A Mass Isotopomer Study of Urea and Glutamine Synthesis from \textsuperscript{15}N-labeled Ammonia in the Perfused Rat Liver\textsuperscript{*}

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This study examines the incorporation of \textsuperscript{15}N from \textsuperscript{15}NH\textsubscript{4}Cl into urea and glutamine, predicts the pattern of isotopomers produced as a function of the \textsuperscript{15}N enrichment of the relevant precursor pools, and presents a means of determining the isotopic enrichment of these pools. Rat livers were perfused, in the nonrecirculating mode, with 0.3 mM \textsuperscript{15}NH\textsubscript{4}Cl, and the isotopomers of urea and of glutamine produced were determined by gas chromatography-mass spectrometry methodology. Three different nitrogen mass isotopomers of urea were found, containing no, one, or two atoms of \textsuperscript{15}N. Four glutamine isotopomers were found, containing no \textsuperscript{15}N, one atom of \textsuperscript{15}N in either the amino or amide position, or two \textsuperscript{15}N atoms. A mathematical relationship was deduced that predicts that the relative proportions of the urea isotopomers depends not only on the relative enrichment of \textsuperscript{15}N in the two precursor pools of urea nitrogen (mitochondrial ammonia and cytoplasmic aspartate) but on their absolute enrichment. This relationship was validated in experiments in which the isotopic enrichment of the substrate, \textsuperscript{15}NH\textsubscript{4}Cl, was varied. The proportions of the urea isotopomers produced can be predicted if one knows the \textsuperscript{15}N enrichment in the two precursor pools. We found that when the \textsuperscript{15}N enrichment of citrulline and aspartate in the perfusate were used as proxies for that in the mitochondrial ammonia and cytoplasmic aspartate pools we could accurately predict the relative proportion of the three isotopomers. The production of the four nitrogen isotopomers of glutamine could be used to determine the \textsuperscript{15}N enrichment in the two precursor pools of glutamine nitrogen, the cytoplasmic ammonia and glutamate pools of the perivenous hepatocytes.

A variety of isotopic approaches have been used to study hepatic nitrogen metabolism. \textsuperscript{13}N has been introduced as a tracer for hepatic ammonia-N metabolism by Cooper et al (1, 2). In a series of elegant studies these workers have demonstrated very rapid uptake of portal ammonia in vivo and extremely rapid reversible transamination among glutamate, aspartate, and alanine. They also showed rapid incorporation of nitrogen from \textsuperscript{15}NH\textsubscript{3} into urea and, to a lesser extent, glutamine. This technique has been extremely useful for obtaining rapid kinetic data in vivo. Geissler et al. (3) have employed \textsuperscript{15}N NMR, which allows them to follow the production of labeled urea by isolated perfused livers in real time. However, the technique is, at present, very insensitive. Our experimental approach employed \textsuperscript{15}NH\textsubscript{3} and GC-MS\textsuperscript{1} to analyze the enrichment of products. In particular, from an analysis of \textsuperscript{15}N enrichment in urea and in glutamine, we sought to develop a theoretical framework and an experimental approach that would enable us to understand the factors that determined label incorporation into these compounds.

The mechanism of urea synthesis is well established. The two nitrogens of urea are introduced from different precursors and in different cellular compartments (4). One nitrogen is derived from ammonia and is incorporated into carbamoyl phosphate by carbamoyl-phosphate synthetase, which occurs in the mitochondrial matrix. This is then transferred to ornithine to give citrulline. The second nitrogen is derived from aspartate and is incorporated into argininosuccinate by argininosuccinate synthetase, which is found in the cytoplasm. The two nitrogens in urea are chemically and sterically identical so that it is not possible to identify the origin of a particular nitrogen atom in urea. Urea synthesis occurs in the liver, where it is a major function of hepatocytes in the perportal and pericentral zones (4). It is absent from the few ultimate hepatocytes in the perivenous zone, about 7% of the total hepatocyte population, which contain glutamine synthetase in the cytoplasm (5, 6). The glutamine produced also contains two atoms of nitrogen. However, they are distinguishable, the amide being derived from ammonia and the amino from glutamate.

The present study examines the labeling of urea and glutamine when \textsuperscript{15}N-labeled ammonia is provided as substrate. We had two objectives in doing this. First, we wished to provide a rigorous theoretical, experimentally verified framework for understanding the determinants of urea isotopomer production. This is particularly relevant, since a number of experimental studies have measured the incorporation of \textsuperscript{15}N-labeled substrates into urea (7–9). One set of studies, in particular, has used these data to suggest that urea synthesis in sheep is fundamentally different from other mammals (8, 9). The second reason for carrying out these studies was to introduce a methodology that would permit the determination of the isotopic enrichment of the two nitrogenous precursor pools involved in urea synthesis. Such methodology will enable us to test Meijs's hypothesis that there is metabolic channeling between glutaminase and carbamoyl-phosphate synthetase I such that the amide nitrogen of glutamine has preferential access to carbamoyl-phosphate synthetase without mixing with the mitochondrial pool of ammonia (10).

We have employed the single-pass isolated perfused liver as our experimental model, since this avoids problems due to

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\textsuperscript{1} The abbreviation used is: GC-MS, gas chromatography-mass spectrometry.
recycling of substrate (such as incorporation of ammonia into glutamine in perivenous hepatocytes and subsequent use of this glutamine nitrogen for urea synthesis in periportal hepatocytes, which could occur either in incubated hepatocytes or in a recirculating perfusion), which could confound the interpretation. When $^{15}$NH$_4$ is provided as substrate the urea formed may have a mass of 60, 61, or 62, depending on whether zero, one, or two $^{15}$N atoms are incorporated. This, in turn, depends on the enrichment of $^{15}$N in the two relevant nitrogen pools, the mitochondrial ammonia pool and the cytoplasmic aspartate pool. We present here a theoretical scheme that predicts the proportions of these four isotopomers of urea as a function of the $^{15}$N enrichment and an experimental means of determining the actual $^{15}$N enrichment of these pools.

We have also considered the synthesis of glutamine isotopomers. When synthesized in the presence of $^{15}$NH$_4$ four separate glutamine mass and positional isotopomers are produced, i.e. one with mass 146 (where no $^{15}$N is present), two with mass 147 (where $^{15}$N is present either in the amino or the amide group), and one with mass 148 (where $^{15}$N is present in both the amino and the amide group). We present here a theoretical scheme that predicts the proportions of these four isotopomers of glutamine as a function of the $^{15}$N enrichment in the precursor pools, i.e. in the cytoplasmic ammonia and glutamate pools of perivenous hepatocytes.

**EXPERIMENTAL PROCEDURES**

Liver Perfusions—Livers from fed, male Sprague-Dawley rats (weighing about 200 g), were perfused in the nonrecirculating mode as described by Sies (11). The perfusion medium was a Krebs' saline continuously gassed with 95% O$_2$, 5% CO$_2$, and containing lactate (2.1 mm) and pyruvate (0.3 mm) as metabolic fuels. Perfusion flow rate, pH, pCO$_2$, and pO$_2$ (in influent and effluent media) were monitored throughout, and oxygen consumption was calculated. After 15 min of perfusion, we changed to a medium containing $^{15}$NH$_4$Cl (final concentration, 0.3 mm), of varying $^{15}$N abundance. Separate perfusate reservoirs, each containing ammonia of different $^{15}$N enrichment, were employed to facilitate changes in perfusion media. Samples were taken from the influent and effluent media for chemical and GC-MS analyses.

At the end of the perfusions livers were freeze-dried with aluminum tongs precooled in liquid N$_2$, the frozen livers were ground into a fine powder and extracted into perchloric acid, and the extracts were used for the analysis of adenine nucleotides by enzymatic techniques (12). Urea and ammonia concentrations in the perfusion media were assayed by standard methods (13, 14). Amino acid concentrations were determined by HPLC, utilizing precolumn derivatization with o-phthalde- hydrazide. A few perfusates were carried out to determine the rate of glutamine production due to proteolysis. For these experiments the rats were pretreated with the glutamine synthetase inhibitor, methionine sulfoximine, and this was included in the perfusate (6).

GC-MS Methodology—GC-MS measurements of $^{15}$N isotopic enrichments were performed on a Hewlett Packard 5970 HP-MS coupled with a 5940 HP- GC. $^{15}$NH$_4$ enrichment was measured after conversion of ammonia to glutamate (16). $^{15}$N enrichment in glutamate and aspartate was determined following separation of these amino acids from glutamine and asparagine. This was done by applying a 250-$\mu$L aliquot of perfusate to an AG-1 column (Cl$^+$-X-8; 200–400 mesh; 0.5 cm). The columns were washed with 3 ml of deionized water. Glutamate and aspartate were eluted with 3 ml of 3 N HCl. $^{15}$N enrichment in citrulline, alanine, and urea was measured following removal of any arginines that might be present, as this can interfere with GC-MS analysis of citrulline. A 500-$\mu$L aliquot of perfusate was added to an equal volume of NaHCO$_3$ buffer (pH 7.1), and this was applied to an AG-50 (Na$^+$; 20–50 mesh, 0.5 cm) column. Aramine bound remained to this 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 cm) column, which was washed with 4 ml of water. Urea and amino acids were eluted with 3 ml of 4 N H$_2$O.

For GC-MS analysis, urea and amino acids were converted into t-butyldimethylsilyl derivatives. The m/z 231, 232, and 233, and 234 of the urea t-butyldimethylsilyl derivative were monitored for singly labeled and doubly labeled urea determination (7, 17). Isotopic enrichment in citrulline, glutamate, aspartate, and alanine was monitored using m/z ratios of 443/442, 432/431, 419/418, and 261/260, respectively (7, 17).

The m/z ratio 259/258 of the t-butyldimethylsilyl derivative of glutamine was used to determine the isotopic enrichment in the amino nitrogen (18). However, the t-butyldimethylsilyl derivative does not provide a measure of $^{15}$N enrichment in the amide nitrogen. Therefore, we utilized the N,N-bis-trifluoroacetyl derivative of glutamine (19). A 500-$\mu$L aliquot of the perfusate was applied to an AG-1 column as indicated above to separate glutamine from glutamate. Glutamine was eluted in the water wash and used to prepare the N,N-bis-trifluoroacetyl derivative, which was monitored at m/z 182/181 for the amino nitrogen and m/z 112/111 for the amide nitrogen as described previously (19). Correction for possible overlapping ions in the MS was as described by Wolve (20). Replicate GC-MS analyses agreed to within 4%.

Data Presentation and Analysis—The data are given as means ± S.D. Statistical analyses between means were by the Student t test or the Newman-Keuls multiple comparison test, as appropriate. Regression analysis was carried out using the Sigma Plot Program.

Materials and Animals—Chemicals were of analytical grade and obtained from Sigma or from Aldrich. Enzymes and cofactors for the analysis of adenine nucleotides and ammonia were obtained from Boehringer Mannheim (Montreal, Canada), and $^{15}$NH$_4$Cl (99 atom% excess) were from Cambridge Isotopes Laboratories Inc. (Andover, MA). Sprague-Dawley rats were obtained from Charles River (Montreal, Canada) and were fed on Agway ProLab Rat Chow.

**THEORY**

Urea Isotopomers—Consider the two separate precursor nitrogen pools for urea synthesis. Let the fractional abundance of $^{15}$N in the mitochondrial ammonia pool be $x$; then the fractional abundance of $^{15}$N in the cytoplasmic aspartate pool be $y$; then the fractional abundance of $^{15}$N in the same pool is 1 – $x$. Similarly, let the fractional abundance of $^{15}$N in the cytoplasmic aspartate pool be $y$; then the fractional abundance of $^{15}$N in the same pool is 1 – $y$. Then the fractional abundance of the urea isotopomers will be,

\[ U_m = (1 – x)(1 – y) \quad (Eq. 1) \]

where $U_m$ is the fraction of urea containing no atom of $^{15}$N,

\[ U_{m-1} = xy \quad (Eq. 2) \]

where $U_{m-1}$ is the fraction of urea containing two atoms of $^{15}$N,

\[ U_{m-2} = (1 – x)(1 – y) \quad (Eq. 3) \]

where $U_{m-2}$ is the fraction of urea containing one atom of $^{15}$N, and $U_{m-1}$ sum to unity. These relationships permit one to predict the fractional abundance of $U_m$, $U_{m-1}$, and $U_{m-2}$ at any given abundance of $^{15}$N in the mitochondrial ammonia and cytoplasmic aspartate pools, i.e. at any values of $x$ and $y$.

One of the most important deductions from these equations is that even in a stable physiological situation the proportions of the different isotopomers need not be constant but will vary depending on the absolute $^{15}$N abundance of the pools. For example, let us consider situations where $x = y$ (i.e. the $^{15}$N abundance in the mitochondrial ammonia pool equals that in the cytoplasmic aspartate pool). For $x = y = 0.1$ liver would produce urea that is 81% $U_m$, 18% $U_{m-1}$, and 1% $U_{m-2}$, whereas when $x = y = 0.9$ the corresponding percentages are 1, 18, and 81%.

In any given experiment the $^{15}$N abundance in each of the relevant nitrogen pools will be determined both by the rate of $^{15}$N incorporation into each pool and by the rates of dilution of this nitrogen by [1$^{14}$N]ammonia and [1$^{15}$N]aspartate, that arise in the liver. It is necessary, therefore, to compute the fractional abundances of the isotopomers for all possible values of $x$ and $y$ (i.e. as fractional $^{15}$N abundances of the mitochondrial ammonia pool and the cytoplasmic aspartate pool are permitted to vary between 0 and 1). Fig. 1, which is a plot of Equations 1, 2, and 3, shows these relationships. An important feature of this figure is its symmetrical nature. It is apparent that the curve
describing $U_{m-1}$ is symmetrical, while those describing $U_m$ and $U_{m+2}$ are images of each other. It is obvious that this figure, on its own, will not permit unique solutions for $x$ and $y$. For example if, in an experiment with $^{15}$NH$_3$ as substrate, a liver produced urea with the fractional isotopomer abundance of $U_m = 0.08$, $U_{m+1} = 0.44$, and $U_{m+2} = 0.48$, this would be satisfied with $^{15}$N abundances of $x = 0.2$ and $y = 0.4$ and equally satisfied with $x = 0.4$ and $y = 0.2$. Additional information is required to give a unique solution.

Glutamine Isotopomers—Four separate isotopomers of glutamine may be synthesized during our experiments: doubly labeled glutamine ($^{15}$N in both the amide and amino positions), glutamine labeled with $^{15}$N only in the amide position, glutamine labeled with $^{15}$N only in the amino position, and unlabeled glutamine. The relative proportions of these isotopomers will vary, in a predictable manner, depending on the $^{15}$N enrichment of the precursor pools of glutamate and of ammonia in the cytoplasm of the perivenous hepatocytes. Let the fractional $^{15}$N abundance of glutamate in this pool be $a$, and then the fractional $^{14}$N abundance is $1 - a$. Let the fractional $^{15}$N abundance of NH$_3$ in the same pool be $b$, and then the fractional $^{14}$N abundance is $1 - b$. Then the fractional abundance of the glutamine isotopomers will be,

$$G_m = (1 - a)(1 - b) \quad \text{(Eq. 4)}$$

where $G_m$ is the fraction of glutamine containing no atom of $^{15}$N

$$G_{m+1(\text{amino})} = a(1 - b) \quad \text{(Eq. 5)}$$

where $G_{m+1(\text{amino})}$ is the fraction of glutamine containing one $^{15}$N atom in the amino position,

$$G_{m+1(\text{amide})} = b(1 - a) \quad \text{(Eq. 6)}$$

FIG. 1. Theoretical relative abundance of the nitrogen isotopomers of urea. These are plotted as a function of the fractional abundance of $^{15}$N in the mitochondrial ammonia pool ($x$) and the cytoplasmic aspartate pool ($y$). $U_m$, $U_{m+1}$, $U_{m+2}$ are, respectively, the isotopomers of urea that contain no, one, and two atoms of $^{15}$N. These are plots of Equations 1-3 (see “Theory”) and were prepared using a Maple computer program (Waterloo Software Foundation, Waterloo, Canada).

FIG. 2. Theoretical relative abundance of the nitrogen isotopomers of glutamine. These are plotted as a function of the fractional abundances of $^{15}$N in the cytoplasmic ammonia ($a$) and glutamate ($b$) pools in glutamine-synthesizing hepatocytes. $G_m$, $G_{m+1(\text{amino})}$, $G_{m+1(\text{amide})}$, and $G_{m+2}$ are, respectively, the isotopomers of glutamine that contain no $^{15}$N atom, one $^{15}$N atom in the amino position, one $^{15}$N atom in the amide position, and two $^{15}$N atoms. These are plots of Equations 4–7 (see “Theory”) and were prepared using a Maple computer program (Waterloo Software Foundation).
where $G_{m+1}(\text{amide})$ is the fraction of glutamine containing one $^{15}$N atom, in the amino position

$$G_{m+2} = ab$$  \hspace{1cm} \text{(Eq. 7)}

where $G_{m+2}$ is the fraction of glutamine containing two $^{15}$N atoms, and $G_{m+1}(\text{amide})$, $G_{m+1}(\text{amino})$, and $G_{m+2}$ sum to unity. Fig. 2, which is a plot of Equations 4, 5, 6, and 7, shows these relationships.

In practice it is much simpler to deduce the enrichments of the precursor pools (ammonia and glutamate) for glutamine synthesis than for urea synthesis. This is because the two monolabeled glutamines are chemically distinct and may be separately determined. Thus, the fractional abundance of $^{15}$N in the ammonia pool used for glutamine synthesis ($b$) is given by the sum of the isotopomer fractions that contain $^{15}$N in the amide position,

$$b = G_{m+1}(\text{amide}) + G_{m+2}$$  \hspace{1cm} \text{(Eq. 8)}

and, similarly the fractional abundance of $^{15}$N in the glutamate pool ($a$) is given by the sum of the isotopomer fractions that contain $^{15}$N in the amino position,

$$a = G_{m+1}(\text{amino}) + G_{m+2}$$  \hspace{1cm} \text{(Eq. 9)}

RESULTS

Characterization of the Perfused Livers—Rat livers were perfused in a single pass mode with Krebs-Henseleit medium containing 2.1 mM lactate and 0.3 mM pyruvate as an energy source (11). The $\text{NH}_4\text{Cl}$ concentration in the perfusate entering the liver remained constant, at 0.3 mM, since this is the concentration found in the rat portal vein (21), but the $^{15}$N enrichment of this $\text{NH}_4\text{Cl}$ was varied in stepwise fashion during the experiment. The basic characteristics of the perfused liver are given in Fig. 3. Viability of the livers over the length of the perfusion is confirmed by the stability of the oxygen uptake (2.4 $\mu$mol/min/g), urea synthesis (300 nmol/min/g), glutamine synthesis (130 nmol/min/g), and ammonia uptake (600 nmol/min/g) (Fig. 3). The rates of release of glutamate (50 nmol/min/g) and alanine (130 nmol/min/g) were also constant between 20 and 75 min of perfusion (data not shown). In addition, the adenine nucleotide content of perfused livers freeze-clamped after 75 min of perfusion (ATP, 3.48 ± 0.32; ADP, 1.02 ± 0.31; and AMP 0.14 ± 0.05 $\mu$mol/g) are similar to levels measured in vivo (12). Although $^{15}$N enrichment in ammonia was varied from 25 to 100%, this did not affect the rates of urea synthesis or of glutamine synthesis, indicating that there is no significant isotope effect due to the $^{15}$NH$_4$Cl that could confound our results.

Urea and Ammonia $^{15}$N Enrichments—The production of the different isotopomers of urea is shown in Fig. 4. Fig. 4a shows the data from those perfusions where the $^{15}$N enrichment was varied from 25 to 100% (three livers) in Fig. 4a and from 100 to 25% in Fig. 4b (three livers). $U_m$, $U_{m+1}$, and $U_{m+2}$, respectively, are urea isotopomers containing no, one, and two atoms of $^{15}$N.

![Fig. 3. Nitrogen metabolism in the perfused liver.](image)

![Fig. 4. Mass isotopomers of urea as a function of the $^{15}$N enrichment of $\text{NH}_4\text{Cl}$.](image)
achieved. Since citrulline is synthesized from carbamoyl phosphate in the mitochondria, we used the 15N abundance in citrulline as an index of the abundance in the mitochondrial carbamoyl phosphate pool. Fig. 6 shows the 15N abundance in hepatic citrulline as a function of that in perfusate citrulline. The data are from six livers that were perfused with 0.3 mM NH4Cl of varying 15N enrichment. The relationship is statistically significant ($r^2 = 0.96; p < 0.0001$) and is described by the equation $y = 0.92x - 4.9$.

The 15N abundance of ammonia leaving the liver (effluent ammonia) is plotted against that in the perfusate (influent ammonia) in Fig. 5. It can be seen that there was very little dilution of the 15N in the NH4Cl as it passed through the liver ($y = 0.90x - 2.5; r^2 = 0.97; p < 0.0001$).

15N Enrichments in Amino Acids—Very small quantities of citrulline were found in the effluent perfusate. To determine whether this citrulline was representative of the general hepatic pool of citrulline, we compared the 15N enrichment of citrulline in the perfusate with that in livers that were freeze-clamped at the same time. Since freeze clamping terminates nitrogen metabolism, we were able to determine the relationship between the 15N abundance in perfusate aspartate and perfusate glutamate ($y = 0.69x - 6.7; r^2 = 0.75; p < 0.001$). The average abundance in glutamate was approximately 80% of that in citrulline.

Glutamate can transfer its amino group to a variety of amino acids by transamination. In these experiments we determined the isotopic enrichment in two of these, alanine and aspartate, so that we were able to determine the relationship between the 15N abundance of perfusate glutamate and that in both of these amino acids. Fig. 7c shows these relationships. This figure shows a nearly identical relationship between the 15N abundance of perfusate glutamate and perfusate alanine ($y = 0.90x - 6.0; r^2 = 0.93; p < 0.001$) indicating that glutamate nitrogen was efficiently transferred to alanine without dilution. There was also a positive relationship between the 15N abundances of [15N]glutamate and [15N]aspartate ($y = 0.78x + 3.5; r^2 = 0.62; p < 0.0001$), but the abundance in aspartate was only about 75% of that in glutamate, indicating a dilution of the isotope.

Validation of the Model—The model we presented for urea mass isotopomer production has predictive consequences. In particular, the proportions of the three isotopomers may be predicted if one knows the isotopic abundances of 15N in the precursor pools i.e. in the mitochondrial ammonia and in the cytoplasmic aspartate pools. We have already indicated that the isotopic enrichment in perfusate citrulline should reflect the mitochondrial ammonia pool. It seems likely that the isotopic enrichment in perfusate aspartate would reflect that in the cytoplasmic pool, since any aspartate released by the liver must pass through the cytoplasm immediately before entering the perfusate. We therefore used the 15N enrichments in effluent citrulline (substituted for x) and aspartate (substituted for y) to predict urea isotopomer distributions using Equations 1–3. In order to ensure that we were dealing with steady state situations we restricted ourselves to data obtained at the end of each 15-min perfusion period with a given 15NH4Cl enrichment (i.e. at 30, 45, 60, and 75 min), and we combined the data from
The two sets of perfusions (those in which \(^{15}\)NH\(_4\)Cl enrichment increased from 25 to 100% and vice versa). This comparison is shown in Table I, which clearly demonstrates very good agreement between the actual and predicted mass isotopomer distributions.

Fig. 8 shows plots of the individual data points. It can be seen that there is an excellent relationship between the predicted and observed isotopomer distributions for \(U_m\) \((r^2 = 0.93; p < 0.0001)\) and good relationships for \(U_{m+1}\) \((r^2 = 0.74; p < 0.0001)\) and \(U_{m+2}\) \((r = -2.0 + 1.19y; r^2 = 0.75; p < 0.0001)\). An important point to be made from a comparison of Table I and Fig. 8 is that, although there is very good agreement between the predicted and observed isotopomer distribution when one compares grouped data (e.g., the largest disagreement between the 12 comparisons of observed and predicted data in Table I is for \(U_{m+1}\) at 25\% \(^{15}\)N abundance, where the observed distribution is 72\% of that predicted) there can be greater variability if one relies on a single datum (e.g., one of the observed estimates of \(U_{m+2}\) is only 50\% of that predicted, and one of the observed estimates of \(U_{m+1}\) is 152\% of that predicted).

**Glutamine Isotopomers**—The percentage distributions of the four mass isotopomers of synthesized glutamine at different \(^{15}\)NH\(_4\)Cl abundances are shown in Table II. To obtain these data we subtracted the rate of glutamine production via prote-
The data shown are means ± S.D., n = 6. The isotopomer distribution was predicted using Equations 1–3 and substituting the $^{15}$N abundance in perfusate citrulline for x and that in perfusate aspartate for y (see “Theory”).

| Percentage distribution | 25%a | 50% | 75% | 100% |
|-------------------------|------|-----|-----|------|
|                         | Observed | Predicted | Observed | Predicted | Observed | Predicted | Observed | Predicted |
| Isotomer                |         |       |       |       |       |       |       |       |
| $U_m$                   | 77.1 ± 4.5 | 68.0 ± 3.7 | 50.5 ± 3.3 | 50.9 ± 4.1 | 35.2 ± 5.4 | 29.8 ± 9.5 | 18.2 ± 4.3 | 15.6 ± 5.3 |
| $U_{m-1}$               | 21.6 ± 3.7 | 29.8 ± 2.9 | 39.1 ± 1.8 | 41.7 ± 2.4 | 47.8 ± 5.1 | 53.4 ± 4.1 | 54.2 ± 5.3 | 52.0 ± 6.7 |
| $U_{m-2}$               | 2.95 ± 0.9 | 2.30 ± 0.9 | 9.4 ± 1.7 | 7.4 ± 2.1 | 17.2 ± 3.2 | 16.9 ± 6.4 | 25.9 ± 3.5 | 32.3 ± 9.3 |
| Precursor enrichment    |         |       |       |       |       |       |       |       |
| x (from citrulline)     | 26.2 ± 3.6 | 35.8 ± 3.1 | 59.4 ± 9.9 | 66.7 ± 13.2 |
| y (from aspartate)      | 8.8 ± 3.1 | 20.7 ± 5.5 | 27.7 ± 7.0 | 41.6 ± 14.3 |

* Values in this row represent $^{15}$NH$_4$ Cl enrichment in perfusate.

| Percentage distribution | 25%a | 50% | 75% | 100% |
|-------------------------|------|-----|-----|------|
|                         | Observed | Predicted | Observed | Predicted | Observed | Predicted | Observed | Predicted |
| Isotomer                |         |       |       |       |       |       |       |       |
| $G_m$                   | 70.6 ± 7.8 | 33.0 ± 1.6 | 19.7 ± 4.2 | 0 |
| $G_{m-1}$ (aamiide)     | 6.3 ± 2.8 | 18.7 ± 2.1 | 20.3 ± 3.4 | 30.8 ± 5.7 |
| $G_{m-1}$ (aamiide)     | 15.8 ± 5.3 | 31.2 ± 8.4 | 29.5 ± 1.9 | 36.9 ± 4.5 |
| $G_{m-2}$               | 7.4 ± 1.7 | 17.4 ± 1.7 | 30.5 ± 2.2 | 34.2 ± 4.8 |

* Values in this row represent $^{15}$NH$_4$ Cl enrichment in perfusate.

dysis (as determined in methionine sulfoxamide-treated, glutamine synthase-inhibited livers). This amounted to 7 nmol/ min/g. Since such glutamine is not enriched in $^{15}$N we could attribute all the $^{15}$N in glutamine to glutamine produced via glutamine synthetase. The data in Table II are restricted to those obtained at the end of each 15-min perfusion period with a given $^{15}$NH$_4$Cl abundance, and we combined the data from the two sets of perfusions. The proportion of all of the $^{15}$N-containing isotopomers increases with increasing $^{15}$N abundance of the perfusate NH$_4$Cl; the proportion of unlabeled molecules decreases to zero at 100% abundance. From these data it is possible to predict the $^{15}$N abundance of the ammonia and glutamate pools, in the cytoplasm of the perivenous cells in which glutamine is synthesized (from Equations 8 and 9) even though those cells only comprise about 7% of the total hepatocyte population. These enrichments are 13.7, 36.1, 50.8, and 70.6, and that in perfusate aspartate for y (see “Theory”).

### DISCUSSION

A long standing problem in the use of isotopic approaches to metabolism has been the determination of true isotopic enrichments of immediate intracellular precursors. Without such information true rates of biosynthesis cannot be measured. A variety of methods have been employed in attempts to assess the intracellular isotopic enrichments of immediate biosynthetic precursors. These have included the direct measurement of cellular isotopic enrichment of the compound in question, but this has required destructive methodology (cells or tissues are extracted) and can be confounded by problems of compartmentation, whether due to different cell types or different compartments within a single cell. Another approach, the “chemical biopsy” has involved the use of xenobiotics that are conjugated and excreted to give information on the isotopic enrichment of the conjugating metabolite. The method of mass isotopomer distribution analysis introduced by Hellerstein and his group (22, 23) represents a significant advance. A similar method, isotopomer spectral analysis, has been introduced by Kelleher and co-workers (24, 25). In this procedure, as initially applied to fatty acid biosynthesis, the frequency distribution of mass isotopomers in fatty acids synthesized from $^{13}$C-labeled acetate could be used to calculate the precise isotopic enrichment of hepatic cytoplasmic acetyl-CoA. Furthermore, since this information is obtained from the biosynthetic product itself (i.e. the fatty acid) one may calculate the isotopic enrichment in the precise acetyl CoA pool from which the fatty acid was synthesized. These techniques have been extended by Hellerstein and by Kelleher to other condensation polymerizations (e.g. steroid synthesis) (26, 27) and even to gluconeogenesis (23).

Hepatic nitrogen metabolism offers several challenges to the application of mass isotopomer distribution analysis. Ammonia may be detoxified to urea, and it is also used to produce glutamine. Although both of these processes occur in hepatocytes, they do not occur in the same cells, since the urea cycle occurs in hepatocytes throughout the hepatic acinus except for the last few cells in the perivenous region where glutamine synthetase is located (5, 6). One must therefore determine precursor enrichments in different cells of intact organs. In addition, there is a special problem with respect to nitrogen metabolism, because both urea and glutamine each contain two nitrogen atoms, but each nitrogen atom arises from different precursor pools. The situation with urea offers a particular challenge in that, once formed, its two nitrogens are chemically indistinguishable. Despite this, the methodology developed here permits one to estimate the isotopic enrichment in both the mitochondrial ammonia pool and the cytosolic aspartate pool in the ureagenic population of hepatocytes. The mitochondrial ammonia isotopic enrichment is given by the enrichment in citrulline. Since there are two enzymatic steps between ammonia and citrulline (carbamoyl-phosphate synthetase and ornithine transcarbamoylase) it is essential, for our purposes, that there be no other significant sources of carbamoyl phosphate or of citrulline or no other means of labeling citrulline. There are two separate carbamoyl-phosphate synthetases in liver. These are carbamoyl-phosphate synthetase I, the mitochondrial enzyme required for urea synthesis that uses NH$_3$ as its nitrogenous substrate (28) and carbamoyl-phosphate synthetase II, a cytoplasmic enzyme required for pyrimidine nu-
deotide biosynthesis, which uses the amide nitrogen of glutamine as its nitrogenous substrate (29). However, there should be no contribution of carbamoyl-phosphate synthetase II to citrulline synthesis in our experiments for the following reasons. (i) Even under conditions of maximal pyrimidine biosynthesis the rate of this process is very small compared with that of ureagenesis. Maximum rates of hepatic pyrimidine biosynthesis of about 2 nmol/min/g have been reported (30) compared with the rates of urea synthesis, 300 nmol/min/g, that occur in our perfusions. (ii) Pyrimidine biosynthesis requires a source of glutamine as substrate, but in the present experiments we have provided no glutamine in the perfusate, and any glutamine synthesized by the liver is produced in the perivenous hepatocytes, downstream from the site of urea synthesis. (iii) Carbamoyl-phosphate synthetase II, aspartate transcarbamoylase, and dihydroorotase exist as a multifunctional enzyme in hepatocyte cytoplasm such that carbamoyl phosphate is efficiently channeled from carbamoyl-phosphate synthetase II to aspartate transcarbamoylase without being released in free form (29). (iv) Citrulline is produced from mitochondrial carbamoyl phosphate.

We must also consider the possibility of citrulline arising from reactions other than ornithine transcarbamoylases. The only such known reaction is nitric-oxide synthetase, which converts arginine to citrulline and nitric oxide and which is known to occur in liver. The activity of this enzyme, in the perfused liver, may be determined from the production of nitric oxide arising from the amide of glutamine has preferred access to carbamoyl-phosphate synthetase with the rates of urea synthesis (300 nmol/min/g) occurring in our experiments. Finally, one must consider whether 15N could be introduced into citrulline other than from carbamoyl phosphate. It is possible that 15N may be introduced into the amino group of ornithine from [15N]glutamate by the action of ornithine aminotransferase to produce labeled ornithine, which may react with [14N]carbamoyl phosphate to produce a citrulline that would contain 15N in the amino position. This scenario cannot occur in our perfusions, however, since ornithine aminotransferase is restricted to the same small population of perivenous hepatocytes that contain glutamine synthetase (32). Since these are distal to the urea-synthesizing perportal hepatocytes it is apparent that labeled citrulline cannot be produced in this manner. However, it is possible that 15N may be introduced into the amino group of citrulline in recirculating perfusions or in hepatocyte incubations so that the procedure we have developed may not be valid in these circumstances unless one is able to use GC-MS methodology that discriminates between 15N in the amino and guanidino groups of citrulline. Thus, since (i) there is no significant source of citrulline in our experiments other than that produced by ornithine transcarbamoylase, (ii) there is no source of carbamoyl phosphate for ornithine transcarbamoylase other than that produced by carbamoyl-phosphate synthetase I, and (iii) 15N label is only introduced into citrulline via ornithine transcarbamoylase, we can accept the 15N abundance in citrulline as a measure of the 15N enrichment in the mitochondrial ammonia pool of urea-synthesizing hepatocytes.

The argument for accepting the 15N abundance in perfusate aspartate as a measure of its 15N enrichment in urea-synthesizing hepatocytes is simple. These cells account for over 90% of the hepatocytes, and aspartate, whether arising in the mitochondria or the cytoplasm, must pass through the cytoplasm before it appears in the perfusate. Validation of these two estimates of precursor isotopic abundance is given by their ability to predict the actual urea isotopomer distribution (Table I).

This approach can give us information on potential channeling in the urea cycle. Perfusion of carbamoyl-phosphate synthetase, presumably a reflection of a general cytoplasmic pool of aspartate, is an accurate measure of the pool of aspartate used for urea synthesis. This implies that aspartate, arising by transamination of glutamate either in the mitochondrion or in the cytoplasm, is not tightly channeled to argininosuccinate synthetase but may equilibrate throughout the cytoplasm. Of interest, also, is the fact that we have two independent estimates of the 15N abundance in the mitochondrial ammonia pool. Citrulline is a reflection of the abundance at carbamoyl-phosphate synthetase, whereas glutamate reflects the abundance at glutamate dehydrogenase. That there is good agreement between these two estimates of the 15N abundance of mitochondrial ammonia (Fig. 7b) is consistent with a single mitochondrial ammonia pool due to the high mobility of this small lipid soluble molecule (33). However, it has been suggested that a metabolic channel exists between glutaminase and carbamoyl-phosphate synthetase such that ammonia arising from the amide of glutamine has preferred access to carbamoyl-phosphate synthetase without mixing with the general mitochondrial ammonia pool (10). The methodology elaborated in this study affords a means of testing this hypothesis, and we are now engaged in doing so.

15N in alanine and aspartate arises by transamination from glutamate; therefore, there should be a precursor-product relationship between 15N enrichment in glutamate and these amino acids (i.e. the enrichment in glutamate greater than or equal to that in the amino acids derived from it). This is borne out in Fig. 7c, where the slope relating [15N]alanine enrichment to that of glutamate is 0.90 and that relating [15N]aspartate enrichment to glutamate is 0.78. The difference between the 15N enrichments of alanine and aspartate deserves comment. Should 15N be transferred without any isotope dilution, then the 15N enrichment of the product should, in steady state, equal that in the precursor. However, although the 15N enrichment in alanine is very similar to that in glutamate (90%), that in aspartate is more diluted (78%). The most likely sources of dilution are unlabeled amino acids that arise via proteolysis. However, the key difference between alanine and aspartate metabolism in these livers is that the rate of alanine production is quite high due to the ready availability of pyruvate, since lactate and pyruvate are provided in the perfusate. Thus, the great bulk of alanine will be derived from transamination of pyruvate by glutamate and will, therefore, reflect the 15N enrichment of glutamate. The rate of aspartate production is very much less than that of alanine; therefore, the effect of isotope dilution by unlabeled aspartate is correspondingly greater. Therefore, the 15N enrichment in alanine is greater than that in aspartate.

Our analysis of urea labeling can also explain some recent, apparently contradictory, experiments on urea production in sheep liver. Lobley et al. (8) examined urea synthesis from 15NH4Cl infused into the portal veins of chronically catheterized sheep. 15N-labeled urea was readily produced, but it was virtually entirely (> 97%) singly labeled urea. This suggested to them that NH3 may be introduced only via carbamoyl phosphate and that the second nitrogen in urea must be provided from amino acids. This, in turn, would have significant implications for the protein economy of these animals, since they produce very large quantities of ammonia due to foregut fermentation. Thus, each molecule of ammonia extracted from the portal venous blood by the liver would obligate the catabolism of an amino acid so as to provide the second urea nitrogen, with
important consequences for protein gain. To examine whether the urea cycle of sheep differed from that in other mammals (i.e. in that NH₃ could not supply both urea nitrogens) they also measured [¹⁵N]urea production in isolated hepatocytes from ⁰⁵NH₄Cl (9) and found very substantial production of doubly labeled urea, indicating that ammonia could indeed supply both nitrogens of urea, even in the presence of physiological concentrations of amino acids. They postulated that this difference between the in vivo and in vitro observations may be related to differences in metabolite channeling. However, these data are readily explained by our model. The key point is that the ratio of singly labeled to doubly labeled urea is not invariant in a given physiological state but depends on the enrichment in the precursor pools. For example, Equations 1-3 show that under circumstances where the enrichment of ¹⁵N in the two precursor pools (ammonia and aspartate) is 10% then the ratio of doubly labeled to singly labeled urea produced is 0.01:0.18 (5.3%), whereas when the precursor enrichment is 90% this ratio increases to 0.81:0.18 (81.8%). In the sheep hepatocyte experiments of Luo et al. (9) ¹⁵NH₄Cl was used as substrate at an isotopic enrichment of 96%, so it is not surprising that substantial amounts of doubly labeled urea (as high as 84%) were produced. However, it is apparent that the ¹⁵NH₄Cl enrichment reaching the liver in the in vivo (8) studies is between 4 and 8% (based on the enrichment of the infused ¹⁵NH₄Cl and its dilution by unlabeled portal ammonia). Therefore, our theory predicts that only very small quantities of doubly labeled urea would be formed. Hence, there is no essential difference in the ability of ammonia to provide both urea nitrogens in sheep compared with other mammals.

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REFERENCES

1. Cooper, A. J. L., Nieves, E., Coleman, A. E., Filic-DeRicco, S., and Gelbard, A. S. (1987) J. Biol. Chem. 262, 1073-1080
2. Cooper, A. J. L., Nieves, E., Rosenspire, K. C., Filic-DeRicco, S., Gelbard, A. S., and Bruslow, S. W. (1988) J. Biol. Chem. 263, 12268–12273
3. Gessler, A., Kanamori, K., and Ross, B. D. (1992) Biochem. J. 287, 813–820
4. Watford, M. (1983) FASEB J. 7, 1448–1474
5. Gebhardt, R., and Mecke, D. (1983) EMBO J. 2, 567–570
6. Häussinger, D. (1983) Eur. J. Biochem. 133, 269–275
7. Nissim, I., Cattano, C., Nissim, I., and Yudkoff, M. (1992) Arch. Biochem. Biophys. 293, 393–401
8. Lobley, G. E., Connel, A., Lomax, M. A., Brown, D. S., Milne, E., Calder, A. G., and Farningham, D. A. H. (1995) Br. J. Nutr. 73, 667–685
9. Luo, Q. J., McAloney, S. A., Lobley, G. E., Calder, A. G., and Lomax, M. A. (1995) Eur. J. Biochem. 228, 912–917
10. Meijer, A. J. (1985) FEBS Lett. 191, 249–251
11. Sies, H. (1978) Methods Enzymol. 52, 48–59
12. Brosnan, J. T., Krebs, H. A., and Williamson, D. H. (1970) Biochem. J. 117, 91–96
13. Geyer, J. W., and Dabich, D. (1971) Anal. Biochem. 39, 412–417
14. Kirsten, E., Gerez, C., and Kirsten, R. (1963) Biochem. Z. 337, 312–319
15. Jones, B. N., and Gillingham, T. P. (1983) J. Chromatogr. 265, 471–482
16. Nissim, I., Yudkoff, M., and Segal, S. (1985) J. Biol. Chem. 260, 13955–13967
17. Nissim, I., Cattano, C., Zhiping, L., and Nissim, I. (1993) J. Am. Soc. Nephrol. 3, 1416–1427
18. Anderson, L. W., Zaharevitz, D. W., and Strong, J. M. (1987) Anal. Biochem. 163, 368–368
19. Nissim, I., Yudkoff, M., and Lapidot, A. (1984) Anal. Biochem. 143, 14–20
20. Wolfe, R. R. (1992) in Radioactive and Stable Isotope Tracers in Biomedicine: Principle and Practice of Kinetic Analysis (Wolfe, R. R., ed) pp. 49–85, Wiley-Liss, New York
21. Brosnan, J. T. (1976) in The Urea Cycle (Grisolia, S., Baguena, R., and Mayor, E., eds) pp. 443–457, John Wiley and Sons, Inc., New York
22. Hälerstein, M. K. (1991) J. Biol. Chem. 266, 10920–10924
23. Hälerstein, M. K., and Neese, R. A. (1992) Am. J. Physiol. 262, E988–E1001
24. Kelleher, J. K., and Masterston, T. M. (1992) Am. J. Physiol. 262, E118–E125
25. Kharroubi, A. K., Masterston, T. M., Aldaghlas, T. A., Kennedy, K. A., and Kelleher, J. K. (1992) Am. J. Physiol. 263, E667–E675
26. Neese, R. A., Faix, D., Kietkows, C., Wu, K., Wang, A. C., Shackleton, C. H., and Hälerstein, M. K. (1993) Am. J. Physiol. 264, E136–E147
27. Kelleher, J. K., Kharroubi, A. T., Aldaghlas, T. A., Shambat, I. B., Kennedy, K. A., Holleran, A. L., and Masterston, T. M. (1994) Am. J. Physiol. 266, E384–E395
28. Cohen, N. S., Kyan, F. S., Kyan, S. S., Cheung, C., and Raji, M. L. (1985) Biochem. J. 229, 205–211
29. Jones, M. E. (1980) Annu. Rev. Biochem. 49, 253–279
30. Pausch, J., Rosemack, J., Hausser, R., and Gerok, W. (1985) Eur. J. Biochem. 150, 189–194
31. Wettstein, M., Gerok, W., and Hausser, R. (1994) Hepatology 19, 641–647
32. Kuo, C., Hwu, W. L., Valle, D., and Karnell, J. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9468–9472
33. Jacobs, M. H. (1940) Cold Spring Harbor Symp. Quant. Biol. 8, 30–39