Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 140-147

ORNITHINE SYNTHESIS FROM GLUTAMATE IN RAT INTESTINAL MUCOSA HOMOGENATES: EVIDENCE FOR THE REDUCTION OF GLUTAMATE TO γ-GLUTAMYL SEMIALDEHYDE*

Gwendolyn Ross, Doris Dunn and Mary Ellen Jones

Departments of Biochemistry, Schools of Medicine, Universities of Southern California, Los Angeles, CA, 90033 and North Carolina, Chapel Hill, NC, 27514

Received September 18,1978

SUMMARY: For 25 years, glutamate has been considered to be the source of the carbon and nitrogen atoms of ornithine and proline synthesized in mammalian tissues. However, the first enzyme reaction for these syntheses, namely the reduction of glutamate to γ-glutamyl semialdehyde, has not been observed in homogenates of mammalian tissues. These studies present evidence for this reaction in rat intestinal mucosa homogenates incubated with [U-^{14}C]glutamate. The ^{14}C-labeled ornithine was separated from other radioactive chemicals by ion-exchange chromatography, and was specifically identified by its capacity to serve as a substrate for ornithine transcarbamylase. The synthesis of ornithine required ATP, Mg^{2+} and NADPH in addition to glutamate.

The reduction of glutamate to glutamic-γ-semialdehyde, the first step in the biosynthesis of proline, can be observed in intact Escherichia coli when a mutant strain which does not metabolize the semialdehyde is used (1-3). This reduction is dependent on ATP and NADPH and it has been postulated that the reduction involves an ATP-dependent γ-glutamyl kinase which forms an enzyme bound γ-glutamyl phosphate which is then reduced to glutamic-γ-semialdehyde by a reductase requiring NADPH (4-6). This enzyme has not been demonstrated in animal tissues, although early nutritional and in vivo isotopic studies strongly suggested its existence (7). The recent observation that citrulline, ornithine and proline were among the products formed from glutamine or glutamate in perfused rat jejunum (8) suggested that the enzyme exists in these cells. Herzfeld and Raper (9) have found that homogenates of intestine from young rats have relatively high levels of the enzymes that interconvert glutamate, ornithine and proline, and in addition that the ornithine aminotransferase of this tissue is more active in the ornithine-forming direction than is the liver enzyme. We chose, therefore, to use intestinal mucosa homogenates from young

* This work was supported by a research grant from the National Institute of Child Health and Development (HD 06536) of the National Institutes of Health.

0006-291X/78/0851-0140$01.00/0

rats to study the conversion of [U-^{14}C]-glutamate to [^{14}C]-ornithine as a measure of the reduction of glutamate to glutamic-γ-semialdehyde.

METHODS: Materials: Conventional male Sprague-Dawley rats approximately 28 days old (90-100 g) were obtained from Simonson Farms, Gilroy, CA. Germ-free male Sprague Dawley rats, which were 32 days old (64 g), were from Charles River Breeding Laboratories, Wilmington, MA. L-[U-^{14}C]glutamic acid (270 Ci/mol) was obtained from Amersham, while L-[U-^{14}C]ornithine (234.5 Ci/mol) and L-[U-^{14}C] proline (290 Ci/mol) were purchased from New England Nuclear. [^{14}C]Carbamoyl phosphate[1] (60.7) mCi/mol) was purchased from New England Nuclear and recrystallized (10). Ion exchange resins, AG 1-X8, 200-400 mesh (acetate form) and AG 50W-X8, 200-400 mesh (hydrogen form), were from Bio-Rad Laboratories. The AG 50W-X8 (H^+ form) was converted to the Na^+ form with 1 M NaOH. 2,5-Diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) were obtained from Beckman Instruments. Ornithine carbamoyl transferase[1] (EC 2.1.3.3) from Streptococcus faecalis A.T.C.C. 8043 was prepared and assayed by the method of Nakamura and Jones (11). Other reagents were purchased from Sigma Chemical Co.

Preparation of intestinal mucosa cell homogenate: Non-fasted rats were killed by cervical dislocation. The intestine was removed and the lumen flushed with cold 0.9% NaCl containing 0.3% glucose. The mucosa was scraped off of sections of the intestine with a glass microscope slide and placed in sufficient 0.1 M phosphate buffer, pH 7.4, to make a 33% suspension (w/v). Homogenization at 0° with a motor-driven Teflon pestle in a glass vessel was followed by sonic disruption for 1 min in a cooled Raytheon sonic disintegrator.

Incubation conditions: The homogenate was incubated with shaking at 37° in 25 ml Erlenmeyer flasks containing 1.0 g wet weight tissue, [U-^{14}C]glutamic acid, and other substrates and cofactors as indicated in a final volume of 4.0 ml. The enzyme reaction was stopped with sufficient concentrated $HClO_4$ to yield a final concentration of 2 M and denatured protein was removed by centrifugation. For the zero-time control, $HClO_4$ was added to the homogenate before the remaining chemicals for the incubation mixture. In order to convert any glutamine present to glutamate, the supernatant was removed and hydrolyzed at 100° for 1 to 2 h in test tubes covered with marbles. The cooled hydrolysate was neutralized with 6 M KOH, maintained at 0° for 30 min and then centrifuged to remove the $KClO_4$. An aliquot of this solution was evaporated to dryness and then dissolved in 1.0 ml of solution containing 0.6 μmol each of ornithine, proline, citrulline and arginine. A portion (0.9 ml) was used for ion exchange chromatography.

Ion exchange chromatography: To separate ornithine from glutamate and its other metabolites, three ion exchange columns (0.7 x 16 cm) were used in sequence (12, 13) as described in Table I. Before applying a fraction to a column, a known portion that had been evaporated to dryness using a Buchler Evapo-Mix was dissolved in a small amount of the solvent with which the column to be used had been equilibrated. The radioactivity of each fraction was determined by placing an aliquot in toluene-Triton X-100 (2:1, v/v) containing 5.5 g PPO and 0.1 g POPOP per l (aqueous samples did not exceed 10% of the scintillant volume). Fraction D (0.4 ml) was diluted to 1.0 ml with water before mixing with scintillant. Samples were counted in a Beckman LS-100 C scintillation spectrometer.

Enzymatic assay for ornithine: Ornithine was assayed by measuring its conversion to citrulline in the presence of OTCase[1] and carbamoyl-P[1]. Approximately 9 ml of fraction D (see Table I) was evaporated to dryness. The dried fraction D was dissolved in 0.7 ml of 0.286 M Tris, pH 11, and then adjusted to pH 7.5 with 10

[1]Abbreviations used: OTCase, ornithine carbamoyl transferase; carbamoyl-P, carbamoyl phosphate.

TABLE I

SEPARATION OF ORNITHINE AND CITRULLINE FROM INCUBATION MIXTURES BY ION EXCHANGE CHROMATOGRAPHY

	Column Used	Elutant	Acids Released	Acids Retained
1. $HClO_4$ denatured supernatant: prepared as described under "incubation conditions" in the Methods. Sample = 0.9 ml	AG1-X8 (acetate)	20 ml H_2O = (A)	neutral and basic amino acids	tricarboxylic and acidic amino acids
2. (A): evaporated to dryness, dissolved in 0.116 M Na citrate, pH 5.3. Sample = 0.5 ml	AG50W-X8 (Na^+)	20 ml 0.116 M Sodium citrate, pH 5.3 = (B)	citrulline (and other neutral amino acids	ornithine and arginine
		20 ml 0.116 M Sodium citrate, pH 5.3 = (C)	ornithine	arginine
3. (B) or (C): acidified to pH 1.5 ($\bar{c}$ 6 N HCl), evaporated to dryness, dissolved in 1 ml of 0.1 M HCl. Sample = 1.0 ml	AG50W-X8 (H^+)	10 ml 0.1 M HCl	-	all applied
		30 ml 1.5 M HCl	neutral amino acids	citrulline or ornithine
		10 ml 6 M HCl = (D)	citrulline or ornithine	-

to 30 µl of 6 M HCl. Two portions, one with and one without 93 mM carbamoyl-P, were incubated for 1 h at 38° in a total volume of 1.0 ml containing 280 units of OTCase. Two samples of standard ornithine (6 µmol, 1.7 µCi/mmol) were also incubated under the same conditions. The reaction was stopped with 0.2 ml of 2 M $HClO_4$; $HClO_4$ was removed after conversion to $KClO_4$ as described above. A measured portion of the supernatant was applied to an AG 50W-X8 (Na^+) column which was eluted as described in Table I to obtain a citrulline and an ornithine fraction. The standard ornithine was always completely converted to citrulline; when all the radioactivity in fraction D was not converted to citrulline, the amount of ornithine formed from glutamate was considered to be the difference in the amount of citrulline formed with and without carbamoyl-P. To further substantiate that the product of the reaction with carbamoyl-P was citrulline and that the material remaining in the absence of carbamoyl-P was ornithine, these two fractions were evaporated, passed through the AG 50W-X8 (H^+) column to remove the citrate buffer and this new fraction D was evaporated to dryness, dissolved in 0.05 ml of H_2O and used for paper electrophoresis as described below.

High voltage paper electrophoresis: Whatman 3 MM chromatography paper (60 cm x 12 cm) was cut into five longitudinal strips separated by channels that shared common end-pieces. Sample (20 to 30 µl) from the enzymatic assay or standards of [U-^{14}C]ornithine and [^{14}C]citrulline were placed on a strip; the paper was wetted (without disturbing the spots) with 0.7 M formic acid buffer, pH 2.0, and then subjected to electrophoresis on a Savant flat plate at 3.5 KV for 30 min. Each longitudinal strip of the dried electropherogram was cut at premarked 1 cm sections which were placed in toluene containing 3 g PPO and 0.1 g POPOP per 1 and counted.

Measurement of ornithine carbamoyl transferase activity: The method used was one previously described (14) with a few modifications: 200 mM triethanolamine, pH 7.5, buffer was used; the 20 min incubation was at 27°; the mucosa cell homogenate added per incubation vial represented 60 mg of tissue.

Synthesis of [^{14}C] Citrulline: [^{14}C]Citrulline was prepared enzymatically from [U-^{14}C]-ornithine by incubating in 1.0 ml: 0.2 mM ornithine; 0.05 µCi (U-^{14}C] ornithine; 0.1 M Tris, pH 7.5; 4.7 mM carbamoyl-P and 280 units (1 unit = 1 µmol product/min) OTCase for 1 h at 38°. The reaction was stopped with 0.2 ml 2 M $HClO_4$; $HClO_4$ was removed after conversion to $KClO_4$ as described above. The supernatant solution was applied directly to an AG 50W-X8 (Na^+) column (Table I). The eluted fraction B was applied to the AG 50W-X8 (H^+) column and then eluted as fraction D which was evaporated to dryness and dissolved in water to the desired concentration. The specific activity of the citrulline so obtained was 0.25 Ci/mol.

RESULTS AND DISCUSSION: At the time these studies were initiated, Windmueller and Spaeth (1975) had reported that a small portion of the glutamine (or glutamate) transported across a perfused jejunum was converted to proline, ornithine and citrulline. Since we did not wish to reduce this limited conversion by using sections of intestinal mucosa that were inactive, we selected the specific activity of OTCase as a reasonable indicator of the potential of the duodenum and ileum to convert glutamate to these amino acids. It was found that all three areas of the small intestine possess OTCase activity. This activity increases from duodenum to ileum (from 2.6 to 4.3 µmol/h/g wet weight tissue); therefore,

TABLE II

ENZYMATIC CONFIRMATION OF THE IDENTITY OF ORNITHINE

Standard ornithine or ornithine fractions from the AG 50W-X8 (H^+) column were incubated, as described in the Methods, with OTCase and carbamoyl-P as indicated. Citrulline denotes radioactivity eluted as fraction B (Table I), while ornithine denotes radioactivity eluted as fraction C.

		Radioactivity (cpm) in sodium citrate buffer eluate from AG 50W-X8 (Na^+) column	
	Carbamoyl-P	Citrulline	Ornithine
Ornithine standard	+	18,000	0
	-	1,100	17,000
Ornithine fraction			
Exp. 1	+	9,400	1,800
	-	1,000	10,000
Exp. 2	+	10,000	0
	-	660	9,300

mucosa from the entire small intestine was used. These values for OTCase activity are similar in magnitude to those reported by Raijman (14) for a homogenate of the entire small intestine, and qualitatively agree with the findings of Herzfeld and Raper (9) that the duodenum and a jejunum-ileum portion have similar OTCase activity. However, the values reported by Herzfeld and Raper (9) were markedly higher than those reported here and by Raijman (14).

When intestinal mucosa homogenates were incubated with [U-^{14}C]glutamate, [^{14}C]ornithine was produced. The identity of the ornithine was confirmed by its conversion to citrulline in the presence of OTCase and carbamoyl-P (Table II). Only in the presence of carbamoyl-P did most (or all) of the counts emerge from the ion-exchange column as citrulline. When aliquots of the citrulline and ornithine fractions were subjected to high voltage electrophoresis (see the methods), the radioactivity migrated as expected from the ion-exchange behavior of the fraction.

The formation of [^{14}C]ornithine from [^{14}C]glutamate requires ATP, $MgCl_2$ and NADPH; 2-mercaptoethanol is only slightly stimulatory (Table III). The re-

TABLE III

REQUIREMENTS FOR ORNITHINE SYNTHESIS IN RAT INTESTINAL MUCOSA HOMOGENATES

	Omissions	Ornithine produced (nmol/g fresh tissue) conventional rat	germ-free rat
A.	none	31.8 (24)	15.9*
	all cofactors	1.4 (0.4)	0.2
	$MgCl_2$	3.6	-
	ATP	1.2	0.5
	NADPH	2.2	0.7
	NADPH; NADH added	2.0	1.6
	2-mercaptoethanol	26.6	-
B.	none	7	12
	none; acetyl CoA added	19	13

The complete incubation system contained in a final volume of 4.0 ml, 3 mM [U-^{14}C]glutamic acid (2 Ci/mol), 5 mM ATP and an ATP regenerating system (15 mM creatine phosphate and 15 enzyme units/ml of creatine phosphokinase), 0.4 mM NADPH and a NADPH regenerating system (10 mM DL-isocitrate, and 0.6 enzyme units/ml of isocitrate dehydrogenase), 25 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. NADPH was replaced by 0.4 mM NADH and a NADH regenerating system (10 mM lactate and 0.6 enzyme units/ml of lactate dehydrogenase) as indicated. The intestinal mucosa homogenate was prepared and added as described in the methods. The incubation at 37° was stopped at the end of 1.5 h. The values are those determined from fraction (C) of Table I; only the complete vessel and the vessel with no cofactors for the conventional rats were carried through to the OTCase assay (see Table II) for ornithine, and the results of this assay are given in parentheses. The values are averages of duplicate flasks.†

For experiment B, the incubation system was the same as described above with 0.5 mM acetyl CoA added as indicated. The reaction was stopped at the end of 3 h; values are averages of duplicate flasks.

†All intestinal mucosa homogenates incubated with ATP, Mg^{2+} and NADPH have produced ornithine. However, the amount of product obtained in 1.5 h has varied from a low value of 3 nmol to a high value of 32 nmol/gm fresh tissue. In a few experiments we have varied the time of incubation from 30 to 180 min and found the synthesis roughly linear for this interval.

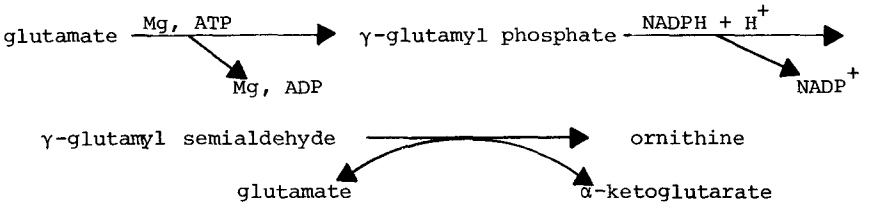
*This value did not change when 8 μM pyridoxal phosphate was added in addition to the other cofactors.

action is very specific for NADPH as electron donor; NADH is ineffective.

The conversion of glutamate to ornithine can occur via one of two pathways: 1) direct reduction to the semialdehyde followed by transamination; or, 2) conversion to N-acetyl glutamate followed by reduction, transamination and deacetylation. The reaction in E. coli occurs via the second pathway, while in eucaryotes the first reaction appeared to be more likely (15). However, since

both small intestine and liver contain N-acetyl glutamate as well as N-acetyl glutamate synthetase (16,17) the second pathway could have occurred in these two tissues (18,17). Our study does not support the acetylated pathway, since acetyl CoA did not stimulate ornithine synthesis in homogenates from germ-free rats (Table IIIB). We conclude that the N-acetylated pathway probably does not function in the rat and that the acetyl CoA stimulation in homogenates from conventional rats was due to enzyme from intestinal E. coli.

From the data of Tables II and III, the following steps probably occur:



This sequence of a phosphorylation step followed by a reduction requiring NADPH is also utilized for the conversion of N-acetyl glutamate to N-acetyl glutamic γ-semialdehyde destined for the synthesis of ornithine in E. coli (19), or glutamate to glutamic-γ-semialdehyde destined for the synthesis of proline in E. coli (6), or for the conversion of aspartate to β-aspartic semialdehyde for the synthesis of homoserine in yeast (20).

The data reported here provide the first evidence for glutamate reduction to the γ-glutamyl semialdehyde in a mammalian tissue homogenate.

REFERENCES

1. Vogel, H.J. and Davis, B.D. (1952) J. Am. Chem. Soc. 74, 109-112.
2. Vogel, H.J. (1955) in Amino Acid Metabolism (McElroy, W.D. and Glass, B., eds.), pp. 335-353, Baltimore.
3. Strecker, H.J. (1957) J. Biol. Chem. 225, 825-834.
4. Baich, A. (1969) Biochim. Biophys. Acta 192, 462-467.
5. Baich, A. (1971) Biochim. Biophys. Acta 244, 129-134.
6. Gamper, H. and Moses, V. (1974) Biochim. Biophys. Acta 354, 75-87.
7. Stetten, M.R. (1955) in Amino Acid Metabolism (McElroy, W.D. and Glass, B., eds.), pp. 277-290, Baltimore.
8. Windmueller, H.G. and Spaeth, A.E. (1975) Arch. Biochem. Biophys., 171, 662-672.
9. Herzfeld, A. and Raper, S.M. (1976 Biochim. Biophys. Acta 428, 600-610.
10. Adair, L. and Jones, M.E. (1972) J. Biol. Chem. 247, 2308-2315.
11. Nakamura, M. and Jones, M.E. (1970) Methods Enzymol. 17A, 286-294.
12. Rojkind, M. and DeLeon, L.D. (1970) Biochim. Biophys. Acta 217, 512-522.
13. Karlin, J.N., Bowman, B.J., and Davis, R.H. (1976) J. Biol. Chem. 251, 3948-3955.

14. Raijman, L. (1974) Biochem. J. 138, 225-232.
15. Vogel, H.J. (1953) Proc. Natl. Acad. Sci., U.S.A. 39, 578-583.
16. Cohen, P.P. and Grisolia, S. (1950) J. Biol. Chem. 182, 747-761.
17. Shigesada, K. and Tatibana, M. (1971) J. Biol. Chem. 246, 5588-5595.
18. Jones, M.E. (1965) Ann. Rev. Biochem. 34, 381-418.
19. Baich, A. and Vogel, H.J. (1962) Biochem. Biophys. Res. Commun. 7, 491-496.
20. Black, S. and Wright, N.G. (1955) J. Biochem. 213, 27-38.