Synthesis of citrulline from glutamine in pig enterocytes

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The synthesis of citrulline from glutamine was quantified in enterocytes from pre-weaning (14–21 days old) and post-weaning (29–58 days old) pigs. The cells were incubated at 37 °C for 30 min in Krebs–Henseleit bicarbonate buffer (pH 7.4) containing 0, 0.5, 2 and 5 mM glutamine. Oxygen consumption was linear during the 30 min incubation period. The rates of citrulline synthesis were low or negligible in enterocytes from 14–21-day-old pigs, but increased 10–20-fold in the cells from 29–58-day-old pigs. This marked elevation of citrulline synthesis coincided with an increase in the activity of pyrroline-5-carboxylate synthase with the animal's post-weaning growth. In contrast, decreases in the activities of phosphate-dependent glutaminase, ornithine aminotransferase, ornithine carbamoyltransferase and carbamoyl-phosphate synthase were observed as the age of the pigs increased. The concentrations of carbamoyl phosphate in

enterocytes from pre-weaning pigs were higher than, or similar to, those in the cells from post-weaning pigs. It is possible that the low rate of citrulline synthesis from glutamine in enterocytes from pre-weaning pigs was due to a limited availability of ornithine, rather than that of carbamoyl phosphate. We suggest that this limited availability of ornithine in pre-weaning-pig enterocytes results from (i) the low rate of pyrroline-5-carboxylate synthesis from glutamate, due to the low activity of pyrroline-5-carboxylate synthase, and (ii) the competitive conversion of pyrroline-5-carboxylate into proline. Our present findings on the developmental aspect of citrulline synthesis in pig enterocytes may offer a biochemical mechanism for the previous observations that arginine is a nutritionally essential amino acid for suckling piglets, but not for adult pigs.

INTRODUCTION

The small intestine is a major organ of glutamine utilization in the post-absorptive adult rat [1]. An important aspect of glutamine metabolism in the intestine is the production of citrulline [2]. The latter is used for the synthesis of arginine in various mammalian cell types, including kidney tubules [3], endothelial cells [4] and macrophages [5]. As arginine is the precursor of NO, a small free radical with versatile physiological importance [6], the synthesis of citrulline in the small intestine plays an important role in whole-body homeostasis. The significance of this endogenous arginine synthesis is epitomized by the findings that, if fed on an arginine-deficient diet, severe metabolic abnormalities and even death immediately occur in such species as chicks [7], cats [8] and ferrets [9], which cannot produce citrulline from glutamine in the gut.

It is often assumed that citrulline is produced from glutamine in the enterocytes of the small intestine [10]. Although this assumption may be correct, we were not aware of published data reporting citrulline synthesis in these cells. For example, Watford et al. [11] were not able to demonstrate the production of citrulline from glutamine in isolated enterocytes from the growing rat. Whether this is due to the low sensitivity of the colorimetric and u.v.-spectrophotometric techniques previously used for citrulline analysis [11] or to the lack of citrulline synthesis in enterocytes is not known. It is noteworthy that the small intestine contains mixed cell populations, including enterocytes and smooth-muscle cells. Since smooth-muscle cells are now known to produce citrulline from arginine via NO synthase [12], they may be the source of the citrulline leaving the intestine in venous blood, therefore accounting for the arteriovenous differences in citrulline concentrations across this organ [2]. Thus, whether citrulline is synthesized from glutamine in enterocytes has yet to be defined.

The objective of this study was to quantify citrulline synthesis from glutamine in enterocytes and to elucidate the pathways involved. The pig was used as an animal model to study the developmental aspect of citrulline synthesis, as previous nutritional studies *in vivo* have suggested that the rates of endogenous arginine synthesis in this species may be age-dependent [13,14].

MATERIALS AND METHODS

Chemicals

L-[U-¹⁴C]Glutamate and [U-¹⁴C]glutamine were obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.). L-Amino acids, D-glucose, BSA (fraction V, essentially fatty-acids-free), Hepes, pyridoxal phosphate, *o*-aminobenzaldehyde, carbamoyl phosphate (dilithium salt), phosphocreatine, creatine kinase, gabaculine, *N*-acetylglutamate and ornithine carbamoyltransferase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dowex AG 1-X8 (acetate form) was obtained from Bio-Rad (Richmond, CA, U.S.A.). ATP, NADPH and α -oxoglutaric acid were obtained from Boehringer Mannheim (Indianapolis, IN, U.S.A.). [U-¹⁴C]Glutamine was purified before use as previously described [15].

Animals and isolation of jejunum

Pigs were offspring of Yorkshire $\times$ Landrace sows and Duroc $\times$ Hampshire boars. They were nursed by their mothers until 21 days of age, and then fed a corn/soybean-meal-based diet (20 % protein), meeting the NRC requirements of all nutrients [16]. Pigs were not fasted before collection of the jejunum sample. Pigs at the ages of 14, 21, 29 and 58 days were intramuscularly injected with atropine (0.05 mg/kg body wt.), followed by ketamine and acetylpromazine at doses of 4.76 and 0.24 mg/kg

Abbreviations used: KHB buffer, Krebs–Henseleit bicarbonate buffer; P-5-C, pyrroline-5-carboxylate.

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body wt. respectively. After this pre-anaesthetic procedure, halothane was administered to the pigs at a 5% concentration via a face mask to achieve a surgical plane of anaesthesia, which was maintained by administration of 1–2% halothane. While pigs were under anaesthesia, the abdomen was opened and a 50 cm jejunal segment was isolated. The experiments were carried out in accordance with the guidelines of the U.S. National Research Council for the care and use of animals, and were approved by Texas A&M University's Institutional Animal Care Committee.

Preparation of enterocytes

Enterocytes were prepared from jejunum essentially as described by Watford et al. [11]. The jejunum (50 cm) was thoroughly rinsed with 0.9% NaCl to remove its luminal contents. It was then rinsed with 3×50 ml of oxygenated (O_2/CO_2 , 19:1) Ca^{2+} -free Krebs–Henseleit bicarbonate (KHB) buffer (119 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 25 mM $NaHCO_3$, pH 7.4) containing 20 mM Hepes (pH 7.4) and 5 mM EDTA (disodium). The jejunum was filled with 60 ml of the oxygenated Ca^{2+} -free KHB buffer and placed in a flask containing 200 ml of this buffer. The flask was gassed with O_2/CO_2 (19:1) for 1 min and incubated at 37 °C in a shaking water bath (70 oscillations/min) for 20 min (for jejunum from 14–21-day-old pigs) or for 50 min (for jejunum from 29–58-day-old pigs). This was based on our preliminary observations that a 20 or 50 min incubation period was required to release enterocytes from the jejunum of 14–21- or 29–58-day-old pigs respectively. At the end of the incubation, the jejunum was gently patted with finger-tips for 1 min, and the lumen was drained into polystyrene tubes. The cells were centrifuged at 400 *g* for 3 min, washed three times with oxygenated (O_2/CO_2 , 19:1) KHB buffer (pH 7.4) containing 2.5 mM $CaCl_2$ and 20 mM Hepes but no EDTA, and resuspended in this buffer. The viability of the prepared enterocytes was over 90%, as assessed by Trypan Blue exclusion before and after a 30 min incubation period.

Measurement of O_2 consumption by enterocytes

O_2 consumption was measured for 30 min at 37 °C with a YSI model 5300 Biological Oxygen Monitor (YSI Inc., Yellow Springs, OH, U.S.A.), which consisted of Clark-type polarographic oxygen probes, four sample chambers, and a circulatory water bath. Medium, in a total volume of 4 ml of oxygenated (O_2/CO_2 , 19:1) KHB buffer (pH 7.4), contained freshly prepared enterocytes (2–3 mg of cell protein), 1% BSA, 2 mM glutamine and 5 mM glucose. O_2 consumption was calculated on the basis of the percentage decrease in the O_2 medium and the solubility of O_2 (22.7 μ l of O_2 /ml) in KHB solution equilibrated with O_2/CO_2 (19:1) at 37 °C and 1 atm (101 kPa) (YSI Inc.).

Incubations of enterocytes

Incubations were performed at 37 °C in 25 ml polypropylene conical flasks placed in a shaking water bath (70 oscillations/min). Enterocytes (4.5–5.5 mg of cell protein) were incubated for 30 min in the presence of 2 ml of KHB buffer (pH 7.4; saturated with O_2/CO_2 , 19:1) containing 20 mM Hepes, 1% BSA and 5 mM glucose. The medium contained 0, 0.5, 2 or 5 mM glutamine. In some experiments, 2 mM ornithine, 2 mM proline or 5 mM NH_4Cl was added to the incubation medium. The gas phase in the flask was O_2/CO_2 (19:1). Incubations were initiated by addition of cells and terminated by addition of 0.2 ml of 1.5 M $HClO_4$. The medium was neutralized with 0.1 ml of 2 M

K_2CO_3 . Amino acids in the neutralized cell extracts were quantified by a fluorimetric h.p.l.c. method involving pre-column derivative formation with *o*-phthalaldehyde [17]. Proline was determined as 4-aminobutan-1-ol by a fluorimetric h.p.l.c. method [18]. In all experiments, parallel incubations in which media were acidified with 0.2 ml of 1.5 M $HClO_4$ before addition of cells (0 min incubation) were run to measure initial cellular concentrations of amino acids. For measurement of glutamine oxidation, enterocytes were incubated at 37 °C for 30 min in the presence of 5 mM [U - ^{14}C]glutamine (150 d.p.m./nmol). At the end of the incubation, the medium was acidified with 0.2 ml of 1.5 M $HClO_4$ and $^{14}CO_2$ was collected in Hyamine hydroxide for measurement of its radioactivity as previously described [15]. It was established in preliminary experiments that the rates of production of citrulline, ornithine, ammonia, glutamate, aspartate and alanine from glutamine were linear for 30 min.

Determination of carbamoyl phosphate content in enterocytes

Enterocytes were incubated at 37 °C for 30 min in the presence or absence of 5 mM glutamine as described above. At the end of the incubation, the cell suspension was centrifuged at 2000 *g* for 2 min. The cell pellet was suspended in 1.4 ml of KHB buffer and transferred to a 1.6 ml silicone-treated microcentrifuge tube. The cells were washed with 3×1.4 ml of KHB buffer by centrifugation (10000 *g*, 1 min), and then suspended in 0.2 ml of KHB buffer. Cells were lysed by three cycles of freezing (solid CO_2 in ethanol) and thawing (37 °C water bath), and the cell extracts were centrifuged at 10000 *g* for 1 min. The supernatants were used for measurement of carbamoyl phosphate by using ornithine carbamoyltransferase and ornithine [19]. The assay mixture (60 μ l), consisting of 0.15 M potassium phosphate buffer (pH 7.5), 6 mM ornithine, 15 units of ornithine carbamoyltransferase and neutralized cell extracts (0.4 mg of protein), was incubated at 37 °C for 0 and 30 min. At the end of the incubation, the assay mixture was acidified with 20 μ l of 1.5 M $HClO_4$, and neutralized with 10 μ l of 2 M K_2CO_3 . The neutralized extracts were used for determination of citrulline by h.p.l.c. [17]. Stoichiometrically, 1 mol of carbamoyl phosphate reacts with 1 mol of ornithine to form 1 mol of citrulline [20].

Measurements of enzyme activities in enterocyte lysates

Enterocyte lysates were prepared by three cycles of freezing (solid CO_2 in ethanol) and thawing (37 °C water bath). The cell extracts were centrifuged for 1 min in a microcentrifuge, and the supernatant was used for enzyme assays. The phosphate-dependent glutaminase (EC 3.5.1.2) activity was measured at 37 °C for 15 min as previously described [21], except that glutamate was quantified by h.p.l.c. [17]. The assay mixture (0.2 ml) contained 20 mM glutamine, 150 mM potassium phosphate buffer (pH 8.2) and cell extracts (0.1 mg of protein). Pyrroline-5-carboxylate (P-5-C) synthase (EC number not assigned) was measured at room temperature for 30 min by using a sensitive radiochemical assay as recommended by Kramer et al. [22], which involved the conversion of [^{14}C]glutamate into [^{14}C]P-5-C. The assay mixture (1 ml) consisted of 20 mM $MgCl_2$, 0.1 M Hepes (pH 7.4), 1 mM gabaculine (an inhibitor of ornithine aminotransferase), 1 mM [U - ^{14}C]glutamate (10^3 d.p.m./nmol), 3 mM ATP, 0.2 mM NADPH, 15 mM phosphocreatine, 10 units of creatine kinase, and cell extracts (1 mg of protein). [^{14}C]P-5-C was separated from [U - ^{14}C]glutamate by Dowex AG 1-X8 anion-exchange chromatography using a 25 mM acetic acid gradient [22], and its radioactivity was measured by a Beckman liquid-scintillation counter. Ornithine aminotransferase (EC 2.6.1.13) activity was measured at 37 °C for 15 min as described by

Table 1 Time course of O₂ consumption by pig enterocytes

Pig enterocytes were prepared as described in the text. Incubation medium contained 2 mM glutamine and 5 mM glucose. The O₂ consumption was monitored for 30 min with Clark-type polarographic oxygen probes. Data are means $\pm$ S.E.M. ($n = 6$): * $P < 0.05$, significantly different from the means for 14–29-day-old pigs within each column as analysed by one-way analysis of variance.

Age of pigs (days)	O ₂ consumption (nmol/mg of protein)						
	1 min	5 min	10 min	15 min	20 min	25 min	30 min
14	24 $\pm$ 1	123 $\pm$ 14	230 $\pm$ 14	359 $\pm$ 14	451 $\pm$ 23	592 $\pm$ 17	710 $\pm$ 31
21	25 $\pm$ 2	124 $\pm$ 7	238 $\pm$ 13	351 $\pm$ 16	463 $\pm$ 19	586 $\pm$ 21	694 $\pm$ 20
29	28 $\pm$ 3	152 $\pm$ 12	295 $\pm$ 21	426 $\pm$ 22	567 $\pm$ 18	730 $\pm$ 24	818 $\pm$ 29
58	43 $\pm$ 1*	214 $\pm$ 6*	418 $\pm$ 13*	602 $\pm$ 24*	827 $\pm$ 16*	987 $\pm$ 19*	1157 $\pm$ 22*

Table 2 Citrulline concentrations in pig enterocyte extracts

Four batches of pig enterocytes from the same cell suspensions were incubated at 37 °C for 30 min in the presence of 0, 0.5, 2 or 5 mM glutamine, as described in the text. Another batch of cells was incubated for 0 min (No incubation). At the end of the incubation, the medium was acidified with 0.2 ml of 1.5 M HClO₄, and neutralized with 0.1 ml of 2 M K₂CO₃. The neutralized extracts were used for citrulline analysis by h.p.l.c. Data are means $\pm$ S.E.M. ($n = 6$), and were statistically analysed by one-way analysis of variance. Means with different superscripts within a column are significantly different ($P < 0.05$). Citrulline concentrations in cell extracts within a row increased ($P < 0.05$) with increasing glutamine concentrations in the medium.

Age of pigs (days)	Citrulline in cell extracts (nmol/mg of protein after 30 min of incubation)				
	No incubation	[Glutamine] in medium (mM)			
		0	0.5	2	5
14	0.22 $\pm$ 0.02 ^a	0.26 $\pm$ 0.01 ^a	0.37 $\pm$ 0.04 ^a	0.46 $\pm$ 0.05 ^a	0.58 $\pm$ 0.05 ^a
21	0.37 $\pm$ 0.04 ^b	0.48 $\pm$ 0.03 ^b	0.65 $\pm$ 0.04 ^b	0.92 $\pm$ 0.05 ^b	1.26 $\pm$ 0.11 ^b
29	2.05 $\pm$ 0.23 ^c	3.18 $\pm$ 0.36 ^c	6.43 $\pm$ 0.43 ^c	8.53 $\pm$ 0.46 ^c	11.2 $\pm$ 0.52 ^c
58	1.64 $\pm$ 0.13 ^c	2.72 $\pm$ 0.29 ^c	4.93 $\pm$ 0.38 ^c	7.34 $\pm$ 0.42 ^c	9.56 $\pm$ 0.47 ^c

Herzfeld and Knox [23], which involved the colorimetric reaction of P-5-C with *o*-aminobenzaldehyde. The assay mixture (2 ml) contained 75 mM potassium phosphate buffer (pH 7.5), 20 mM ornithine, 0.45 mM pyridoxal phosphate, 3.75 mM α -oxoglutarate, 5 mM *o*-aminobenzaldehyde, and cell extracts (1 mg of protein). Ornithine carbamoyltransferase (EC 2.1.3.3) activity was measured at 37 °C for 15 min, as described by Ceriotti [24], except that citrulline was quantified by h.p.l.c. [17]. The assay mixture (0.2 ml) consisted of 0.1 M potassium phosphate buffer (pH 7.5), 20 mM ornithine, 10 mM carbamoyl-phosphate, and cell extracts (0.3 mg of protein). Carbamoyl-phosphate synthase (EC 6.3.4.16) activity was determined at 37 °C for 25 min as previously described [19], except that citrulline was measured by h.p.l.c. [17]. The assay mixture (0.5 ml) contained 0.15 M potassium phosphate buffer (pH 7.5), 25 mM ATP, 25 mM MgCl₂, 5 mM *N*-acetylglutamate, 10 mM NH₄Cl, 5 mM ornithine, 5 mM NaHCO₃ and 10 units of ornithine carbamoyltransferase. The activities of the enzymes were expressed on the basis of cell protein, which was determined by the modified Lowry procedure [25]. It was established in preliminary experiments that the activities of the above five enzymes were linear with the amount of protein and the incubation time used.

Statistical analysis

Data were analysed by paired *t*-test or one-way analysis of variance with the Student–Newman–Keuls multiple-range test [26]. Probability values less than 0.05 were taken to indicate statistical significance.

RESULTS

O₂ consumption by enterocytes

The consumption of O₂ by enterocytes from all ages of the pigs used was linear for 30 min (Table 1). The rates of O₂ consumption by enterocytes from 58-day-old pigs were greater ($P < 0.05$) than those from 14-, 21- and 29-day-old pigs at each time point. There were no significant differences ($P > 0.05$) in O₂ consumption among the cells from 14-, 21- and 29-day-old pigs throughout the 30 min incubation period.

Citrulline synthesis from glutamine in enterocytes

The synthesis of citrulline from glutamine is shown in Table 2. A small amount of citrulline was synthesized by 14–21-day-old pig enterocytes in the presence of 0.5–5 mM glutamine. Interestingly, the rate of citrulline production markedly increased ($P < 0.05$) in enterocytes from 29–58-day-old pigs, as compared with the cells from pre-weaning pigs. Increasing glutamine concentrations in the medium from 0 to 5 mM resulted in increased synthesis of citrulline ($P < 0.05$) in enterocytes from all ages of the pigs used. Note that a small amount of citrulline was formed in enterocytes incubated in the absence of added glutamine. This resulted from the presence of pre-existing cellular glutamate, whose concentrations increased ($P < 0.05$) with increasing glutamine concentrations in the medium (Table 3).

Ornithine synthesis from glutamine in enterocytes

The net accumulation of ornithine in pig enterocytes incubated

Table 3 Glutamate concentrations in pig enterocyte extracts

Four batches of pig enterocytes from the same cell suspensions were incubated at 37 °C for 30 min in the presence of 0, 0.5, 2 or 5 mM glutamine, as described in the text. Another batch of cells was incubated for 0 min (No incubation). At the end of the incubation, the incubation medium was acidified with 0.2 ml of 1.5 M HClO₄, and neutralized with 0.1 ml of 2 M K₂CO₃. The neutralized extracts were used for glutamate analysis by h.p.l.c. Data are means $\pm$ S.E.M. ($n = 6$), and were statistically analysed by one-way analysis of variance. Means with different superscripts within a column are significantly different ($P < 0.05$). Glutamate concentrations in cell extracts within a row increased ($P < 0.05$) with increasing glutamine concentrations in the medium.

Age of pigs (days)	Glutamate in cell extracts (nmol/mg of protein after 30 min of incubation)				
	No incubation	[Glutamine] in medium (mM)			
		0	0.5	2	5
14	14.5 $\pm$ 1.1 ^a	17.4 $\pm$ 2.3 ^a	34.5 $\pm$ 3.2 ^a	67.3 $\pm$ 6.2 ^a	95.3 $\pm$ 8.4 ^a
21	13.3 $\pm$ 1.7 ^a	15.5 $\pm$ 1.4 ^a	43.3 $\pm$ 3.9 ^a	75.7 $\pm$ 6.1 ^a	102.6 $\pm$ 7.2 ^a
29	27.5 $\pm$ 1.5 ^b	29.7 $\pm$ 2.1 ^b	47.1 $\pm$ 4.5 ^a	78.4 $\pm$ 5.7 ^a	97.4 $\pm$ 4.0 ^a
58	26.8 $\pm$ 2.2 ^b	31.5 $\pm$ 2.6 ^b	44.8 $\pm$ 3.2 ^a	63.0 $\pm$ 3.6 ^a	83.8 $\pm$ 6.7 ^a

Table 4 Ornithine concentrations in pig enterocyte extracts

Four batches of pig enterocytes from the same cell suspensions were incubated at 37 °C for 30 min in the presence of 0, 0.5, 2 or 5 mM glutamine, as described in the text. Another batch of cells was incubated for 0 min (no incubation). At the end of the incubation, the incubation medium was acidified with 0.2 ml of 1.5 M HClO₄, and neutralized with 0.1 ml of 2 M K₂CO₃. The neutralized extracts were used for ornithine analysis by h.p.l.c. Data are means $\pm$ S.E.M. ($n = 6$), and were statistically analysed by one-way analysis of variance. Means with different superscripts within a column are significantly different ($P < 0.05$). Ornithine concentrations in cell extracts within a row increased ($P < 0.05$) with increasing glutamine concentrations in the medium.

Age of pigs (days)	Ornithine in cell extracts (nmol/mg of protein after 30 min of incubation)				
	No incubation	[Glutamine] in medium (mM)			
		0	0.5	2	5
14	0.26 $\pm$ 0.03 ^a	0.31 $\pm$ 0.04 ^a	0.35 $\pm$ 0.03 ^a	0.48 $\pm$ 0.04 ^a	0.61 $\pm$ 0.05 ^a
21	0.28 $\pm$ 0.02 ^a	0.34 $\pm$ 0.02 ^a	0.44 $\pm$ 0.05 ^a	0.56 $\pm$ 0.04 ^a	0.68 $\pm$ 0.06 ^a
29	0.36 $\pm$ 0.02 ^b	0.52 $\pm$ 0.03 ^b	0.78 $\pm$ 0.06 ^b	1.07 $\pm$ 0.10 ^b	1.40 $\pm$ 0.11 ^b
58	0.44 $\pm$ 0.01 ^b	0.67 $\pm$ 0.05 ^b	0.85 $\pm$ 0.05 ^b	1.13 $\pm$ 0.08 ^b	1.53 $\pm$ 0.07 ^b

Table 5 Glutamine metabolism and carbamoyl phosphate content in pig enterocytes

Two batches of pig enterocytes from the same cell suspensions were incubated at 37 °C for 30 min in the presence of 0 or 5 mM glutamine (Gln), and the concentrations of glutamine metabolites in enterocyte extracts as well as CO₂ production were measured as described in the text. Data are means $\pm$ S.E.M. ($n = 6$): * $P < 0.01$, significantly different from the control (0 Gln), as analysed by paired t test. The rate of CO₂ production from glutamine progressively increased ($P < 0.05$) with increasing age, as analysed by one-way analysis of variance. The rates of glutamine utilization and concentrations of ammonia, alanine and carbamoyl phosphate (CP) in cell extracts in enterocytes of 21–58-day-old pigs were different ($P < 0.05$) from those in the cells from 14-day-old pigs. Units: † nmol/30 min per mg of protein; ‡ products in cell extracts (nmol/mg of protein after 30 min of incubation).

Gln utilization or products from Gln	14-day-old pigs		21-day-old pigs		29-day-old pigs		58-day-old pigs	
	Medium [Gln]		Medium [Gln]		Medium [Gln]		Medium [Gln]	
	0 mM	5 mM	0 mM	5 mM	0 mM	5 mM	0 mM	5 mM
Gln utilization†	—	218 $\pm$ 14	—	298 $\pm$ 17	—	286 $\pm$ 16	—	309 $\pm$ 20
CO ₂ †	—	9.0 $\pm$ 0.7	—	36 $\pm$ 3.7	—	68 $\pm$ 5.7	—	98 $\pm$ 6.7
Ammonia‡	28 $\pm$ 3	242 $\pm$ 16	35 $\pm$ 4	338 $\pm$ 21	30 $\pm$ 4	323 $\pm$ 28	38 $\pm$ 4	356 $\pm$ 25*
Arg‡	3.8 $\pm$ 0.4	4.1 $\pm$ 0.3	3.3 $\pm$ 0.4	3.6 $\pm$ 0.3	3.2 $\pm$ 0.3	5.1 $\pm$ 0.6*	4.4 $\pm$ 0.5	6.4 $\pm$ 0.7*
Asp‡	11 $\pm$ 2.1	35 $\pm$ 4.3*	8.3 $\pm$ 1.5	48 $\pm$ 5.1*	8.5 $\pm$ 0.7	35 $\pm$ 1.8*	11 $\pm$ 1.6	38 $\pm$ 6.8*
Ala‡	11 $\pm$ 0.7	27 $\pm$ 2.0*	21 $\pm$ 1.3	107 $\pm$ 9.2*	28 $\pm$ 2.5	128 $\pm$ 8.7*	34 $\pm$ 3.2	132 $\pm$ 9.6*
Pro‡	1.7 $\pm$ 0.2	6.9 $\pm$ 0.9*	2.0 $\pm$ 0.2	5.5 $\pm$ 0.6*	1.7 $\pm$ 0.2	1.8 $\pm$ 0.1	1.9 $\pm$ 0.3	1.8 $\pm$ 0.3
CP‡	38 $\pm$ 2.7	53 $\pm$ 4.0*	25 $\pm$ 2.0	37 $\pm$ 2.5*	25 $\pm$ 1.9	34 $\pm$ 2.6*	27 $\pm$ 2.3	38 $\pm$ 2.3*

in the presence of 0–5 mM glutamine is shown in Table 4. Since some ornithine was converted into citrulline (Table 2), the rate of ornithine synthesis was the sum of the citrulline produced and the net accumulation of ornithine. Ornithine synthesis was low in enterocytes from 14–21-day-old pigs, but markedly increased

($P < 0.05$) in the cells from 29–58-day-old pigs. Increasing glutamine concentrations in the medium resulted in increased ($P < 0.05$) ornithine synthesis in enterocytes from all ages of the pigs used, and the increment was greater in enterocytes from 29–58-day-old pigs than in cells from pre-weaning pigs. A small

Table 6 Citrulline concentrations in the extracts of pig enterocytes incubated in the presence of ornithine, ammonia or proline

Six batches of pig enterocytes from the same cell suspensions were incubated at 37 °C for 30 min in the presence of 2 mM ornithine, 5 mM NH₄Cl or 2 mM proline as indicated in the text. At the end of the incubation, the incubation medium was acidified with 0.2 ml of 1.5 M HClO₄, and neutralized with 0.1 ml of 2 M K₂CO₃. The neutralized extracts were used for citrulline analysis by h.p.l.c. Data are means $\pm$ S.E.M. ($n = 6$), and were statistically analysed by one-way analysis of variance. Means with different superscripts within a row are significantly different ($P < 0.05$). The concentrations of all measured parameters in the cell extracts are higher ($P < 0.05$) in enterocytes from 29–58-day-old pigs than in the cells from 14–21-day-old pigs.

Age of pigs (days)	Addition to incubation medium...	Citrulline in cell extracts (nmol/mg of protein after 30 min of incubation)					
		None	2 mM Orn	2 mM Pro	5 mM NH ₄ Cl	2 mM Orn, 5 mM NH ₄ Cl	2 mM Pro, 5 mM NH ₄ Cl
14		0.21 $\pm$ 0.03 ^a	0.21 $\pm$ 0.03 ^a	0.22 $\pm$ 0.03 ^a	0.29 $\pm$ 0.03 ^b	0.47 $\pm$ 0.05 ^c	0.28 $\pm$ 0.02 ^b
21		0.44 $\pm$ 0.03 ^a	0.46 $\pm$ 0.03 ^a	0.42 $\pm$ 0.02 ^a	0.73 $\pm$ 0.07 ^b	0.98 $\pm$ 0.08 ^c	0.72 $\pm$ 0.06 ^b
29		2.69 $\pm$ 0.16 ^a	2.52 $\pm$ 0.18 ^a	2.79 $\pm$ 0.20 ^a	7.24 $\pm$ 0.43 ^b	12.3 $\pm$ 0.73 ^c	7.37 $\pm$ 0.38 ^c
58		2.33 $\pm$ 0.14 ^a	2.60 $\pm$ 0.19 ^a	2.49 $\pm$ 0.15 ^a	6.60 $\pm$ 0.30 ^b	10.7 $\pm$ 0.69 ^c	6.82 $\pm$ 0.40 ^b

Table 7 Activities of the enzymes involved in citrulline synthesis from glutamine in pig enterocytes

The activities of enzymes were measured as described in the text. Data are means $\pm$ S.E.M. ($n = 6$). Means with different superscripts within a column are significantly different ($P < 0.05$) as analysed by one-way analysis of variance.

Age of pigs (days)	Phosphate-dependent glutaminase (nmol of Glu/min per mg of protein)	P-5-C synthase (pmol of P-5-C/min per mg of protein)	Ornithine amino-transferase (nmol of Orn/min per mg of protein)	Ornithine carbamoyl-transferase (nmol of Cit/min per mg of protein)	Carbamoyl phosphate (CP) synthase (nmol of CP/min per mg of protein)
14	23.6 $\pm$ 1.9 ^a	12.4 $\pm$ 0.93 ^a	332.9 $\pm$ 56.9 ^a	129.0 $\pm$ 12.1 ^a	2.20 $\pm$ 0.20 ^a
21	24.4 $\pm$ 2.9 ^a	16.7 $\pm$ 2.1 ^a	122.9 $\pm$ 11.1 ^b	96.9 $\pm$ 5.2 ^b	2.31 $\pm$ 0.24 ^a
29	9.5 $\pm$ 0.71 ^b	272.2 $\pm$ 32.6 ^b	64.8 $\pm$ 5.3 ^c	59.7 $\pm$ 2.6 ^c	1.08 $\pm$ 0.08 ^b
58	8.4 $\pm$ 0.56 ^b	239.5 $\pm$ 25.8 ^b	27.9 $\pm$ 1.9 ^d	25.5 $\pm$ 1.7 ^d	1.18 $\pm$ 0.05 ^b

amount of ornithine was produced by enterocytes incubated in the absence of added glutamine, due to the presence of pre-existing cellular glutamate (Table 3).

Glutamine metabolism and cellular carbamoyl phosphate content in enterocytes

These data are presented in Table 5. The rates of glutamine utilization were greater ($P < 0.05$) in enterocytes from 21–58-day-old pigs than in cells from 14-day-old pigs, as were the rates of production of ammonia and alanine in the presence of 5 mM glutamine. There were no differences ($P > 0.05$) in aspartate production from glutamine among enterocytes from 14–58-day-old pigs, but the rates of alanine synthesis in enterocytes from 21–58-day-old pigs were 7–10-fold greater than those in the cells from 14-day-old pigs. The rate of glutamine oxidation in enterocytes progressively increased ($P < 0.05$) with the pig's growth. Similar results were obtained in the presence of 0.5–2 mM glutamine (results not shown). Proline synthesis from glutamine occurred in enterocytes from 14–21-day-old pigs, but not from 29–58-day-old pigs. In contrast, there was synthesis of arginine from glutamine only in the cells from post-weaning pigs. The concentrations of carbamoyl phosphate in enterocytes from 14-day-old pigs were greater than those in the cells from older pigs, whereas there were no differences among the cells from 21–58-day-old pigs. Addition of 5 mM glutamine to the incubation medium increased ($P < 0.05$) the concentrations of carbamoyl phosphate in enterocytes from all ages of the pigs used.

Effects of ornithine, proline and ammonia in the medium on citrulline synthesis

In the absence of NH₄Cl, the addition of 2 mM ornithine or proline to the incubation medium had no effect ($P < 0.05$) on citrulline synthesis in enterocytes from all ages of the pigs used (Table 6). The addition of 5 mM NH₄Cl alone increased ($P < 0.05$) citrulline production in enterocytes from 29–58-day-old pigs to a greater extent than in the cells from 14–21-day-old pigs. Addition of 2 mM ornithine, but not 2 mM proline, together with 5 mM NH₄Cl further increased ($P < 0.05$) citrulline synthesis in enterocytes.

In contrast with citrulline formation, the addition of 2 mM ornithine alone increased proline synthesis in pig enterocytes. The concentrations of proline (nmol/mg of protein after incubation for 30 min; means $\pm$ S.E.M., $n = 6$) in the extracts of enterocytes from 14-, 21-, 29- and 58-day-old pigs were 1.50 $\pm$ 0.03, 1.62 $\pm$ 0.06, 1.86 $\pm$ 0.07 and 1.95 $\pm$ 0.08 respectively, in the absence of ornithine, and increased ($P < 0.01$) to 27.4 $\pm$ 0.59, 24.6 $\pm$ 1.37, 24.5 $\pm$ 1.22 and 26.6 $\pm$ 0.68 respectively, in the presence of 2 mM ornithine. The addition of 2 mM ornithine plus 5 mM NH₄Cl did not further increase the rates of proline synthesis in enterocytes, as compared with those in the presence of 2 mM ornithine alone (results not shown).

Enzyme activities

Table 7 shows the activities of phosphate-dependent glutaminase, P-5-C synthase, ornithine aminotransferase, ornithine carba-

moyltransferase and carbamoyl-phosphate synthase in enterocyte extracts. The phosphate-dependent glutaminase activity was similar in 14–21-day-old pigs, but decreased significantly ($P < 0.05$) in cell extracts from 29–58-day-old pigs. Similar results were obtained with carbamoyl-phosphate synthase. In contrast, P-5-C activity was low in 14- and 21-day-old pigs, but increased ($P < 0.05$) by 15–20-fold in 29–58-day-old pigs. Interestingly, the activities of both ornithine aminotransferase and ornithine carbamoyltransferase were greater than those of glutaminase at all ages of the pigs used, and markedly decreased ($P < 0.05$) with the increase in the animal's age.

DISCUSSION

The biochemical viability of incubated enterocytes depends, to a significant extent, on the animal species used. For example, incubated enterocytes from the chick remain viable for a longer period of time than do cells from the rat under the same experimental conditions [11]. We found that pig enterocytes were viable for 30 min under the incubation conditions used, as assessed by O_2 consumption (Table 1). This is also consistent with the linearity of production of citrulline, glutamate, ammonia, aspartate, alanine, CO_2 and lactate from the pig enterocytes during the 30 min incubation period (results not shown), and with the cell viability assessed by Trypan Blue exclusion. Using the present sensitive fluorimetric h.p.l.c. method, we demonstrated the production of ornithine and citrulline from glutamine in enterocytes from both the pig (Tables 3 and 4) and the growing (200–350 g) rat (results not shown). Our data suggest that the previous inability of detecting citrulline synthesis in incubated rat enterocytes [11] may result from the relatively low sensitivity of the analytical techniques used.

A novel finding from this study is that the rate of citrulline synthesis markedly increased in enterocytes from post-weaning pigs as compared with those from pre-weaning pigs (Table 2). Interestingly, Blachier et al. [27] recently reported synthesis *de novo* of arginine from glutamine in enterocytes from 0–8-day-old pigs. However, it is unlikely that the low rate of production of citrulline by enterocytes from 14–21-day-old pigs (Table 2) was due to a possible conversion of citrulline into arginine, as there was no measurable amount of arginine synthesized from glutamine in these cells (Table 5). As arginine synthesis from glutamine occurred in enterocytes from 29–58-day-old pigs, our values of citrulline concentrations in cell extracts underestimate the rates of citrulline synthesis in these cells. It is worth pointing out that, since the mass of the small intestine and enterocytes increases with the pig's growth [28], the total amounts of citrulline synthesized from glutamine would increase to a much greater extent in the small intestine of 29–58-day-old pigs than in that of 14–21-day-old pigs. Similarly, more citrulline would be produced by the small intestine of 58-day-old pigs than that of 29-day-old pigs, because of the 45% larger mass of the small intestine and enterocytes in the former than in the latter [28].

The synthesis of citrulline requires ornithine and carbamoyl phosphate [20]. It is worth pointing out that glutamine can provide all these precursors for citrulline synthesis in enterocytes (Table 5). It is also noteworthy that citrulline, as compared with ammonia and alanine, is only a minor nitrogenous end-product of glutamine metabolism in incubated pig enterocytes (Table 5). The finding that the addition of 5 mM NH_4Cl alone increased citrulline synthesis to a greater extent in enterocytes from post-weaning pigs than in the cells from pre-weaning pigs (Table 6) is consistent with the increased synthesis of ornithine in the former

than in the latter (Table 4). Interestingly, in the absence of NH_4Cl , the addition of 2 mM ornithine resulted in synthesis of a relatively large amount of proline, but not citrulline, in enterocytes from all ages of the pigs used. This may result from (i) the negligible or low rate of carbamoyl phosphate synthesis and (ii) the conversion of ornithine into proline, thus limiting the availability of intracellular carbamoyl phosphate and ornithine for citrulline synthesis under the experimental conditions used. The observation that addition of 2 mM ornithine plus 5 mM NH_4Cl only slightly increased citrulline synthesis in enterocytes from 14–21-day-old pigs may be explained, in part, by the competitive conversion of ornithine into proline.

The conversion of glutamine into citrulline in the small intestine involves four enzymes, namely phosphate-dependent glutaminase, P-5-C synthase, ornithine aminotransferase and ornithine carbamoyltransferase, in addition to carbamoyl-phosphate synthase for synthesis of carbamoyl phosphate from ammonia, HCO_3^- and ATP [29]. Glutaminase catalyses the hydrolysis of glutamine to glutamate, which is subsequently converted into the γ -glutamic semialdehyde by P-5-C synthase. Ornithine aminotransferase catalyses the transamination of γ -glutamic semialdehyde with glutamate to form ornithine, which is converted into citrulline by ornithine carbamoyltransferase. The activities of all these enzymes but P-5-C synthase (Table 7) were much greater than the capacity of the enterocytes to synthesize citrulline from glutamine (Table 2) in pig enterocytes, and decreased with the animal's growth (Table 7). Our data are consistent with those reported by Wakabayashi and Jones [30] that P-5-C synthase activity is low in rat intestinal mucosa. Interestingly, the increase in citrulline synthesis from glutamine in enterocytes from 29–58-day-old pigs coincided with 15–20-fold increases in the activity of P-5-C synthase (Table 7). In light of our findings that the concentrations of carbamoyl phosphate in enterocytes from pre-weaning pigs were either higher than, or similar to, those in the cells from post-weaning pigs (Table 5), it is likely that the low rate of citrulline synthesis in enterocytes from pre-weaning pigs in the presence of glutamine was due to a limited availability of ornithine rather than that of carbamoyl phosphate. This limited availability of ornithine may result from (i) the low rate of P-5-C synthesis from glutamate, due to the low activity of P-5-C synthase, and (ii) the competitive conversion of P-5-C into proline.

The present findings on the developmental aspect of citrulline synthesis in pig enterocytes are interesting, and may help to provide a biochemical mechanism for explaining the differential qualitative requirements of arginine by suckling and adult animals, including humans. It has been reported that arginine is a nutritionally essential amino acid for suckling and young rats, but not for adult rats [31,32]. Similarly, dietary arginine has been shown to be required for the growth of suckling piglets [13], but not for the post-pubertal growth and the pregnancy of pigs [14]. Furthermore, adult humans do not appear to require dietary arginine for maintaining a positive nitrogen balance [33], suggesting an adequate rate of endogenous arginine synthesis. In light of our present findings, it is possible that suckling animals are not able to synthesize enough arginine relative to its requirements by the body. This probably results from the low or negligible citrulline synthesis from glutamine in enterocytes of the suckling animal. It has been demonstrated that the kidney is the major organ for arginine synthesis from citrulline in the rat [3]. Thus one can surmise that such a pathway also exists in the pig, thereby providing endogenous arginine for metabolic utilization [14]. On the basis of the stoichiometry of arginine synthesis from citrulline [3], we suggest that the synthesis of citrulline from glutamine in enterocytes (Table 2), but not the formation of

citrulline from arginine in intestinal smooth-muscle cells [12], contributes to the net synthesis of arginine via the intestinal-renal axis. This further underscores the physiological and nutritional significance of intestinal glutamine metabolism and citrulline synthesis in whole-body homeostasis.

In conclusion, there was an interesting developmental aspect of citrulline synthesis from glutamine in pig enterocytes. We suggest that the rate of citrulline synthesis in these cells may be limited by P-5-C synthase activity, which converts glutamate into γ -glutamic semialdehyde. The increased synthesis of citrulline from glutamine in enterocytes with the post-weaning growth of pigs may directly contribute to an increased availability of citrulline for endogenous synthesis of arginine. This may offer a biochemical mechanism for the previous observations that arginine is a nutritionally essential amino acid for suckling piglets, but not adult pigs. A similar explanation may also be applied to the differential dietary arginine requirements by suckling and adult rats, and perhaps by humans as well.

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