Regulation of [15N]Urea Synthesis from [5-15N]Glutamine

ROLE OF pH, HORMONES, AND PYRUVATE*

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We have utilized both [5-15N]glutamine and [3-13C]pyruvate as metabolic tracers in order to: (i) examine the effect of pH, glucagon (GLU), or insulin on the precursor-product relationship between [15N]glutamine, [15N]citrulline, and, thereby, [15N]urea synthesis and (ii) elucidate the mechanism(s) by which pyruvate stimulates [15N]urea synthesis. Hepatocytes isolated from rats were incubated at pH 6.8, 7.4, or 7.6 with 1 mM [5-15N]glutamine and 0.1 mM [15N]citrulline in the presence or the absence of [3-13C]pyruvate (2 mM). A separate series of experiments was performed at pH 7.4 in the presence of insulin or GLU. [15N]citrulline enrichment exceeded or was equal to that of [15N]citrulline under all conditions except for pH 7.6, when the [15N] enrichment in citrulline exceeded that in ammonia. The formation of [15N]citrulline (atom % excess) was increased with higher pH. Flux through phosphate-dependent glutaminase (PDG) and [15N]urea synthesis were stimulated (p < 0.05) at pH 7.6 or with GLU and decreased (p < 0.05) at pH 6.8. Insulin had no significant effect on flux through PDG or on [15N]urea synthesis. Increased [15N]urea production at pH 6.8 was associated with depleted aspartate and glutamate levels. Pyruvate attenuated this decrease in the aspartate and glutamate pools and stimulated [15N]urea synthesis. Production of Asp from pyruvate was increased with increasing medium pH. Approximately 80% of Asp was derived from [3-13C]pyruvate regardless of incubation pH or addition of hormone. Furthermore, approximately 20, 40, and 50% of the mitochondrial N-acetylglutamate (NAG) pool was derived from [3-13C]pyruvate at pH 6.8, 7.4, and 7.6, respectively. Both the concentration and formation of [15C]NAG from [3-13C]pyruvate were increased (p < 0.05) with glucagon and decreased (p < 0.05) with insulin or at pH 6.8. The data suggest a correlation between changes in [15N]urea synthesis and alterations in the level and synthesis of [15C]NAG from pyruvate. The current observations suggest that the stimulation of [15N]urea synthesis in acute alkalosis is mediated via increased flux through PDG and subsequent increased utilization of [5-15N] of glutamine for [15N]citrulline synthesis and/or increased synthesis of NAG from glutamate and pyruvate. The opposite may have occurred in acute acidosis. Glucagon, but not insulin, stimulated [15N]urea synthesis via increased flux through PDG and synthesis of NAG. Pyruvate stimulated urea synthesis via increased availability of aspartate and/or increased synthesis of NAG. The formation of NAG and aspartate from pyruvate are both pH-sensitive processes.

Although numerous investigations have shown that pH, hormones, and substrates such as pyruvate modify hepatic glutamine metabolism and urea synthesis, the mechanism(s) of such modulation and the site(s) of action are not clearly understood (1–8).

Previous studies from our laboratory (9, 10) and others (5, 8, 12, 14) have demonstrated that glutamine serves as a chief precursor for urea–N. The metabolism of glutamine by hepatocytes is mediated primarily via phosphate-dependent glutaminase (PDG), which is located in the mitochondria of periportal hepatocytes and is sensitive to small pH changes, [NH4+], and glucagon (5, 8, 11–20). It has been proposed that NH3, derived via PDG from the amide–N of glutamine, is the major source for carbamyl phosphate synthesis and that the amide–N of glutamine could be directly channeled to carbamyl phosphate synthetase (21). However, none of the previous studies has demonstrated a possible precursor-product relationship between the amide–N of glutamine, NH3, citrulline, and the subsequent urea–N. In the current study we have utilized isolated hepatocytes as a model system and [5-15N]glutamine as a substrate for urea synthesis to examine the effect of pH and hormones on precursor-product relationships between [5-15N]glutamine, [15N]citrulline, and [15N]urea, using gas chromatography-mass spectrometry (GC-MS) methodology as described previously (9, 10, 22).

In addition to NH3, glutamate is the second product of the PDG pathway (9, 10). Glutamate–N can be transaminated to form other amino acids, primarily aspartate and alanine (9, 10). Alanine formation may serve as an acceptor of ammonia nitrogen when urea synthesis is diminished or insufficient to detoxify the ammonia load (10, 23). Aspartate–N provides the second nitrogen of urea (5, 9, 12, 20). The availability of aspartate in the cytosol may play a significant role in hepatic ureagenesis (9, 12, 20). It has been suggested that aspartate is generated in the mitochondria and then transported to the cytosol where it is used for the synthesis of argininosuccinate (12, 20). Therefore, the availability of oxaloacetate (Oxa) in the mitochondria may regulate the formation of aspartate and, hence, urea synthesis.

The abbreviations used are: PDG, phosphate-dependent glutaminase; GC-MS, gas chromatography-mass spectrometry; Oxa, oxaloacetate; PC, pyruvate carboxylase; NAG, N-acetylglutamate; PCA, perchloric acid; t-BDMS, t-butyldimethyl-silyl.

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Oxaloacetate can be formed from pyruvate in the mitochondrial via direct conversion of pyruvate to Oxa through pyruvate carboxylase (PC). However, isotopically labeled carbon atoms from pyruvate may be incorporated into Oxa both by PC or via the pyruvate dehydrogenase pathway and subsequent flux of carbons through the tricarboxylic acid cycle (24–26). Regardless whether the PC reaction or tricarboxylic acid cycle metabolism is the primary site for furnishing the carbon moieties of aspartate, it has been shown that supplementation with pyruvate stimulated ureagenesis (25, 26), but the exact mechanism(s) is uncertain.

Metabolism of pyruvate via the pyruvate dehydrogenase pathway may stimulate urea synthesis secondary to formation of acetyl-CoA, a precursor of N-acetylglutamate (NAG), the primary activator of carbamyl phosphate synthetase 

\[ \text{NAG} \] and an additional 40-min incubation. A separate series of incubations 

\[
\text{PC} \text{reaction or tricarboxylic acid cycle metabolism is the primary site for furnishing the carbon moieties of aspartate, it has been shown that supplementation with pyruvate stimulated ureagenesis (25, 26), but the exact mechanism(s) is uncertain.}
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Metabolism of pyruvate via the pyruvate dehydrogenase pathway may stimulate urea synthesis secondary to formation of acetyl-CoA, a precursor of N-acetylglutamate (NAG), the primary activator of carbamyl phosphate synthetase (6, 4, 27–32). Therefore, an understanding of the role of pyruvate supplementation in hepatic ureagenesis requires an evaluation of the precursor-product relationship between pyruvate, aspartate, and NAG. In the current study we have used 

\[ [3-^{15}N]\text{pyruvate and GC-MS to measure the transfer of [3-^{15}N]pyruvate carbon to aspartate, tricarboxylic acid cycle intermediates, glutamate, and NAG at various pH levels of the incubation medium or in the presence of insulin or glucagon. We have investigated the action of glucagon or insulin on hepatic ureagenesis because these hormones are key regulators of hepatic nitrogen and carbohydrate metabolism in normal and disease states (8, 16–20, 22, 33–35). 

The current study suggests that the production of 

\[ [5-^{15}N]\text{citrulline from [5-^{15}N]glutamine and/or NAG from pyruvate may play a key role in the regulation of urea synthesis at various acid base or hormonal states.}
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**EXPERIMENTAL PROCEDURES**

**Materials**

Male Sprague-Dawley rats weighing 250–300 g were obtained from Charles River Breeding Laboratories (Wilmington, DE). [5-15N]Glutamine (I-6634), collagenase type IV (C-5138), and hyaluronidase Type II (H-6076) were purchased by Cambridge-Isotopes (Andover, MA). All enzymes and hormones were purchased from Sigma as follows: glucagon (G-2015), bovine insulin (I-6634), collagenase type IV (C-5138), and hyaluronidase Type II (H-2126).

**Methods**

Hepatocytes were prepared as described previously by Mariusz et al. (36). Viability of hepatocytes was approximately 85% as determined by the trypan blue staining. Suspension of hepatocytes was prepared in Krebs-Henseleit bicarbonate (KHB) buffer, pH 7.4, containing 2% (w/v) bovine serum albumin. After the preparation, the hepatocytes were kept on ice for 20 min of recovery and then used for the following studies.

**Effect of Extracellular pH on the Metabolism of [5-15N]Glutamine—**KHB buffer of pH 6.8 (acidosis), 7.4 (control), or 7.6 (alkalosis) was prepared by increasing the NaHCO₃ in the buffer to 49 mM (pH 7.6) or prepared by increasing the NaHCO₃ in the buffer to 49 mM (pH 7.6) or at 104°C for 3 min. The supernatant was removed and used for extracellular metabolite determinations. To 4 ml of extracellular fluid, 1 ml of 25% perchloric acid (PCA) was added. Bovine serum albumin was removed by centrifugation, and the supernatant was neutralized with KOH and used for intracellular determination of amino acid levels as well as 15N or 13C abundance.

**Analytical Methods**

**Metabolite Levels—**Total urea nitrogen was determined by the indophenol adsorption method (Sigma Diagnostic Kit 640-5). Amino acids were determined by a Varian 5060 high pressure liquid chromatography system as described previously (9, 10, 37). Ammonia was determined spectrophotometrically by the reductive amination of α-ketoglutarate via glutamate dehydrogenase reaction (38). GC-MS Determination of 15N or 13C-Labeled Metabolites—GC-MS measurements of 15N isotopic enrichment were performed on a Hewlett-Packard 5970 MSD coupled with a 5890 HP-GC. 13C enrichment was measured after addition of ammonia to glutamate (37). Isotopic enrichment in glutamine, aspartate, or N-acetylglutamate was determined following separation of these amino acids from glutaminase and asparagine. This was done by applying a 500-μl aliquot of medium or cellular PCA extract to an AG-1 column (CI-X8; 100–200 mesh; 0.5 × 2.5 cm). The column was washed with 3 ml of deionized water. Glutamate, aspartate, and NAG were eluted with 3 ml of 1 N HCl (see Fig. 1).

15N enrichment in citrulline, alanine, urea, and glutamine was measured using a GC-MS modified with 450 µl aliquots of the medium or cellular PCA extract was added to an equal volume of NaHCO₃ buffer (pH 7.1), and this was applied to an AG-50 (Na⁺; 50–50 mesh, 0.5 × 2.5 cm) column. Arginine remained bound to the resin, whereas citrulline, urea, and other amino acids were eluted with 3 ml of water. The effluent was collected and then applied to an AG-50 (H⁺; 100–200 mesh; 0.5 × 2.5 cm) column, which was washed with 4 ml of water and amino acids were eluted with 3 ml of 1 N HCl (see Fig. 1).

For GC-MS analysis of 15N enrichment, urea and amino acids were converted into t-butyldimethylsilyl (t-BDMS) derivatives. The m/z 231, 232, 233, and 234 of the urea t-BDMS derivative were monitored for singly labeled and doubly labeled urea determination (9, 10, 39). Possible overlapping ions were corrected for as described by Wolfe (40). Isotopic enrichment in citrulline, glutamine, glutamate, aspartate, and alanine was monitored using ratios of ions at m/z of 442/442, 432/432, 433/432, 419/418, and 261/260, respectively (9, 10, 22).

In experiments with [3-15C]pyruvate, we have also monitored the appearance of singly and doubly labeled aspartate or glutamate using m/z 418, 419, 420, and 421 for aspartate and m/z 432, 433, 434, and 435 for glutamate. 15C-Labeled alanine was monitored using the m/z 261/260 ratio. 13C-Labeled NAG was determined using m/z 360, 361, 362, and 363 (Fig. 1). 13C doubly labeled tricarboxylic acid cycle intermediates were monitored using m/z 593/591, 421/420, 289/287, and 291/289 for citrate, malate, fumarate, and succinate, respectively.

**Measurement of N-Acetylglutamate**

To determine the level of NAG in hepatocytes following incubations with [5-15N]glutamine as outlined above, we have used GC-MS and a modification of the conventional isotope dilution technique (41, 42). Following the initial determination of 15N abundance in NAG (I₁), we have spiked 1 ml of the hepatocytes PCA extract with 100pmol of unlabeled NAG (C₁). Then, samples were passed through an ion exchange resin with N₃ and deproteinized with t-BDMS derivatization as described above. Then, a second measurement of isotopic abundance (I₂) was performed in each sample. The concentration of NAG in the sample (C₂) was calculated as:

\[
C_2 = C_1 / (I_2 / I_1 - 1)
\]

(Eq. 1)
The formation of 15N- or 13C-labeled metabolites was determined by the product of their isotopic enrichment (atom % excess/100) times concentration (nmol/g wet weight) and is expressed as nmol 15N-metabolite/g wet weight. Flux through the PDG pathway was considered to be the sum of [15N]ammonia formation and [15N]urea production from [5-15N]glutamine. Statistical analyses were carried out by the use of a nonparametric (Mann-Whitney) test for unpaired data using the Instant Software Package for Macintosh. A p value less than 0.05 was taken as indicating a statistically significant difference.

RESULTS

Flux through the Glutaminase Pathway—Formation of 15NH₃ glutamine metabolism and ammonia formation are mediated essentially via flux through the PDG pathway (5, 8–10). In the current study we estimated the flux through PDG as the sum of 15NH₃ and [15N]urea production following a 40-min incubation. We have found that the formation of other amino acids from [5-15N]glutamine accounted for less than 5% of [5-15N]glutamine consumption. Therefore, it is reasonable to define the flux through the PDG pathway as the sum of 15NH₃ and [15N]urea production.

Table I summarizes the calculated rate of PDG flux at various extracellular pH or in the presence of hormones, with or without supplementation with pyruvate. At pH 6.8 plus pyruvate, the flux through PDG was significantly lower compared with pH 7.4. However, without pyruvate, there was no difference between pH 6.8 and 7.4. Both pH 7.6 and glucagon remarkably augmented (p < 0.05), whereas insulin tended to decrease the flux through PDG by about 15%, but this effect was not significant (Table I).

Without pyruvate, the accumulation of 15NH₃ (µmol/g wet weight) was significantly higher at pH 7.6, with little change at pH 6.8 compared with incubation at pH 7.4 (Table I). Glucagon mimics the effect of alkalinosis, whereas insulin had no effect on 15NH₃ accumulation (Table I). In the presence of pyruvate, the accumulation of 15NH₃ was decreased compared with experiments without pyruvate regardless of experimental conditions, but the decrease was statistically significant only at pH 7.6 or in the presence of glucagon (Table I).

Fig. 2 represents the 15N abundance in ammonia, citrulline, and urea in experiments without pyruvate. Comparisons of data in Table I and Fig. 2 suggest a correlation between 15NH₃ accumulation (Table I) and 15NH₃ abundance (Fig. 2) in all experimental groups except at pH 7.6, where 15NH₃ enrichment was smaller compared with experiments at either pH 6.8 or 7.4. The isotopic enrichment of intracellular [5-15N]glutamine following a 40-min incubation was approximately 33, 31, and 25% of the total ammonia accumulated in the incubation medium was derived from [5-15N]glutamine at pH 6.8, 7.4, and 7.6, respectively. The ratio between the isotopic enrichment in 15NH₃ and intracellular [5-15N]glutamine indicates that approximately 33, 31, and 25% of the total ammonia accumulated in the incubation medium was derived from [5-15N]glutamine at pH 6.8, 7.4, and 7.6, respectively. In the presence of hormones, the isotopic enrichment of intracellular [5-15N]glutamine showed little difference compared with pH 7.4. The calculated fraction of 15NH₃...
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Isolated hepatocytes were preincubated for 5 min with 0.1 mM $^{14}$NH$_4$Cl in the presence or the absence of 2 mM pyruvate and then for an additional 40 min following the addition of 1 mM [5-$^{15}$N]glutamine. Experimental conditions are as outlined under "Experimental Procedures." Results are mean ± S.E. of 5–12 experiments.

| Experiment | Pyruvate | Pyruvate | Pyruvate | Pyruvate |
|------------|----------|----------|----------|----------|
| pH 7.4     | 2.2 ± 0.2 | 1.8 ± 0.1 | 1.48 ± 0.1 | 1.48 ± 0.1 |
| pH 6.8     | 2.3 ± 0.2 | 2.3 ± 0.2 | 1.55 ± 0.05 | 1.55 ± 0.05 |
| pH 7.6     | 3.6 ± 0.1 | 2.4 ± 0.2 | 2.80 ± 0.28 | 2.80 ± 0.28 |
| pH 7.4     | 3.9 ± 0.7 | 2.3 ± 0.1 | 1.32 ± 0.08 | 2.37 ± 0.26 |
| (++) Insulin| 1.9 ± 0.2 | 1.5 ± 0.2 | 0.59 ± 0.05 | 1.24 ± 0.13 |

a Sum of single and double labeled $^{15}$Nurea.
b Sum of $^{15}$NH$_3$ and $^{15}$Nurea production/min.

d $P < 0.05$ compared with incubation at pH 7.4.

e $P > 0.05$ compared with the same experiment without pyruvate.
f $P < 0.05$ compared with the same experiment without pyruvate.

Fig. 2. Labeling (atom % excess) in $^{15}$Nammonia, $^{15}$Ncitrulline, and $^{15}$Nurea following incubation with [5-$^{15}$N]glutamine in the absence of pyruvate. Following 5 min of preincubation with 0.1 mM $^{14}$NH$_4$Cl, [5-$^{15}$N]glutamine (1 mM, final concentration) was added, and hepatocytes were incubated for an additional 40 min at the indicated pH or in the presence of indicated hormones. Values are means ± S.E. of 5–12 separate incubations. *, $P < 0.05$ compared with experiment at pH 7.4 without hormone. GLU, glutagon; INS, insulin.

TABLE I

| Experiment | $^{15}$NH$_3$ | $^{15}$Nurea$^a$ | Flux through glutaminase$^b$ |
|------------|---------------|-----------------|-----------------------------|
| Pyruvate   |               |                 |                             |
| pH 7.4     | 62.8 ± 5.6    | 74.5 ± 6.2     |                             |
| pH 6.8     | 62.8 ± 5.6    | 74.5 ± 6.2     |                             |
| pH 7.6     | 62.8 ± 5.6    | 74.5 ± 6.2     |                             |
| pH 7.4     | 62.8 ± 5.6    | 74.5 ± 6.2     |                             |

Experimental Condition

FIG. 2. Labeling (atom % excess) in $^{15}$Nammonia, $^{15}$Ncitrulline, and $^{15}$Nurea following incubation with [5-$^{15}$N]glutamine in the absence of pyruvate. Following 5 min of preincubation with 0.1 mM $^{14}$NH$_4$Cl, [5-$^{15}$N]glutamine (1 mM, final concentration) was added, and hepatocytes were incubated for an additional 40 min at the indicated pH or in the presence of indicated hormones. Values are means ± S.E. of 5–12 separate incubations. *, $P < 0.05$ compared with experiment at pH 7.4 without hormone. GLU, glutagon; INS, insulin.

derived from the amide–N was approximately 44 and 29% in the presence of glucagon and insulin, respectively.

In the presence of 2 mM pyruvate, $^{15}$NH$_3$ enrichment was higher (Fig. 3) compared with experiments in the absence of pyruvate (Fig. 2), except in experiments with glucagon, where only little change in $^{15}$NH$_3$ abundance was observed. Furthermore, $^{15}$NH$_3$ enrichment was considerably higher at pH 7.6 compared with pH 7.4 and exceeded the isotopic abundance noted in experiments with glucagon. The calculated contribution of the amide–N to $^{15}$NH$_3$ formation in the presence of pyruvate was also higher compared with experiments without pyruvate, e.g. approximately 45, 42, 58, 53, and 52% of ammonia present in the incubation medium was derived from N-5 of glutamine at pH 6.8, 7.4, or 7.6 in the presence of glucagon or insulin, respectively. Therefore, the fraction of the ammonia pool that was derived from the amide–N was greater in the presence of pyruvate than in the absence of pyruvate.

Formation of $^{15}$NCitrulline—Figs. 2 and 3 illustrate an increase in $^{15}$NCitrulline abundance with increasing pH of the incubation medium. In the absence of pyruvate, $^{15}$N enrichment in citrulline was decreased at pH 6.8 and increased at pH 7.6 compared with pH 7.4 ($P < 0.05$). Furthermore, at pH 7.6 $^{15}$N abundance in citrulline was higher ($P < 0.05$) than that of ammonia (Fig. 2), indicating no precursor-product relationship between $^{15}$NH$_3$ and $^{15}$NCitrulline. Glucagon had little effect on the formation of $^{15}$NCitrulline. However, the presence of insulin significantly decreased formation of $^{15}$NCitrulline (atom % excess) compared with incubation at pH 7.4 (Fig. 2). When pyruvate was added to the incubation medium, $^{15}$NH$_3$ enrichment exceeded that of $^{15}$NCitrulline or $^{15}$Nurea under all experimental conditions (Fig. 3), suggesting a precursor-product relationship.

Formation of $^{15}$Nurea—The $^{15}$NCitrulline formed in the mitochondria is expected to be utilized in the cytosol for production of $^{15}$Nurea. The current measurements illustrate a precursor-product relationship between $^{15}$NCitrulline and $^{15}$Nurea under all experimental conditions (Figs. 2 and 3). It should also be recognized that some of the $^{15}$N in singly labeled urea will be introduced via aspartate rather than citrulline, but this will make a minor contribution due to the low $^{15}$N enrichment in aspartate compared with citrulline (Figs. 2–4). $^{15}$N enrichment in singly labeled urea is significantly higher at pH 7.4 and 7.6 compared with pH 6.8 (Figs. 2 and 3). Without pyruvate, $^{15}$N abundance in urea was significantly lower in the presence of insulin, whereas glucagon had only little effect on the abundance of $^{15}$Nurea (Fig. 2). The addition of pyruvate almost doubled the synthesis of $^{15}$Nurea from [5-$^{15}$N]glutamine in all experimental groups (Table I). Moreover, in the presence of pyruvate the difference in $^{15}$Nurea formation at pH 6.8 compared with 7.4 in the absence of pyruvate was
narrowed to only 20% \( (p < 0.05) \), indicating that pyruvate augmented urea synthesis at all pH values.

Levels and Formation of \(^{15}N\) Amino Acid—GC-MS determination of the intracellular \(^{15}N\) amino acids demonstrates that extracellular pH had little effect on formation of \(^{15}N\)-labeled glutamate, aspartate, or alanine (Fig. 4). Table II indicates that the intracellular pool of aspartate was significantly reduced following incubation at pH 6.8 with little change at pH 7.6 compared with pH 7.4. This observation is in good accord with previous in vivo observations demonstrating decreased aspartate and other amino acids in the liver of acidotic rats (43) and in isolated hepatocytes (10, 12, 20). In the current study, supplementation with pyruvate elevated the intracellular level of aspartate at all pH values, although at pH 6.8 the aspartate level remained significantly lower than at pH 7.4 or 7.6 (Table II). Similarly, intracellular levels of glutamate and alanine were lower at pH 6.8 and higher at pH 7.6 compared with pH 7.4 without pyruvate, with little difference upon the addition of pyruvate (Table II). Insulin or glucagon had little effect on amino acid levels compared with experiments at pH 7.4 (data not shown). The most striking observation is that the extracellular pool of alanine was significantly higher at pH 6.8 compared with pH 7.4 or 7.6, regardless of pyruvate supplementation. Therefore, in acidosis there was a significantly higher production and translocation of alanine from cell to incubation medium. However, some alanine may also have been formed via alanine aminotransferase that leaked from damaged hepatocytes during the course of the incubation.

Transfer of [\(^{3-13}C\)]Pyruvate to Carbon Moiety of Amino Acids—To evaluate the relative contribution of pyruvate carbon to the synthesis of Oxa and, hence, to aspartate, we have measured the transfer of [\(^{3-13}C\)]pyruvate to aspartate, glutamate, and alanine. Fig. 5 shows that approximately 80% of aspartate, that is, the sum of singly and doubly labeled aspartate, was derived from pyruvate regardless of the pH of the incubation. However, the portion of doubly labeled aspartate is significantly \( (p < 0.05) \) higher at pH 7.4 and 7.6 compared with pH 6.8. The product of \(^{13}C\) enrichment (Fig. 5) and concentration (Table II) indicates that approximately 224, 312, and 501 nmol/g wet weight of singly labeled aspartate and 32, 162, and 220 nmol/g wet weight doubly labeled aspartate accumulated from pyruvate at pH 6.8, 7.4, and 7.6, respectively. Therefore, the transfer of pyruvate carbons to aspartate was increased with increasing medium pH.

Transfer of [\(^{3-13}C\)]Pyruvate to Tricarboxylic Acid Cycle Intermediates—To examine the possible interaction between tricarboxylic acid cycle metabolism, formation of aspartate, and, hence, urea synthesis, we have determined the transfer of [\(^{3-13}C\)]pyruvate into tricarboxylic acid cycle intermediates. Fig. 6 demonstrates that the formation of doubly labeled \(^{13}C\)citrate was significantly higher at pH 7.4 and 7.6 compared with pH 6.8. Similarly, the \(^{13}C\) abundance in doubly labeled fumarate and malate increased with increasing pH of the incubations. However, \(^{13}C\) abundance in succinate, the product of \( \alpha \)-ketoglutarate dehydrogenase (25), was higher by approximately 25% in acidosis. The data in Fig. 5 and 6 illustrate an isotopic equilibrium between doubly labeled succinate, fumarate, malate, and aspartate, regardless of the \( H^+ \) level.

Formation of \( N \)-Acetylglutamate from Pyruvate—In addition to its role as a main donor of the carbon moiety of aspartate, pyruvate may have an important role in synthesis of NAG. \( N \)-Acetylglutamate is exclusively formed in the mitochondria from acetyl-CoA and glutamate via \( N \)-acetylglutamate synthetase (EC 2.3.1.1) (27). Fig. 7 illustrates the formation of \(^{13}C\)NAG isotopomers when [\(^{3-13}C\)]pyruvate and unlabeled glutamine were added to the incubation medium. Although the
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Experiments were carried out as detailed in Table I and under “Experimental Procedures.” Results are the means ± S.E. of 4–12 experiments. ND, not detectable.

| Experiment | Aspartate (μmol/g wet weight) | Glutamate (μmol/g wet weight) | Alanine (μmol/g wet weight) |
|------------|-------------------------------|-------------------------------|-----------------------------|
| Intracellular | Pyruvate | + Pyruvate | Pyruvate | + Pyruvate | Pyruvate | + Pyruvate |
| pH 7.4 | 0.31 ± 0.04 | 0.67 ± 0.04 | 0.48 ± 0.02 | 0.63 ± 0.02 | 0.34 ± 0.02 | 0.38 ± 0.01 |
| pH 6.8 | 0.23 ± 0.02 | 0.33 ± 0.03 | 0.37 ± 0.03 | 0.45 ± 0.05 | 0.22 ± 0.01 | 0.43 ± 0.03 |
| pH 7.6 | 0.42 ± 0.04 | 0.88 ± 0.10 | 0.75 ± 0.04 | 0.68 ± 0.03 | 0.41 ± 0.02 | 0.33 ± 0.01 |
| Extracellular | Pyruvate | + Pyruvate | Pyruvate | + Pyruvate | Pyruvate | + Pyruvate |
| pH 7.4 | ND | 0.14 ± 0.02 | 1.85 ± 0.12 | 1.54 ± 0.15 | 1.14 ± 0.12 | 3.97 ± 0.5 |
| pH 6.8 | ND | 0.06 ± 0.09 | 0.82 ± 0.04 | 0.86 ± 0.07 | 1.89 ± 0.15 | 5.34 ± 0.78 |
| pH 7.6 | ND | 0.18 ± 0.02 | 1.98 ± 0.04 | 3.40 ± 0.14 | 1.23 ± 0.10 | 3.66 ± 0.26 |

*p < 0.05 compared with incubation at pH 7.4.
*p > 0.05 compared with the same experimental condition without pyruvate.

**Fig. 5.** Formation of 13C isotopomers of aspartate and glutamate from [3-13C]pyruvate. Hepatocytes were preincubated at the indicated pH for 5 min with 2 mM [3-13C]pyruvate and 0.1 mM NH4Cl, and then 1 mM unlabeled glutamine (final concentration) was added, and incubation continued for additional 40 min. M+1, singly labeled; M+2, doubly labeled. Values are means ± S.E. of four experiments.

**Fig. 6.** Labelling (atom % excess) in 13C doubly labeled tricarboxylic acid cycle intermediates following incubation with [3-13C]pyruvate. Experimental conditions are as described in the legend to Fig. 5. Values are means ± S.E. of four experiments. *p < 0.05 compared with incubation at pH 7.4.

The current methodology does not identify which carbons are labeled, the singly labeled NAG may arise either by the condensation of [2-13C]acetyl-CoA (that has been derived from [3-13C]pyruvate via the pyruvate dehydrogenase pathway) with unlabeled glutamate or of unlabeled acetyl-CoA with singly labeled glutamate. Doubly labeled NAG may arise either by the condensation of [2-13C]acetyl-CoA with singly labeled glutamate or of unlabeled acetyl-CoA with doubly labeled glutamate. It is not possible to determine the relative contributions of these possibilities with GC-MS methodology. The current observations demonstrate an increased formation of [13C]NAG (mol % excess) with increased pH, being significantly higher at pH 7.6 and significantly lower at pH 6.8 compared with pH 7.4 (Fig. 7). Glucagon remarkably stimulated the formation of [13C]NAG from [3-13C]pyruvate (Fig. 7), whereas insulin had little effect on the formation of [13C]-labeled NAG compared with incubation at pH 7.4 (data not shown).

The sum of [13C]NAG isotopomers, that is, the fraction of 13C-labeled NAG of the total mitochondrial pool, shows a significant elevation with increased pH and a further increase in the presence of glucagon (Fig. 7). Similarly, the product of isotopic enrichment (Fig. 7) and the calculated level of NAG (Table III) indicates a significant elevation in the synthesis of 13C-labeled NAG from [3-13C]pyruvate with increasing pH and a further increase in the presence of glucagon. Therefore, the current data suggest that in addition to production of aspartate from pyruvate, the latter may stimulate urea synthesis by increasing the formation of NAG, especially at acute alkalosis or in the presence of glucagon.

**Level of N-Acetylglutamate in Isolated Hepatocytes**—A major difficulty in our understanding of the role of NAG in the regulation of urea synthesis is the lack of a reliable and precise method for determination of the NAG level in tissues (3, 29). In the current study, we have used the t-BDMS derivative of NAG and GC-MS to directly measure this metabolite with a stable isotope dilution method (41, 42). The current analysis provides an excellent separation between NAG, aspartate, and glutamate (Fig. 1), as well as a simultaneous measurement of isotopic enrichment in each of these metabolites. Table III illustrates that the level of NAG at physiologic pH is in good agreement with the level obtained in previous studies using GC-MS or indirect chemical determination (3, 29). However, Lund and Wiggins (3) did not detect a correlation between NAG level and rates of urea synthesis from glutamine, whereas our current data suggest a correlation between changes in [15N]urea synthesis from [5-15N]glutamine (Table I) and changes in the level of NAG with alteration of the incubation pH or in the presence of glucagon (Table III). However, in experiments with insulin, NAG levels decreased by approximately 60% with only 15% decrease in [15N]urea synthesis (Tables I and III).
Regulation of Hepatic Urea Synthesis

A unique feature of the current studies is the utilization of [5-15N]glutamine and [3-13C]pyruvate in order to examine: (i) a precursor-product relationship between 15NH3, [15N]citrulline and [15N]urea; (ii) the pH-dependent and pH-independent effects on the precursor-product relationship, metabolite production, and, thereby, synthesis of urea; and (iii) the mechanism(s) by which pyruvate stimulates urea synthesis.

15NH3 from [5-15N]glutamine is predominantly formed via the mitochondrial PDG reaction (8–10, 22, 37, 44). This 15NH3 may be utilized for formation of carbamyl phosphate via carbamyl phosphate synthetase (4, 28, 45, 46) or glutamate via the glutamate dehydrogenase reaction. Both pathways are located intracellularly (5, 47). In addition, this 15NH3 in theory should move freely across the mitochondria or cellular membrane and equilibrate with the cytosolic as well as extracellular ammonia. In the current experiments we have added 0.1 mM unlabeled NH4Cl to the incubation medium as a catalyst for the PDG reaction (13–15). [5-15N]Glutamine was added following a 5-min preincubation. Therefore, 15NH3 enrichment (atom % excess) in the incubation medium (Figs. 2 and 3) probably represents the equilibrium reached between 15NH3 formed in the mitochondria and 14NH3 that was present in the incubation medium. Table I demonstrates a significant stimulation of the flux through the PDG pathway with increasing pH. Glucagon mimics the stimulatory action of acute alkalosis, whereas insulin decreased flux through PDG by approximately 15%. These observations are in agreement with numerous publications demonstrating the sensitivity of hepatic PDG to the H+ level (6, 5, 11–15) or glucagon (5, 8, 12, 20, 22).

At pH 6.8 or 7.4 or with insulin, there was insignificant change in 15NH3 accumulation in the absence or the presence of pyruvate but approximately a 2-fold increase in [15N]urea synthesis in the presence of pyruvate (Table I). Furthermore, the current data indicate an equal or greater 15NH3 accumulation than [15N]urea production regardless of the experimental condition (Table I). Although the current experiments were carried out without addition of ornithine to the incubation medium, the accumulation of 15NH3 cannot be attributed to the lack of ornithine for two reasons. First, our measurements have shown that intracellular ornithine was between 120 and 160 nmol/g wet weight at the end of a 45-min incubation, an increase from approximately 90 nmol/g wet weight at the start of the incubation. This increase in ornithine occurred regardless of [H+] or hormones. Second, previous studies by Lund and Wiggins (48) have shown that urea synthesis from glutamine is independent of added ornithine, although this amino acid is required for maximal urea synthesis when a superphysiologic concentration of ammonia is utilized as substrate (48). Therefore, the current investigation implies that in addition to the flux through the PDG pathway, other factors may have a role in production of 15NH3 and regulation of [15N]urea synthesis from [5-15N]glutamine.

The current observations indicate that the extracellular pH significantly altered the production of [15N]citrulline from [5-15N]glutamine. Regardless of pyruvate supplementation, [15N] enrichment in citrulline was increased with increasing medium pH (Figs. 2 and 3). Furthermore, in the absence of pyruvate the [15N] enrichment in citrulline exceeded that in ammonia at pH 7.6 (Fig. 2), indicating an apparent absence of a precursor-product relationship between the pool of 15NH3 and formation of [15N]citrulline in the mitochondria. This seeming anomaly could have occurred following the synthesis of 15N labeled carbamoyl phosphate before an equilibrium with the

**DISCUSSION**

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**TABLE III**

Isolated hepatocytes were incubated (in the presence of 2 mM pyruvate) as outlined in Table I. NAG levels were determined as described under “Experimental Procedures.” Results are the means ± S.E. of four experiments.

| pH | N-Acetylglutamate (nmol/g wet weight) |
|----|-------------------------------------|
| 7.4 | 177.8 ± 4.0                           |
| 6.8 | 39.8 ± 3.9*                          |
| 7.6 | 221.7 ± 67.1*                        |

* p < 0.05 compared with experiments at pH 7.4 without hormone.
The primary pathway for hepatic amino acid production from ammonia is the formation of glutamate via reductive amination of α-ketoglutarate in the mitochondria (44, 47). The data in Fig. 4 support this pathway by demonstrating a possible precursor-product relationship between glutamate, alanine, and aspartate. It is also possible that a portion of alanine was derived via the glutamine aminotransferase pathway (Gln II) as previously indicated (10, 49). However, any alanine produced via the Gln II pathway would be unlabeled in experiments using [5-15N]glutamine as substrate. Regardless of the pathway by which alanine was formed, the current observations indicate that in acute acidosis, when urea synthesis was decreased, there was a remarkable intracellular production of alanine and its translocation into the incubation medium (Table II). This observation is consistent with an *in vivo* study demonstrating that hepatic amino acids uptake was decreased, whereas the release of amino acids into the hepatic vein, especially alanine, was increased in acute acidic compared with alkalotic rats (7). Therefore, it is possible that hepatic alanine production and its removal from hepatocytes might have a significant implication for urea synthesis and ammonia detoxification as well as nitrogen retention and sparing in cases of perturbed acid base homeostasis and/or activity of urea cycle.

An important question we have addressed in the current investigation is related to the mechanism(s) by which pyruvate stimulates urea synthesis. The stimulatory effect of pyruvate on the synthesis of [15N]urea could come about via the following nonexclusive mechanism(s): (i) furnishing the carbon moiety of aspartate in the mitochondria; (ii) stimulating carbon flux through the tricarboxylic acid cycle, thereby forming CO₂ and ATP needed for carbamyl phosphate synthetase; and/or (iii) formation of acetyl-CoA required for synthesis of NAG.

When comparing the formation of [15N]urea synthesis in the presence and the absence of pyruvate (Table I) with the formation of [15C]aspartate (Table II and Fig. 5), the observations imply that the production of Oxa and therefore aspartate is nonexclusive mechanism(s): (i) furnishing the carbon moiety of aspartate in the mitochondria; (ii) stimulating carbon flux through the tricarboxylic acid cycle, thereby forming CO₂ and ATP needed for carbamyl phosphate synthetase; and/or (iii) formation of acetyl-CoA required for synthesis of NAG.
vate is metabolized to \([13C]\)aspartate. Presumably, a major portion of pyruvate was converted to Oxa via the PC pathway because singly labeled aspartate was markedly higher than doubly labeled regardless of the incubation pH. The ratio between singly labeled aspartate to total \([13C]\)-labeled aspartate (single plus double) may provide information regarding the relative flux through the PC pathway versus formation of doubly labeled aspartate through the tricarboxylic acid cycle following metabolism of pyruvate via the pyruvate dehydrogenase pathway. These ratios are calculated to be 0.83, 0.65, and 0.69 at pH 6.8, 7.4, and 7.6, respectively, suggesting that at pH 6.8 approximately 83% of aspartate was derived from pyruvate via PC and 17% via scrambling of \([3-13C]\)pyruvate through tricarboxylic acid cycle metabolism versus 65% via PC and 35% via tricarboxylic acid cycle metabolism at pH 7.4 or 7.6.

It is clear that increased intracellular aspartate is associated with the effect of pyruvate in increasing urea synthesis. This could come about via increased flux through PC. However, metabolism of pyruvate via the pyruvate dehydrogenase pathway may have a significant role in the synthesis of NAG and, thereby, urea synthesis. The current data demonstrate a remarkable increase in the formation of \([13C]\)NAG from \([3-13C]\)pyruvate with increased extracellular pH or in the presence of glucagon (Fig. 7 and Table III). The data suggest that approximately 20, 40, and 50% of the mitochondrial NAG were derived from \([3-13C]\)pyruvate at pH 6.8, 7.4, and 7.6, respectively. These observations illustrate a correlation between the synthesis of NAG and \([15N]\)urea synthesis at various pH or in the presence of glucagon (Figs. 2, 3, and 7 and Tables I and III). Furthermore, the current data indicate a correlation between intracellular glutamate levels (Table II) and formation of \([13C]\)NAG (Fig. 7), in agreement with previous observations, indicating that NAG synthesis may be mediated by both high pH and/or increased glutamate level (28, 48, 50). Therefore, the diminished synthesis of \([13C]\)urea in acidosis may be in part related to decreased formation of NAG. The opposite may have occurred in alkalosis or in the presence of glucagon, when stimulation of NAG synthesis was associated with stimulation of \([15N]\)urea synthesis. Increased synthesis of NAG most likely occurred secondary to the formation of Acetyl-CoA via the pyruvate dehydrogenase pathway (50). Hence, metabolism of pyruvate via both pyruvate dehydrogenase and PC pathways seem to be an important site of regulation of \([15N]\)urea synthesis from \([5-13N]\)glutamine.

In summary, the current investigation illustrates that the control of urea synthesis by pH or hormones may be mediated via various nonexclusive mechanisms including: alteration of citrulline synthesis; formation of aspartate from pyruvate primarily via the PC pathway; and/or modulation of NAG synthesis from pyruvate, mediated via the pyruvate dehydrogenase pathway.

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