

# Pathways of Glutamine and Organic Acid Metabolism in Renal Cortex in Chronic Metabolic Acidosis

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**ABSTRACT** The metabolism of labeled glutamine and of several labeled organic acid anions was compared in tissue slices of renal cortex from chronically acidotic and alkalotic littermate dogs.  $^{15}\text{NH}_3$  formation and  $^{15}\text{N}$ -amide-glutamine utilization were significantly increased by slices from acidotic animals providing further evidence for the similarity of the metabolic responses seen in the tissue slice system and the physiologic effects produced by chronic metabolic acidosis on renal metabolism in the intact animal. Slices from acidotic dogs formed more  $^{14}\text{CO}_2$  and glucose- $^{14}\text{C}$  than did slices from alkalotic animals when labeled glutamine, citrate, or malate was used as substrate but  $^{14}\text{CO}_2$  production from pyruvate-1- $^{14}\text{C}$  was slightly reduced in acidotic tissue. With most of the substrates used glucose- $^{14}\text{C}$  formation was small compared with  $^{14}\text{CO}_2$  formation. Using the amount of glucose- $^{14}\text{C}$  formed, the expected  $^{14}\text{CO}_2$  production was calculated based on the hypothesis that the primary site of action of metabolic acidosis is on a cytoplasmic step in gluconeogenesis. The actual difference in  $^{14}\text{CO}_2$  production between slices from acidotic and alkalotic animals always greatly exceeded this predicted amount, indicating that stimulation of gluconeogenesis represents a minor metabolic response to chronic metabolic acidosis. Evidence from experiments with citrate labeled in various positions showed that metabolic acidosis has its principal effect on an early step in substrate metabolism which must be intramitochondrial in location.

## INTRODUCTION

In chronic metabolic acidosis renal utilization of glutamine is increased, and increased amounts of  $\text{NH}_3$  are

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formed from the amide and amine groups of glutamine (1, 2). The resulting increase in the renal  $\text{NH}_3$ - $\text{NH}_4^+$  pool causes more  $\text{NH}_3$  to enter the tubular fluid where it combines with secreted hydrogen ions to form  $\text{NH}_4^+$ , enabling the kidney to excrete more  $\text{H}^+$  in order to maintain acid-base homeostasis (3). This metabolic alteration in acidosis is accompanied by decreased concentrations of glutamine and glutamate and of several citric acid cycle intermediates in the renal cortex of the intact animal (1, 4, 5), indicating that the biochemical response is more widespread than a direct effect on glutamine utilization alone. Previous *in vitro* experiments have shown that  $\text{CO}_2$  formation and glucose synthesis from glutamine are accelerated in tissue slices obtained from chronically acidotic animals (6-8); however the amounts of these two products of glutamine metabolism have not been directly compared. In the present investigation formation of labeled  $\text{CO}_2$ , glucose, and  $\text{NH}_3$  from  $^{14}\text{C}$ - and  $^{15}\text{N}$ -labeled glutamine and from  $^{14}\text{C}$ -labeled organic acid substrates has been measured in tissue slices from chronically acidotic and alkalotic dogs in order to assess the relative contributions of various metabolic pathways to the increased substrate metabolism stimulated by metabolic acidosis.

The results show that slices of renal cortex from acidotic animals utilize more glutamine and form more  $\text{NH}_3$ ,  $\text{CO}_2$ , and glucose from this amino acid than do slices from alkalotic animals. In addition  $\text{CO}_2$  production and glucose formation by acidotic slices are increased when citrate or malate is used as substrate, but pyruvate oxidation to  $\text{CO}_2$  is slightly decreased. The relative magnitude of  $\text{CO}_2$  and glucose formation from any of the substrates tested indicates that glucose formation is a minor pathway of metabolism from these substrates since the quantity of carbon atoms converted to  $\text{CO}_2$  greatly exceeds the amount incorporated into glucose. The results also indicate that the primary site at which chronic acid-base changes act to regulate renal metabolism is

intramitochondrial in location; although increased formation of phosphoenolpyruvate (PEP)<sup>1</sup> in the cytoplasm undoubtedly occurs in chronic metabolic acidosis, this change by itself cannot account for the observed changes in CO<sub>2</sub> formation.

## METHODS

**Incubation technique.** Pairs of littermate dogs were prepared for 9–14 days before sacrifice by feeding 8–15 mEq/kg NH<sub>4</sub>Cl to one animal and 18 mEq/kg NaHCO<sub>3</sub> to the other. Plasma bicarbonate levels were measured several days before sacrifice and on the day of sacrifice. In the NH<sub>4</sub>Cl-treated animals [HCO<sub>3</sub><sup>-</sup>]<sub>p</sub> averaged 15 mEq/liter and was always less than 18 mEq/liter; in the NaHCO<sub>3</sub>-treated animals the average value was 27 mEq/liter and the minimum was 23 mEq/liter. Tissue was prepared for incubation as previously described (8). 100 mg renal cortical slices were incubated in 2.0 ml medium containing 107 mM NaCl, 20 mM NaHCO<sub>3</sub>, 10 mM KCl, 1 mM sodium phosphate, 1.25 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 2 mM Na malate, 2 mM Na pyruvate, 0.5 mM Na citrate, and 0.5 mM glutamine. Incubation was carried out in 25-ml Erlenmeyer flasks closed with a serum stopper which supported a plastic cup (Kontes Glass Co., Vineland, N. J.). Before adding the tissue the flask was warmed to 37°C, and gassed with 96% O<sub>2</sub>, 4% CO<sub>2</sub>. At the start of incubation the gassing was discontinued, the flask briefly opened, the tissue inserted, and the flask quickly closed. The flasks were incubated at 37°C in a reciprocating water bath shaker for 15 min. The experiment was terminated by injecting 0.5 ml of 1.0 N perchloric acid through the serum stopper into the medium.

**<sup>14</sup>C analysis.** When <sup>14</sup>C-labeled substrates were used 0.25 ml ethanolamine was added at the end of incubation to the suspended plastic cup and CO<sub>2</sub> absorbed for 2 hr while shaking was continued. The ethanolamine was then quantitatively transferred to a 10 ml volumetric flask, and diluted with ethanolamine. 1 ml of this solution was transferred to a glass vial, 10 ml of scintillation solution was added, and the radioactivity measured by liquid scintillation counting; counting efficiency was determined by adding toluene-<sup>14</sup>C internal standard. Scintillation solution contained 800 ml toluene, 200 ml methyl-Cellosolve (Union Carbide Corp., New York), 4.8 g 2,5-diphenyloxazole (PPO), and 0.4 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP), per liter; all reagents were scintillation grade.

**Glucose-<sup>14</sup>C analysis.** Several techniques for isolation of radioactive glucose were tried, but initially none proved satisfactory for the purposes of these experiments. In particular the standard phenylglucosazone technique provided capricious yields and the highly colored derivatives resulted in extremely low counting efficiencies. On the other hand, isolation of the potassium gluconate salt provided good, reproducible yields of a colorless compound which could be counted with high efficiency; however when glutamine-<sup>14</sup>C or citrate-<sup>14</sup>C was used in the medium, serious contamination of the gluconate by these radioactive compounds occurred and could not be eliminated by repeated crystallizations. In order to overcome this drawback cations and anions were removed from the medium before formation of the gluconate by passage through ion exchange columns as described below.

<sup>1</sup> Abbreviations used in this paper: dimethyl-POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PPO, 2,5-diphenyloxazole.

After absorption of the CO<sub>2</sub>, 2.0 ml of acidified medium was removed from the incubation flask and the pH carefully adjusted to 5.5–6.0 with 1 N KOH. The solution was then diluted with water to 5 ml in a volumetric flask, chilled in ice, and centrifuged at 2°C to remove the potassium perchlorate precipitate. 100 mg carrier glucose was added to 2.0 ml of the supernate and the solution acidified to pH 2.0–2.5 with 1 N HCl and placed on a 0.9 cm diameter column of Dowex (Bio-Rad Labs, Richmond, Calif.) 50 × 8, 200–400 mesh in 0.2 M citrate buffer, pH 3.1; a 2 cm column height was used when <sup>14</sup>C-labeled organic acid substrates were used and 5 cm column height was used with glutamine-<sup>14</sup>C. Two 1 ml rinses of 0.01 N HCl were used to transfer the solution to the column and the column was then washed with 4 ml 0.01 N HCl. The entire column effluent was collected, the pH adjusted with 1 N KOH to 6.0, and the solution placed on a 0.9 × 5 cm Dowex 1 × 8, 200–400 mesh formate column. Two 2-ml rinses with water were used for transfer and the column then rinsed with an additional 10 ml water. The entire effluent was collected in a small evaporating dish and dried over P<sub>2</sub>O<sub>5</sub> in a vacuum oven at 40°C, 40 mm Hg. The potassium gluconate derivative was then prepared and recrystallized twice as described by Blair and Segal (9). 40 mg of the dried gluconate were placed in a counting vial and 20 ml of scintillation solution added; the latter contained 750 ml toluene, 250 ml Triton X-100, 4.5 g PPO, 0.375 g dimethyl-POPOP per liter.

**<sup>15</sup>NH<sub>3</sub> assay.** When <sup>15</sup>N-amide-glutamine was used in the incubation medium, <sup>15</sup>NH<sub>3</sub> and glutamine-<sup>15</sup>N were measured in a mass spectrometer using an adaptation of the method of Rittenberg (10). This method enables the estimation of 0.1 μmoles or more of <sup>15</sup>NH<sub>3</sub>, extending the range of the mass spectrometer to a level practical for <sup>15</sup>N measurements in *in vitro* metabolic experiments. Although the results are sufficiently precise to demonstrate significant differences in NH<sub>3</sub> production and glutamine utilization in the experiments to be reported, certain methodologic problems persist which limit the precision of the technique compared to measurements of <sup>14</sup>C-labeled compounds. One of these problems which is especially pertinent to the present study is the instability of glutamine in either acid or alkaline solutions (11). In our experiments the medium was acidified with perchloric acid to kill the tissue and incubation continued in order to absorb NH<sub>3</sub> from the gas phase of the flask. During this absorption period, some deamidation of glutamine to pyrrolidone carboxylic acid occurred resulting in release of <sup>15</sup>NH<sub>3</sub>. Despite inclusion of control flasks to estimate the contribution of this nonmetabolic <sup>15</sup>NH<sub>3</sub>, variations in the amount of glutamine breakdown during ammonia absorption undoubtedly contributed to fluctuations in the final <sup>15</sup>NH<sub>3</sub> measurements. Another problem encountered was the occasional appearance of abnormal peaks in the mass spectrometer tracing. Some of these could be traced to contamination of the vapor leaving the flasks with sodium hydroxide, but other randomly generated fluctuations still occasionally resulted in mass 29 peaks of excessive heights. The method finally adopted by us is as follows:

The L-glutamine in the medium described in the section on incubation technique was replaced by <sup>15</sup>N-amide-L-glutamine, 95 atoms per cent excess, 0.5 μmoles/ml. Three flasks were prepared for each tissue with this medium and a fourth flask containing no glutamine-<sup>15</sup>N in the medium was also set up as a control. After incubation the reaction was terminated in the usual manner with perchloric acid; 1.0 μmole glutamine-<sup>15</sup>N was then added immediately to the control flask. Shaking of the flasks continued for an hour in order

TABLE I  
Comparison of  $^{15}\text{NH}_3$  and  $^{14}\text{CO}_2$  Production and Glutamine- $^{15}\text{N}$  Utilization by Slices of Dog Renal Cortex

Incubation time	Flask No.	$^{15}\text{NH}_3$ produced	Glutamine- $^{15}\text{N}$ used	$^{14}\text{CO}_2$ produced
		$\mu\text{moles}$	$\mu\text{moles}$	$\text{dpm} \times 10^3$
10 min	1	0.177	0.199	14.3
	2	0.203	0.234	16.1
	3	0.199	0.234	16.2
Average		0.193	0.222	15.5
20 min	4	0.285	0.347	29.6
	5	0.304	0.324	27.8
	6	0.347	0.395	34.4
Average		0.312	0.355	30.6
0	Control	0.023	0.890	

Each flask except the control contained 1.0  $\mu\text{mole}$   $^{15}\text{N}$ -amide-L-glutamine, 95 atoms per cent excess, and  $465 \times 10^3$  dpm L-glutamine-U- $^{14}\text{C}$  at the start of incubation. 1.0  $\mu\text{mole}$  of glutamine- $^{15}\text{N}$  was added at the end of the incubation period to the control flask, which was otherwise identical to the other flasks.

to permit  $\text{NH}_3$  absorption from the gas phase. The flasks were then opened and 2.0 ml of acidified medium removed, neutralized, diluted, chilled, and centrifuged as described in the section on glucose- $^{14}\text{C}$  analysis. A portion of this solution was placed on a small column of Dowex 50 and the  $\text{NH}_4^+$  and glutamine separated as previously described except that the  $\text{NH}_4^+$  was eluted from the column with 2.0 ml 0.1 N NaOH and collected in 0.4 ml of 1 N HCl (12). 5.0  $\mu\text{moles}$  of carrier  $\text{NH}_4\text{Cl}$  were added to the eluate and the solution transferred to a gas conversion tube (Eck and Krebs Inc., Long Island City, N. Y.) for eventual oxidation of the  $\text{NH}_3$  to  $\text{N}_2$  with hypobromite (10). After evacuation and freezing of the ammonium solution, the tubes were transferred to a manifold connected to an MS-103 mass spectrometer (Consolidated Engineering Co., Pasadena, California), the output of which was recorded on a 1185 Mk2 ultraviolet recorder (Honeywell, Fort Washington, Pa.). The tracing obtained was evaluated by measuring the height of the mass 28, 29, and 30 peaks. Total  $^{15}\text{N}$  was obtained as the sum of the mass 29 peak plus twice the mass 30 peak;  $^{14}\text{N}$  was similarly calculated as the sum of the mass 29 peak plus twice the mass 28 peak. The amount of  $^{15}\text{N}$  present in the sample was then determined by comparing the  $^{15}\text{N}:^{14}\text{N}$  ratio for the sample to the ratios obtained from a standard curve run with each set of samples using  $^{15}\text{NH}_4\text{Cl}$ , 95 atoms per cent excess; the samples for the standard were also passed through Dowex 50 columns and handled in exactly the same manner as the experimental samples themselves.

For determination of the glutamine- $^{15}\text{N}$  remaining in the medium after incubation, the eluate from the Dowex 50 columns obtained before elution with sodium hydroxide was collected in 0.5 ml sodium acetate buffer, pH 4.9, and incubated with 0.04 ml of glutaminase solution, 4 mg/ml, for 30 min at 37°C. The  $^{15}\text{NH}_3$  produced was absorbed on a second Dowex 50 column as previously described and the procedure for the  $^{15}\text{N}$  assay described in the preceding paragraph was

then repeated. A standard curve using known amounts of  $^{15}\text{N}$ -amide-glutamine was run with each set of samples.

Table I shows a control experiment in which  $^{15}\text{NH}_3$  production and glutamine- $^{15}\text{N}$  utilization by slices of dog renal cortex were measured after 10- and 20-min incubation periods. In this experiment glutamine-U- $^{14}\text{C}$  was also added to the medium and the  $^{14}\text{CO}_2$  produced during the incubation was determined. The results indicate the validity and precision of the methods used for determining  $^{15}\text{NH}_3$  and glutamine- $^{15}\text{N}$ .

**Materials.**  $^{14}\text{C}$ -labeled isotopes were obtained from New England Nuclear Corp., Boston, Mass., except for malic-4- $^{14}\text{C}$  acid, obtained from Calbiochem, Los Angeles, Calif.  $^{15}\text{N}$ -amide-L-glutamine, 95 atoms per cent excess and  $^{15}\text{NH}_4\text{Cl}$ , 95 atoms per cent excess were purchased from Isotopes, a Teledyne Co., Westwood, N. J. *Escherichia coli* glutaminase was obtained from Worthington Biochemical Corp., Freehold, N. J. Unlabeled glutamine and organic acid substrates were Calbiochem, A grade, products. Ion exchange resins were products of Bio-Rad Labs, Richmond, Calif.

## RESULTS

*Glutamine metabolism in chronic metabolic acidosis and alkalosis.* Previously we reported that when slices of renal cortex were incubated with L-glutamine-U- $^{14}\text{C}$  more  $^{14}\text{CO}_2$  was produced by tissue from acidotic than from alkalotic dogs (8). The experiments shown in Table II again demonstrate this effect with increases in  $^{14}\text{CO}_2$  production by the acidotic tissue of 46, 38, and 82% being recorded in the three experiments shown. Table II also shows that  $^{14}\text{C}$  incorporation into glucose was simi-

TABLE II  
 $\text{CO}_2$  and Glucose Formation from Glutamine-U- $^{14}\text{C}$  by Slices of Renal Cortex from Littermate Dogs with Chronic Metabolic Acidosis or Alkalosis

Systemic acid-base state	Flask No.	$^{14}\text{CO}_2$		Glucose- $^{14}\text{C}$	
		$\text{dpm} \times 10^{-3}$	Average	$\text{dpm} \times 10^{-3}$	Average
Experiment 1. $4.58 \times 10^6$ dpm per flask					
Acidotic	1	478	496	169	176
	2	513		183	
Alkalotic	3	355	340	120	125
	4	324		130	
	Difference		156		51
Experiment 2. $4.08 \times 10^6$ dpm per flask					
Acidotic	1	426	423	100	98
	2	419		96	
Alkalotic	3	296	307	51	53
	4	318		54	
	Difference		116		45
Experiment 3. $4.02 \times 10^6$ dpm per flask					
Acidotic	1	621	598	168	178
	2	575		188	
Alkalotic	3	330	332	67	67
	4	334		67	
	Difference		266		111

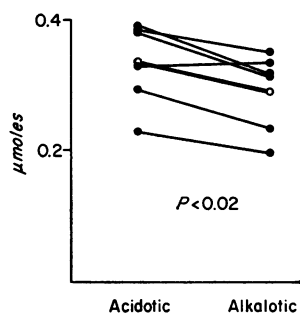


FIGURE 1  $^{15}\text{NH}_3$  production from  $^{15}\text{N}$ -amide-glutamine by slices of renal cortex from pairs of littermate dogs with chronic metabolic acidosis or alkalosis.  $1.0\ \mu\text{mole}$  labeled glutamine ( $95\%$   $^{15}\text{N}$  excess) was present in  $2.0\ \text{ml}$  medium at the start of the experiment. Incubation time was  $16\ \text{min}$ . The open circles represent the average  $^{15}\text{NH}_3$  production from the six experiments shown. Each closed circle is the mean  $^{15}\text{NH}_3$  produced by three flasks containing slices of renal cortex from a single dog and corrected for nonmetabolic  $^{15}\text{NH}_3$  production from glutamine.

larly enhanced with 40, 85, and 166% more dpm present in the glucose formed by the acidotic slices in the three examples. In the first experiment  $156 \times 10^3$  more dpm were present in  $\text{CO}_2$  from the acidotic slices than in that from the alkalotic ones and  $51 \times 10^3$  more dpm were present in glucose. The ratio of the increase in dpm in  $\text{CO}_2$  to that in glucose is thus 3:1; in the second and third experiments this ratio is 2.5:1. These ratios indicate that acidosis stimulated the conversion of far more molecules of glutamine to  $\text{CO}_2$  than to glucose (see Discussion).

Figs. 1 and 2 show the results of six experiments in which  $^{15}\text{NH}_3$  production and glutamine- $^{15}\text{N}$  utilization were measured in slices of renal cortex obtained from

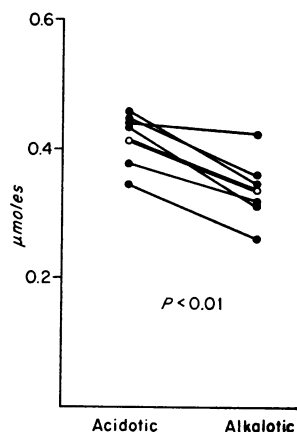


FIGURE 2  $^{15}\text{N}$ -amide-glutamine utilization in six experiments described in Fig. 1. Each closed circle is the mean glutamine- $^{15}\text{N}$  utilization in three flasks in a single experiment.

pairs of acidotic and alkalotic littermate dogs. In Fig. 1 each point represents the average of  $^{15}\text{NH}_3$  determinations on three flasks minus the  $^{15}\text{NH}_3$  formed in a control flask. Glutamine- $^{15}\text{N}$  utilization was calculated by subtracting the measured amount of glutamine- $^{15}\text{N}$  remaining from the amount initially present ( $0.95\ \mu\text{moles}$  per flask); each point in Fig. 2 is the average of glutamine- $^{15}\text{N}$  measurements from three flasks. Glutamine deamidation was significantly greater ( $P < 0.02$ ) in the slices from acidotic dogs (Fig. 1) and glutamine utilization was increased ( $P < 0.01$ ) in these slices (Fig. 2). The magnitude of the effect on  $^{15}\text{NH}_3$  was somewhat less than that obtained with  $^{14}\text{CO}_2$  measurements, which may reflect primarily the much lower precision of the  $^{15}\text{NH}_3$  analytical method compared to that for  $^{14}\text{CO}_2$ . However, the average amount of glutamine- $^{15}\text{N}$  utilized conformed closely to the  $^{15}\text{NH}_3$  produced, providing a further check on the validity of the  $^{15}\text{N}$  measurements.

These results demonstrate that glutamine utilization and  $\text{CO}_2$ , glucose, and  $\text{NH}_3$  formation from glutamine are all increased in tissue slices from renal cortex of chronically acidotic dogs. Thus this system accurately reproduces in vitro the physiologic effects produced by metabolic acidosis in the intact animal on glutamine utilization and  $\text{NH}_3$  formation.

**Citrate- $^{14}\text{C}$  metabolism.** Recently it has been shown that citrate-1,5- $^{14}\text{C}$  oxidation to  $\text{CO}_2$ , like that of glutamine, is increased in slices of renal cortex from acidotic rats (13). Table III confirms this observation in dog tissue and indicates increased  $^{14}\text{C}$  incorporation into glucose in this situation. In the first experiment shown  $102 \times 10^3$  more dpm were incorporated into  $\text{CO}_2$  by the acidotic than by the alkalotic slices, an increase of 60%; in the second experiment  $^{14}\text{CO}_2$  formation increased by 117% in the acidotic tissue. Acidosis increased glucose- $^{14}\text{C}$  formation by  $6.7 \times 10^3$  dpm or 92% in the first experiment and by  $5.4 \times 10^3$  dpm or 174% in the second. The ratio of the increase in  $^{14}\text{C}$  in  $\text{CO}_2$  to that in glucose stimulated by acidosis in the two experiments was 15:1 and 19:1, again indicating many times more  $\text{CO}_2$  than glucose formed from the substrate.

Citrate-6- $^{14}\text{C}$  metabolism was also measured in tissue from the same animals used for the citrate-1,5- $^{14}\text{C}$  studies (Experiment 1, Table III). Oxidation to  $\text{CO}_2$  was increased by  $58 \times 10^3$  dpm, or 58%, in the acidotic slices in this experiment.  $^{14}\text{C}$  incorporation into glucose was very low with this isotopic form of citrate since only citrate acted upon by cytoplasmic citrate lyase will lead to  $^{14}\text{C}$  in glucose when citrate-6- $^{14}\text{C}$  is metabolized (see Discussion). Thus less than 3% as many dpm were present in glucose as in  $\text{CO}_2$ . However, acidosis still caused an increase of over 75% in the dpm present in glucose.

In the second experiment shown in Table III citrate-1,5- $^{14}\text{C}$  and citrate-2,4- $^{14}\text{C}$  metabolism is compared. The

TABLE III  
*CO<sub>2</sub> and Glucose Formation from Citrate-<sup>14</sup>C by Slices of Renal Cortex from Littermate Dogs with Chronic Metabolic Acidosis or Alkalosis*

Labeled substrate	Systemic acid-base state	Flask No.	<sup>14</sup> CO <sub>2</sub>			Glucose- <sup>14</sup> C	
			dpm × 10 <sup>-3</sup>	Average	R*	dpm × 10 <sup>-3</sup>	Average
Experiment 1							
Citrate-1, 5- <sup>14</sup> C 7.72 × 10 <sup>6</sup> dpm per flask	Acidosis	1	272	270	13	12.7	14.0
		2	267			15.3	
	Alkalosis	3	171	168		7.2	7.3
		4	166	102		7.4	6.7
Difference							
Citrate-6- <sup>14</sup> C 2.94 × 10 <sup>6</sup> dpm per flask	Acidosis	5	164	158	20	3.6	3.4
		6	151			3.2	
	Alkalosis	7	100	100		1.7	1.9
		8	100	58		2.0	1.5
Difference							
Experiment 2							
Citrate-1, 5- <sup>14</sup> C 4.21 × 10 <sup>6</sup> dpm per flask	Acidosis	1	183	187	24.0	9.2	8.5
		2	191			7.8	
	Alkalosis	3	84.2	86		2.9	3.1
		4	87.3	101		3.3	5.4
Difference							
Citrate-2, 4- <sup>14</sup> C 5.68 × 10 <sup>6</sup> dpm per flask	Acidosis	5	35.2	36.3	4.0	68.0	75.3
		6	37.5			82.6	
	Alkalosis	7	13.9	14.0		22.7	23.9
		8	14.0	22.3		25.2	51.4
Difference							

$$* R = \frac{\text{dpm in CO}_2}{\text{total dpm in flask}} \times 10^3.$$

difference between <sup>14</sup>CO<sub>2</sub> production in acidotic and in alkalotic tissue was 101 × 10<sup>3</sup> dpm when the former compound was used as substrate and 22.3 × 10<sup>3</sup> dpm with the latter compound. These values must be divided by the total dpm in each flask in order to allow comparison between results with the two different substrates. The results of this calculation, shown in column R, indicate the relative effects of acidosis on the metabolism of these labeled citrate compounds. Acidosis stimulated the formation of six times as much <sup>14</sup>CO<sub>2</sub> from citrate-1,5-<sup>14</sup>C as from citrate-2,4-<sup>14</sup>C. As described in the Discussion this result is useful in determining the approximate location of the major site of action of metabolic acidosis on renal metabolism.

*Malate-4-<sup>14</sup>C metabolism.* Table IV shows that metabolism of malate like that of glutamine and citrate is increased in slices from acidotic dog kidney, both <sup>14</sup>CO<sub>2</sub> and glucose-<sup>14</sup>C production being enhanced. Acidosis increased CO<sub>2</sub> labeling by 200 × 10<sup>3</sup> and 332 × 10<sup>3</sup> dpm, or 43 and 62%, in the two experiments shown. <sup>14</sup>C incorporation into glucose was greater by 40 × 10<sup>3</sup> and 61 × 10<sup>3</sup> dpm in the acidotic slices, giving percentage increases of 71 and 94%. The ratio of <sup>14</sup>C in CO<sub>2</sub> to that in glucose was about 5:1 in both experiments.

*Pyruvate-1-<sup>14</sup>C metabolism.* The above experiments indicate increase in conversion of glutamine, citrate, and malate to CO<sub>2</sub> and glucose by slices from acidotic animals. In contrast oxidation of pyruvate-1-<sup>14</sup>C to <sup>14</sup>CO<sub>2</sub> shown in Table V, was not increased in acidotic

TABLE IV  
*Effect of Chronic Metabolic Acidosis and Alkalosis on CO<sub>2</sub> and Glucose Formation by Slices of Dog Renal Cortex from Malate-4-<sup>14</sup>C*

Systemic acid-base state	Flask No.	<sup>14</sup> CO <sub>2</sub>		Glucose- <sup>14</sup> C	
		dpm × 10 <sup>-3</sup>	Average	dpm × 10 <sup>-3</sup>	Average
Experiment 1. 6.43 × 10 <sup>6</sup> dpm per flask					
Acidotic	1	673	696	94.3	96.1
	2	719		98.0	
Alkalotic	3	505	496	57.9	55.8
	4	487	200	53.7	40.3
Difference					
Experiment 2. 6.21 × 10 <sup>6</sup> dpm per flask					
Acidotic	1	828	864	124	126
	2	900		179	
Alkalotic	3	543	542	73	65
	4	540	332	57	61
Difference					

TABLE V  
*CO<sub>2</sub> and Glucose Formation from Pyruvate-1-<sup>14</sup>C by Slices of Renal Cortex from Littermate Dogs with Chronic Metabolic Acidosis or Alkalosis*

Systemic acid-base balance	Flask No.	<sup>14</sup> CO <sub>2</sub>		Glucose- <sup>14</sup> C	
		dpm × 10 <sup>-3</sup>	Average	dpm × 10 <sup>-3</sup>	Average
Experiment 1. 5.54 × 10 <sup>6</sup> dpm per flask					
Acidotic	1	549	558	17.7	17.8
	2	567		17.9	
Alkalotic	3	713	694	15.5	15.0
	4	675		14.5	
Difference		-136		2.8	
Experiment 2. 5.64 × 10 <sup>-4</sup> dpm per flask					
Acidotic	1	642	617	40.7	33.8
	2	592		27.0	
Alkalotic	3	607	627	14.1	15.0
	4	648		15.9	
Difference		-10		18.8	
Experiment 3. 2.19 × 10 <sup>6</sup> dpm per flask					
Acidotic	1	253	247	5.24	5.30
	2	240		5.35	
Alkalotic	3	296	300	2.90	2.90
	4	304			
Difference		-53		2.40	

kidney tissue; in fact <sup>14</sup>CO<sub>2</sub> formation by acidotic slices was slightly lower than that by alkalotic tissue. Incorporation of label into glucose was still increased by acidosis although the total number of dpm incorporated was small. In the first experiment in Table V <sup>14</sup>CO<sub>2</sub> formation by the acidotic slices was 20% lower than in the alkalotic slices; glucose-<sup>14</sup>C formation was increased about 20% by acidosis, but total dpm in glucose represented about 3% or less of the total dpm in CO<sub>2</sub>. Similar results were obtained in the other two experiments shown except that the difference between <sup>14</sup>CO<sub>2</sub> formation by acidotic and alkalotic slices was negligible in the second experiment.

## DISCUSSION

Attempts to find a biochemical explanation for the increase in renal glutamine utilization and NH<sub>3</sub> production produced by metabolic acidosis have resulted in several previous theories regarding this regulatory phenomenon. The first of these was based on the observation that glutaminase levels were increased in the kidney of acidotic rats (14-16), a possibility which had the attraction of providing a regulatory site at the initial step in glutamine metabolism. However, several observations have made glutaminase induction an unlikely cause for the effect of acidosis on glutamine metabolism. No increase in glutaminase occurs in dog kidney in acidosis (17, 18), although increased glutamine utilization occurs in this situation in dog as well as rat, and glutaminase induction in the rat can be blocked

with actinomycin D without interfering with the increased ammonium excretion which accompanies metabolic acidosis (19, 20). Finally, the decreased cortical concentrations of glutamate, and other substances demonstrated in acidotic dog and rat kidney (1, 4, 5) cannot be accounted for by the glutaminase hypothesis.

The theory which has enjoyed most favor recently is that the primary site of regulation of renal metabolism in response to acid-base change is in the pathway of gluconeogenesis, a hypothesis based on observations that glucose synthesis from glutamine and citric acid cycle substrates is increased in tissue slices of acidotic rats (6, 7). Evidence that phosphoenolpyruvate carboxykinase (PEPCK) levels in the cytoplasm are increased in metabolic acidosis in renal cortex of rat (21, 22) and dog<sup>a</sup> has suggested a specific site at which regulation of gluconeogenesis may occur. This theory suggests that increased conversion of cytoplasmic oxalacetate to phosphoenolpyruvate (PEP) occurs in acidosis and that this "drags" citric acid cycle intermediates out of mitochondria, producing decreased levels of various substrates inside mitochondria; the decrease in glutamate level thus produced then increases by mass action the utilization of glutamine by the mitochondria. While there is considerable evidence which makes this action-at-a-distance concept at least a plausible basis for the increase in glutamine utilization in acidosis, some recent results suggest the need for investigation of other alternatives. For example, increased net renal glucose production either does not occur in the intact kidney of the acidotic dog or occurs only to a very limited extent (23-26). Species differences in the metabolic response to acidosis do exist, as noted in the preceding paragraph, and it is possible that the dog is unique in its response to acidosis. However in vitro both dog (7, 28) and rat (6, 27) respond to acidosis by increasing glucose formation from glutamine. Little information is currently available on in vivo changes in gluconeogenesis in the rat during chronic acidosis. However, even if gluconeogenesis is small in this situation, PEPCK could still be the regulatory site, as discussed below, if the PEP formed was largely converted to pyruvate and subsequently metabolized intramitochondrially.

The above discussion indicates that the major site at which acid-base changes exert their effect on renal metabolism is still uncertain. In previous work we showed that slices of renal cortex from chronically acidotic dogs are able to oxidize more glutamine-<sup>14</sup>C to CO<sub>2</sub> than are slices from alkalotic littermates (8). In the present study we have shown that NH<sub>3</sub> production from the amide group of glutamine as well as glutamine-<sup>15</sup>N utilization is increased in slices from acidotic

<sup>a</sup> Simpson, D. P. Unpublished observation.

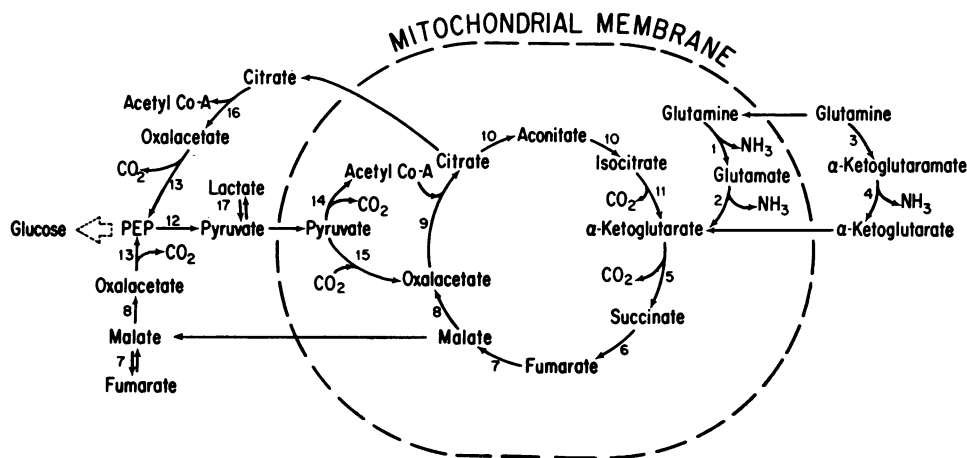


FIGURE 3 Major intermediates, products, and pathways of metabolism of glutamine and citric acid cycle compounds within mitochondria and in the cytoplasm. Numbers designate enzymes referred to in the text.

animals, providing further documentation that this *in vitro* system retains the essential characteristics of the *in vivo* phenomenon under investigation. We have used this system to study the effect of metabolic acidosis on the utilization of various labeled metabolic intermediates in order to obtain stoichiometric data on the conversion of these compounds to  $\text{CO}_2$  and glucose. By the use of labels in various positions the contribution of certain metabolic pathways to the effect of metabolic acidosis on renal metabolism can be defined.

With respect to the effect of acidosis on metabolism of various substrates, the results of these experiments show that oxidation of glutamine, citrate, or malate to  $\text{CO}_2$  and to glucose is increased by acidosis, but oxidation of pyruvate is slightly decreased. Another recent study indicates that formation of glucose is also enhanced by slices from acidotic dogs when  $\alpha$ -ketoglutarate is used as substrate (28). These findings can be compared with the results obtained on intact tissue when concentrations of these and other intermediates were measured in control and acidotic rats. The levels of citrate,  $\alpha$ -ketoglutarate, malate, and glutamine in renal cortex are significantly lower in acidotic animals, whereas those of pyruvate are unchanged and those of PEP are increased (1, 4, 5). Thus the substrate specificities are similar in the *in vivo* and *in vitro* systems. It is also of interest to compare the results of this study with the effect of acute acid-base changes on mitochondrial substrate metabolism. In previous investigations (8, 29) we noted that decreasing pH and  $[\text{HCO}_3^-]$  in the medium stimulated the utilization by renal mitochondria of citrate,  $\alpha$ -ketoglutarate, and glutamine, and we related this effect to the decrease in citrate clearance produced by acute metabolic alkalosis.

In other unpublished studies the metabolism of pyruvate-1- $^{14}\text{C}$  to  $\text{CO}_2$  was shown to be decreased by decreasing pH and  $[\text{HCO}_3^-]$ , the only substrate tested where this occurred.<sup>3</sup> Thus acute and chronic metabolic acidosis affect different substrates similarly and it may be that the underlying mechanisms are closely related.

In order to interpret the results of the experiments with variously labeled compounds certain assumptions are necessary regarding the distribution of various enzymes in the cells of renal cortex. The basis for these assumptions has been reviewed by Greville (30) and the following statements are supported by the data cited by him, or elsewhere in the literature (31) (the numbers in italics refer to the sites shown in Fig. 3): (a)  $\alpha$ -ketoglutarate dehydrogenase (5), succinate dehydrogenase (6), pyruvate dehydrogenase (14), glutamate dehydrogenase (2), glutaminase (1), and citrate synthase (9) are exclusively located within mitochondria. (b) malate dehydrogenase (8), fumarase (7), PEPCK (13), isocitrate dehydrogenase (11), aconitase (10), and pyruvate carboxylase (15) are present both in the cytoplasm and in mitochondria. (c) citrate lyase (16), glutamine transaminase (3),  $\omega$ -oxidase (4), pyruvate kinase (12), lactic dehydrogenase (17), and the enzymes of gluconeogenesis beyond PEPCK are considered to be exclusively cytoplasmic in location. In addition to these enzyme locations, it should be noted that the major transport compounds from intra- to extramitochondrial sites are generally believed to be malate and citrate, although other compounds such as aspartate may be involved also. Oxalacetate is considered to penetrate the mitochondrial membranes

<sup>3</sup> Simpson, D. P. Unpublished observation.

poorly and hence plays a negligible role in substrate transport out of mitochondria.

Using the above information based on current knowledge of enzyme locations and mitochondrial transport the experiments in this paper provide data on the magnitude of certain pathways. When a molecule of glutamine is metabolized,  $\alpha$ -ketoglutarate is formed either by a mitochondrial route involving glutaminase (1) and glutamate dehydrogenase (2) (or glutamate oxalacetate transaminase) or in the cytoplasm by means of glutamine transaminase (3) and  $\omega$ -oxidase (4). The next steps in  $\alpha$ -ketoglutarate metabolism occur within mitochondria since  $\alpha$ -ketoglutarate dehydrogenase (5) and succinate dehydrogenase (6) are both intramitochondrial enzymes. The former enzyme produces the first  $\text{CO}_2$  derived from glutamine. If glucose is the major metabolic product of glutamine metabolism, after succinate is formed then fumarate, malate, and oxalacetate will be produced and the latter will be decarboxylated to PEP, producing a second  $\text{CO}_2$  molecule, and the remaining three carbons from glutamine will proceed through the cytoplasmic pathways of gluconeogenesis. As a result of these reactions if a molecule of glutamine- $\text{U-}^{14}\text{C}$  is converted to glucose two molecules of  $^{14}\text{CO}_2$  will be formed and three labeled carbon atoms will appear as glucose- $^{14}\text{C}$ . Thus if the effect of metabolic acidosis were entirely that of stimulating gluconeogenesis, the ratio of the increase in  $^{14}\text{CO}_2$  to the increase in glucose- $^{14}\text{C}$  formation from glutamine- $\text{U-}^{14}\text{C}$  in the acidotic tissue should be 2:3. However this difference ratio in the experiments of Table II is 2:1 or greater, indicating that acidosis caused far more glutamine carbons to be converted to  $\text{CO}_2$  than can be accounted for by an effect limited to stimulation of gluconeogenesis.<sup>4</sup> Similar results were obtained when citrate-1,5- $^{14}\text{C}$  was used as substrate. When two molecules of this isotopic form of citrate are metabolized to glucose, the five carbons are removed by  $\alpha$ -ketoglutarate dehydrogenase, yielding two molecules of  $^{14}\text{CO}_2$ . The 1-carbons will label succinate in the 1 and 4 positions, subsequently resulting in oxalacetate-1,4- $^{14}\text{C}$ . Formation of PEP will remove the 4-carbon as  $\text{CO}_2$ , leaving PEP-1- $^{14}\text{C}$ , which will eventually produce labeled glucose. As a result for each  $^{14}\text{C}$  incorporated into glucose, three labeled carbons should appear as  $\text{CO}_2$ . How-

<sup>4</sup> For evaluation of the site of action of acidosis on renal substrate metabolism the critical result is the ratio of the difference between  $^{14}\text{CO}_2$  production by acidotic and alkalotic slices and the difference in  $^{14}\text{C}$  incorporation into glucose by the same slices, not the ratio of  $^{14}\text{CO}_2$  to glucose- $^{14}\text{C}$  in either tissue preparation by itself. Regardless of the amount of  $^{14}\text{CO}_2$  or glucose- $^{14}\text{C}$  produced from glutamine- $\text{U-}^{14}\text{C}$  by alkalotic slices, if the primary effect of acidosis is on cytoplasmic gluconeogenesis the difference ratio of  $^{14}\text{CO}_2$  production to glucose- $^{14}\text{C}$  formation will be 2:3.

ever when citrate-1,5- $^{14}\text{C}$  was metabolized, instead of a  $^{14}\text{CO}_2$  to glucose- $^{14}\text{C}$  difference ratio of 3:1, ratios of 15:1 or greater were obtained (Table III). Thus far more substrate was oxidized to  $^{14}\text{CO}_2$  than can be accounted for by an effect of acidosis confined to stimulation of gluconeogenesis.

However it would still be possible for the primary effect of acidosis to be induction of cytoplasmic PEPCK provided that most of the PEP produced by this enzyme was converted to pyruvate and subsequently metabolized by mitochondria or converted to lactate. Consideration of the results obtained with citrate labeled in various positions (Table III) provides more definitive evidence on the site of action of acidosis on renal metabolism. When citrate-6- $^{14}\text{C}$  is metabolized by mitochondria the labeled carbon is removed as  $\text{CO}_2$  by isocitrate dehydrogenase (11) leaving behind only unlabeled carbon atoms for further metabolism. Any labeled carbon incorporated into glucose from citrate-6- $^{14}\text{C}$  must be derived entirely from extramitochondrial metabolism of citrate. In the cytoplasm citrate lyase (16) can convert citrate-6- $^{14}\text{C}$  to acetyl Co A and oxalacetate-1- $^{14}\text{C}$  which can form labeled PEP and glucose. Thus the magnitude of glucose- $^{14}\text{C}$  formation from this labeled form of citrate indicates the amount of citrate being metabolized to glucose by the citrate lyase route. The results of Experiment 1 in Table III show that the amount of glucose- $^{14}\text{C}$  formed from citrate-6- $^{14}\text{C}$  is only a few per cent of the amount of  $^{14}\text{CO}_2$  formed from this substrate. Consequently extramitochondrial metabolism of citrate is negligible compared to mitochondrial citrate oxidation. Recent evidence from studies with liver mitochondria suggests that citrate is the major intermediate in the transfer of citric acid cycle compounds into the cytoplasm (32). If such is the case in dog renal cortex little of the citrate exiting from mitochondria enters the reactions involved in gluconeogenesis. Examination of the first experiment in Table III also demonstrates that although labeled glucose formation from citrate-6- $^{14}\text{C}$  is small, acidosis increased the amount of glucose- $^{14}\text{C}$  by over 75%. Since this incorporation of  $^{14}\text{C}$  can only result from extramitochondrial metabolism of citrate this finding shows that the cytoplasmic reactions leading to glucose formation are increased by acidosis independently of any effect of acidosis on mitochondrial metabolism. Presumably induction of cytoplasmic PEPCK accounts for this result by increasing the conversion of oxalacetate to PEP (21, 22).

Experiment 2 in Table III describes the metabolism of citrate-1,5- $^{14}\text{C}$  and of citrate-2,4- $^{14}\text{C}$ . Comparison of the amount of  $^{14}\text{CO}_2$  produced from these two forms of labeled citrate enables determination of the relative effects of acidosis on early and late steps in citrate



metabolism. When citrate-1,5-<sup>14</sup>C is metabolized the 5-carbon is removed as CO<sub>2</sub> by α-ketoglutarate dehydrogenase (5), forming succinate-1-<sup>14</sup>C as the other reaction product; because of the symmetry of succinate, labeling of both the 1 and 4 positions of this compound results. When citrate-2,4-<sup>14</sup>C is metabolized none of the labeled carbons is removed before succinate formation so that succinate-1,2,3,4-<sup>14</sup>C is produced. Thus metabolism of either citrate-1,5-<sup>14</sup>C or citrate-2,4-<sup>14</sup>C produces succinate with both carboxyl groups labeled. Whenever <sup>14</sup>CO<sub>2</sub> is subsequently produced from succinate derived from citrate-1,5-<sup>14</sup>C metabolism, a labeled CO<sub>2</sub> will also be produced from succinate formed from citrate-2,4-<sup>14</sup>C. Consequently <sup>14</sup>CO<sub>2</sub> production from citrate-2,4-<sup>14</sup>C will be equal to or greater than that from citrate-1-<sup>14</sup>C and therefore the difference between <sup>14</sup>CO<sub>2</sub> produced from citrate-1,5-<sup>14</sup>C and that produced from citrate-2,4-<sup>14</sup>C will provide a minimum estimate of decarboxylation of the citrate-5 position; i.e., (<sup>14</sup>CO<sub>2</sub> from citrate-5) is greater than or equal to (<sup>14</sup>CO<sub>2</sub> from citrate-1,5-<sup>14</sup>C) minus (<sup>14</sup>CO<sub>2</sub> from citrate-2,4-<sup>14</sup>C). The second experiment of Table III shows that acidosis stimulated 6 times as much <sup>14</sup>CO<sub>2</sub> from citrate-1,5-<sup>14</sup>C as from citrate-2,4-<sup>14</sup>C. By the above reasoning therefore over 80% of the <sup>14</sup>CO<sub>2</sub> from citrate-1,5-<sup>14</sup>C came from the 5-position and only the small remainder from the 1-position. Thus the total metabolism of citrate beyond succinate by all pathways contributed only slightly to <sup>14</sup>CO<sub>2</sub> formation from citrate-1,5-<sup>14</sup>C. Chronic metabolic acidosis therefore must stimulate renal substrate metabolism at a mitochondrial site (or sites) which precedes any reactions which decarboxylate products of succinate metabolism. The precise nature of this stimulatory effect remains to be determined.

*Note added in proof.* Utilization of glutamine-<sup>14</sup>C and its conversion to CO<sub>2</sub> and glucose by the intact kidney of the dog have recently been measured by Pitts, R. F., L. A. Pilkington, M. B. MacLeod, and E. Leal-Pinto. 1972. Metabolism of glutamine by the intact functioning kidney of the dog. Studies in metabolic acidosis and alkalosis. *J. Clin. Invest.* **51**: 557. On the basis of their in vivo experiments these authors conclude, as we have from our in vitro studies, that stimulation of gluconeogenesis can account for only a small fraction of the increased glutamine metabolism observed in chronic metabolic acidosis.

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