In Vitro Determination of the Extent of Hydrolysis of Homoarginine by Arginase in the Small Intestine of the Growing Rat

Gerard Schuttert, Paul J. Moughan,* and Felicity Jackson

Department of Animal Science, Massey University, Palmerston North, New Zealand

The extent of hydrolysis of free homoarginine, catalyzed by arginase, in the small intestine of the growing rat was determined in an in vitro experiment. Synthetic L-homoarginine was added to digesta collected from the small intestine of growing rats or to a supernatant prepared from digesta. The material was incubated under CO_2 at pH 7.3 and a temperature of 38 °C for 4 h. Following incubation, there was no change in the concentrations of homoarginine or lysine, in either the supernatant or the digesta samples. The lysine concentration did not alter, regardless of whether or not homoarginine was added to the material before incubation. Further evidence for a lack of arginase activity under these conditions was the constancy of the arginine concentration. The result supports the use of guanidinated proteins for determining endogenous ileal lysine flows in animals.

INTRODUCTION

Completely guanidinated proteins can be given to animals to allow a direct determination of the endogenous flow of lysine at the terminal ileum (Moughan and Rutherfurd, 1990). The latter measure is required for calculation of true estimates of lysine absorption or for the factorial estimation of lysine requirements. Recently, the virtually complete conversion of lysine in gelatin to homoarginine has been reported (Rutherfurd and Moughan, 1990).

The approach of using completely guanidinated proteins to allow determination of endogenous lysine losses relies on the assumption that there is negligible hydrolysis of homoarginine, catalyzed by arginase (EC 3.5.3.1), in the digestive tract of the animal. Arginase of body origin may be present in the small intestinal contents of mammals and may also be produced by microorganisms in the small bowel (Stevens and Bush, 1950; Edmonds et al., 1987; Siriwan et al., 1987).

The aim of the present study was to determine if there was a significant degree of hydrolysis of homoarginine when added free homoarginine was incubated with small-intestinal contents collected from the growing rat. Changes in the concentrations of homoarginine and lysine in total digesta or digesta supernatants were used to indicate the degree of hydrolysis of homoarginine. Arginine concentrations were also determined, to examine whether arginase was active in hydrolyzing this amino acid. Arginase catalyzes the hydrolysis of homoarginine to lysine and urea and of arginine to ornithine and urea.

MATERIALS AND METHODS

Materials. L-Homoarginine hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of reagent grade. All reagents were prepared with deionized water, and all equipment was sterilized before use.

Procedure. Twelve 225-g body weight Sprague-Dawley female rats were kept singly in stainless steel wire bottomed cages at an ambient temperature of 22 ± 2 °C, with a 12-h light/dark cycle. The rats were given unrestricted access to a standard 10% crude protein casein based diet for 3 h (8:30–11:30 a.m.) each day over an 8-day period. On average the rats consumed 12.50 g of diet daily. Fresh water was available at all times. On the eighth day and 4 h after the start of feeding, each rat was asphyxiated with carbon dioxide gas and decapitated. The entire small intestine was immediately removed and washed with physiological saline. The small intestinal contents were gently flushed out by using 14 nL of 0.15 M isotonic phosphate buffer (pH 7.3) from a syringe. It was found in preliminary work that the highest pH in the small intestine was 7.3, at the ileum. The pH optimum for arginase is 9.5 (range 6.0–11.10) (Mohamed and Greenberg, 1945). The digesta collected from the 12 rats were pooled and kept on ice. The digesta were thoroughly mixed with a high-speed mechanical mixer, then halved, and half was centrifuged (1000*g*) for 30 min. The supernatant was decanted and recentrifuged (2000*g*) for 30 min. The resulting supernatant, which allowed investigation of the free (nonbacterial) arginase activity, was then divided into two equal fractions. To one of the supernatant subsamples was added free homoarginine monohydrochloride (0.062 mg of 0.05 M homoarginine solution/nL of digesta supernatant), while no addition to the other fraction was made. The supernatant fractions were thoroughly mixed and four subsamples taken from each and frozen for subsequent determination of homoarginine, lysine, and arginine levels. The remaining material in each supernatant fraction (approximately 40 mL) was divided equally into four incubation vessels and incubated under CO_2 gas. pH was measured before and each hour during incubation. Incubation was at 38 °C [the deep-body temperature of the rat (Farris and Griffith, 1949; Short and Woodnott, 1969)] for 4 h [the mean retention time of digesta in the small intestine of the 225-g rat (Varga, 1976; Warner, 1981)]. Reaction was stopped by reducing the pH to 3.0 by adding 3 M hydrochloric acid. In addition to the supernatant samples, four samples of the phosphate buffer with homoarginine added at the same rate as for the supernatants (blanks) were prepared and subsampled for analysis of homoarginine and were then incubated under the above-described conditions. Postincubation, duplicate subsamples of material were taken from each vessel and were frozen while awaiting analysis for homoarginine (blank samples), arginine, and lysine (supernatant without homoarginine) and arginine, lysine, and homoarginine (supernatant with added homoarginine). The blanks were included to give an indication of the loss of free homoarginine during incubation. The supernatant samples not containing homoarginine served to indicate changes in the concentrations of lysine not associated with the hydrolysis of homoarginine.

Lysine, arginine, and homoarginine were determined by using ion-exchange chromatography. Analysis was conducted on a Beckman 119BL amino acid analyzer using 0.5-mL samples. The material was hydrolyzed in 1.0 mL of 6 M glass-distilled HCl with phenol added, for 24 h at 110 ± 1 °C in glass tubes sealed under vacuum.

The other half of the original mixed digesta was also divided into fractions, to allow investigation of the total (free plus bacterial) arginase activity. These digesta samples were treated

* Author to whom correspondence should be addressed.

0021-8561/91/1439-0511$02.50/0

Table I. Effect of a 4-h Incubation on the Mean Concentration of Free Homoarginine Added either to Fresh Rat Ileal Digesta, to a Supernatant Prepared from Rat Ileal Digesta, or to a Phosphate Buffer Solution (Blank)

	homoarginine concn, μmol/g			
	preincubation		postincubation	
	mean[a]	CV[b]	mean[a]	CV[b]
supernatant	2.93	3.3	2.85	4.02
digesta	2.56	4.7	2.47	2.10
blank	2.83	1.8	2.69	4.80

[a] Means of four replicate samples, each analyzed in duplicate. [b] Coefficient of variation (%) between replicates (n = 4) within a sample.

Table II. Effect of a 4-h Incubation of a Rat Ileal Digesta Supernatant with or without Added Free Homoarginine on the Mean Concentrations of Lysine and Arginine

	concn, μmol/g			
	preincubation		postincubation	
	mean[a]	CV[b]	mean[a]	CV[b]
lysine				
supernatant + homoarginine	0.64	8.9	0.64	4.8
supernatant - homoarginine	0.69	1.3	0.67	3.9
arginine				
supernatant + homoarginine	0.35	4.2	0.36	2.1
supernatant - homoarginine	0.32	1.0	0.38	2.3

[a] Means of four replicate samples, each analyzed in duplicate. [b] Coefficient of variation (%) between replicates (n = 4) within a sample.

as described above for the supernatant samples. Incubation of blanks was not repeated.

RESULTS AND DISCUSSION

The pH of the digesta material following collection was 7.35 and decreased during the 4-h incubation. For the supernatant samples, pH dropped by 0.28 of a pH unit while for the digesta samples it decreased by 0.52 of a unit. The decrease in pH was independent of the presence of homoarginine.

The overall average differences (each difference expressed as a proportion of the mean), between duplicates within samples after incubation, were 5.0, 2.6, and 6.3% for lysine, homoarginine, and arginine, respectively.

The free homoarginine concentrations of the digesta and supernatant samples and of the blank, before and after a 4-h incubation, are shown in Table I. There was no change in the homoarginine concentration of the blank sample, indicating that there was no breakdown of homoarginine due to hydrolysis per se. Nor was there any change in the homoarginine concentrations of the supernatant or total digesta samples. Further, there was no increase in the supernatant lysine concentration or decrease in supernatant arginine (Table II), regardless of whether or not homoarginine was added to the supernatant. That there was no change in the supernatant lysine concentration when homoarginine was not added indicates that there was no net synthesis or degradation of this amino acid during incubation. Moreover, the addition of homoarginine did not lead to an increase in lysine concentration, which would have occurred if there was significant hydrolysis of the homoarginine. The constancy of the arginine concentration also indicates minimal arginase activity in the digesta supernatant.

The pre- and postincubation concentrations of lysine and arginine in fresh untreated rat ileal digesta are given in Table III. The overall result was the same as that found for the supernatant samples. The pre- and postincubation concentrations of lysine and arginine for the total digesta samples were somewhat higher than the comparable supernatant concentrations, but the differences were not as great as expected. The small discrepancies between the preincubation lysine and arginine concentrations for the samples containing homoarginine or those devoid of homoarginine may indicate a lack of homogeneity in the mixed total digesta sample, which was the source for these samples. It is concluded that at around a pH of 7.0, which corresponds to the higher pH found in the small intestine of mammals, and closest to the pH optimum for arginase, there is no appreciable breakdown of homoarginine by either free arginase or arginase of bacterial origin. This finding is not in accord with that of Siriwan et al. (1987), who reported a 22% loss of free homoarginine after in vitro anaerobic incubation with gut contents from the chicken but complete recovery of homoarginine with aerobic conditions. The present result, however, is consistent with what would be expected on the basis of knowledge of the pH optimum for arginase and the relatively low activity of arginase in intestinal mucosa tissue (Edmonds et al., 1987). That there is negligible hydrolysis of homoarginine to lysine in the small intestine also receives support from the work of Stevens and Bush (1950) with growing rats, whereby there was only a small growth response to homoarginine added to a lysine-deficient diet, which could be completely explained by the liver metabolism of absorbed homoarginine to lysine.

Table III. Effect of a 4-h Incubation of Total Rat Ileal Digesta with or without Added Free Homoarginine on the Mean Concentrations of Lysine and Arginine

	concn, μmol/g			
	preincubation		postincubation	
	mean[a]	CV[b]	mean[a]	CV[b]
lysine				
digesta + homoarginine	0.72	9.7	0.72	0.96
digesta - homoarginine	0.82	1.0	0.82	5.4
arginine				
digesta + homoarginine	0.39	4.5	0.38	4.1
digesta - homoarginine	0.46	4.7	0.44	8.5

[a] Means of four replicate samples, each analyzed in duplicate. [b] Coefficient of variation (%) between replicates (n = 4) within a sample.

The greater decrease in pH during incubation of the total digesta compared to the supernatant suggests that bacteria are active in the rat small intestine. Buraczewska and Buraczewski (1985) determined the extent of bacterial deamination of amino acids in vitro, using fresh ileal digesta from pigs. They reported negligible change in the concentration of lysine in unsupplemented material but a 20% decrease in lysine concentration after 4 h incubation of digesta supplemented with a casein hydrolysate. The latter workers concluded that there may be a significant degree of bacterial metabolic activity in the mammalian small intestine.

Care was taken in the present study to simulate the natural conditions found in the rat small intestine. The free homoarginine was added to the digesta to give a final concentration similar to that obtained if a guanidinated protein containing diet had been fed to the rat. The pH was within the range of pH found in the small intestine of the rat, incubation time simulated small-intestinal transit time, and incubation conditions were near anaerobic. The incubation temperature was maintained close to the deep-body temperature of the rat. Under these conditions, no net metabolism of homoarginine was detected, and it is concluded that homoarginine is not metabolized in the rat small-intestinal digesta to any significant degree. Thus, the use of completely guanid-

inated proteins (Moughan and Rutherfurd, 1990) should provide accurate estimates of gut endogenous lysine loss.

ACKNOWLEDGMENT

We thank Dr. J. C. McIntosh for his advice and J. Reid and Dr. G. G. Midwinter for assistance with the amino acid analyses.

LITERATURE CITED

Buraczewska, L.; Buraczewski, S. Deamination of amino acids in the intestinal digesta of pigs. In *Digestive Physiology in the Pig*; Just, A., Jorgensen, H., Fernandez, J. A., Eds.; National Institute of Animal Science: Copenhagen, 1985; pp 268–271.

Edmonds, M. S.; Lowry, K. R.; Baker, D. H. Urea cycle metabolism: Effects of supplemental ornithine or citrulline on performance, tissue amino acid concentrations and enzymatic activity in young pigs fed arginine-deficient diets. *J. Anim. Sci.* **1987**, *65*, 706–716.

Farris, E. J.; Griffith, J. Q. *The rat in laboratory investigations*, 2nd ed.; Hafner Press: New York, 1949; 542 pp.

Mohamed, M. S.; Greenberg, D. M. Liver arginase, I. Preparation of extracts of high potency, chemical properties, activation-inhibition, and pH activity. *Arch. Biochem.* **1945**, *8*, 349–376.

Moughan, P. J.; Rutherfurd, S. M. Endogenous flow of total lysine and other amino acids at the distal ileum of the protein-fed or peptide-fed rat—The chemical labelling of gelatin protein by transformation of lysine to homoarginine. *J. Sci. Food Argic.* **1990**, *52*, 179–192.

Rutherfurd, S. M.; Moughan, P. J. Guanidination of lysine in selected dietary proteins. *J. Agric. Food Chem.* **1990**, *38*, 209–211.

Short, D. J.; Woodnott, D. P. *T.A.T manual of laboratory animal practice and techniques*, 2nd ed.; Crosby Lockwood: London, 1969; 462 pp.

Siriwan, P.; Bryden, W. L.; Annison, E. F. The use of homoarginine to correct ileal digestibility values for endogenous amino acids. *Proc. Nutr. Soc. Aust.* **1987**, *12*, 102.

Stevens, C. M.; Bush, J. A. New syntheses of α-amino-ϵ-Guanidino-n-Caproic acid (homoarginine) and its possible conversion in vivo into lysine. *J. Biol. Chem.* **1950**, *183*, 139–147.

Varga, F. Transit time changes with age in the gastrointestinal tract of the rat. *Digestion* **1976**, *14*, 319–324.

Warner, I. A. C. The rate of passage of digesta through the gut of mammals and birds. *Nutr. Abstr. Rev.* **1981**, *51*, 789–820.

Received for review March 2, 1990. Accepted September 19, 1990.

Registry No. Homoarginine, 156-86-5; arginase, 9000-96-8; lysine, 56-87-1; arginine, 74-79-3.