New Zealand Patent Application 272486.

S0021-8561(95)00032-X CCC: $12.00 © 1996 American Chemical Society

in a 40 mL centrifuge tube. The solution was left to cool for 30 min before centrifuging at 6400*g* for 10 min. The supernatant was retained and the precipitate washed with approximately 2 mL of distilled deionized water before recentrifuging. The washings were added to the supernatant, and the pH was checked. If the pH of the solution was lower than 12, then it was assumed that conversion of the sulfate salt to the free base was incomplete and the solution was remade. However, if the pH was above 12, then the pH was adjusted to the appropriate pH for guanidination (pH 10.6−11.2) and made up to 20 mL with distilled deionized water.

Preparation of a Heated Lactose/Casein Mixture. A heat-treated lactose/casein mixture, which contained 250 g of lactose and 750 g of lactic casein, was prepared by mixing the two components in 4 L of distilled deionized water and then freeze-drying the suspension and autoclaving the dried mixture for 3.5 min at 121 °C. The autoclaved sample was ground through a 1 mm mesh. The resultant mixture simulated a protein having undergone early to late Maillard damage (Moughan *et al.*, 1996).

Optimization of Reaction Time for Guanidination of Unheated Partially Purified Proteins. Samples of lysozyme and casein (5−10 mg) were incubated for 1, 2, and 3 days in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C in a shaking water bath with the reagent to lysine ratio greater than 1000. The samples were then reduced to dryness and the homoarginine and lysine contents determined.

Optimization of the Reaction Time for Guanidination of Heated Lactose/Casein. Samples (5−10 mg) of heated lactose/casein were incubated for 1, 3, 7, and 14 days in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C in a shaking water bath, with the reagent to lysine ratio greater than 1000. The samples were subsequently reduced to dryness, and the homoarginine content was determined.

Optimization of the Reaction Time and pH for Guanidination of Digesta. The optimal incubation time was determined after 5−10 mg samples of rat ileal digesta were incubated in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C in a shaking water bath for 1, 3, 7, 14, and 21 days, with the reagent to lysine ratio greater than 1000. The samples were reduced to dryness, and the homoarginine content was determined. The ileal digesta had been obtained from rats given either an unheated casein-based diet or a heated lactose/casein-based diet.

The optimal reaction mixture pH was determined after 5−10 mg samples of ileal digesta from rats fed unheated casein and from rats fed heated lactose/casein were incubated in 0.6 M *O*-methylisourea at pH 9.8, 10.2, 10.6, 11.0, 11.4, at 21 ± 2 °C in a shaking water bath, with the reagent to lysine ratio being greater than 1000, for 1 and 7 days, respectively. The samples were then reduced to dryness, and the homoarginine content was determined.

Digestibility Study. Ethics approval for the animal trial was obtained from the Animal Ethics Committee, Massey University, Palmerston North, New Zealand. Sprague-Dawley male rats, of approximately 150 g body weight, were housed individually in stainless steel wire-bottomed cages in a room maintained at 22 ± 2 °C, with a 12 h light/dark cycle. Two semisynthetic test diets were formulated to each contain 100 g/kg crude protein. Two enzymatically hydrolyzed casein (EHC)-based diets were also formulated to allow determination of endogenous ileal amino acid flows (Moughan *et al.*, 1990; Butts *et al.*, 1991). Chromic oxide was included in each diet as an indigestible marker. The ingredient compositions of the diets are given in Table 1. The diets were randomly allocated to the rats such that there were six rats on each diet. The rats were given the diets for a 14 day period. On each day each rat received its respective diet as nine meals given hourly (8:30 a.m. to 4:30 p.m.). At each meal time the diet was freely available for a 10 min period. The feed containers were weighed after each meal. Water was available at all times. On the 14th day of the study, from 5.5 to 7 h after the start of feeding, the rats were asphyxiated in carbon dioxide gas and then decapitated. The 20 cm of ileum immediately anterior to the ileo-caecal junction was dissected out. The dissected ileum was washed with distilled deionized water to remove any blood and hair and carefully dried on an absorbent paper towel. The digesta were then gently flushed from the ileum section with distilled deionized water from a syringe. The digesta from the rats fed the test diets were then freeze-dried ready for chemical analysis. The pH of the digesta of rats fed the EHC diet was adjusted to approximately pH 3 with 6 M HCl, to minimize protease activity. The EHC digesta were then centrifuged at 1400*g* for 30 min at 3 ± 1 °C, and the precipitate was washed and recentrifuged. The washings were pooled with the supernatant. The supernatant underwent ultrafiltration in a Centriprep 10 disposable ultrafiltration device, after which the filtrate was discarded and the retentate washed and subjected to ultrafiltration for a second time. The resulting retentate was added to the precipitate from the centrifugation step and freeze-dried, ready for chemical analysis.

Table 1. Ingredient Compositions (Grams per Kilogram of Air-Dry Weight) of the Experimental Diets Given to the Laboratory Rat[a]

	EHC1[b]	unheated casein	EHC2[c]	heated lactose/casein[d]
wheat starch	625.7	639.7	583	584.9
soybean oil	50	50	50	50
purified cellulose	50	50	50	50
sucrose	100	100	100	100
vitamin/mineral mix[e]	39.3	39.3	39.3	39.3
lactose			42.7	
casein		116		
heated lactose/casein				170.8
EHC	130		130	
chromic oxide	5	5	5	5

[a] All diets were formulated to contain equal crude protein contents. [b] Enzymatically hydrolyzed casein-containing diet for determining endogenous amino acid loss for the unheated casein diet. [c] Enzymatically hydrolyzed casein-containing diet for determining endogenous amino acid loss for the heated lactose/casein diet. [d] Heated lactose/casein was prepared as described under Experimental Procedures. [e] Vitamin/mineral mix was formulated to meet the requirements for vitamins and minerals as described by the National Research Council (National Academy of Sciences, 1972).

Chemical Analysis. Amino acids contents were determined in triplicate 5 mg digesta samples and quadruplicate 5 mg diet samples using a Waters ion-exchange HPLC system, utilizing postcolumn *o*-phthalaldehyde derivatization and fluorescence detection, following hydrolysis in 6 M glass-distilled HCl containing 0.1% phenol for 24 h at 110 ± 2 °C in evacuated sealed tubes. Cysteine, methionine, proline, and tryptophan were not determined. When appropriate, the weight of each amino acid was calculated using free amino acid molecular weights.

For the determination of reactive lysine, the samples were incubated for 7 days in 0.6 M *O*-methylisourea (pH 10.6) (pH 11.0 for the digesta samples) at 21 °C in a shaking water bath, with the reagent to lysine ratio greater than 1000, before being dried down and analyzed as described above.

The chromium contents of diet and ileal digesta samples were determined in duplicate on an Instrumentation Laboratory atomic absorption spectrophotometer following the method of Costigan and Ellis (1987).

Data Analysis. Conversion of lysine to homoarginine was calculated as

$$\text{conversion of lysine to homoarginine} = \frac{\text{mol of homoarginine}}{\text{mol of unreacted lysine} + \text{mol of homoarginine}} \times 100$$

Endogenous amino acid flows at the terminal ileum were calculated using the following equation [units are μg g^{-1} dry matter intake (DMI)]:

$$\text{ileal amino acid flow} = \text{amino acid concn in ileal digesta} \times \frac{\text{diet chromium}}{\text{ileal chromium}}$$

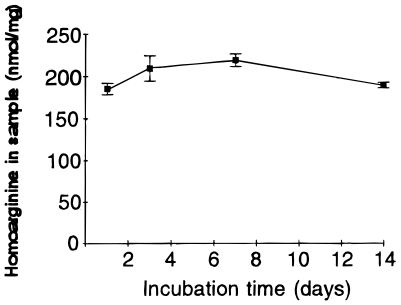

Figure 1. Homoarginine (reactive lysine) content of heated lactose/casein incubated with 0.6 M *O*-methylisourea (pH 10.6) in a shaking water bath at 21 ± 2 °C for 1−14 days, with the reagent to lysine ratio greater than 1000.

True ileal amino acid digestibility was calculated using the following equations (units are μg g^{-1} DMI):

$$\text{true digestibility} = [\text{dietary amino acid intake} - (\text{ileal amino acid flow} - \text{endogenous amino acid flow})]/\text{dietary amino acid intake} \times 100$$

True ileal reactive lysine digestibility was calculated using the following equation (units are μg g^{-1} DMI):

$$\text{true reactive lysine digestibility} = [\text{dietary reactive lysine intake} - (\text{ileal reactive lysine flow} - \text{endogenous lysine flow})]/\text{dietary reactive lysine intake} \times 100$$

The amino acid digestibility data were subjected to a one-way analysis of variance for each amino acid singly (GLM Procedure, SAS Institute Inc., Cary, NC).

RESULTS

Optimization of Reaction Time for Guanidination of Unheated Partially Purified Proteins. In preliminary studies investigating the optimal time for guanidination of two unheated protein sources (lysozyme and unheated casein), carried out over 1, 2, and 3 day incubation periods, near complete conversion of lysine to homoarginine (greater than 98%) was achieved in all cases.

Optimization of the Reaction Time for Guanidination of Heated Lactose/Casein. The conversion of lysine to homoarginine in heated lactose/casein was investigated using incubation times ranging from 1 to 14 days. The yield of homoarginine over the 14 day period is shown in Figure 1. Maximal guanidination was achieved after 3−7 days incubation in the *O*-methylisourea solution, although there was no statistically significant difference between homoarginine yields observed after 1, 3, 7, or 14 days of incubation.

Optimization of the Reaction Time and pH for Guanidination of Digesta. The optimum incubation times for maximal guanidination of digesta from rats fed unheated casein and heated lactose/casein were determined (Figure 2A). Maximal conversion of lysine to homoarginine in the digesta of rats fed unheated casein was achieved with a 1 day incubation, after which time the levels appeared to decline slightly, although this trend was not statistically significant. In contrast, a 7 day incubation time was required to achieve maximal guanidination of lysine in the digesta of rats fed the heated lactose/casein, although there was no statistically significant difference between homoarginine yields for the reaction mixtures after 3, 7, or 14 days of incubation. There was also no significant difference in the amount of homoarginine determined in the heated lactose/casein digesta after incubation in 0.6 M *O*-methylisourea for 1 and 3 days.

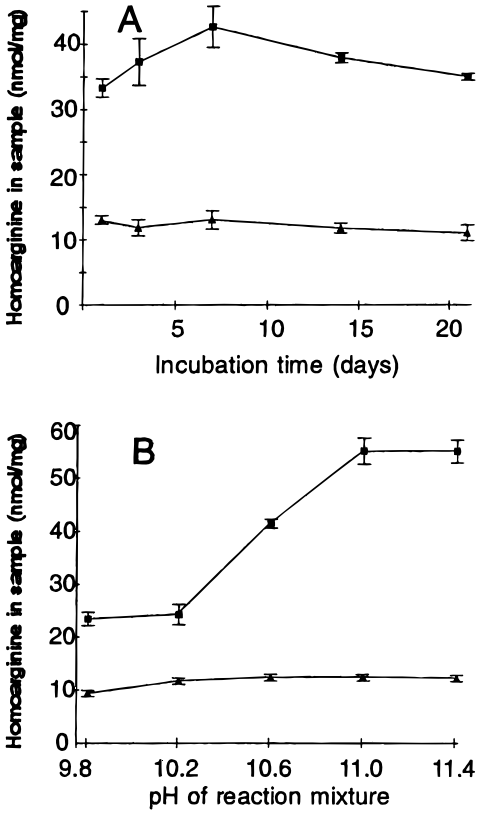

Figure 2. Amount of homoarginine (reactive lysine) present in the digesta of rats fed unheated casein (▲) and heated lactose/casein (nmol/mg of sample) (■) determined using the guanidination reaction varying reaction time (A) and reaction mixture pH (B). (A) Guanidination conditions were incubation in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C for 1−21 days, with the reagent to lysine ratio greater than 1000, followed by conventional amino acid analysis. (B) Guanidination conditions for the digesta of rats fed unheated casein were incubation for 1 day at 21 ± 2 °C in 0.6 M *O*-methylisourea (pH 9.8−11.4), with the reagent to lysine ratio greater than 1000, followed by conventional amino acid analysis. The guanidination conditions for the digesta of rats fed heated lactose/casein were incubation for 7 days at 21 ± 2 °C in 0.6 M *O*-methylisourea (pH 9.8−11.4), with the reagent to lysine ratio greater than 1000, followed by conventional amino acid analysis. (A) n = 13 for unheated casein, 1 day incubation; n = 8 for heated lactose/casein, 1 day incubation; n = 7 for unheated casein, 3 and 7 day incubations, and heated lactose/casein, 7 day incubation; n = 5 for heated lactose/casein, 7 day incubation; n = 3 for both proteins at 14 and 21 day incubations. For (B) n = 3 for all analyses. Values are means ± SE.

The optimum reaction mixture pH for maximal guanidination of digesta of rats fed unheated casein and heated lactose/casein was also determined (Figure 2B). The pH optimum for the guanidination of lysine in digesta of rats fed the unheated casein was approximately 10.6, although the amounts of homoarginine obtained from guanidination mixtures at pH values ranging from 10.2 to 11.4 were not significantly different. From the pH range examined in this experiment, the pH required for optimal guanidination of digesta from rats fed the heated lactose/casein was between 11.0 and 11.4.

Reactive Lysine in Unheated Casein and Heated Lactose/Casein. The reactive lysine contents of the unheated casein and the heated lactose/casein were compared using the guanidination method (in which homoarginine levels were equated to reactive lysine

Table 2. Reactive Lysine Content in Unheated Casein and Heated Lactose/Casein Determined Using the FDNB and Guanidination Methods and "Total Lysine" Levels Determined by Conventional Amino Acid Analysis

	lysine content [mmol (g of casein)$^{-1}$]		
	FDNB[a]	guanidination[b]	total[c]
unheated casein	0.51	0.55	0.50
heated lactose/casein	0.31	0.33	0.38

[a] FDNB analyses used a correction factor of 1.05 for both samples. [b] Guanidination analyses consisted of a 24 h incubation in 0.6 M *O*-methylisourea (pH 10.6) in a shaking water bath at 21 ± 2 °C with the reagent to lysine ratio greater than 1000, followed by conventional acid analysis. Reactive lysine values were corrected to an optimal 7 day incubation time using Figure 1. [c] Total analyses consisted of conventional acid hydrolysis and amino acid quantitation. All values are means of triplicate analyses.

levels), the FDNB-reactive lysine method, and conventional amino acid analysis. The guanidination conditions used were incubation for 24 h in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C in a shaking water bath, with the reagent to lysine ratio greater than 1000. The reactive lysine level of the heated lactose/casein was then extrapolated using Figure 1 to determine the reactive lysine content using the optimal 7 day incubation period. The results are shown in Table 2. The amount of reactive lysine in the unheated casein ranged from 0.5 mmol (g of casein)$^{-1}$ determined using conventional amino acid analysis to 0.55 mmol (g of casein)$^{-1}$ determined using the guanidination method. Generally, the amounts of lysine in the unheated casein, where it can be assumed that all of the lysine is available, compared quite well between methods, with a less than 10% difference among the three methods. The reactive lysine content of the heated lactose/casein determined using the FDNB and guanidination methods also agreed well [0.31 and 0.33 mmol (g of casein)$^{-1}$, respectively] and differed by less than 7%. In contrast, the total lysine level determined using conventional amino acid analysis was considerably higher (almost 20%) than for the other two methods.

Recovery of Acid Stable Amino Acids during Guanidination. The amounts of other acid stable amino acids were compared when using the guanidination method and conventional amino acid analysis for a range of protein sources, with the aim of determining if the guanidination method interfered with the quantitation of these other amino acids. The recovery of acid stable amino acids is shown in Figure 3. Again, conversion of lysine to homoarginine in the relatively unprocessed proteins (lysozyme, soy protein isolate, skim milk powder, lactic casein, whey protein concentrate, soy protein concentrate, rotary dried blood meal and soybean meal) was high, ranging from 97 to 100%. In contrast, in wheat meal and the more severely processed protein sources such as meat and bone meal and cottonseed meal, the conversion of lysine to homoarginine was considerably lower. The recoveries of almost all of the amino acids in all protein sources examined were close to 100%. The main exception was histidine, for which, in skim milk powder and wheat and soybean meals, recoveries well above 100% were observed.

Determination of True Ileal Amino Acid Digestibility in Unheated Casein and Heated Lactose/Casein. The rats appeared to be healthy throughout the 14 day study. There was no sign of fecal particles in the stomach contents of the rats at post-mortem, indicating that coprophagy had not occurred, at least on the last day of the study. Meal intakes were relatively constant over the first five meals on the last day of study, and therefore a relatively constant flow of digesta through the gut should have been achieved (Figure 4).

Table 3. Mean True Ileal Amino Acid Digestibility (Percent) of Unheated Casein and Heated Lactose/Casein in the Growing Rat Determined Using an Ileal Amino Acid Digestibility Assay Based on Conventional Amino Acid Analysis

	unheated casein	heated lactose/ casein[a]	overall SE	significance[b]
aspartic acid	96.0	87.3	1.37	**
threonine	93.6	88.0	0.96	**
serine	89.7	85.0	1.03	**
glutamic acid	93.1	89.4	0.74	**
glycine	86.0	81.2	2.70	NS
alanine	97.2	93.0	0.67	NS
valine	96.7	92.4	0.73	**
isoleucine	94.8	90.1	0.82	**
leucine	99.1	97.1	0.27	**
tyrosine	100.4	97.1	0.29	***
phenylalanine	100.4	98.0	0.30	NS
histidine	95.8	86.0	0.88	*
arginine	98.1	96.1	1.68	NS

[a] Heated lactose/casein was prepared as described under Experimental Procedures. [b] NS, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The endogenous amino acid flows at the terminal ileum, determined using the "lactose-free" EHC1 diet, were used to correct apparent amino acid digestibilities to true ones for the unheated casein diet. The lactose-containing EHC2 diet was used to correct apparent digestibilities to true digestibilities for the heated lactose/casein diet. Although the absolute endogenous amino acid flows appeared to be higher with the lactose-containing EHC diet compared to the lactose-free EHC diet, there was no statistical difference ($P < 0.05$) between the two diets for all amino acids determined, with the exception of histidine (P. J. Moughan and S. M. Rutherfurd, unpublished results). The variation in endogenous flows observed in the rats fed the lactose-containing EHC diet was considerably greater than that observed for the rats fed the lactose-free EHC diet.

The true ileal amino acid digestibility (conventional assay) of the unheated casein was very high, with a mean digestibility (excluding lysine) of about 95% (Table 3). The digestibility of the heated lactose/casein was significantly lower for all amino acids except glycine, alanine, phenylalanine, and arginine. The mean decrease in digestibility between the unheated casein and the heated lactose/casein (excluding lysine) was 3 percentage units but was as high as 10 percentage units for histidine (Table 3).

The true ileal digestibility of lysine determined using the traditional ileal digestibility assay with conventional amino acid analysis was very high in the unheated casein (99%) (Table 4). The same coefficient in the heated lactose/casein was considerably lower, at 71%. In contrast, the true ileal digestibility of reactive lysine determined using the traditional ileal amino acid digestibility assay coupled with the guanidination reaction (new method) yielded a digestibility of 85%, significantly higher than the digestibility found using conventional methods.

DISCUSSION

Recently, there has been renewed interest in the effects of food processing on the availability of amino

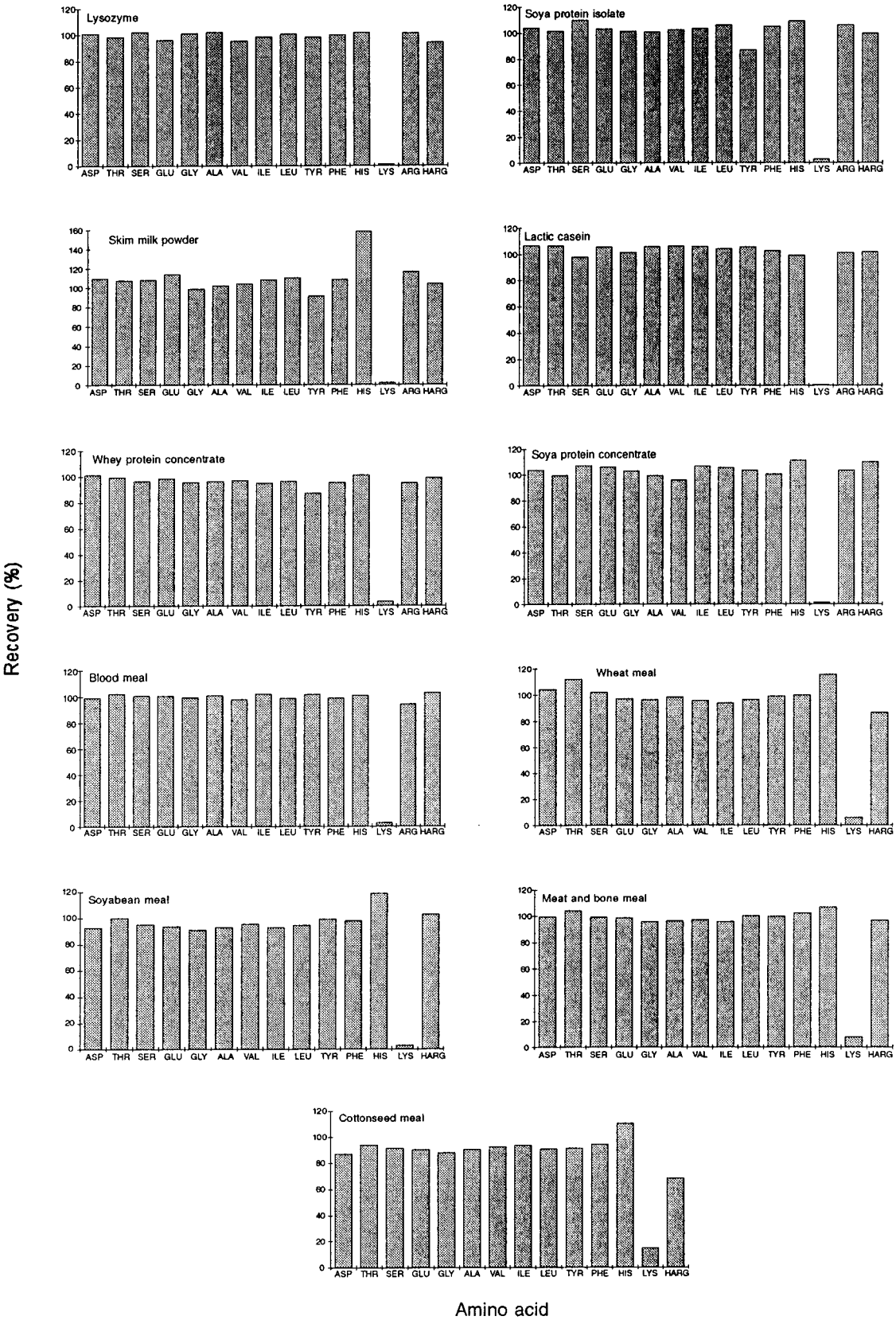

Figure 3. Recovery of amino acids from various protein sources after guanidination with *O*-methylisourea. Recoveries were calculated as

$$\text{recovery (\%)} = \frac{\text{mol of amino acid determined in the guanidinated protein}}{\text{mol of amino acid determined in the unreacted protein}} \times 100$$

acids, in particular, lysine (Knipfel, 1981; Batterham, 1992). This has stemmed from a realization that conventional lysine digestibility assays and assays for determining chemically available lysine do not adequately describe the effects of processing on lysine bioavailability (Moughan, 1991). There has been an emphasis in the literature on describing the chemical transformations that lysine undergoes during food

Table 4. Mean True Ileal Lysine Digestibility ($n = 6$) for an Unheated Casein Determined Using the Rat Ileal Digestibility Assay Based on Conventional Amino Acid Analysis and for a Heated Lactose/Casein ($n = 6$) Using the Rat Ileal Digestibility Assay Coupled with either Conventional Amino Acid Analysis or the Guanidination Method (Reactive Lysine Digestibility Coefficient)[a]

		heated lactose/casein[b]			
	unheated casein	conventional amino acid analysis	guanidination method	overall SE	significance
digestibility of lysine	98.8	70.5	85.9[c]	2.01	***

[a] Endogenous amino acid flows at the terminal ileum were corrected for, using the EHC/ultrafiltration method (Butts *et al.*, 1991) and the appropriate EHC diet (see Table 1). [b] Heated lactose/casein was prepared as described under Experimental Procedures. [c] The reactive lysine digestibility was calculated as follows (units are micrograms per gram DMI):

$$\frac{\text{reactive lysine in the diet} - (\text{reactive lysine in the digesta} - \text{endogenous lysine})}{\text{reactive lysine in the diet}} \times 100$$

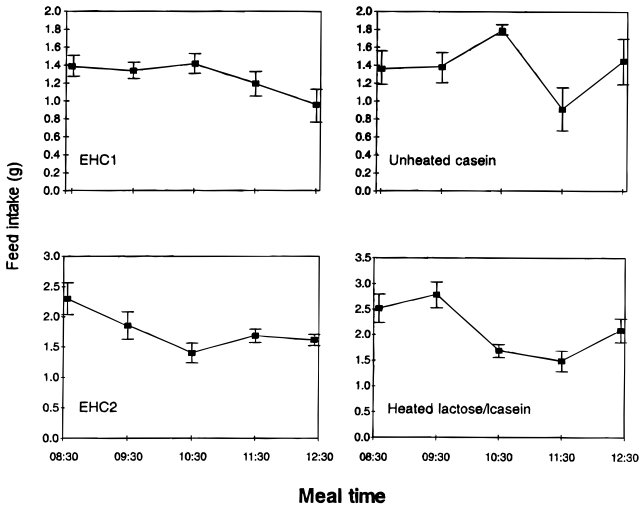

Figure 4. Mean (±SE) food intakes for the first five meals for rats on the last day of trial.

processing and the fate of the altered residues during digestion and metabolism. The approach taken here, however, was to regard the altered lysine residues as "lost" to the animal for protein synthesis and to attempt to directly determine the absorption of the "unaltered" or "reactive" lysine residues remaining in a processed feedstuff.

The ileal reactive lysine digestibility assay described here is a modification of the traditional ileal amino acid digestibility assay. However, with the reactive lysine digestibility assay the reactive lysine contents of the diets and digesta, rather than the total lysine contents, are directly determined, thereby eliminating the problem of overestimated lysine values due to interference from heat-induced lysine derivatives reverting back to lysine during acid hydrolysis. Several key criteria must be met for this new method to be valid. First, the derivatizing agent used to determine the reactive lysine in diets and digesta must be specific for the ϵ-amino group of lysine, to allow the determination of both free and bound reactive lysine that may be present in digesta. Second, the derivatized lysine compound (homoarginine in the case of the guanidination reaction) must be acid stable. Third, derivatization must be quantitative. FDNB, the most commonly used reagent for determining reactive lysine, fails as a suitable reagent as it can also react with the α-amino group of lysine and will therefore not detect free reactive lysine. Furthermore, DNP-lysine is not particularly acid stable, necessitating the use of correction factors. The derivatizing agent, *O*-methylisourea, is specific for the ϵ-amino group of lysine, as in a preliminary study 95% of lysine was recovered as homoarginine or residual unguanidinated lysine from a mixture of lysine and 0.6 M OMIU at pH 10.6 incubated for 24 h at 20 ± 2 °C in a shaking water bath (P. J. Moughan and S. M. Rutherfurd, unpublished results). Furthermore, *O*-methylisourea appears to be acid stable, as in a preliminary study 96% of homoarginine was recovered when homoarginine was incubated in 6 M HCl in a sealed evacuated glass tube for 24 h at 110 ± 2 °C (P. J. Moughan and S. M. Rutherfurd, unpublished results). Therefore, *O*-methylisourea shows promise as a suitable reagent for the ileal reactive lysine digestibility assay.

The present study first investigated the reaction time required to achieve complete conversion of lysine to homoarginine in lysozyme and lactic casein. For both proteins, near complete conversion was routinely achieved using a 24 h incubation in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C in a shaking water bath with the reagent to lysine ratio greater than 1000. Similarly, in the relatively unheated partially purified proteins (skim milk powder, whey protein concentrate, and soy protein concentrate and isolate), near complete guanidination was also achieved using the above guanidination conditions. It was therefore concluded that these conditions were also suitable for complete guanidination of unheated proteins, and no further optimization was required for application of the guanidination method to partially purified proteins.

In contrast, a longer incubation period (3−7 days) was required to achieve maximal guanidination of the heated lactose/casein. However, this may be an artifact of the larger particle size (1 mm mesh) of the heated lactose/casein, as other, more finely ground (0.5 mm mesh), heated protein sources including blood meal, soybean meal, meat and bone meal, and cottonseed meal required only a 24 h incubation to achieve maximal guanidination (P. J. Moughan, S. M. Rutherfurd, and L. van Osch, unpublished results). The reaction mixture pH of 10.6 was deemed to be optimal for guanidination of the heated lactose/casein since this pH was optimal for the guanidination of other partially purified proteins as well as other heated protein sources (P. J. Moughan and S. M. Rutherfurd, unpublished results). Guanidination conditions consisting of a 24 h incubation in 0.6 M *O*-methylisourea (pH 10.6) at 21 ± 2 °C in a shaking water bath where the reagent to lysine ratio is greater than 1000 may to be suitable for determining reactive lysine in a wide range of unprocessed and processed protein sources.

It was also important in the present work to investigate the reaction conditions required for guanidination of lysine in digesta. The optimal guanidination conditions for the digesta of rats fed unheated casein were 24 h of incubation in 0.6 M *O*-methylisourea (pH 10.6)

at 21 ± 2 °C in a shaking water bath with a reagent to lysine ratio of greater than 1000. In contrast, the digesta of rats fed the heated lactose/casein required a 3−7 day incubation in 0.6 M *O*-methylisourea in a shaking water bath at 21 ± 2 °C at pH 11.0 to achieve maximal guanidination. This longer reaction time required for optimal guanidination of digesta samples compared to ground protein sources may be caused by the presence of larger particles in the digesta. At the optimal pH and incubation time, the reactive lysine content in the digesta of the rats fed the heated lactose/casein contained approximately 5 times the amount of reactive lysine present in the unheated casein digesta. This results from a significantly lower digestibility of the heated lactose/casein compared to the unheated casein. For the purpose of determining the ileal reactive lysine digestibility in the heated lactose/casein, the following guanidination conditions were employed: 7 day incubation in 0.6 M *O*-methylisourea (pH 11.0) at 21 ± 2 °C in a shaking water bath with a reagent to lysine ratio greater than 1000. These conditions were deemed to be suitable for digesta from animals fed either unheated casein or heated lactose/casein.

The amount of lysine in the unheated casein determined using the FDNB, guanidination, and conventional amino acid analysis methods compared favorably, with all values being within 10% of each other. It was noted that the reactive lysine determined using the guanidination method was slightly higher than the values determined using the FDNB and amino acid analysis methods. The reason for this is difficult to discern. However, it cannot be explained by incomplete guanidination, a common criticism of the guanidination method, since the homoarginine levels were higher than the FDNB-reactive lysine level and total lysine level. The amount of reactive lysine in the heated lactose/casein determined using the guanidination method agreed well with the amount of reactive lysine determined using the FDNB method, with less than a 7% difference between the two values. In contrast, both values were considerably lower (20% lower) than the total lysine content determined using conventional amino acid analysis. This is most likely due to an overestimate in total lysine determined using conventional amino acid analysis, due to the reversion of lactulosyllysine to lysine during acid hydrolysis (Hendriks *et al.*, 1994). Both the FDNB and guanidination methods also detected that 40% of the original lysine in the heated lactose/casein was destroyed or modified by heat treatment, again demonstrating the good agreement between these two methods. In contrast, using conventional amino acid analysis, only 24% of the original lysine in the heated lactose/casein was found to have been destroyed or modified by heat treatment.

When the amino acid levels in 11 different protein sources, both unprocessed and processed, were determined after guanidination and compared to those determined using conventional amino acid analysis, the recoveries of most amino acids were close to 100%, the exception being histidine for which, in skim milk powder and wheat, soybean, and cottonseed meal, the recovery was much greater than 100%. This may have been associated with the chromatographic procedure and may have been due to column aging. An analogue of *O*-methylisourea, *S*-methylisothiourea, has also been examined as a guanidinating agent. However, the recoveries of serine were low (50−60%) (P. J. Moughan and S. M. Rutherfurd, unpublished results) in all protein sources examined when *S*-methylisothiourea was used, which would render it unsuitable for application in a general amino acid digestibility assay. The present study shows that the guanidination reaction using *O*-methylisourea does not cause significant modification of any of the acid stable amino acids besides lysine, and therefore quantitation of all acid stable amino acids including reactive lysine is possible in processed feedstuffs using the guanidination procedure alone.

True ileal amino acid digestibility coefficients for all of the acid stable amino acids were determined by conventional amino acid analysis of the diet and digesta samples. As expected, most of the amino acids in the unheated casein were highly digestible. There was, however, a significant difference between amino acid digestibility in the unheated casein compared to the heated lactose/casein for most amino acids, with the amino acid digestibility in the heated lactose/casein being lower for all amino acids except tyrosine. The decreases in digestibility resulting from heat treatment (excluding lysine) ranged from approximately 2 percentage units for arginine and leucine to 10 percentage units for histidine, with the average decrease being about 5 percentage units. This decrease may be at least in part due to a decreased efficiency of proteolytic enzymes in cleaving the peptide bonds in the vicinity of structurally altered lysine residues (Boctor and Harper, 1968), leading to the presence of limit peptides. Furthermore, the decrease in digestibility from the unheated casein to the heated lactose/casein for a particular amino acid may depend upon proximity to the structurally altered lysine residues (Swaisgood and Catignani, 1991).

Lysine was found to be highly (99%) digestible in the unheated casein. The determined lysine digestibility coefficient in the heated lactose/casein, when lysine content was quantified using conventional amino acid analysis, was 71%, which was 28 percentage units lower than for the unheated casein. This decrease in digestibility was considerably greater than the 2−10 percentage unit decrease observed for the other amino acids. In contrast, the true reactive lysine digestibility coefficient in the heat-damaged casein obtained using guanidination was significantly higher at 86%, approximately 13 percentage units lower than in the unheated casein. This shows better agreement with the decrease in digestibility observed for the other amino acids.

While it is clear that the use of conventional amino acid analysis significantly underestimates lysine digestibility, it is also possible that the reactive lysine digestibility, determined using the guanidination method, is overestimated. However, since the guanidination reaction conditions have been optimized for the reaction time and reaction mixture pH and the reagent to lysine ratio has been maintained at a very high level, it is likely that nearly complete guanidination has been achieved in both digesta and diets, and therefore the reactive lysine digestibility estimate obtained using the guanidination method would provide more reliable estimates than those obtained using conventional amino acid analysis.

There are many methods for determining reactive lysine in feeds, all with their inherent advantages and disadvantages, but currently there is no ideal method that allows for the routine determination of digestible reactive lysine in heat-processed feeds. The new true ileal reactive lysine digestibility method described here may be a means by which this can be achieved.

Furthermore, the use of the rat as an experimental animal may allow for a relatively routine assessment of available lysine in processed feedstuffs. Although work is required to further validate this new assay, preliminary findings suggest that the method gives rise to meaningful estimates of the true digestibility of reactive lysine.

ACKNOWLEDGMENT

We acknowledge W. Hendriks for assistance with FDNB analysis and R. Watson for assistance with chromium analysis.

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Received for review January 13, 1995. Accepted May 7, 1996.[⊗] We acknowledge the financial support of the Australian Pig Research and Development Corp.

JF950032J

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1996.