Use of guanidinated dietary protein to measure losses of endogenous amino acids in poultry

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Guanidinated proteins when fed to non-ruminants provide values for both endogenous amino acid losses and amino acid digestibilities, provided that the homoarginine residues in the treated protein are randomly distributed. Earlier studies have established that guanidination has only minor effects on the structure of the protein and, in particular, on its susceptibility to proteolysis. Furthermore, we have confirmed that homoarginine behaves as a typical amino acid in the small intestine. Lysine residues in casein and soya-bean protein, and in the proteins of cotton-seed meal, meat meal, soya-bean meal, maize, sorghum and wheat were converted to homoarginine by guanidination, the extent of conversion ranging from 37–68%. Sequential proteolysis *in vitro* of these guanidinated materials showed that the ratios of homoarginine to other amino acids remained unchanged for casein and soya-bean protein, indicating random distribution of homoarginine residues, but not for all the amino acids in meals and cereals. The use of guanidinated casein as the sole protein source in diets fed to broiler chickens allowed measurement of endogenous losses of amino acids under normal feeding conditions and calculation of true digestibilities of dietary amino acids at the ileum. Endogenous amino acid losses measured by the use of guanidinated casein (15·3 g/kg dry matter (DM) intake) were significantly higher ($P < 0·001$) than values obtained by feeding a N-free diet (5·4 g/kg DM intake), or by regression analysis to zero N intake (7·2 g/kg DM intake).

Endogenous amino acids: Amino acid digestibility: Homoarginine: Guanidination: Poultry

The use of dietary protein labelled with homoarginine to measure endogenous amino acid losses was proposed by Hagemeister & Erbersdobler (1985). Conversion of the lysine residues of dietary protein into homoarginine was achieved by guanidination. Homoarginine, an amino acid not utilized for protein synthesis, is normally absent from feedstuffs and body tissues, and is converted to lysine in the liver (Ryan *et al.* 1968). These properties ensure that absorbed homoarginine is not re-cycled into the small intestine.

An assumption implicit in the use of guanidinated proteins to study protein digestibility and endogenous amino acid losses is that the introduction of the homoarginine label has only minor effects on the structure of the protein, and that the release and absorption of homoarginine is essentially similar to that of other amino acids. Indirect evidence supporting these assumptions is that guanidination, which is carried out under mild conditions of pH (10·5) and ionic strength (0·2 mM), has little effect on the biological activity of growth hormone, lactogenic enzymes and lysozyme (*EC* 3.2.1.17; see Schmitz *et al.* 1991). The ready absorption of homoarginine from the small intestine has been

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demonstrated in chickens (Siriwan *et al.* 1989) and pigs (Schmitz *et al.* 1991), and confirmed in the present studies. Furthermore, we have confirmed that homoarginine, when incubated with digesta from the small intestine, is no more susceptible to microbial activity or to the activities of tissue enzymes than other amino acids, as reported earlier (Siriwan *et al.* 1989; Schuttert *et al.* 1991). Evidence that guanidination has only a minor influence on protein digestion was obtained by Schmitz *et al.* (1991), who showed that guanidinated and native casein are hydrolysed by chymotrypsin (*EC* 3.4.21.1) at similar rates.

A major issue addressed in the present paper is whether or not the replacement of lysine with homoarginine in partially guanidinated proteins occurs randomly. In the absence of any selective conversion of lysine to homoarginine, the ratio of the homoarginine content to that of the other amino acids that make up the protein should remain constant during the sequential proteolysis of the guanidinated protein. If the ratio remains unchanged the true digestibility of a guanidinated dietary protein, and that of its constituent amino acids, will be identical to the measured digestibility of homoarginine. In the present studies the ratios remained unchanged during the sequential digestion of casein and soya-bean protein, but not for a number of other protein sources used in animal feeds. In diets in which guanidinated casein was the sole protein source, comparison of the ratios of homoarginine to other amino acids in dietary protein with the same ratios in ileal digesta allowed calculation of endogenous amino acid losses.

MATERIALS AND METHODS

Management of birds

Male broiler chickens, aged 5 weeks, were housed at 24° under continuous lighting and had access to water at all times. Commercial diets were provided *ad lib.* to all birds, except when fed on the experimental diets.

The chickens were ranked according to weight, and randomly allocated to experimental groups containing five birds of similar means and ranges of liveweight.

Incubation of homoarginine and other amino acids with digesta from the small intestine

Broiler chickens (5 weeks of age) reared on standard diets were anaesthetized with sodium pentobarbitone (Syntex Australia Ltd., Castle Hill, NSW, Australia), the abdominal cavity opened and the intestinal tract removed. The contents of the jejunum (duodenum to vitelline diverticulum) and ileum (diverticulum to ileocaecal junction) were collected immediately by washing out each section with 0·2 M-phosphate buffer (5 ml, pH 6·0) into a beaker immersed in ice water (Skurray & Cumming, 1975). The intestinal contents from two birds were pooled, and jejunal and ileal samples were examined in quadruplicate.

The contents (about 10 g) from each section were weighed into a 100 ml conical flask and 5 ml of either 12·5 mM-homoarginine or an amino acid mixture (2·5 mM each of threonine, valine, leucine, isoleucine, phenylalanine, histidine, lysine, arginine and methionine) was added. The contents of each flask were made up to 50 ml with 0·2 M-phosphate buffer (pH 5·6 for jejunal contents, pH 6·8 for ileal contents). After mixing, followed by low speed centrifugation, a portion (2 ml) of supernatant was retained for amino acid analysis. The jejunal and ileal contents were incubated under aerobic and anaerobic conditions.

Aerobic incubation

The contents of each flask were gassed with O_2–CO_2 (95: 5; v/v) for 3 min and incubated in a shaking water bath at 41° for 3 h. The contents were then centrifuged, and 2 ml supernatant placed in a 10 ml centrifuge tube. Sulphosalicylic acid (300 g/l; 1 ml) and

1 mM-norleucine (3 ml) were added, and the mixture was well mixed before centrifugation (1600 *g*, 15 min). The deproteinized supernatant containing internal standard (norleucine) was adjusted to pH 2·1 with 1 M-NaOH, passed through a Millipore filter (0·22 μm) and analysed for amino acids as described earlier (Siriwan *et al.* 1993).

Anaerobic incubation

Flasks containing ileal and jejunal contents were gassed with N_2 for 3 min and placed in an anaerobic jar under a mixture of H_2 and CO_2 generated by a commercial anaerobic gas pack (BBL Gas Pak; Becton Dickerson Microbiology Systems, Corkeysville, MD, USA). The jar was sealed and incubated at 41°, with occasional shaking, for 3 h. After incubation the contents were prepared for amino acid analysis as described for contents incubated aerobically.

Absorption of homoarginine and other amino acids from sections of small intestine in vivo

Broiler chickens (6 weeks of age) reared on standard diets were starved for 12 h and anaesthetized with a mixture of O_2 and N_2O (1:1, v/v) and halothane (2%; Fluothane; ICI Australia Ltd) administered by tracheal intubation (Bryden, 1989). The birds were surgically prepared for the study of intestinal absorption as described by Bryden (1989). A section of both jejunum and ileum extending 25 mm on either side of the vitelline diverticulum was loosely ligated by linen thread. A further 100 mm from the first ligatures, further ligatures were placed to isolate sections of jejunum and ileum. The sections were flushed with Krebs-Ringer phosphate buffer (pH 7·4, 41°) to remove digesta, and the second set of ligatures was secured tightly.

A solution of amino acids was administered into both sections of the small intestine through the loose ties, and both ligatures were then tightened. The intestinal tract was covered with a gauze pad kept moist with warm physiological saline (9 g NaCl/l) during the period of each experiment (15 min).

Immediately before removing the sections of intestinal tract and recovering contents by washing out with physiological saline, blood samples were collected by heparinized needle and syringe from a major mesenteric vein that drained both sections. Blood samples and intestinal contents were analysed for amino acids.

The absorption of homoarginine, lysine and arginine (2·5 mM in Krebs-Ringer phosphate buffer, pH 7·4) from the tied-off sections of jejunum and ileum was examined singly, or in combination in experiments carried out in triplicate. Results were obtained on both the loss of amino acids from the jejunal and ileal sections, and their appearance in mesenteric blood.

In further experiments the absorption of homoarginine in the presence of the amino acids used in the incubation experiments described above was examined over a range of homoarginine concentrations.

Guanidination of proteins

Lysine residues in commercial sources of casein, soya-bean protein, cotton-seed meal, sunflower meal, meat meal, soya-bean meal, maize, sorghum and wheat were converted to homoarginine by guanidination based on the procedures of Markland *et al.* (1975) and Maga (1981). Protein (about 1 g) was weighed into a 100 ml conical flask containing 0·2 M-glycine-NaOH buffer (10 ml, pH 10·5) and treated at 4° with 10 ml of 0·6 M-*O*-methylisourea (freshly prepared; Sigma Chemical Co., St Louis, MO, USA) adjusted to

pH 10·5 with NaOH. The mixture was maintained at 4°, with continuous stirring, for 24 h, before dissolved protein was precipitated with sulphosalicylic acid. The precipitate and residual insoluble protein were separated by centrifugation and washed twice with distilled water (10 ml) to remove sulphosalicylic acid, which was assayed during subsequent amino acid analysis of the hydrolysed guanidinated proteins by ion-exchange chromatography. Residual sulphosalicylic acid concentrations were below 1 mg/l.

Preliminary studies had established earlier that the extent of guanidination of casein was not increased by extending the reaction time beyond 24 h.

Sequential enzymic digestion of guanidinated proteins

Guanidinated proteins (casein, soya-bean isolate, cotton-seed meal, soya-bean meal, sunflower meal, meat meal, maize and wheat) were digested for 30 min with pepsin (*EC* 3.4.23.1), followed by incubation with pancreatin for 1, 2, 3 or 4 h.

Guanidinated protein (about 0·5 g) was weighed into a 100 ml conical flask to which was added 900 units of pepsin (Sigma Chemical Co., 4000 units/mg) in 15 ml 0·1 M-NH_4Cl containing thimerosal (50 μg/ml) as preservative. The flasks were incubated in a shaking water bath at 41° for 30 min, and immediately adjusted to pH 8·0 with 0·2 M-NaOH.

Pancreatin (1 mg; Grade IV; Sigma) in 10 ml phosphate buffer (0·1 M, pH 8·0) was added to the pepsin and incubation at 41° was resumed, as before. Flasks were removed after 1, 2, 3 and 4 h, and treated with 15 ml of sulphosalicylic acid (300 mg/ml) to precipitate undigested protein. The residual protein was collected by centrifugation, washed twice with water, freeze-dried and analysed for amino acids.

The extent of digestion of each protein source varied, ranging from 65% for casein to 30% for meat meal after 4 h.

Each two step incubation was carried out in triplicate.

Amino acid analysis

Amino acids in diets and intestinal digesta were analysed by standard ion-exchange column chromatographic procedures, with fluorimetric detection of amino acids using *O*-phthaldialdehyde, as described earlier (Siriwan *et al.* 1993). In the present studies there was usually insufficient ileal sample to determine cystine and methionine after oxidation, and as a consequence no values for cystine are reported and the values determined for methionine are likely to be underestimated due to loss during hydrolysis.

Blood plasma (3 ml) was deproteinized by treatment with an equal volume of sulphosalicylic acid (100 mg/ml) after the addition of DL-norleucine (0·5 ml, 2·5 mM) as internal standard. The treated samples were centrifuged (3000 ***g***, 20 min) and the supernatant stored at −20°. Before analysis, the samples were thawed and the pH adjusted to 2·2–2·3 with 3 M-LiOH before filtration using a 0·22 μm Millipore filter.

The amino acids in the filtrate were separated on a column (ID 4·00 mm, length 180 mm) packed with Interaction IC-1011-6 resin (Activon Scientific Products Co., Pennant Hills, NSW, Australia), and eluted with lithium-based buffers. Homoarginine is well separated from other amino acids in this system.

Measurement of endogenous amino acids in the small intestine

Endogenous amino acid losses in the small intestine were measured by using the guanidinated casein procedure, and also by the conventional methods of extrapolation to zero N intake by regression analysis, and by feeding a N-free diet. This involved the formulation of six diets (Table 1) containing graded amounts of casein (0–294 g casein/kg) and formulated to be approximately isoenergetic (13·3 MJ/kg). The macroingredients were

Table 1. *Composition (g/kg) of diets based on casein, guanidinated casein or zero casein*

Composition	Dietary protein (g/kg)					
	0	50	100	150	200	250
Casein, or guanidinated casein	—	59	118	177	236	294
Maize starch	567	500	434	367	300	235
Vegetable oil	50	50	50	50	50	50
Glucose	200	200	200	200	200	200
Solkafloc	90	90	90	90	90	90
Alkathene	26	36·5	46	56	66·5	75·5
Celite	20	20	20	20	20	20
NaCl	2·5	2·5	2·5	2·5	2·5	2·5
$CaCO_3$	11	12	13	14	15	16
$CaHPO_4$	28·5	25	21·5	18·5	15	12
Broiler premix	5	5	5	5	5	5

Table 2. *Conversion (%) of lysine to homoarginine in nine guanidinated protein sources*
(Means with their standard errors for three determinations)

Protein	Lysine (g/kg)		Conversion*			
			A		B	
	Untreated	Guanidinated	Mean	SEM	Mean	SEM
Casein	78·1	24·8	68·2	0·78	66·1	1·32
Soya-bean protein	57·7	18·5	68·0	0·11	68·0	0·47
Cotton-seed meal	24·1	13·9	42·2	0·90	36·7	0·83
Soya-bean meal	38·5	12·0	68·8	0·49	63·1	1·31
Sunflower meal	15·3	7·8	49·2	0·72	49·3	0·84
Meat meal	27·7	10·7	61·6	1·76	50·0	0·42
Maize	2·3	1·1	53·7	0·90	57·3	1·04
Sorghum	2·5	1·0	61·3	0·95	61·2	0·87
Wheat	3·4	1·0	69·0	1·63	62·6	0·65

* A, Conversion (%) based on loss of lysine; B, conversion (%) based on the transformation of lysine to homoarginine (molar basis).

supplemented with a premix to meet vitamin and mineral requirements. Celite (Johns-Manville Sale Corporation, Lomco, CA, USA), a source of acid-insoluble ash, was used as a dietary marker, and powdered polyethylene (Alkathene: ICI Australia Ltd.) was added to decrease nutrient density to that of normal commercial diets (Siriwan *et al.* 1993).

Use of guanidinated casein

The test diet (Table 1) contained guanidinated casein (236 g/kg) in which two thirds of the lysine had been converted to homoarginine (Table 2). A diet of the same general composition as the test diet, but based on untreated casein (236 g/kg), was fed *ad lib.* for 1 week to twenty male broiler chickens (aged 6 weeks). The birds were then allowed access to glucose for 24 h before being precision-fed (Sibbald, 1987) 25 g of the test diet. After 3 h the birds were killed by injection with sodium pentabarbitone, the small intestine

exposed and the duodenum, jejunum and ileum tied off to allow collection and processing of contents for amino acid analysis, as described earlier (p. 517). Intestinal contents from five birds were pooled to provide enough material for amino acid analysis.

Levels of endogenous amino acids in the duodenum, jejunum and ileum were calculated from changes in the ratios of homoarginine to each individual amino acid in guanidinated casein from those measured in intestinal digesta.

The true digestibilities of individual amino acids were assumed to be identical to that of homoarginine, in view of the constancy of the ratios of homoarginine to individual amino acids during the sequential digestion of guanidinated casein, the sole protein source in the test diet.

Use of nitrogen-free diets and regression analysis

The measurement of endogenous amino acids by the collection and analysis of ileal contents of birds fed either a N-free diet or diets containing graded levels of N was reported earlier (Siriwan *et al.* 1993). In the latter procedure, extrapolation to zero N intake by regression analysis provides a measure of endogenous amino acid losses.

Comparison of the three techniques was based on feeding semi-purified diets containing either guanidinated casein (236 g/kg) or graded amounts of untreated casein (0, 59, 118, 177, 236 and 294 g/kg; Table 1).

Fifty-six male broiler chickens (aged 5 weeks) were fed on the semi-purified diet containing casein (236 g/kg) for 5 d. The diet was then removed and glucose was supplied for 12 h before the birds were randomly assigned into four replicates of two birds for each experimental treatment.

Each bird was precision-fed with 25 g of experimental diet containing either guanidinated casein, one of five levels of casein, or zero casein. At 3 h after feeding, ileal contents were collected and analysed for amino acids, including homoarginine, and acid-insoluble ash.

Endogenous amino acid losses were calculated from the levels of homoarginine and other amino acids in the ileal digesta of birds given either guanidinated casein (see above), varying levels of untreated casein (regression analysis) or the N-free diet.

RESULTS

Guanidination of protein sources

The extent of conversion of lysine residues to homoarginine in nine protein sources varied from about 37% for cotton-seed meal to about 68% for a soya-bean protein isolate (Table 2). For most proteins there was reasonable agreement between the extent of conversion based on loss of lysine with that calculated from yield of homoarginine, expressed on a molar basis (Table 2). The average conversion of lysine to homoarginine for all protein sources was about 60%.

Incubation of homoarginine and other amino acids with intestinal digesta

There were no significant losses of homoarginine, or of threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine or arginine when mixtures of these amino acids were incubated with jejunal and ileal digesta under either aerobic or anaerobic conditions (Table 3).

Absorption of homoarginine from the jejunum and ileum

The absorption of homoarginine from the small intestine relative to that of lysine or homoarginine was examined by placing the amino acid solutions (5 ml, 2·5 μmol/ml) in ligated sections of jejunum and ileum and measuring losses over 15 min. The amino acids

Table 3. *Homoarginine and amino acid concentrations (μg/ml) (A) before and (B) after aerobic and anaerobic incubation with intestinal contents of broiler chickens for 3 h**

(Means with their standard errors for four determinations)

	Aerobic incubation								Anaerobic incubation							
	Jejunum				Ileum				Jejunum				Ileum			
	A		B		A		B		A		B		A		B	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Threonine	35	0·6	36	0·6	34	1·3	33	1·8	33	0·6	34	0·7	33	0·6	32	0·2
Valine	40	0·5	40	0·8	37	1·6	36	2·2	39	0·8	40	1·4	39	0·9	39	0·6
Methionine	47	1·1	45	0·5	46	1·7	48	2·1	48	1·1	49	1·6	46	0·6	4	1·0
Isoleucine	46	0·7	46	0·7	39	1·7	39	2·4	39	1·2	38	1·0	39	0·6	39	1·2
Leucine	37	0·6	38	0·7	36	1·5	36	1·8	36	0·8	37	0·8	36	0·4	36	0·2
Phenylalanine	52	0·8	52	1·0	49	1·9	49	2·4	52	1·2	52	1·9	50	0·6	50	0·4
Histidine	48	0·2	49	1·6	46	2·3	46	2·9	46	3·4	44	2·8	41	0·9	41	1·0
Lysine	53	0·6	53	0·9	50	1·8	50	2·5	51	1·4	52	1·6	50	0·9	50	0·5
Arginine	53	1·0	53	0·5	50	2·0	50	2·1	53	1·3	53	1·2	49	0·4	50	0·5
Homoarginine	289	0·6	293	2·4	292	10·2	297	9·9	285	7·0	285	6·2	290	7·8	293	5·1

* For details of procedures, see pp. 516–518.

Table 4. *Absorption (% of dose) of arginine, lysine and homoarginine from ligated intestinal segments of broiler chickens**

(Means with their standard errors for three determinations)

	Absorption			
	Jejunum		Ileum	
Amino acid	Mean	SEM	Mean	SEM
Single administration				
Arginine	57·2	1·87	62·8	0·87
Lysine	59·0	1·94	57·5	0·78
Homoarginine	59·1	5·39	54·1	5·10
Mixture				
Arginine	28·5	0·62	28·8	1·44
Lysine	36·5	1·83	37·8	1·30
Homoarginine	51·4	1·50	51·1	0·79

* For details of procedures, see p. 517.

were examined singly or in combination (Table 4), and blood was taken from a mesenteric vein about 1 min before the intestinal sections were removed for recovery of contents.

The absorption of all three amino acids from both jejunum and ileum was not significantly different. Homoarginine absorption was largely unaffected by the presence of lysine and arginine, but the extent of absorption of the latter two amino acids from the mixture were lower than when examined singly. The ready absorption of homoarginine was confirmed by its appearance in mesenteric venous blood following its administration to the small intestine, either alone or in combination with lysine and arginine (Table 5).

In further studies the absorption of homoarginine added at varying concentrations (1·0,

Table 5. *Mesenteric venous plasma amino acid concentrations (nmole/ml) following administration of lysine, arginine and homoarginine to chickens, either singly or as a mixture**

(Means with their standard errors for three determinations)

	Control		Single amino acid		Mixture	
Amino acid	Mean	SEM	Mean	SEM	Mean	SEM
Lysine	582	95	676	87	751	45
Arginine	325	6	463	97	437	48
Homoarginine	—		105	10	90	6

* For details of procedures, see p. 517.

Table 6. *Absorption (%) from the jejunum of chickens of homoarginine (1 mM; A), and of mixtures of amino acids (each at 2·5 mM) without homoarginine (B), and containing homoarginine at 1 mM (C), 1·5 mM (D), 2·0 mM (E) and 2·5 mM (F)**

(Mean values for four determinations)

Amino acid	A	B	C	D	E	F
Threonine	—	72	76	74	84	88
Valine	—	56	60	60	71	80
Methionine	—	88	89	85	91	94
Isoleucine	—	88	89	85	93	94
Leucine	—	86	88	83	92	93
Phenylalanine	—	78	81	79	87	89
Histidine	—	67	70	68	79	83
Lysine	—	77	77	72	83	85
Arginine	—	65	67	65	77	81
Homoarginine	78	—	82	77	87	90

* For details of procedures, see pp. 516–517.

1·5, 2·0 or 2·5 mM) to ligated segments of the jejunum and ileum was examined in the presence or absence of a mixture of threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine, lysine and arginine (each at 2·5 mM). The proportion of the dose of homoarginine added to the jejunum and ileum that was absorbed was unaffected by either the concentration of homoarginine or the presence of the amino acid mixture (Tables 6 and 7). The extent of absorption of homoarginine, relative to that of the other amino acids examined, was similar in both segments of small intestine. The apparent trends towards increased amino acid absorption at higher homoarginine concentrations were not statistically significant.

Constancy of ratios of homoarginine to other amino acids in partially digested protein sources

The ratios of homoarginine content to that of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine in guanidinated casein and guanidinated soya-bean protein

Table 7. *Absorption (%) from the ileum of chickens of homoarginine (1 mM; A)and of mixtures of amino acids (each at 2·5 mM) without homoarginine (B), and containing homoarginine at 1 mM (C), 1·5 mM (D), 2·0 mM (E) and 2·5 mM (F)*

(Mean values for four determinations)

Amino acid	A	B	C	D	E	F
Threonine	—	73	73	73	83	85
Valine	—	57	59	59	76	77
Methionine	—	86	86	80	87	91
Isoleucine	—	86	85	84	90	91
Leucine	—	85	86	80	88	90
Phenylalanine	—	78	78	78	83	86
Histidine	—	66	68	64	79	79
Lysine	—	76	76	70	82	80
Arginine	—	66	66	62	78	78
Homoarginine	80	—	80	74	85	85

Table 8. *Homoarginine: amino acid ratios in guanidinated casein before and after enzymic digestion for 1, 2, 3 and 4 h**

(Means with their standard errors for four determinations)

	Before digestion		Time of digestion (h)							
			1		2		3		4	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	1·3	0·20	1·3	0·04	1·3	0·03	1·4	0·02	1·4	0·04
Threonine	2·2	0·02	2·4	0·04	2·4	0·02	2·5	0·06	2·4	0·01
Serine	1·5	0·01	1·6	0·01	1·7	0·01	1·8	0·03	1·8	0·06
Glutamic acid	0·4	0·004	0·4	0·00	0·4	0·01	0·4	0·004	0·4	0·01
Glycine	5·4	0·06	5·4	0·06	5·4	0·11	5·7	0·07	5·3	0·05
Alanine	3·2	0·04	3·2	0·02	3·1	0·07	3·3	0·04	3·1	0·05
Valine	1·5	0·07	1·6	0·07	1·6	0·07	1·5	0·15	1·5	0·17
Methionine	2·9	0·11	2·7	0·04	2·8	0·12	3·1	0·24	3·1	0·29
Isoleucine	2·1	0·07	2·3	0·08	2·3	0·10	2·2	0·25	2·2	0·22
Leucine	1·0	0·01	1·0	0·01	1·0	0·01	1·0	0·02	0·9	0·01
Tyrosine	1·6	0·01	1·6	0·02	1·6	0·04	1·5	0·04	1·6	0·05
Phenylalanine	1·6	0·01	1·6	0·01	1·5	0·02	1·6	0·04	1·6	0·02
Histidine	3·6	0·04	3·6	0·03	3·5	0·03	3·6	0·08	3·6	0·05
Lysine	7·3	0·21	7·3	0·21	7·2	0·05	7·3	0·11	7·3	0·14
Arginine	3·1	0·02	3·2	0·07	3·2	0·05	3·3	0·09	3·2	0·06

* For details of procedures, see p. 518.

isolate remained essentially unchanged during sequential enzymic digestion for up to 4 h (Tables 8 and 9).

The studies were repeated with nine guanidinated protein sources (Table 10) including casein and soya-bean protein, but in each instance digestion was continued for 4 h. The ratios of homoarginine to other amino acids confirmed the relative constancy of ratios for casein and soya-bean protein, which are not shown in Table 10. The ratios were sensibly constant for cotton-seed meal, but for soya-bean, sunflower and meat meals, and the

Table 9. *Homoarginine: amino acid ratios in guanidinated soya-bean protein before and after enzymic digestion for 1, 2, 3 and 4 h**

(Means with their standard errors for four determinations)

	Before digestion		Time of digestion (h)							
			1		2		3		4	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	0·7	0·04	0·6	0·01	0·6	0·01	0·6	0·02	0·6	0·02
Threonine	2·1	0·09	2·0	0·01	2·0	0·01	2·1	0·05	2·1	0·06
Serine	1·4	0·09	1·3	0·02	1·3	0·02	1·3	0·05	1·2	0·04
Glutamic acid	0·4	0·02	0·4	0·003	0·4	0·002	0·4	0·01	0·3	0·01
Glycine	2·0	0·14	1·8	0·04	1·8	0·04	1·8	0·08	1·7	0·07
Alanine	1·9	0·13	1·8	0·04	1·8	0·04	1·8	0·06	1·8	0·03
Valine	1·8	0·26	1·6	0·11	1·7	0·04	1·9	0·21	1·9	0·09
Methionine	10·8	0·74	11·1	0·61	10·9	1·60	10·9	2·90	11·1	0·86
Isoleucine	1·8	0·33	1·6	0·12	1·7	0·05	1·9	0·24	1·9	0·08
Leucine	0·9	0·02	0·9	0·01	0·9	0·00	0·9	0·01	0·9	0·01
Tyrosine	1·9	0·04	1·9	0·03	2·0	0·01	2·1	0·07	2·0	0·05
Phenylalanine	1·3	0·02	1·3	0·01	1·3	0·01	1·4	0·01	1·4	0·02
Histidine	3·0	0·04	2·9	0·03	2·9	0·02	2·8	0·02	2·8	0·03
Lysine	7·7	0·15	7·4	0·33	7·8	0·23	7·3	0·15	7·5	0·09
Arginine	1·1	0·01	1·1	0·01	1·1	0·01	1·1	0·01	1·0	0·01

* For details of procedures, see p. 518.

proteins in maize, sorghum and wheat, the ratios for some amino acids changed during partial digestion (Table 10).

Measurement of endogenous amino acids and amino acid digestibilities in broiler chickens

Endogenous amino acid levels in the duodenum, jejunum and ileum of broiler chickens, calculated from the ratios of homoarginine to individual amino acids in the respective digesta, are shown in Table 11. These values reflect the balance between entry of endogenous amino acids into the small intestine, which is highest in the duodenum, and their uptake following digestion. Losses of endogenous amino acids are normally equated with ileal values. Glutamic acid and serine were the most abundant amino acids of endogenous origin in ileal digesta (Table 11).

The digestibility of homoarginine, which may be equated with the true digestibility of all of the amino acids in guanidinated casein, was 97% in broiler chickens.

Comparison of methods for measurement of endogenous amino acid losses based on guanidinated casein, regression analysis and nitrogen-free diets

Endogenous amino acid losses measured by the three methods are shown in Table 12. Values obtained by feeding guanidinated casein, which were based on the concentrations of homoarginine in ileal digesta, were significantly higher ($P < 0·001$) than those given by feeding a N-free diet, or regression analysis to zero N intake (Table 13). The same data were used to calculate the apparent and true amino acid digestibilities of guanidinated casein (Table 13). There were no significant differences between apparent and true digestibilities when endogenous amino acid losses were determined using a N-free diet, or by regression analysis. Use of values based on homoarginine, however, resulted in highly significant differences ($P < 0·001$) between apparent and true amino acid digestibilities.

Table 10. *Homoarginine:amino acid ratios (A) before digestion: (B) after digestion, for cotton-seed meal (CSM), soya-bean meal (SOB), sunflower meal (SFM), meat meal (MM), maize (MAZ), sorghum (SOG) and wheat (WHT) before and after enzymic digestion (4 h)**

(Mean values with their standard errors for three determinations)

	CSM				SOB				SFM				MM				MAZ				SOG				WHT			
	A		B		A		B		A		B		A		B		A		B		A		B		A		B	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	0·3	0·00	0·3	0·01	0·6	0·00	0·6	0·00	0·3	0·01	0·3	0·00	0·6	0·01	0·5	0·00	0·4	0·00	0·3	0·00	0·2	0·02	0·2	0·01	0·6	0·01	0·6	0·02
Threonine	0·8	0·01	0·9	0·01	1·7	0·01	1·6	0·00	0·7	0·01	0·8	0·01	1·3	0·01	1·0	0·00	0·7	0·00	0·4	0·01	0·6	0·01	0·3	0·01	0·9	0·01	1·1	0·01
Serine	0·6	0·00	0·6	0·02	1·1	0·01	1·1	0·01	0·6	0·02	0·6	0·01	1·0	0·00	0·9	0·00	0·5	0·00	0·3	0·00	0·3	0·02	0·2	0·01	0·5	0·00	0·9	0·02
Glutamic acid	0·1	0·00	0·2	0·00	0·3	0·00	0·4	0·00	0·1	0·00	0·1	0·00	0·4	0·00	0·3	0·00	0·1	0·00	0·1	0·00	0·1	0·00	0·0	0·00	0·1	0·00	0·2	0·00
Glycine	0·6	0·00	0·7	0·01	1·6	0·01	1·5	0·01	0·5	0·01	0·5	0·00	0·3	0·00	0·5	0·00	0·7	0·00	0·5	0·01	0·6	0·05	0·4	0·02	0·7	0·00	0·9	0·01
Alanine	0·7	0·00	0·7	0·01	1·6	0·00	1·5	0·01	0·6	0·01	0·6	0·01	0·7	0·00	0·7	0·00	0·3	0·00	0·2	0·00	0·2	0·01	0·1	0·00	0·8	0·01	0·8	0·02
Valine	0·5	0·01	0·8	0·01	1·5	0·00	1·2	0·00	0·5	0·02	0·7	0·01	0·9	0·00	0·6	0·00	0·5	0·00	0·3	0·00	0·4	0·04	0·2	0·01	0·7	0·01	0·8	0·05
Methionine	2·1	0·09	1·7	0·08	5·6	0·09	4·4	0·08	1·7	0·05	1·3	0·02	4·1	0·27	2·5	0·01	1·1	0·02	0·6	0·02	1·1	0·05	0·7	0·01	2·0	0·12	2·8	0·14
Isoleucine	0·7	0·01	1·1	0·01	1·6	0·01	1·2	0·01	0·6	0·03	0·8	0·01	1·3	0·00	1·0	0·00	0·6	0·00	0·4	0·01	0·7	0·09	0·2	0·01	0·9	0·02	1·1	0·08
Leucine	0·5	0·00	0·5	0·00	0·8	0·00	0·8	0·00	0·4	0·00	0·4	0·00	0·7	0·00	0·5	0·00	0·2	0·00	0·1	0·00	0·1	0·00	0·0	0·00	0·4	0·01	0·5	0·00
Tyrosine	0·9	0·01	1·0	0·01	1·8	0·01	1·7	0·01	0·9	0·01	0·9	0·01	1·7	0·01	1·2	0·00	0·5	0·00	0·3	0·00	0·4	0·00	0·2	0·01	0·9	0·01	1·2	0·01
Phenylalanine	0·5	0·00	0·7	0·01	1·2	0·00	1·2	0·01	0·5	0·00	0·6	0·00	1·2	0·00	0·9	0·00	0·4	0·00	0·3	0·00	0·3	0·01	0·1	0·01	0·5	0·01	0·9	0·01
Histidine	1·1	0·02	1·2	0·02	2·8	0·02	2·6	0·01	1·1	0·01	1·2	0·01	2·8	0·03	2·2	0·04	1·0	0·01	0·6	0·01	0·9	0·01	0·4	0·02	1·4	0·02	1·7	0·01
Lysine	1·1	0·01	1·1	0·01	4·5	0·05	4·2	0·06	1·8	0·03	2·2	0·03	2·3	0·03	1·6	0·00	3·6	0·02	2·7	0·01	5·5	0·15	3·7	0·12	3·7	0·10	3·2	0·10
Arginine	0·3	0·00	0·4	0·01	1·0	0·00	1·1	0·01	0·3	0·00	0·4	0·00	0·7	0·00	0·7	0·00	0·7	0·00	0·5	0·01	0·6	0·00	0·4	0·02	0·7	0·00	0·7	0·01

* For details of procedures, see p. 518.

Table 11. *Endogenous amino acid levels (g/kg dry matter intake) in the duodenum, jejunum and ileum of broilers fed on a semi-purified diet containing guanidinated casein**

(Means with their standard errors for three chickens)

	Duodenum		Jejunum		Ileum	
Amino acid	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	4·2	0·43	2·7	0·18	1·8	0·10
Threonine	2·1	0·19	1·0	0·09	0·7	0·07
Serine	3·2	0·34	3·8	0·05	3·3	0·28
Glutamic acid	6·5	0·61	5·9	0·21	4·8	0·40
Glycine	1·8	0·18	0·9	0·08	0·6	0·04
Alanine	1·7	0·24	1·0	0·10	0·7	0·04
Valine	2·9	0·35	1·8	0·10	1·4	0·06
Methionine	0·3	0·08	0·2	0·06	0·4	0·02
Isoleucine	2·5	0·34	0·6	0·16	1·6	0·05
Leucine	2·3	0·43	0·8	0·14	0·5	0·09
Tyrosine	1·2	0·20	0·4	0·07	0·2	0·05
Phenylalanine	1·5	0·22	0·3	0·08	0·1	0·01
Histidine	1·0	0·11	0·4	0·05	0·2	0·03
Lysine	2·2	0·34	0·9	0·11	0·4	0·09
Arginine	1·7	0·29	0·8	0·10	0·3	0·05
Total	35·1		21·5		17·0	

* For details of diets and procedures, see Table 1 and pp. 518–520.

Table 12. *Endogenous amino acid levels (g/kg dry matter intake) in ileal digesta of broiler chickens obtained using a nitrogen-free diet (NFD), regression analysis (REG) and guanidinated casein (GC)*

(Mean values for four assays)

				SEM
Amino acid	NFD	REG	GC	(df 9)
Aspartic acid	0·61	0·81	1·69	0·08
Threonine	0·54	0·61	1·48	0·10
Serine	0·51	0·97	2·11	0·15
Glutamic acid	0·74	1·78	3·08	0·24
Glycine	0·29	0·31	0·72	0·05
Alanine	0·27	0·27	0·69	0·04
Valine	0·42	0·50	1·02	0·07
Methionine	0·07	0·08	0·11	0·01
Isoleucine	0·20	0·52	0·91	0·06
Leucine	0·47	0·48	1·04	0·08
Tyrosine	0·32	0·15	0·49	0·04
Phenylalanine	0·29	0·21	0·51	0·04
Histidine	0·14	0·13	0·31	0·03
Lysine	0·24	0·19	0·56	0·05
Arginine	0·25	0·18	0·54	0·05
Total	5·36	7·19	15·26	0·55

Table 13. *Apparent and true digestibilities (%) of amino acids in a casein-based diet fed to broiler chickens*

(Mean values with their standard errors for four chickens)

	Apparent digestibility		True digestibility*					
			NFD		REG		GC	
Amino acid	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Aspartic acid	85·9	0·95	88·8	0·96	89·4	0·95	96·7	0·95
Threonine	80·8	1·17	85·4	1·27	85·0	1·17	96·6	1·17
Serine	80·9	1·88	83·3	1·88	85·2	1·88	96·5	1·88
Glycine	77·4	1·18	82·5	1·17	82·4	1·18	96·7	1·17
Alanine	86·0	0·67	88·9	0·68	88·6	0·68	96·8	0·68
Valine	91·4	0·85	92·9	0·85	93·3	0·85	96·3	0·85
Methionine	94·5	0·73	95·2	0·73	95·4	0·73	96·8	0·72
Isoleucine	91·1	1·18	92·5	1·18	93·5	1·18	96·6	1·18
Leucine	91·7	0·44	93·2	0·44	93·2	0·44	96·4	0·44
Tyrosine	92·8	0·59	94·5	0·75	93·6	0·59	96·8	0·59
Phenylalanine	92·7	0·57	94·2	0·57	93·7	0·57	96·8	0·57
Histidine	91·7	0·74	93·2	0·74	93·0	0·74	96·7	0·74
Lysine	86·4	0·60	89·4	0·64	88·6	0·68	96·8	0·60
Arginine	89·3	0·72	91·6	0·68	90·9	0·69	96·8	0·70
Mean	88·0		90·4		90·4		96·7	
SEM	0·78	(df 12)						

* True digestibility values obtained by correction for endogenous losses measured by nitrogen-free diet (NFD), regression analysis (REG) and guanidinated casein (GC) procedures. For details, see pp. 518–520.

DISCUSSION

The incubation of homoarginine and other amino acids with digesta from the small intestine under either aerobic or anaerobic conditions revealed that homoarginine is not selectively degraded, or incorporated into other materials, in confirmation of earlier studies (Siriwan *et al.* 1987; Schuttert *et al.* 1991). Furthermore, *in vivo* studies showed that homoarginine is readily absorbed from the small intestine in the presence or absence of other amino acids. The loss of homoarginine from the small intestine was accompanied by its appearance in mesenteric venous blood at concentrations roughly similar to those of other amino acids placed in the small intestine at the same concentrations. These results indicate that homoarginine behaves in the small intestine like a typical amino acid.

The use of guanidinated food proteins to measure endogenous protein losses or amino acid availability is not dependent on the level of conversion of lysine residues to homoarginine provided that the conversion is random. Rutherford & Moughan (1990) have pointed out that when measuring the contribution of lysine to endogenous amino acid losses a high degree of guanidination results in improved precision. In the present studies the extent of guanidination for nine protein sources varied from 37% to 68%. With heterogeneous feed protein sources such as vegetable protein meals and cereals, low levels of guanidination may reflect the inaccessibility of some lysine residues in proteins closely associated with other polymeric materials. In any event, it was essential to establish whether or not the conversion of lysine residues to homoarginine was random, or non-random. This was examined by following the ratios of homoarginine to other amino acids during sequential digestion *in vitro* of guanidinated protein using enzymes which stimulated digestion *in vivo*. Guanidinated casein and soya-bean protein showed little variation in

amino acid ratios during stepwise digestion (Tables 7 and 8) indicating that both guanidinated proteins, when incorporated into diets as the sole protein source, would provide unequivocal data on endogenous losses of amino acids by comparing homoarginine: amino acid ratios in feed and in ileal digesta. Again, if the guanidinated protein is digested at the same rate as the normal protein the digestibility of homoarginine measured at the ileum will be identical with that of the other amino acids in the protein. The available evidence suggesting that guanidination has little effect on the physical or biological properties of the protein has been summarized by Schmitz *et al.* (1991), who reported that homoarginine labelling is suitable for determination of protein absorption in miniature pigs.

In contrast, the homoarginine:amino acid ratios in several vegetable protein meals and in meat meal and cereals (Table 9) changed enough in some instances to warrant caution in the use of these guanidinated materials to measure either endogenous losses or amino acid digestibility. Further studies of different protein sources are required as the optimum conditions of guanidination vary between proteins (Maga, 1981; Rutherford & Moughan, 1990) and in some instances maximum conversion of lysine may be necessary to ensure random distribution of homoarginine. It is important to establish, however, that such treatments should not affect digestibility.

Values for endogenous amino acid losses obtained by the use of guanidinated casein were about twofold greater than those given either by feeding a N-free diet or by extrapolation to zero N intake (Table 12). The lower values given by the latter two procedures probably reflect the responses of birds to zero or low intakes of dietary N. In these unphysiological situations the inflow of digestive secretions into the small intestine is likely to be much reduced. In contrast, values based on guanidinated casein were obtained under normal feeding conditions. The latter values are of similar magnitude to those measured using the ^{15}N-dilution technique (de Lange *et al.* 1990; Roos *et al.* 1990). Use of these values to correct apparent digestibilities of amino acids, based on the analysis of ileal digesta, to true digestibility values revealed that the guanidinated casein method gave significantly higher figures (Table 13). The virtually identical values for true digestibility given by the latter procedure were consistent with the view that the digestibility of homoarginine cannot differ from that of other amino acids in guanidinated casein, given the random distribution of homoarginine residues established by sequential digestion.

It is implicit in the use of guanidinated proteins to measure endogenous amino acid losses that there is no significant recycling of absorbed homoarginine back into the small intestine. Direct evidence of absence of recycling has not been obtained, but the presence of high levels of arginase (*EC* 3.5.3.1) in the kidneys of chickens (Smith & Lewis, 1963; Austic & Nesheim, 1970) will ensure the continuous removal of circulating homoarginine. Also, the amino acid specificities of the enzymes involved in protein synthesis almost certainly precludes incorporation of homoarginine into proteins secreted into the small intestine. The effect of homoarginine recycling into the small intestine would be to reduce the measured digestibility of homoarginine. The latter value is used to calculate true digestibilities of all dietary amino acids. The high values (about 97%) for amino acid digestibility recorded in Table 13 indicate that when guanidinated casein was fed, re-entry of homoarginine was insignificant.

In summary, the use of semi-purified diets containing guanidinated casein as the sole source of amino acids makes it possible to measure, under normal feeding conditions, endogenous amino acid losses. This technique will facilitate study of the effects of factors such as dietary protein level and source, dietary fibre and anti-nutritional components of feed which are believed to influence the magnitude of endogenous losses. More importantly, values for the digestibility of homoarginine in any guanidinated protein source in which

there is random conversion of lysine residues are identical with the true digestibility of other constituent amino acids. Since there is good evidence that guanidinated proteins and their untreated counterparts are digested at similar rates, it would appear that we have, for the first time, a relatively simple procedure for the measurement of true digestibility values for amino acids in certain protein sources.

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REFERENCES

Austic, R. E. & Nesheim, M. C. (1970). Role of kidney arginase in variations of the arginine requirements of chicks. *Journal of Nutrition* **100**, 855–869.

Bryden, W. L. (1989). Intestinal distribution and absorption of biotin in the chicken. *British Journal of Nutrition* **62**, 389–398.

de Lange, C. F. M., Souffrant, W. L. & Sauer, W. C. (1990). Real ileal protein and amino acid digestibilities in feedstuffs for growing pigs as determined with the ^{15}N-isotope dilution technique. *Journal of Animal Science* **68**, 409–418.

Hagemeister, H. & Erbersdobler, H. (1985). Chemical labelling of dietary protein by transformation of lysine to homoarginine: new technique to follow intestinal digestion and absorption. *Proceedings of the Nutrition Society* **44**, 133A.

Maga, J. A. (1981). Measurement of available lysine using the guanidination reaction. *Journal of Food Science* **46**, 132–134.

Markland, F. S., Bacharach, A. D. E., Weber, B. H., O'Grady, T. C., Saunder, G. C. & Umeura, N. (1975). Chemical modification of yeast 3-phosphoglycerate kinase. *Journal of Biological Chemistry* **250**, 1301–1310.

Roos, N., Hagemeister, H. & Scholtissek, J. (1990). Protein digestibility measured by ^{15}N and homoarginine. *Proceedings of the Nutrition Society* **49**, 48A.

Rutherford, S. M. & Moughan, P. J. (1990). Guanidination of lysine in selected dietary proteins. *Journal of Agricultural and Food Chemistry* **38**, 209–211.

Ryan, W. L., Barak, A. J. & Johnson, R. J. (1968). Lysine, homocitrulline and homoarginine metabolism by the isolated perfused rat liver. *Archives of Biochemistry and Biophysics* **123**, 294–297.

Schmitz, M., Hagemeister, H. & Erbersdobler, H. F. (1991). Homoarginine labelling is suitable for determination of protein absorption in miniature pigs. *Journal of Nutrition* **121**, 1575–1580.

Schuttert, G., Moughan, P. J. & Jackson, F. (1991). *In vitro* determination of the extent of hydrolysis of homoarginine by arginase in the small intestine of the growing rat. *Journal of Agricultural and Food Chemistry* **39**, 511–513.

Sibbald, I. R. (1987). Estimation of bioavailable amino acids in feedstuffs for poultry and pigs: a review with emphasis on balance experiments. *Canadian Journal of Animal Science* **67**, 221–300.

Siriwan, P., Bryden, W. L. & Annison, E. F. (1987). The use of homoarginine to correct ileal digestibility values for endogenous amino acids. *Proceedings of the Nutrition Society of Australia* **12**, 103.

Siriwan, P., Bryden, W. L. & Annison, E. F. (1989). The use of homoarginine to determine true amino acid digestibility. *Proceedings of the Nutrition Society of Australia* **14**, 97.

Siriwan, P., Bryden, W. L., Mollah, Y. & Annison, E. F. (1993). Measurement of endogenous amino acid losses in poultry. *British Poultry Science* (In the Press).

Skurray, G. R. & Cumming, R. B. (1975). Deamination of amino acids in the small intestine of chickens fed meat meal. *Poultry Science* **54**, 1689–1692.

Smith, G. H. & Lewis, D. (1963). Arginine in poultry nutrition. 2. Chick arginase. *British Journal of Nutrition* **17**, 415–444.

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