

# Effects of Glutamine Deamination on Glutamine Deamidation in Rat Kidney Slices

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**ABSTRACT** Glutamate is known to inhibit the activity of isolated glutaminase I; however, its actual physiologic importance in regulating renal ammonia-genesis has not been established. To determine the regulatory role of glutamate on the metabolism of glutamine by rat kidney slices, we followed the effects on glutamine (2 mM) deamidation of increased removal of glutamate via augmented deamination. Three agents (malonate, 2,4-dinitrophenol, and methylene blue) were known to and shown here to hasten exogenous glutamate deamination. In slices from 10 control rats,  $21.5 \pm 1.7$  (SEM)  $\mu\text{mol/g}$  of ammonia were formed from amide nitrogen and  $9.3 \pm 0.5$  (SEM)  $\mu\text{mol/g}$  from the amino nitrogen of glutamine in vitro. Over 90% of the glutamine deamidated formed glutamate at one point in its catabolism. After addition of malonate (10 mM), 2,4-dinitrophenol (0.1 mM), or methylene blue (0.5 mM), the production of ammonia from the amino group rose to  $29.3 \pm 6.0$  (SEM)  $\mu\text{mol/g}$ ,  $20.0 \pm 1.8$  (SEM)  $\mu\text{mol/g}$ , and  $15.5 \pm 4.2$  (SEM)  $\mu\text{mol/g}$ , respectively; ammonia production from the amide nitrogen rose also,  $45.1 \pm 7.3$  (SEM)  $\mu\text{mol/g}$ ,  $39.7 \pm 2.6$  (SEM)  $\mu\text{mol/g}$ , and  $41.9 \pm 3.7$  (SEM)  $\mu\text{mol/g}$ . In the case of the former two, a minimum of 99% and 75% of the glutamine catabolized formed glutamate. Despite increased glutamine catabolism, there was no build up of glutamate in the media. A correlation between the formation of ammonia from the amino and amide nitrogen was apparent. Since

none of the three agents selected affected phosphate activated glutaminase I activity directly or appeared to affect glutamine transport, we interpret the increase in deamidation as an expression of deinhibition of glutaminase I activity secondary to lowered glutamate concentrations at the deamidating sites through more rapid removal of glutamate via hastened deamination. Interestingly, slices removed from acidotic rats produced more ammonia from both the amino  $29.1 \pm 3.8$  (SEM) and amide nitrogens  $45.9 \pm 4.3$  (SEM) of glutamine, without a buildup of glutamate in the medium. At least 90% of the glutamine deamidated formed glutamate. A common mechanism is proposed to explain these results and the previous ones.

## INTRODUCTION

The regulatory mechanisms increasing renal glutamine extraction, deamidation and deamination during metabolic acidosis play a major protective role in acid-base homeostasis by permitting increased excretion of hydrogen ions in the form of ammonium ( $\text{NH}_4^+$ ) (1). Accordingly, the carbon skeleton remaining after nitrogen removal provides a major source of fuel for the kidneys during acidosis (2). Despite many attempts to gain understanding of this adaptive phenomenon, confusion still exists as to the role various mechanisms play. We favor the hypothesis that one rate limiting reaction controlling renal glutamine metabolism is the rate of glutamine deamination via the glutamate dehydrogenase pathway (3-6).

Our theory holds that faster removal of glutamate at ammonia-producing sites during acidosis lowers glutamate concentrations, deinhibits phosphate-dependent glutaminase, and results in faster formation of ammonia via hastened glutamine deamidation. Such a proposal seems reasonable for glutamate is a known inhibitor of

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TABLE I  
Ammonia Formation from Glutamine in Rat Kidney Slices  
(Exp. 1, Glutamine Added 1.97  $\mu\text{mol/ml}$ )

Flask	TNH <sub>2</sub>	ENH <sub>2</sub>	SNH <sub>2</sub>	G*	$\Delta G^\dagger$	Amide H <sub>2</sub> §
				$\mu\text{mol/ml}$		%
1	0.73	0.23	0.50	1.60	0.37	74%
2	0.70	0.25	0.45	1.58	0.39	87%
3	0.68	0.16	0.52	1.59	0.38	73%
4	0.73	0.27	0.46	1.59	0.38	83%
	TNH <sub>2</sub>	ENH <sub>2</sub>	SNH <sub>2</sub>	Amide NH <sub>2</sub>	Amino NH <sub>2</sub>	
				$\mu\text{mol/g per 90 min}$		
1	53.4	16.7	36.7	27.0	9.7	
2	50.4	18.3	32.1	28.0	4.1	
3	71.7	17.2	54.5	39.8	14.7	
4	49.8	18.6	31.2	25.9	5.3	
Ave	56.3	17.7	38.6	30.2	8.4	

\* Glutamine recovered after 75 min incubation with slices.

† Amount of amide nitrogen disappearing during incubation, also the maximal amount of ammonia formed from the amide nitrogen.

§ Percent of ammonia formed from glutamine derived from the amide nitrogen.

the activity of the enzyme glutaminase I (7). However, recent investigations have turned up several inconsistencies which cast doubt that under physiologic conditions glutamate concentrations play a major regulatory role in renal ammoniogenesis (8, 9). Therefore, our purpose was to determine if glutamate concentrations at ammonia-producing sites do indeed affect glutamine deamidation.

## METHODS

Sprague-Dawley rats, 200–250 g, were fed Purina Rat Chow and water ad lib. To produce a metabolic acidosis, rats drank 1% (wt/vol) ammonium chloride solution instead of water for 7 days. Rats were sacrificed with a blow to the

head, and the kidneys were rapidly removed and placed in cold saline solution. Kidney slices were cut to approximately 0.4 mm thickness with a Stadie-Riggs microtome (10), weighed, and placed in 7 ml of incubation medium composed of (millimolar) 120.0 NaCl, 5.0 KCl, 1.2 Mg SO<sub>4</sub>, 1.0 CaCl<sub>2</sub>, and 10.0 NaH<sub>2</sub>PO<sub>4</sub> buffer (pH 7.4). In addition, some flasks also contained glutamine (2.0 or 4.0 mM), glutamate (10.0 mM), 2,4-dinitrophenol (0.1 mM), methylene blue (0.5 mM), and/or malonate (10.0 mM). Slices were incubated at 37°C with a 100% oxygen gas phase. After incubation, medium was deproteinized with cold 1.2 N perchloric acid. After centrifugation, the perchloric acid was precipitated by the addition of KOH-phosphate buffer mixture (final pH 7.4), and ammonia, glutamine and glutamate were determined on the supernatant by methods described previously (11, 12).

To study the effects of various agents on glutamate deamination by slices, cortical slices were bisected and each half weighed. One half (approximately 50 mg) was incubated in medium containing 10.0 mM glutamate and the other half slice of similar weight was incubated in medium containing 10.0 mM glutamate plus 2,4-dinitrophenol, methylene blue, or malonate. After 75 min incubation, ammonia was determined on the deproteinized medium. Results are expressed as  $\mu\text{moles ammonia per gram wet weight of kidney slice}$ , and statistics are by paired analysis using Student's *t* test.

To study the direct effects of 2,4-dinitrophenol, methylene blue, and malonate on glutaminase I activity, 2½% homogenates of two kidneys were analyzed in duplicate. These determinations were kindly performed in the laboratory of Dr. R. H. Janicki.<sup>1</sup> His procedure for this assay is well described in a recent publication (13). Results are expressed as  $\mu\text{moles ammonia per milligram protein per hour}$ .

Mitochondrial swelling studies were carried out in a manner similar to those described by Phaolain and O'Donovan (14). Mitochondria from 100 g of cortical tissue were incubated in 0.3 M sucrose buffered to pH 7.4 with 0.02 M Tris. To some flasks addition of malonate and 2,4-dinitrophenol were added to make the final concentration 10 mM and 0.1 mM, respectively. We read optical densities of the concentrated mitochondrial suspensions initially upon their addition to the medium and after 30 min incubation at 37°C in a Coleman Jr. spectrophotometer (Coleman Instruments

<sup>1</sup> Cellular and Radiation Biology Laboratories, Allegheny General Hospital, Pittsburgh, Pa.

TABLE II  
Ammonia Formation from Glutamine (2.0 mM) by Rat Kidney Slices ( $\mu\text{mol/g per 75 min}$ )\*

	TNH <sub>2</sub>	ENH <sub>2</sub>	SNH <sub>2</sub>	Amide NH <sub>2</sub>	Amino NH <sub>2</sub>	Amide/amino†
						%
1	56.3 (49.8–71.7)	17.7 (16.7–18.6)	38.6 (31.2–54.5)	30.2 (25.9–39.8)	8.4 (4.1–14.7)	79/21
2	47.2 (44.1–49.4)	18.2 (17.3–18.9)	29.0 (26.8–30.5)	19.1 (18.0–21.7)	9.9 (8.8–10.7)	66/34
3	59.0 (50.8–73.8)	30.1 (21.9–39.4)	28.9 (17.3–51.9)	17.0 (12.5–30.3)	11.9 (3.7–21.6)	59/41
4	61.9 (51.6–67.8)	26.7 (19.5–38.7)	35.2 (27.7–45.9)	25.8 (20.2–33.5)	9.4 (7.5–12.4)	73/27
5	54.4 (45.7–66.5)	17.2 (12.7–23.8)	37.2 (33.0–42.7)	30.5 (22.4–35.6)	6.7 (3.3–9.1)	82/18
6	47.4 (42.9–52.2)	18.6 (16.5–21.6)	28.8 (26.3–30.6)	17.3 (15.8–18.4)	11.5 (10.5–12.2)	60/40
Ave	54.4	21.4	33.0	23.3	9.7	70/30

\* Each value is the average result for each experiment obtained from four observations (four rats). Range of observation is in parentheses.

† Percent of ammonia formed from the amide and amino nitrogens of glutamine.

TABLE III  
*Ammonia Formation from Glutamine (2.0 mM) by Rat Kidney Slices in the Presence of 2,4-Dinitrophenol (0.1 mM)*  
( $\mu\text{mol/g}$  per 75 min)

	TNH <sub>s</sub>	ENH <sub>s</sub>	SNH <sub>s</sub>	Amide NH <sub>s</sub>	Amino NH <sub>s</sub>	Amide/amino %
1	83.2 (78.5– 92.3)	11.3 (9.4–12.4)	71.9 (66.8–79.9)	43.9 (38.1–48.7)	28.0 (24.2–31.2)	61/39
2	64.5 (63.4– 65.2)	13.4 (12.9–13.8)	51.1 (50.2–51.8)	31.2 (30.5–35.1)	19.9 (15.1–20.4)	64/36
3	87.7 (81.0– 95.9)	31.8 (28.0–34.8)	55.9 (51.3–64.6)	44.2 (40.3–46.5)	11.7 (8.8–18.1)	79/21
4	106.6 (85.7–127.6)	35.7 (15.6–65.4)	70.9 (58.0–93.3)	52.5 (36.3–63.4)	18.4 (10.5–29.9)	74/26
5	79.9 (72.4– 84.5)	26.9 (24.7–29.1)	53.0 (43.3–59.8)	29.7 (22.5–34.1)	23.3 ( 9.2–33.6)	56/44
Ave	84.4*	23.8	60.6*	40.3†	20.3†	67/33

See table II for details

\*  $P < 0.01$  compared to controls in Table II.

†  $P < 0.05$  compared to controls in Table II.

Div., Perkin Elmer Corp., Maywood, Ill.) at 520 nm. We performed all studies in duplicate.

In studies concerned with formation of ammonia from the amide and amino nitrogens of glutamine, kidney cortical slices from the same rat were incubated either in flasks containing medium without substrate or in medium containing 2 mM glutamine. 2,4-dinitrophenol, methylene blue, or malonate were added to both sets of flasks as required by the protocol. Slices were weighed before incubation and the weight of the paired slices were kept within 10% of one another. Slice weight in all flasks approximated 100–200 mg. In the first series of experiments (Tables I–VI) four flask pairs (four rats) were used to determine ammonia formation by slices without substrate and ammonia formation in the presence of glutamine. In the second series of experiments (Table VII) four flask pairs (two rats) were used to determine ammonia formation from amide and amino nitrogen of glutamine. The only significant variation in protocol between the two series was that the glutamate accumulation was followed in the last. Ammonia and glutamate concentrations were determined on the deproteinized medium from all flasks and glutamine concentrations on the deproteinized medium from those flasks to which glutamine was added. Two additional flasks with substrate were incubated without slices in order to determine nonenzymatic glutamine breakdown from incubation and also from the assay procedure for ammonia. Under the conditions of the experiment, 75 min incubation at 37°C and by the method used to assay ammonia, nonenzymatic glutamine breakdown

approximated less than 2% of the total ammonia formed in the presence of slices incubating in substrate. Thus, the medium from these flasks served as a standard to determine the concentration of glutamine added in each experiment. Results from the four flask pairs were averaged.

Our method of estimating formation of ammonia from the amide and amino nitrogens of glutamine is as follows:

Endogenous Ammonia (ENH <sub>s</sub> )	Ammonia formed by kidney slices in the presence of no substrate.
Total Ammonia (TNH <sub>s</sub> )	Ammonia formed from kidney slices in the presence of substrate glutamine, includes ammonia formed endogenously as well as from substrate.
Substrate Ammonia (SNH <sub>s</sub> )	The ammonia formed by slices from substrate glutamine estimated by subtracting ENH <sub>s</sub> from TNH <sub>s</sub> . This would include ammonia formed from both the amide and amino nitrogens of glutamine.
Amide Ammonia	The maximal amount of ammonia produced from gluta-

TABLE IV  
*Ammonia Formation from Glutamine (2.0 mM) by Rat Kidney Slices in the Presence of Methylene Blue (0.5 mM)*  
( $\mu\text{mol/g}$  per 75 min)

	TNH <sub>s</sub>	ENH <sub>s</sub>	SNH <sub>s</sub>	Amide NH <sub>s</sub>	Amino NH <sub>s</sub>	Amide/amino %
1	90.0 (65.2–101.6)	21.4 (19.0–25.5)	68.6 (41.2–90.0)	50.8 (34.2–63.0)	17.8 (7.0–27.0)	74/26
2	80.2 (75.7– 86.5)	17.8 (16.4–20.9)	62.4 (54.8–69.9)	44.3 (39.3–47.0)	18.1 (8.2–22.9)	71/29
3	74.2 (52.6– 83.3)	22.0 (19.3–26.1)	52.2 (32.0–60.7)	38.6 (26.0–46.6)	13.6 (6.0–19.5)	74/26
4	87.1 (81.1– 93.3)	41.0 (33.7–49.4)	46.1 (37.5–53.5)	33.7 (30.7–37.2)	12.4 (5.4–21.6)	73/27
Ave	82.9*	25.6	57.3*	41.9*	15.5*	73/27

See Table II for details.

\*  $P < 0.01$  compared to controls in Table II.

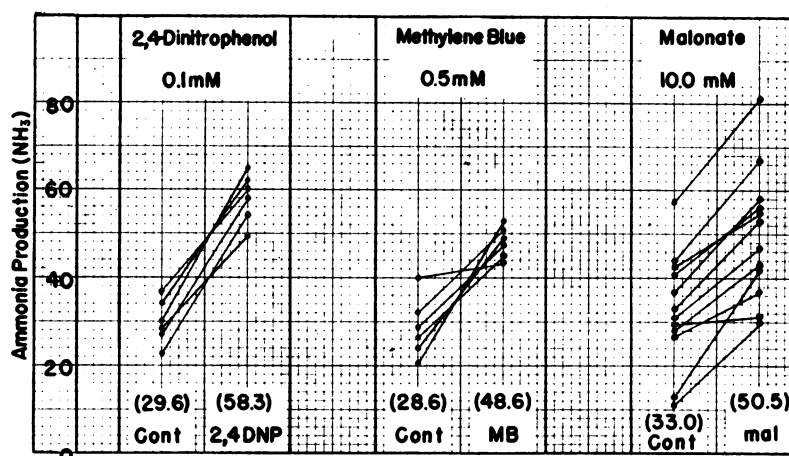


FIGURE 1 Ammonia production in the presence of glutamate. Paired slice halves from rat kidneys are followed in control medium or medium containing 0.1 mM, 2,4-dinitrophenol, 0.5 mM methylene blue, or 10.0 mM malonate. Slice pairs are connected by lines. Average of each group is shown in parentheses. Values are in terms of  $\mu\text{mol/g}$  per 75 min.

#### Amino Ammonia

mine in the medium, calculated from the glutamine added to the flask minus the glutamine remaining at the end of incubation.

Amount of ammonia formed from the amino group of glutamine estimated by subtracting ammonia formed from the amide nitrogen of glutamine from the ammonia formed from glutamine ( $\text{SNH}_3$ ).

Statistics are by group analysis using Student's *t* test.

## RESULTS

*Effects of agents on ammonia production from monosodium glutamate by rat kidney slices.* Achieving the final objective depended on finding agents which would hasten deamination, i.e., removal of glutamate formed as glutamine is deamidated. As a first approximation, we followed the effects of 2,4-dinitrophenol, methylene blue, and malonate on ammonia formation starting with exogenous glutamate as substrate. These studies were performed under the conditions used later for the glutamine studies. The results with all three agents are presented together in Fig. 1. Without exception, every slice half incubated in one of these agents increased deamination above that of its control partner. While the results in both control and test flasks represent endogenous as well as substrate ammonia production ( $\text{TNH}_3$ ), the increases in the test flasks are undoubtedly secondary to a rise in ammonia formation from glutamate itself ( $\text{SNH}_3$ ) as the ensuing studies with glutamine will show that these agents have little effect on endogenous ammonia formation ( $\text{ENH}_3$ ).

*Effect of various agents on phosphate-dependent glutaminase I activity.* Addition of 2,4-dinitrophenol, methylene blue, and malonate in the concentrations used in the slices studies to 2½% kidney homogenates resulted in no apparent stimulation in phosphate-dependent glutaminase activity. The following results were obtained when each assay was run in duplicate: the homogenates alone released  $6.28 \mu\text{mol}$  ammonia/milligram protein per hour from glutamine, and in the presence of 0.1 mM 2,4-dinitrophenol released  $5.90 \mu\text{mol}$  ammonia/milligram protein per hour; with 0.5 mM methylene blue,  $6.01 \mu\text{mol}$ /milligram protein per hour and with 10 mM malonate,  $6.04 \mu\text{mol}$ /milligram per hour. This fulfilled a criterion that the chosen agents themselves should not stimulate phosphate-dependent glutaminase activity directly.

*Mitochondrial swelling studies.* In five control experiments concerned with mitochondrial swelling, the change in optical density (OD) after 30 min at  $37^\circ\text{C}$  in 0.3 M sucrose and 0.02 M Tris (pH 7.4) averaged  $98.4 \pm 4.8$  (SEM) with a range of OD change from 97 to 120. In the presence of 0.1 M 2,4-dinitrophenol, the mitochondrial swelling in two runs was less, OD changes equal 77 and 55. This proved the case also when the concentration of malonate in the medium was 10 mM, OD changes equal 65 and 90.

*Effects of various agents on ammonia production from the amide and amino nitrogens of glutamine by rat kidney slices.* To clarify how we estimated amide and amino ammonia formation from glutamine, the first experiment in the initial series is detailed in Table I. In two flasks incubated without slices but with substrate, the recovery of glutamine after 75 min incubation was  $1.97 \mu\text{mol/ml}$ . (In the first 23 experiments, the

TABLE V  
*Ammonia Formation from Glutamine (2.0 mM) by Rat Kidney Slices in the Presence of Malonate (10.0 mM)*  
( $\mu\text{mol/g}$  per 75 min)

	TNH <sub>3</sub>	ENH <sub>3</sub>	SNH <sub>3</sub>	Amide NH <sub>3</sub>	Amino NH <sub>3</sub>	Amide/amino %
1	138.8 (114.2–171.5)	30.0 (26.0–37.3)	108.8 (92.1–142.9)	57.7 (39.3– 67.2)	51.1 (26.3–75.7)	53/47
2	108.1 (83.8–133.6)	26.4 (17.5–33.2)	81.7 (50.6–105.0)	51.3 (33.9– 70.6)	30.4 (16.7–55.6)	63/37
3	90.3 (61.7–106.1)	14.8 (7.7–17.9)	75.5 (43.8– 89.8)	57.4 (33.3– 73.6)	18.1 (10.5–26.6)	76/24
4	145.9 (118.7–175.5)	21.3 (11.1–27.9)	124.6 (97.1–150.8)	72.3 (51.5–101.3)	52.3 (31.4–79.9)	58/42
Ave	120.8*	23.1	97.7*	59.7*	38.0*	63/37

See Table II for details.

\*  $P < 0.01$  compared to controls in Table II.

average recovery of glutamine after incubation was 2.0  $\mu\text{mol/ml}$  with a range in 1.87  $\mu\text{mol/ml}$ –2.11  $\mu\text{mol/ml}$ .) The total ammonia concentration found in the medium of the four flasks containing glutamine ranged from 0.68 to 0.73  $\mu\text{mol/ml}$ . In the flasks with paired slices of similar weight incubated without substrate the concentrations of ammonia were 0.16–0.27  $\mu\text{mol/ml}$ . Therefore, slices formed ammonia from glutamine to the extent that medium concentration increased anywhere from 0.45 to 0.52  $\mu\text{mol/ml}$ . The glutamine recovered in the medium at the end of incubation was 1.58–1.60  $\mu\text{mol/ml}$ . In the flasks containing slices but not substrate, no glutamine could be measured in the medium after 75 min of incubation. Therefore, this determination was not routinely performed. Based on these results, we estimated that in each milliliter of medium 0.37–0.39  $\mu\text{mol}$  of glutamine amide formed ammonia. To look at it another way, 73%–87% of the ammonia derived from glutamine came from the amide nitrogen. Therefore, 13%–27% must have come from the amino nitrogen of glutamine. Admittedly, these are estimates; however, since little free ammonia in the medium is lost in slice metabolism (4), these estimates seem reasonable. Ammonia production from glutamine in each flask was expressed in terms of  $\mu\text{moles}$  formed per gram wet weight of tissue. Using the percentages calculated above,

the total amount of ammonia formed from the amide and amino nitrogens of glutamine, also were estimated. In Table II, we present data from six control studies. Overall, the ratio of amide to amino ammonia averaged 70%–30% here.

In Table II, IV, and V we present data from the studies with 2,4-dinitrophenol, methylene blue, and malonate. An increase in total ammonia production from glutamine is always seen with the addition of these substances to the incubation media. 2,4-dinitrophenol and methylene blue almost doubled ammoniogenesis and malonate more than tripled ammoniogenesis. While ammonia production from the amino nitrogen of glutamine increased significantly in the presence of each agent, a significant increase in ammonia production from the amide nitrogen was also seen. In the studies using 2,4-dinitrophenol and methylene blue, the ratio of amide ammonia to amino ammonia formation remained close to the 70–30% ratio seen in the control experiments. In the case of 10 mM malonate, amino ammonia production approached 40% of the total. Table VI depicts the results when ammonia production in slices from chronically acidotic rats were studied. Compared to control, these slices produced significantly more ammonia from the amino and amide nitrogens of glutamine.

TABLE VI  
*Ammonia Formation from Glutamine (2.0 mM) by Kidney Slices from Acidotic Rats ( $\mu\text{mol/g}$  per 75 min)*

	TNH <sub>3</sub>	ENH <sub>3</sub>	SNH <sub>3</sub>	Amide NH <sub>3</sub>	Amino NH <sub>3</sub>	Amide/amino %
1	95.3 (71.7–112.6)	26.4 (18.2–39.5)	68.9 (53.5– 88.4)	53.8 (42.8–67.2)	15.1 (10.7–21.2)	78/22
2	126.5 (116.6–146.0)	15.9 (10.1–23.7)	110.6 (102.8–122.3)	64.2 (57.6–73.4)	46.4 (44.9–48.9)	58/42
3	113.5 (104.6–128.0)	24.7 (20.0–28.7)	88.8 (78.5–100.1)	53.3 (39.5–60.0)	35.5 (25.4–40.1)	60/40
4	89.0 (80.5– 98.3)	20.1 (15.4–24.6)	68.9 (60.0– 77.6)	36.5 (28.0–41.1)	32.4 (24.0–37.1)	62/38
Ave	106.1*	21.8	84.3*	52.0*	32.3*	62/38

See Table II for details.

\*  $P < 0.01$  compared to controls in Table II.

We performed a second series of similar experiments almost 1 yr after the first to gain additional information, an estimation of glutamate accumulation which occurred as glutamine was catabolized during incubation.

Results are summarized in Table VII. In these repeat experiments, amide and amino ammonia formation from glutamine was similar to previous results in the control and 2,4-dinitrophenol studies, while in the studies performed with acidotic slices, the values were slightly lower. However, ammonia production from the slices incubating in malonate this time, for some unexplained reason, was only half that previously found. Nevertheless, amide and amino ammonia formation from glutamine by slices increased once more in the presence of malonate compared to the control situation.

These newer studies (Table VII) show that at least 92% of the glutamine metabolized by kidney slices under control conditions is catabolized with the formation of glutamate, i.e. with the disappearance of 18.7  $\mu$ mol glutamine via deamidation, 17.2  $\mu$ mol of glutamate can be accounted for—8.8  $\mu$ mol remaining as glutamate and 8.4  $\mu$ mol of the glutamate deaminated to  $\alpha$  ketoglutarate. The 1.5  $\mu$ mol of catabolized glutamine unaccounted for (8%) might still have formed glutamate which transaminated with ketoacids to form another amino acid such as aspartic acid; or a second possibility, this small amount of glutamine was deamidated via glutaminase II (1) without the formation of glutamate. In the studies performed with malonate and with slices from acidotic rats, again a minimum of 90–99% of the glutamine

TABLE VII  
*Ammonia Production in Kidney Slices from Rats ( $\mu$ mol/g per 90 min)\**

	Amide NH <sub>3</sub>	Amino NH <sub>3</sub>	Glutamate	Amide/Amino	Amino NH <sub>3</sub> + Glutamate
					Amide NH <sub>3</sub>
				%	%
Glutamine (2.0 mM) in control rats					
1	18.2	8.7	7.5	68/32	89
2	16.5	7.4	8.3	69/31	95
3	18.6	6.6	8.7	75/25	82
4	21.7	10.7	10.7	67/33	99
Ave	18.7	8.4	8.8	70/30	92
Glutamine (2.0 mM) + malonate (10.0 mM) in control rats					
1	27.9	17.7	5.6	61/39	81
2	22.6	18.3	7.3	55/45	113
3	26.5	17.5	10.5	60/40	103
Ave	25.7	18.5	7.8	59/41	99
Glutamine (2.0 mM) + 2,4-dinitrophenol (0.1 mM) in control rats					
1	36.0	17.1	8.8	68/32	72
2	40.6	24.5	11.7	62/38	89
3	39.3	17.2	8.2	70/30	64
Ave	38.6	17.2	9.6	67/33	75
Glutamine (2.0 mM) in acidotic rats					
1	34.7	25.5	8.7	58/42	99
2	43.4	23.6	8.8	65/35	75
3	35.3	25.4	8.3	58/42	95
Ave	37.8	24.8	8.7	60/40	90
Glutamine (4.0 mM) in control rats					
1	34.9	15.7	18.2	69/31	97
2	46.7	15.0	14.0	66/34	62
Ave	40.8	15.4	16.1	67/33	80
Glutamine (2.0 mM) and glutamate (10.0 mM)					
1	1.9	10.8			
2	3.4	14.3			
3	0.0	17.7			
Ave	1.8	14.3			

\* Each value is the average result obtained from four observations (two rats).

breakdown resulted in the subsequent formation of glutamate. In the presence of 2,4-dinitrophenol, less of the glutamine catabolized (75%) could be definitely judged to have formed glutamate. Despite the marked increase in glutamine catabolized by acidotic slices and control slices in the presence of malonate and 2,4-dinitrophenol, it is of interest that the glutamate accumulation in the media did not increase significantly above that seen in the control situation—no doubt due greatly to increased deamination.

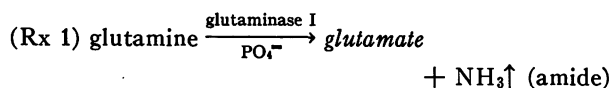
Doubling the concentration of glutamine in the incubation medium to 4 mM produced both more amide and amino ammonia. Of importance, compared to the results in the second series of studies (Table VII), while amide ammonia formation exceeded that of slices incubating in 2 mM glutamine in malonate and 2,4-dinitrophenol and was similar to that formed by acidotic slices, amino ammonia formation was less, and the accumulation of glutamate was greater. These findings become important when considering whether glutamine catabolism is driving glutamate deamination or vice versa. Addition of glutamate 10 mM to slices incubating in 2 mM glutamine produced an increase in amino ammonia formation (14.3  $\mu\text{m/g}$ ) over control but a resounding drop in amide ammonia production (1.8  $\mu\text{m/g}$ ).

## DISCUSSION

That glutamate concentrations at ammonia producing sites within mitochondria might be physiologically important in regulating renal ammoniogenesis is hypothesized because:

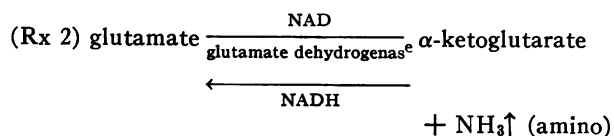
(a) Glutamine is the major precursor of renal ammonia (15, 16).

(b) A primary pathway involved in renal ammoniogenesis is via glutaminase I, an enzyme which is phosphate-dependent and present in mitochondria (17). After glutamine deamidation, glutamate, and ammonia (amide) are formed (Rx 1).



(c) Glutamate inhibits glutaminase I enzyme (7).

(d) Much glutamate formed after deamidation is removed by oxidative deamination through the glutamate dehydrogenase pathway within mitochondria with the formation of a  $\alpha$ -ketoglutarate and ammonia (amino) (Rx 2).



Theoretically, increased removal of glutamate via Rx 2 would lower the concentration of formed glutamate at the deamidating site, would de/inhibit glutaminase I, and would result in greater formation of ammonia from amide nitrogen.

Two observations have been used to support this sequence of events: first, in response to acute or chronic acid challenge, renal tissue concentrations of glutamate decrease, while renal ammoniogenesis increases (18); and second, intraperitoneal injections of glutamate into rats, which supposedly increase renal concentrations of this amino acid, prevent adaptive increases in renal ammoniogenesis in response to acidosis (19). Neither finding lends conclusive support to the theory. An argument lodged against the first finding is that glutamate can effect ammonia production only at ammonia-producing sites, i.e., somewhere within mitochondria. Measurements of cortical or whole tissue glutamate concentrations may not necessarily reflect glutamate concentrations within the mitochondria; thus, it is not too surprising that others cannot corroborate these previous findings and that poor correlation between ammonia production and whole tissue glutamate concentrations have been found (9). As for the second observation, how can one be sure that when injected or infused, glutamate actually reaches ammonia-producing sites? While Goldstein (19) found that after glutamate injections into rats ammonia excretion during acidosis decreased, Churchill and Malvin (8) found that glutamate infused into dogs neither increased nor decreased ammonia production. We have attempted to explain the latter by showing that barriers (actual and relative) to glutamate penetration into ammonia-producing sites probably exist, both in vivo and in vitro (5). If one accepts that barriers exist then extramitochondrial glutamate concentrations may not have a direct regulatory role in ammonia production.

While exogenous glutamate may not penetrate the sites of ammonia production to any extent, glutamate may still accumulate in mitochondria after glutamine deamidation (Rx 1). Since this formed glutamate could regulate further glutamine deamidation, a comparison of mitochondrial concentrations of glutamate and ammonia production would be important. However, because of the techniques of isolating mitochondria, measurements of glutamate concentrations within mitochondria prepared from the kidney might not necessarily represent a true condition. Also, ammonia-producing sites in mitochondria may be contained in subcompartments whose concentrations might not be reflected accurately by measuring total mitochondrial concentrations.

We gathered the data depicted in Tables I–VII in an attempt to answer this important question by using another approach. Kidney slices from control rats form am-

monia from amide nitrogen, principally with the formation of glutamate which may go on to form more ammonia from the amino nitrogen (Tables I, II, and VII). Three agents—2,4-dinitrophenol (4), methylene blue (4), and malonate (11) (Fig. 1), we knew, increased ammonia from exogenous glutamate, but we now found that they enhanced both amide and amino ammonia production from glutamine (Tables III, IV, and V). The key question, which of two possibilities (or two sequences of reactions) explains these latter findings better: first, does increased deamidation (Rx 1) elevate glutamate concentrations resulting in more amino ammonia production (Rx 2); or second, does augmented glutamate deamination (Rx 2) decrease glutamate concentrations at ammonia-producing sites, deinhibit glutaminase I and result in increased amide ammonia formation (Rx 1)? In other words, does reaction 1 drive reaction 2 (Rx 1 > Rx 2) or vice versa (Rx 2 > Rx 1)?

We considered the first possibility. Increased deamidation increases glutamate accumulation and results in enhanced deamination (Rx 1 > Rx 2). That such a chain of events can occur is concluded from comparing data in Tables II and VII. Increasing the concentration of glutamine from 2 mM to 4 mM obviously increases amide ammonia production, amino ammonia formation, and glutamate accumulation via the first set of reactions. Therefore, do our three agents under study as well as acidosis increase deamidation (Rx 1) and by this mechanism lead to increased amino ammonia formation (Rx 2)? To accomplish this our agents must increase Rx 1, and two ways exist to increase glutamine deamidation: (a) these agents and acidosis increase the activity of the isolated enzyme glutaminase I by a direct action or (b) these agents and acidosis increase penetrance of glutamine into the mitochondrial site of ammoniogenesis and hasten Rx 1 by mass action.

While acidosis increases glutaminase I activity (Rx 1) in rats (20) we were unable to show that 2,4-dinitrophenol, malonate, and methylene blue at the concentrations used in the slice studies increase glutaminase I activity directly. Further, this suggests that these agents are not increasing mitochondrial swelling; and thus, possibly enhancing glutamine entrance into mitochondria in this manner. This is based on data presented by Fhaolain and O'Donovan (14) which suggest that phosphate activated glutaminase is dependent on mitochondrial swelling. These two correlated glutaminase activity with mitochondrial swelling and none of our agents in concentrations used in our studies directly stimulated glutaminase I activity even when checked in another laboratory. Also, we like Tapley (21) could not show that malonate or 2,4-dinitrophenol cause mitochondrial swelling. Still could each agent directly increase transport of glutamine into renal cells and directly or

indirectly into mitochondria? We have no answer to this. As far as transport studies go, malonate and 2,4-dinitrophenol have been known to decrease rather than increase other transport systems; for example, 2,4-dinitrophenol and malonate decrease para-aminohippurate transport (22, 23). While these agents may have different and unexplained effects, that is exactly why they were chosen. It seems unlikely that all these agents could enhance glutamine transport into mitochondria. While we have no evidence that our reagents enhance amide and amino ammoniogenesis by the first sequence of reactions, there is a good deal of suggestive data to indicate that they can increase ammonia production from glutamine through the second sequence of reactions (Rx 2 > Rx 1). Although deamidation, when the glutamine concentration was increased to 4 mM (Table VII), approaches that in the 2,4-dinitrophenol, malonate, and acidosis studies in the presence of 2 mM glutamine (Table VII), deamination in the presence of these agents far exceeded that found in the presence of 4 mM glutamine indicating an enhancement of Rx 2 over and above simply supplying more glutamate after deamidation.

This is important since in acidosis increased glutaminase I activity does occur in the rat, and we could explain increased amide and amino ammonia formation as secondary to reaction sequence one. However, the doubling in amino ammonia formation and the lowering of medium glutamate concentrations certainly could not be explained this way, again compared with the results of the studies using 4 mM glutamine (Table VII).

The inability to explain all our data on the basis of the first sequence of reactions, leads us to consider the importance of the second alternative.

While we have no proof that malonate, 2,4-dinitrophenol, and methylene blue enhance glutaminase I activity or glutamine transport, there is much evidence that these agents effect the glutamate dehydrogenase pathway. This is based on the above findings as well as the fact that each agent tested and also acidosis (we shall refer to slice removed from acidotic rats in this manner) augment exogenous glutamate deamination (Rx 2) by kidney slices directly (Fig. 1) (4, 11). Last, addition of 10 mM glutamate to slices incubating in 2.0 mM glutamine, allows deamination to proceed at the same rate as in the 4 mM studies but almost completely halts deamidation. Although measurement of glutamate concentrations in media cannot tell us intramitochondrial concentrations, a final point that must be mentioned is that despite increased glutamine (2.0 mM) catabolism wrought by 2,4-dinitrophenol, malonate, and acidosis, there was no accumulation of glutamate in the incubation medium when compared to control as there was when glutamine concentrations were raised to 4.0 mM.



While we cannot prove that faster deamination occurring in slices from acidotic rats stimulates deamination, it is an intriguing speculation when we compare these results with those using 2,4-dinitrophenol, methylene blue, and malonate (Tables III, IV, V, and VII). When ammonia formation from the amide nitrogen of glutamine is plotted against the ammonia formation from the amino nitrogen of glutamine in all the studies, a positive correlation exists (Fig. 2). Therefore, those slices from the acidotic rats fit into the schema of ammonia production from glutamine in the same manner as those exposed to 2,4-dinitrophenol, methylene blue, and malonate. Excluding the control studies, one can almost imagine a 1:1 correlation between amide and amino ammonia formation when studying Fig. 2. We feel that these findings are consistent with a hypothesis that faster deamination of glutamate during acidosis (16) not only increases formation of amino ammonia but also sparks deamidation to increase amide ammonia formation. One intriguing analogy in support of the above is that guinea pigs that lack an active glutamate dehydrogenase pathway cannot adapt renal ammonia production in response to chronic acid challenge (6).

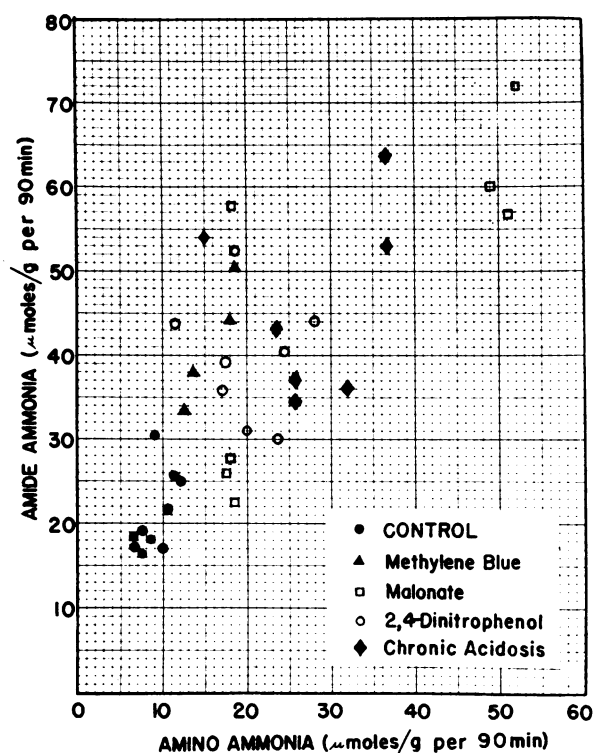


FIGURE 2 Comparison of ammonia production from the amide and amino nitrogens of glutamine. The production of ammonia from the amide nitrogen of glutamine is plotted against the production of ammonia from the amino nitrogen of glutamine in all the studies.

Some of the major fuels through which the kidney cortices derive oxidative energy are lactate, palmitate, and glutamine (2). In the case of the former two, oxidative decarboxylation is involved; in the latter, oxidative deamination precedes oxidative decarboxylation of glutamine's carbon skeleton. Both in dogs and rats, these agents seem to compete for oxidation (3, 4). How may all this relate to augmented renal ammoniogenesis? Since we have felt that the glutamate dehydrogenase pathway may be key to the renal ammonia adaptation, we have postulated that oxidative deamination through the glutamate dehydrogenase pathway could compete more successively during acid challenge with the oxidative decarboxylation of lactate and/or palmitate or for that matter other Krebs' cycle reactions which require oxidation. The possible ways this might occur have been summarized previously (4).

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