This study examines the role of glucagon and insulin in the incorporation of $^{15}$N derived from $^{15}$N-labeled glutamine into aspartate, citrulline and, thereby, $[^{15}N]$urea isotopomers. Rat livers were perfused, in the nonrecirculating mode, with 0.3 mM NH$_4$Cl and either $^{2-15}$N- or $^{5-15}$N-labeled glutamine (1 mM). The isotopic enrichment of the two nitrogenous precursor pools (ammonia and aspartate) involved in urea synthesis as well as the production of $[^{15}N]$urea isotopomers were determined using gas chromatography-mass spectrometry. This information was used to examine the hypothesis that 5-N of glutamine is directly channeled to carbamyl phosphate (CP) synthesis. The results indicate that the predominant metabolic fate of $[2-^{15}$N] and $[5-^{15}$N]glutamine is incorporation into urea. Glucagon significantly stimulated the uptake of $^{15}$N-labeled glutamine and its metabolism via phosphate-dependent glutaminase (PDG) to form $U_{m+1}$ and $U_{m+2}$ (urea containing one or two atoms of $^{15}$N). However, insulin had little effect with control. The $[5-^{15}$N]glutamine primarily entered into urea via ammonia incorporation into CP, whereas the $[2-^{15}$N]glutamine was predominantly incorporated via aspartate. This is evident from the relative enrichments of aspartate and of citrulline generated from each substrate. Furthermore, the data indicate that the $^{15}$NH$_3$ that was generated in the mitochondria by either PDG (from $5-^{15}$N) or glutamate dehydrogenase (from $2-^{15}$N) enjoys the same generated in the mitochondria by either PDG (from $5-^{15}$N) or glutamate dehydrogenase (from $2-^{15}$N) enjoys the same

We have previously demonstrated that glutamine is the chief precursor for urea-N (1–3), following its metabolism via the phosphate-dependent glutaminase (PDG)$^1$ pathway to provide NH$_3$ and glutamate (1–3). A smaller fraction of ammonia may be derived via the glutamate dehydrogenase (GDH) reaction (1). However, glutamate rapidly transaminated to aspartate to provide the second nitrogen of urea (1–3). More recently, we developed a theoretical framework that described the incorporation of $^{15}$N from $^{15}$NH$_4$Cl into urea and that predicted the proportions of $U_m$, $U_{m+1}$, and $U_{m+2}$ isotopomers of urea produced (containing no, one, or two atoms of $^{15}$N) as a function of the isotopic enrichment of the two nitrogenous precursor pools for urea. We experimentally validated this model in the isolated perfused rat liver (4). We have also examined the incorporation of $^{15}$N from $[5-^{15}$N]glutamine into urea in isolated hepatocytes and examined effects of pH and hormones on this process (3). These latter studies showed that our theoretical framework for prediction of the labeling patterns of urea was also valid in the hepatocyte model and that alkalosis and glucagon were powerful stimuli for increased flux through hepatic glutaminase and the urea cycle. They also showed important effects of hormones and of pH on the hepatocyte concentration of N-acetylglutamate (N-AG), the obligatory activator of carbamyl-phosphate synthetase-I (CPS-I) (5–8).

In this study we used this framework to explore the role of insulin or glucagon in the production of mass isotopomers of urea. We used $^{2-^{15}$N}- or $^{5-^{15}$N}-labeled glutamine and GC-MS to address the following questions: (i) What is the relative incorporation of $^{15}$NH$_3$, formed from $^{15}$N-labeled glutamine via the PDG (from $5-^{15}$N) and/or GDH (from $2-^{15}$N) pathway, into citrulline or aspartate, and thereby, $[^{15}N]$ urea isotopomers? (ii) Is the hepatic intramitochondrial pool of $^{15}$NH$_3$ (formed via either PDG or GDH) in equilibrium with the perfusate NH$_3$ pool? (iii) Does production of $[^{15}N]$urea isotopomers depend on the species of $^{15}$N-labeled glutamine, i.e. amino versus amido $^{15}$N? The results of these determinations were used to examine the hypothesis that the 5-N of glutamine is directly channeled to carbamyl phosphate synthesis (9).

Our methodological employed the stable isotope, $^{15}$N, and GC-MS provides an excellent approach to the quantitation and identification of $^{15}$N enrichment in metabolic intermediates (1–4, 10, 11). The use of $^{15}$N as a metabolic tracer is pivotal to the precise definition of precursor-product relationships and quantitation of N-flux from either the 2-N or 5-N of glutamine to NH$_3$, carbamyl phosphate, aspartate, citrulline, and, thereby, urea (1–4).

In a separate series of perfusions, we have examined the role

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1 The abbreviations used are: PDG, phosphate-dependent glutaminase; GDH, glutamate dehydrogenase; N-AG, N-acetylglutamate; CPS-I, carbamyl-phosphate synthetase-I; GC-MS, gas chromatography-mass spectrometry; APE, atom percent of excess.
of glucagon or insulin in the regulation of the 15N enrichment of the two nitrogenous precursor pools involved in urea synthesis as well as the production of mass isotopomers of [15N]urea. These hormones are key players in the regulation of hepatic nitrogen and carbohydrate metabolism in normal and disease states (3, 12–16). The data demonstrate that glucagon stimulated the flux through the PDG pathway and the formation of [15N]urea mass isotopomers from [2-15N]glutamine or [5-15N]glutamine. However, insulin has little effect on the flux through the PDG pathway and the formation of [15N]urea. The increased urea synthesis with glucagon was coupled with an increased hepatic level of N-Ag. There was no evidence for a metabolic channeling between glutaminase and carbamylphosphate synthetase.

EXPERIMENTAL PROCEDURES

Materials and Animals—Chemicals were of analytical grade and obtained from Sigma-Aldrich. Enzymes and cofactors for the enzymatic analyses were obtained from Roche Molecular Biochemicals. The 2-15N- and 5-15N-labeled glutamine were from Cambridge Isotopes Laboratories, Inc. (Andover, MA). Harlan Sprague-Dawley rats were from the Memorial University colony and were fed on Agway ProLab rat chow.

Liver Perfusion.—Livers from fed, male Harlan Sprague-Dawley rats (weighing about 200–270 g) were perfused in the nonrecirculating mode as described by Sies (17). The perfusion medium was a Krebs’ saline continuously gassed with 95% O2, 5% CO2 and containing lactate (2.1 mM) and pyruvate (0.3 mM) as metabolic fuels. Perfusion flow rate, pH, pCO2, and pO2 (in influent and effluent media) were monitored throughout and O2 consumption was determined. After 10 min of perfusion, glutamine (1 mM), either [5-15N]glutamine or [2-15N]glutamine, 99 atom percent of excess (APE), and NH4Cl (0.3 mM) were added to the medium. Perfusion continued for another 60 min with hormones (10 μM insulin) infused between 40 and 70 min. Control perfusions had saline infusion from 40 to 70 min.

Samples were taken at the indicated times from influent and effluent media for chemical and GC-MS analysis. At the end of the perfusion (70 min), livers were harvested, washed in ice-cold saline, and homogenized in liquid N2. The frozen livers were ground to a fine powder and extracted into perchloric acid, and the neutralized extracts were used for analysis by high pressure liquid chromatography, using precolumn derivatization with o-phthalaldehyde (19). Urea and ammonia were determined using conventional methods (20, 21). The level of N-Ag in freeze-clamped livers was determined using GC-MS and a modification of the conventional isotope dilution technique as we have previously described (3).

GC-MS Determination of [15N]-Labeled Metabolites—GC-MS measurements of 15N-isotopic enrichment were performed on a Hewlett Packard model 5970 high resolution instrument coupled with a 5890 HP-GC, as described previously (3, 4). Briefly, 15N-enrichment was measured after conversion of ammonia to glutamate (22). Isotopic enrichment in glutamate, aspartate, or N-acetylglutamate was determined following separation of these amino acids from glutamine and asparagine. 15N enrichment in citrulline, alanine, urea, and glutamine was determined following removal of any arginine that might be present at this time by interference with the GC-MS analysis of citrulline. A 500-μl aliquot of the medium was first applied to an AG-50 (Na+ form, 2.5 cm) column. Arginine remained bound to the resin, whereas urea, citrulline, alanine, and other amino acids were eluted with 3 ml of water. The effluent was collected and then applied to an AG-50 (H+ form, 100–200 mesh; 0.5 × 2.5 cm) column that was washed with 4 ml of water. Further and alanine acids were eluted with 3 ml of 4 N NaOH (3, 4).

For measurement of 15N enrichment, urea and amino acids were converted into t-butyldimethylsilyl derivatives. The m/z 231, 232, 233, and 234 of the urea t-butyldimethylsilyl derivative was monitored for singly labeled and doubly labeled urea determination (3, 4). Isotopic enrichment in citrulline, glutamine, glutamate, aspartate, and alanine was monitored using ratios of ions at m/z of 443/442, 439/438, 443/432, 419/418, and 261/260, respectively (3).

Data Presentation and Analysis—The formation of 15N-labeled metabolites was determined by the product of their isotopic enrichment (APE/100) times concentration (nmol/g wet wt) and is expressed as nmol 15N-metabolite/g wet wt. Flux through the PDG pathway during the course of the perfusion was calculated from the sum of 15N-labeled urea, ammonia, alanine, and glutamate formation from [5-15N]glutamine (5).

The endogenous production of glutamine (Pd) during the course of perfusion was calculated according to the equation: 
Pd = I × (E1/E2) - 1
where I is the rate of 15N-labeled glutamine infusion (nmol/min/g), E1 is the isotopic enrichment of the influent glutamine, and E2 is the 15N enrichment of effluent glutamine at the steady state most cases between 0.7 and 0.75.

The distribution of 15N[urea mass isotopomers was calculated using the mathematical model we have previously described (4). Briefly, when 15N-labeled glutamine is provided as substrate the urea formed may have a mass of 60, 61, or 62 molecular weight depending on whether zero, one, or two 15N atoms are incorporated. This is in turn dependent on the enrichment of glutamine in the two relevant nitrogen pools, i.e., the mitochondrial ammonia pool and the cytoplasmic aspartate pool. Let the fractional abundance of 15N in the mitochondrial ammonia pool be x; then the fractional abundance of 15N in the same pool is 1 - x. Similarly, let the fractional abundance of 15N in the cytoplasmic aspartate pool be y; then the fractional abundance of 15N in the same pool is 1 - y. Then the fractional abundance of the urea isotopomers will be:

U1 = (1 - x)(1 - y), where U1 is the fraction of urea containing no atom of 15N; U2 = 1 - (1 - x)(1 - y), where U2 is the fraction of urea containing one atom of 15N; U3 = x, where U3 is the fraction of urea containing two atoms of 15N.

Therefore, U1/U2, U2/U3, and U3/U1 sum to unity. This relationship permits one to predict the fractional abundance of U1, U2, and U3 at any given abundance of 15N in the mitochondrial ammonia and cytoplasmic aspartate pools, i.e., at any value of x and y.

Statistical analyses were carried out by the use of Student’s t test or analysis of variance test, as appropriate. A p value less than 0.05 was taken as indicating a statistically significant difference. Regression analysis was carried out using the Sigma Plot Program.

RESULTS

Characterization of the Perfused Livers—A total of eighteen perfusions were carried out: nine with [5-15N] glutamine, of which three each were infused with saline, glucagon, or insulin from 40 to 70 min, and nine with [2-15N] glutamine, of which three each were infused with saline, glucagon, or insulin from 40 to 70 min. Because all perfusions were presented with the same concentrations of substrates (1 mM glutamine, 0.3 mM NH4Cl, 2.1 mM lactate, and 0.3 mM pyruvate), it was possible to combine those with [2-15N] and those with [5-15N] glutamine when basic perfusion characteristics were assessed. As illustrated in Fig. 1, in the control perfusions (saline infused) O2 consumption was at about 2.5 μmol min⁻¹ g⁻¹ from 25 to 70 min. Likewise the outputs of alanine, glutamate, and urea were stable as well as the uptake of ammonia and glutamine (the apparent increase in urea output between 50 and 70 min was not significant). At 70 min the measured uptake of nitrogen (from glutamine and ammonia) was about 100 nmol of nitrogen min⁻¹ g⁻¹, whereas the output of nitrogen (in urea, alanine, and glutamate) was about 150 nmol of nitrogen min⁻¹ g⁻¹, indicating that endogenous sources contributed about a third of the nitrogen measured in the perfusion effluent (Fig. 1A).

When glucagon was infused from 40 to 70 min (Fig. 1B), there was a significant increase in O2 consumption as well as a significantly increased uptake of glutamine and output of urea and glutamate. At 70 min the measured uptake of nitrogen was about 145 nmol min⁻¹ g⁻¹, and output was about 214 nmol min⁻¹ g⁻¹. Fig. 1C shows that when insulin was infused there was stable O2 consumption of 2.5 μmol min⁻¹ g⁻¹. In addition, nitrogen uptake was about 100 nmol of nitrogen min⁻¹ g⁻¹, and output was about 161 nmol of nitrogen min⁻¹ g⁻¹.

In control, the liver content of adenine nucleotides after 70 min perfusion (ATP, 2.58 ± 0.29 μmol/g; ADP, 1.34 ± 0.18 μmol/g; and AMP, 0.29 ± 0.09 μmol/g) were quite similar to levels found in vivo (18). There is no significant difference following glucagon or insulin infusion. Similarly, there is no difference in the levels of aspartate and citrulline. The only significant (p < 0.05) differences found were increased levels of

Phylogenetic Analysis—Phylogenetic trees were constructed from 16S rDNA sequences using the neighbor joining method (23). Bootstrap analysis indicated strong support for most clades.
glutamine uptake. The uptake of 15N-labeled glutamine during the course of perfusion is depicted in Fig. 2A, where the curves of 15N glutamine uptake (Fig. 2A) are derived from experiments with [2-15N]glutamine and [5-15N]glutamine, and flux through the PDG was estimated from experiments with [5-15N]glutamine as detailed under "Experimental Procedures." Bars are the means ± S.D for three livers. *p < 0.05 compared with saline perfusions.

When we examine the hormonal regulation of glutamine metabolism, we must take into account the rate of hepatic glutamine synthesis (recycling) as indicated previously (2, 23). The initial 15N enrichment of effluent glutamine (at 15 min) is 93.5 ± 1.3 APE (mean ± S.D, n = 18), regardless of the experimental condition. However, the 15N enrichment of glutamine in the effluent at 70 min is decreased to 89.3 ± 3.1 APE (p < 0.001). There are two possibilities to explain this result. One possibility is that because of slow uptake of perfusate glutamine, there is little opportunity for equilibration, but after 70 min of perfusion this seems unlikely. The second possibility is that intracellular isotopic enrichment is diluted by the endogenous production of unlabeled glutamine. From the isotopic enrichment of 15N-labeled glutamine at the steady state (50–70 min), we were able to calculate the rate of the endogenous glutamine production. In perfusions with [5-15N]glutamine, these rates are 191 ± 109, 204 ± 108, and 274 ± 22 nmol·min⁻¹·g⁻¹ (mean ± S.D, n = 3) in the control, glucagon-infused, and insulin-infused livers, respectively. These rates are not significantly different from each other. The estimated rates of the endogenous glutamine synthesis are about 30% higher in the livers perfused with [5-15N]glutamine compared with those perfused with [2-15N]glutamine. We consider the data obtained with [5-15N]glutamine to be more reliable because the APE of effluent glutamate in these perfusions is less than 10, whereas in livers perfused with [2-15N]glutamine it ranged between 25–50 APE. Thus, if uptake of glutamate by
the perivenous hepatocytes were a significant source of glutamate for glutamine synthesis in these cells, there would be an appreciable underestimation of the rate of glutamine recycling if this glutamate were already appreciably labeled.

Production of \(^{15}\text{N}\)-Labeled Products—Fig. 4 shows the production of labeled products in livers perfused with \([5-^{15}\text{N}]\)glutamine and infused with saline (Fig. 4A), glucagon (Fig. 4B), or insulin (Fig. 4C). As indicated above, the PDG pathway will convert \([5-^{15}\text{N}]\)glutamine to \(^{15}\text{NH}_{3}\), which may be released as \(^{15}\text{NH}_{4}^+\) or incorporated into glutamate by glutamate dehydrogenase or into carbamyl phosphate via CPS-I (and then, into urea). \(^{15}\text{N}\) in glutamate may be transaminated to other amino acids that are released by the liver (e.g. alanine) or transaminated to aspartate, which can be incorporated into urea. As indicated in Fig. 4, the principal nitrogenous product released was urea. After 40 min of perfusion, \([^{15}\text{N}]\)urea production amounted to about 100–150 nmol min\(^{-1}\)g\(^{-1}\) in each of the perfusions and continued to increase to 240–300 nmol min\(^{-1}\)g\(^{-1}\) at 70 min in the perfusions with saline or insulin infusions. In the case of the glucagon perfusions, however, \([^{15}\text{N}]\)urea production increased to about 650 nmol min\(^{-1}\)g\(^{-1}\). The \([^{15}\text{N}]\)ammonia production profiles showed a similar response to glucagon: \(^{15}\text{NH}_{3}\) production plateaued at about 80 nmol min\(^{-1}\)g\(^{-1}\) in the control and insulin-perfused livers but increased to 130 nmol min\(^{-1}\)g\(^{-1}\) in the glucagon perfusions. Production of alanine and of glutamate were minor (5–20 nmol min\(^{-1}\)g\(^{-1}\)).

Fig. 5 shows the production of labeled products in livers perfused with \([2-^{15}\text{N}]\)glutamine and infused with saline (Fig. 5A), glucagon (Fig. 5B), and insulin (Fig. 5C). Glutaminase will convert \([2-^{15}\text{N}]\)glutamine to \([^{15}\text{N}]\)glutamate within hepatic mitochondria, and this glutamate can then be transaminated to form \([^{15}\text{N}]\)aspartate or alanine or be deaminated via glutamate dehydrogenase to yield \(^{15}\text{NH}_{3}\). \(^{15}\text{N}\) from \([2-^{15}\text{N}]\)glutamine may be incorporated into urea either through aspartate or through carbamyl-phosphate synthetase. As in the experiments with \([5-^{15}\text{N}]\)glutamine, experiments with \([2-^{15}\text{N}]\)glutamine demonstrate that in the control perfusions (Fig. 5A) the principal labeled product was urea that was produced at a rate of about 120–200 nmol min\(^{-1}\)g\(^{-1}\) at 40 min, and this increased to 320, 760, and 277 nmol min\(^{-1}\)g\(^{-1}\) at 70 min in the control, glucagon-infused, and insulin-infused perfusions, respectively. The other \([^{15}\text{N}]\)labeled products were relatively minor, but there were important differences between them. At 70 min in the control perfusions, the production of \([^{15}\text{N}]\)-labeled ammonia, glutamate, and alanine were 27, 19, and 19 nmol min\(^{-1}\)g\(^{-1}\). In the glucagon-infused livers, these rates were 66, 81, and 12 nmol min\(^{-1}\)g\(^{-1}\) and in the insulin-perfused livers rates of 40, 16, and 29 nmol min\(^{-1}\)g\(^{-1}\) were found. The increased production of \([^{15}\text{N}]\)labeled ammonia and glutamate in the glucagon-infused livers were significantly different from controls, as was the increased production of \([^{15}\text{N}]\)alanine in the insulin-infused perfusions.

Comparison of \(^{15}\text{N}\) Enrichment in Perfusate with That in the Liver—An important aim of the current study is to address the following question: Is the \(^{15}\text{N}\) labeling of intracellular metabolites in equilibrium with the effluent metabolites? To this end, we determined the \(^{15}\text{N}\) enrichment in liver metabolites after freeze-clamping at 70 min and in samples taken simultaneously in the perfusate outflow. These data are shown in Fig. 6, demonstrating a highly significant relationship \((p < 0.0001)\) between \(^{15}\text{N}\) enrichment in perfusate and liver for citrulline, aspartate, and glutamate.

Fig. 6 also shows the differing degrees to which these amino acids were labeled in the different experimental situations. It is apparent that aspartate and glutamate were more heavily labeled by \([2-^{15}\text{N}]\)glutamine than by \([5-^{15}\text{N}]\)glutamine. This is to be expected as glutaminase will produce \([^{15}\text{N}]\)glutamate, which will be readily transaminated to aspartate. Such a product-precursor relationship is borne out by the slightly higher \(^{15}\text{N}\) enrichment in the precursor (glutamate) than in the products (aspartate). Of course the \(^{15}\text{N}\) enrichment in glutamate is considerably lower than in its precursor, glutamine. Citrulline was more heavily labeled by \([5-^{15}\text{N}]\)glutamine. This is consistent with the \(^{15}\text{NH}_{3}\) produced through the PDG pathway being efficiently removed by carbamyl-phosphate synthetase.

Fig. 7 shows a plot of the \(^{15}\text{N}\) enrichment of perfusate ammonia with that of perfusate citrulline. Data points from the perfusions with \([2-^{15}\text{N}]\)glutamine and \([5-^{15}\text{N}]\)glutamine are denoted with separate symbols. It is evident that there was a highly significant relationship \((p < 0.0001)\) between \(^{15}\text{N}\) enrichment in perfusate ammonia and citrulline for either substrate, and furthermore, it is clear that the points obtained with \([2-^{15}\text{N}]\)glutamine fall on the same line as those obtained with \([5-^{15}\text{N}]\)glutamine. This will be discussed in more detail.
under “Discussion.”

Production of Urea Mass Isotopomers—Fig. 8 illustrates the actual distribution of urea mass isotopomers when [2-15N]glutamine or [5-15N]glutamine was used as labeled precursor. The data indicate that glucagon rapidly and remarkably stimulated \( U_m^{11} \) and \( U_m^{12} \) production regardless of the position of 15N in glutamine. However, insulin had little effect on the formation of [15N]urea mass isotopomers compared with control perfusions. It should be emphasized that a steady state level of isotopomer production was not achieved immediately, and indeed, the proportions of \( U_m^{11} \) and \( U_m^{12} \) continued to increase, and the proportion of \( U_m \) continued to decrease until about 60 min. We therefore used the 15N enrichment in the effluent citrulline and aspartate to calculate [15N]urea isotopomer distribution at 70 min using the mathematical model we have previously described (4).

In our previous work we showed that during perfusions with 15NH4Cl, the 15N enrichment in perfusate citrulline and aspartate were reliable proxies for the enrichment, respectively, of mitochondrial ammonia and cytoplasmic aspartate (4). It is important, therefore, to determine whether these relationships would also hold when 15N-labeled glutamine was the precursor. These data are shown in Table I. In this table we provide data at 40 min (when \( n = 9 \) for each group) and at 70 min (\( n = 3 \) for each group). It is quite apparent that we were able to predict, very accurately, the proportion of unlabeled urea produced (\( U_m \)) and also the proportion of singly labeled produced (\( U_m^{11} \)); however, in the case of doubly labeled urea (\( U_m^{12} \)), the agreement was not good, because there was always a larger proportion of this isotopomer found than predicted.

Hormonal Regulation of N-Acetylglutamate Levels—In a study of nitrogen metabolism in isolated hepatocytes, we observed that glucagon increased and insulin decreased N-acetylglutamate levels (3). In the present study we measured N-AG in livers freeze-clamped at the end of the 70-min perfusions. The current measurements substantiate our previous observation and demonstrate that glucagon caused a significant increase in N-AG, 130 \( \pm \) 62 nmol min\(^{-1}\) g\(^{-1}\) compared with 61 \( \pm \) 8 nmol min\(^{-1}\) g\(^{-1}\) for the control (means \( \pm \) SD, \( n = 6 \)). A decrease of approximately 20% was observed in the insulin perfusions (48 \( \pm \) 12 nmol min\(^{-1}\) g\(^{-1}\)), but this did not quite reach statistical significance (\( p = 0.06 \)) from the control values. Previous investigations have indicated a short term regulation...
of urea synthesis through rapid changes in N-AG levels, because the mitochondrial carbamyl-phosphate synthetase-I is inactive when N-AG is absent (6, 7, 25). The current study indicates a linear correlation between hepatic N-AG levels and glutaminase flux based on conversion of [5-15N]glutamine to labeled ammonia and glutamate dehydrogenase, and the aminotransferases are reversible so that the incorporation of 15N into ammonia and glutamate dehydrogenase equilibration. It is well established that the glutamate side (28), but this holds true only for the glutamate dehydrogenase equilibration. The labeling of ammonia and of citrulline in the experiments that utilize [2-15N]glutamine and the increase in glutamate. The fact that glutamine and the amino acids by these enzymes will reflect isotopic exchange as well as net flux. Our results supply clear evidence for the reversibility of these enzymes. For example, the labeling of glutamate, alanine, and aspartate in the experiments that utilize [5-15N]glutamine and of citrulline in the experiments that utilize [2-15N]glutamine are urea isotopomers containing no, one, or two 15N, respectively, for saline-infused livers (○), glucagon-infused livers (△), and insulin-infused livers (□). Each data point represents the mean ± S.D for three livers. *, p < 0.05 compared with saline perfusions.

DISCUSSION

In the current study we used a series of liver perfusion experiments to examine the role of glucagon or insulin in the regulation of the 15N enrichment of the two nitrogenous precursor pools involved in urea synthesis as well as the production of mass isotopomers of [15N]urea from either 2-15N- or 5-15N-labeled glutamine. These hormones have a pivotal role in the regulation of hepatic nitrogen and carbohydrate metabolism (3, 12–16). Elucidation of the hormonal regulation of N-AG synthesis may play a key role in ureagenesis from glutamine.

FIG. 8. Production of [15N]urea mass isotopomers during the course of perfusion. Livers were perfused with 1 mM [5-15N]glutamine or [2-15N]glutamine and other nutrients as indicated under Experimental Procedures. *U, U0, and U1 are urea isotopomers.

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<td>Comparison of experimental and predicted isotopomer percentages</td>
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Atoms. The amide nitrogen of glutamine was primarily incorporated into urea via carbamyl phosphate, whereas the amino nitrogen was primarily incorporated via aspartate, as is evident from the relative enrichments of aspartate and citrulline generated from each substrate.

Glucagon stimulated flux through the PDG pathway (p < 0.05), as has been previously reported (3, 12, 16, 23). The activation of glutaminase is confirmed by the decrease in liver glutamine and the increase in glutamate. The fact that glutaminase is irreversible permits reliable estimates of glutaminase flux based on conversion of [5-15N]glutamine to labeled products. However, the enzymes subsequent to glutaminase, glutamate dehydrogenase, and the aminotransferases are reversible so that the incorporation of 15N into ammonia and amino acids by these enzymes will reflect isotopic exchange as well as net flux. Our results supply clear evidence for the reversibility of these enzymes. For example, the labeling of glutamate, alanine, and aspartate in the experiments that utilized [5-15N]glutamine depends on the incorporation of ammonia into glutamate via glutamate dehydrogenase acting in the direction of reductive animation. The labeling of ammonia and of citrulline in the experiments that utilize [2-15N]glutamine rely on the deamination of glutamate via glutamate dehydrogenase.

These differences of product enrichment in perfusions with the differently labeled glutamines are germane to the question of glutamate dehydrogenase equilibration. It is well established that the equilibrium of the GDH enzyme activity lies far to the glutamate side (28), but this holds true only for the purified enzyme in which the concentration of ammonia is in the high millimolar range, well beyond any expected in vivo concentration (28, 29). Indeed, recent investigation with the hyperammonemia, hyperinsulinemia syndrome, which is caused by a mutant GDH that is no longer subject to tight inhibition by GTP, suggests that the enzyme can function primarily for the purpose of glutamate oxidation, at least in liver.
and pancreas (29). The current data indicate that the glutamate dehydrogenase is reversible; the incorporation of 15N from [5-15N]glutamine into glutamate and from [2-15N]glutamine into ammonia occurs via theamination and deamination reactions, respectively, of glutamate dehydrogenase. However, the flux through the glutamate dehydrogenase is not so great, in comparison with the flux of other relevant pathways (1–3), that full 15N equilibration occurs. Glutaminase produces equal quantities of glutamate and ammonia. If the equilibrating effect of glutamate dehydrogenase were absolute, then glutamate and ammonia would become equally labeled within mitochondria regardless of whether [2-15N]glutamine or [5-15N]glutamine were substrates, and there would be no difference between these substrates in the labeling of other metabolites such as alanine, aspartate, or citrulline. There are, however, substantial differences in the labeling of these other metabolites, which is most likely explained by “metabolic competition,” i.e., that aspartate and alanine aminotransferases compete effectively with glutamate dehydrogenase for [15N] glutamate that arises from [2-15N]glutamine metabolism and carbamyl-phosphate synthetase competes effectively with glutamate dehydrogenase for [15N]H3 that arises from [5-15N]glutamine metabolism. Therefore, it is not necessary to invoke metabolic channeling, merely metabolic competition, to account for the different labeling patterns that are found when these two positional isotomers of 15N glutamate are employed as substrates.

The glutamate dehydrogenase reaction may have also a key role in the regulation of glutamine synthesis. It is possible that a significant portion of the endogenous glutamine production is mediated via de novo synthesis, in which a net flux of carbons into glutamine occurs. In this case α-ketoglutarate formed in the tricarboxylic acid cycle (from pyruvate and lactate added to the perfusate) would be converted to glutamate via the glutamate dehydrogenase reaction and then to glutamine. Our previous investigation with isolated hepatocytes and [3-13C] pyruvate indicated that approximately 15% of glutamate carbons were derived from [3-13C]pyruvate (3). However, further study with 13C-labeled precursor would be required to determine whether a similar portion of glutamate carbon is derived during liver perfusion.

Fig. 3 shows that the 15N enrichment of glutamine in the cellular pool is always lower than in the effluent perfusate and that the enrichment of the cellular pool is much higher when [2-15N]glutamine is the substrate. That the enrichment in the cellular pool is lower than in the perfusate means that some of the intracellular glutamine arises from unlabeled sources, i.e., via proteolysis, glutamine recycling, or de novo glutamine synthesis (as indicated above). We previously reported that glutamine production via proteolysis amounted to 7 nmol/min/g (4), that is, approximately 3% of the endogenous glutamine production calculated in the current study. Therefore, proteolysis seems unlikely to be the major source for endogenous glutamine production, and thus the dilution of 15N enrichment in the perfusate glutamine must occur following production of glutamine from other unlabeled sources. In the case of [5-15N]glutamine, unlabeled glutamate and 15NH3 will be produced via the PDG pathway. In the mitochondria, this 15NH3 can be metabolized to [15N]glutamate via the GDH pathway. If 15N-labeled ammonia and glutamate are used for glutamine synthesis, then glutamine would be labeled at 2-N and 5-N (4). However, in the current study no doubly labeled glutamine was detected regardless of the experimental conditions, indicating that glutamine was formed from either unlabeled ammonia, unlabeled glutamate, or both. It is possible that the unlabeled mitochondrial glutamate (formed via the PDG pathway) is transported to the cytosol and then recycled to glutamine in the perivenous hepatocytes. Therefore, during perfusion with [5-15N] glutamine, mainly unlabeled glutamine is produced and simultaneously dilutes the [5-15N]glutamine enrichment in the liver (Fig. 3). However, in the case of perfusions with [2-15N]glutamine, [15N]glutamate (between 30–50 APE, depending upon the experimental condition) is formed so that the glutamine produced would be labeled, and thereby, the dilution of the intrahepatic [2-15N]glutamine would be less significant than in the case of perfusions with [5-15N]glutamine. Haußinger et al. (24) have shown that an intrahepatic glutamine cycle exists whereby there is simultaneous catabolism and synthesis of glutamine (in periportal and perivenous hepatocytes, respectively. Haußinger (23) has also shown an effective uptake mechanism for glutamate in the perivenous hepatocytes. We therefore envisage that some glutamate produced in periportal hepatocytes by glutaminase is taken up by the perivenous hepatocytes and converted to glutamine. In this case, the endogenous production of glutamine is mediated primarily via recycling of perfusate glutamine as indicated by Haußinger et al. (23, 24).

The data in Fig. 7 are of considerable interest in the light of the suggestion that a metabolic channel exists between PDG and CPS-I such that ammonia that arises from 5-15N of glutamine enjoys preferential access to carbamyl-phosphate synthetase (9). Both [5-15N]glutamine and [2-15N]glutamine give rise to ammonia in the same compartment (the mitochondria of glutaminase-containing hepatocytes) but as a result of the action of two different enzymes. 15NH3 will be produced from [5-15N]glutamine by the PDG pathway. It will also be produced from [2-15N]glutamine by the GDH pathway, which acts on [15N]glutamate that arises via PDG. By comparing the metabolic fate of 15NH3 that is produced in the same compartment by these two different enzymes, we can determine whether ammonia that arises via the PDG pathway has preferential access to carbamyl-phosphate synthetase. Ammonia that arises in the mitochondria can have three immediate metabolic fates: (i) to be incorporated into carbamyl phosphate by CPS-I, (ii) to be incorporated into glutamate by glutamate dehydrogenase, and (iii) to leave the mitochondria. 15NH3 that is incorporated into carbamyl phosphate will be reflected in perfusate [15N]citrulline, whereas 15NH3 that leaves the mitochondria will be reflected in perfusate NH3 (4). Fig. 7 shows the correlation between perfusate [15N]NH3 and perfusate [15N]citrulline at all of the time points in all of the perfusions. An excellent correlation was found between the isotopic enrichment of perfusate ammonia and citrulline, and it is clear that the data from the perfusions with [2-15N]glutamine and [5-15N]glutamine fall on the same line. Thus, 15NH3 molecules that arise within the mitochondria by the agency either of glutaminase or of glutamate dehydrogenase enjoy the same partition between incorporation into carbamyl phosphate by means of CPS-I or leaving the mitochondria. Thus, in these experiments, there is no evidence for preferential access for ammonia that arises by the action of glutaminase to carbamyl-phosphate synthetase. To the contrary, we provide strong evidence that such ammonia is metabolized without any such metabolic channel. Rajiman and co-workers (31) have shown a channeling of urea cycle intermediates at each of the three cytoplasmic enzymes of the urea cycle. However, there are fundamental differences between the current investigation and that of Rajiman and co-workers (31). First, the current observation deals with the first mitochondrial reaction in the urea cycle (the channeling of ammonia derived via the PDG to CPS-I), whereas the observations of Rajiman and co-workers deals with the cytoplasmic enzymes of the urea cycle. Second, in the current study we used 15N-labeled glutamine and liver perfusion system, whereas
Rajiman and co-workers (31) used [14C]HCO\textsubscript{3} and isolated hepatocytes.

The production of the urea isotopomers requires comment. The time course for the three isotopomers (Fig. 8) shows that the pattern of their production varied throughout the perfusions, and even in the control perfusions, a steady state did not appear to be achieved until about 60–70 min. This is attributable to a slow increase in PDG activity that occurred during the perfusions (Fig. 2A), except in the case of the glucagon-infused livers, where there was a large activation of glutaminase. But, even there, the activation is progressive, and a steady state is not achieved until about 60 min. Thus the progressive decrease in the proportion of \textit{U}\textsubscript{m} and increase in the proportion of \textit{U}\textsubscript{m+1} and \textit{U}\textsubscript{m+2} throughout the perfusions are attributable to the increased provision of 15N-labeled urea precursors with time. The comparison with experimental and predicted isotopomer production (Table I) showed very good agreement for the proportion of \textit{U}\textsubscript{m} and \textit{U}\textsubscript{m+1}. However, there was quite a difference between the predicted and experimental values of \textit{U}\textsubscript{m+2}, with the experimental value invariably being greater. We do not know precisely why this is so, but we can make one suggestion. A possible explanation is that the enzymes of the urea cycle and, presumably, ureagenesis occur throughout the liver acinus, except for the last few perivenous cells, which contain glutamine synthetase (24). However, the glutaminase location does not exactly parallel that of the urea cycle in that it is expressed only in the early portion of the peri-portal region (24). In the experiments reported in this paper, urea synthesis will occur in the early part of the acinus from perfusate NH\textsubscript{4}Cl and 15N-labeled glutamine and in the later part of the acinus largely from NH\textsubscript{3}Cl. The urea isotopomers measured in the perfusate will be the sum of those produced in early and later portions of the acinus. Support for this explanation is provided in our previous study demonstrating a significant correlation between observed and predicted \textit{U}\textsubscript{m+1} and \textit{U}\textsubscript{m+2} when various 15NH\textsubscript{4}Cl enrichments were used (4). Brunengraber et al. (30) have emphasized the errors that can occur in measuring bio-syntheses by means of mass isotopomer distribution when there are variations in the enrichment of the labeled precursor because of compartmentation. A similar explanation may underlie the differences we find between the predicted and experimentally determined values for the \textit{U}\textsubscript{m+2} isotopomer of urea.

In conclusion, the current investigation indicates that there is no channeling of [5-15N]glutamine toward the synthesis of mitochondrial carbamyl phosphate. Ammonia formed from glutamine, whether from [5-N] via PDG or from [2-N] via GDH, enjoys the same partition between incorporation into carbamyl phosphate or leaving the mitochondria. This observation is of importance in terms of understanding perturbations of hepatic glutamine metabolism and ureagenesis in vivo, as we have previously demonstrated (32).

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Studies of Hepatic Glutamine Metabolism in the Perfused Rat Liver with $^{15}$N-Labeled Glutamine
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