We have utilized [15N]alanine or [15NH₄] as metabolic tracers in order to identify sources of nitrogen for hepatic ureagenesis in a liver perfusion system. Studies were done in the presence and absence of physiologic concentrations of portal venous ammonia in order to test the hypothesis that, when the NH₄⁺:aspartate ratio is >1, increased hepatic proteolysis provides cytoplasmic aspartate in order to support ureagenesis. When 1 mM [15N]alanine was the sole nitrogen source, the amino group was incorporated into both nitrogens of urea and both nitrogens of glutamine. However, when studies were done with 1 mM alanine and 0.3 mM NH₄Cl, alanine failed to provide aspartate at a rate that would have detoxified all administered ammonia. Under these circumstances, the presence of ammonia at a physiologic concentration but ammonia uptake was accompanied by the uptake of almost equimolar quantities of alanine, which presumably provided the α-amino group to aspartate. Alanine would serve this role following transamination to glutamate and then to aspartate for incorporation into urea. Since alanine is not produced by intestinal metabolism in these fasting dogs, it must be provided by peripheral tissues. One possibility is that proteolysis, perhaps in the liver itself, is a metabolic price that must be paid to compensate the inability of ammonia to serve as a source of nitrogen for cytoplasmic aspartate for incorporation into urea (3). The experiments of Lopez et al. (4) are also germane in this regard. These workers investigated amino acid, ammonia, and urea fluxes across the liver and intestines of fed and postabsorptive rats. In both of these physiological states amino acid uptake exceeded ammonia uptake. Alanine was the principal amino acid extracted by the liver, accounting for 33 and 25% of total hepatic amino acid extraction, respectively, in the fed and postabsorptive animals. In both situations there was a prominent hepatic output of glutamine (in the postabsorptive state glutamine output exceeded that of urea), which these researchers interpreted as a salvage process that conserves nitrogen arising from hepatic amino acid metabolism, especially that of alanine (4).

The process of urea synthesis involves equimolar consumption of NH₄⁺ and aspartate-N. However, in pathological situations such as cirrhosis (5), cancer (6), renal failure (7), or chronic bacterial hydrolysis of urea and glutamine in the small intestine (3, 5, 8–10), there is a high rate of production of NH₄⁺. Under these conditions, the portal blood does not provide the liver with an equimolar amount of aspartate-N to support the needs of hepatic ureagenesis. Therefore, in this study our aim was to explore the hypothesis that when ammonia in the portal venous system is present at physiologic concentration but nitrogen supply differs from the 1:1 (NH₄⁺:aspartate-N) stoichiometry, there is increased hepatic proteolysis to provide cytoplasmic aspartate so that formation of cytosolic argininosuccinate can keep pace with the rapid incorporation of ammonia into mitochondrial carbamyl phosphate.

To test this hypothesis, we have used our previously reported
experimental and theoretical methodology that involves the use of 15N-labeled substrates (11–14) to explore hepatic nitrogen metabolism and, in particular, to determine the contribution of alanine nitrogen to urea synthesis. We also were able to discriminate between incorporation into urea from the mitochondrial ammonia and cytoplasmic aspartate pools as well as the incorporation of nitrogen into 2-N and 5-N of glutamine. We perfused liver with [15N]alanine in the presence and absence of physiological portal venous concentrations of ammonia. The results show that alanine nitrogen can be used for incorporation into both nitrogens of urea and both nitrogens of glutamine. However, alanine is more effective in providing nitrogen for urea synthesis via cytosolic aspartate than through the mitochondrial ammonia. Similarly, alanine-N was a more effective source of the amino nitrogen of glutamine than of the amide nitrogen. We also found that the presence of physiological concentrations of ammonia increased hepatic alanine uptake and intra-hepatic proteolysis.

MATERIALS AND METHODS

Liver Perfusions—Livers from fed male Harlan Sprague-Dawley rats (weighing about 11–13 g) were perfused in the non-recirculating mode as described by Sies (15). The basic 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 oxygen consumption was calculated. After 20 min of pre-perfusion we changed to a medium that contained, in addition to the lactate and pyruvate, either [15N]alanine (1 mM), [15N]alanine (1 mM), and NH4Cl (0.3 mM) or alanine (1 mM) and 15NH4Cl (0.3 mM). Perfusions continued for a total of 70 min. When [15N]alanine was present it was at an isotopic enrichment of 50 mol % excess (MPE)1 from 20 to 45 min and 100 MPE from 45 to 70 min. 15NH4Cl was present at 100% isotopic enrichment. Separate perfusate reservoirs, each containing different media, were used to facilitate changes in perfusions. Samples were taken from the influent and effluent media for chemical and GC-MS analyses. At the end of the perfusions livers were freeze-clamped with aluminum tongs precooled in liquid N2, and the frozen livers were ground into a fine powder, extracted into perchloric acid, and the extracts used for the analysis of adenine nucleotides by enzymatic techniques (16). Amino acids were determined by high pressure liquid chromatography, utilizing pre-column derivatization with o-phthalaldehyde (17). Ammonia and urea were assayed by standard methods (18, 19).

GC-MS Methodology, Determination of 15N-Labeled Metabolites—GC-MS measurements of 15N isotopic enrichment were performed on a Hewlett-Packard 5970 MSD and/or 5971 MSD coupled with a 5890 series II Gas Chromatograph-mass spectrometry. Hewlett-Packard 5973 MSD and 5971 MSD are detectors. The fraction of 15N-labeled precursor is provided, the urea formed may have a mass of 60 (U0), 61 (U1), or 62 (U2) depending on whether 0, 1, or 2 15N atoms of urea are labeled. 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 215N in the cytoplasmic aspartate pool be y, then the fractional abundance of 15N in the same pool is 1 − y. Then the fraction of the urea isotope containing no 15N will be U0 = (1 − x)(1 − y), the fraction of urea containing 1 atom of 15N will be U1 = xy + (1 − x)(1 − y), and the fraction of urea containing 2 atoms of 15N will be U2 = xy. Therefore, U0, U1, and U2 sum to unity. This relationship permits us to calculate the fraction of U0, U1, and U2 at any given abundance of 15N in the mitochondrial ammonia and cytoplasmic aspartate pools, i.e. at any values of x and y, as we have described (14).

**RESULTS**

Characterization of the Perfused Livers—Viability of the perfused liver model is verified by the concentration of adenine nucleotides at the end of the 70 min of perfusion (Table I). There were no significant differences between the adenine nucleotide concentrations during the different experimental conditions. These values are similar to those we previously reported in perfused livers (13, 14) and to in vivo levels (16). Fig. 1, panels A and B, shows the changes in urea, ammonia, alanine, glutamine, and glutamate in the effluent under the various experimental conditions. Oxygen consumption is also shown. The constancy of oxygen consumption is an indication of the stability of the preparations.

It is apparent that, at all times, the mean uptake of alanine was greater in the presence of ammonia than in its absence. These differences reached statistical significance at 25, 30, 45, 65, and 70 min. Glutamine output was significantly greater in the presence of ammonia than in its absence at all time points except at 25 min. Of course, urea synthesis was always appreciably greater in the presence of ammonia than in its absence. The effluent alanine, glutamine, glutamate, ammonia, and urea represent the major nitrogenous metabolites in these experiments.
Fig. 1. Total nitrogen balance and \( \text{O}_2 \) consumption in perfused liver. Livers were perfused with 1 \( \text{mM} \) \([^{15}\text{N}]\)alanine plus 0.3 \( \text{mM} \) \( \text{NH}_4\text{Cl} \), \([^{15}\text{N}]\)alanine plus 0.3 \( \text{mM} \) \( ^{15}\text{NH}_4\text{Cl} \), or only with 1 \( \text{mM} \) \([^{15}\text{N}]\)alanine as a sole nitrogen source. The data are the means \( \pm \) S.D. for perfusions with alanine plus ammonia (panel A, \( n = 8 \)) and perfusions with alanine alone (panel B, \( n = 4 \)). For values of total nitrogen balance indicated in the text, we multiplied by 2 the urea and glutamine output (nanomoles/min/g wet weight, shown in this figure), to account for total nitrogen atoms. Symbols used are as follows: \( \bullet \), urea; \( \triangle \), ammonia; \( \square \), glutamate; \( \odot \), glutamine.

The production and output of \([^{15}\text{N}]\)labeled glutamine (\([5-^{15}\text{N}]\)glutamine, \([2-^{15}\text{N}]\)glutamine, and \([2,5-^{15}\text{N}]\)glutamine) are shown in Fig. 4. It is apparent that there was substantial production of all three isotomers in the presence of \( ^{15}\text{NH}_4\text{Cl} \), with \([5-^{15}\text{N}]\)glutamine being most abundant, followed by \([2-^{15}\text{N}]\)glutamine, and then by doubly labeled glutamine (Fig. 4, panel C). The pattern produced from \([^{15}\text{N}]\)alanine was quite different. The 2-\( ^{15}\text{N} \) isotomer of glutamine was the predominant form produced (Fig. 4, panel A) and was virtually the exclusive form in the presence of \( ^{15}\text{NH}_4\text{Cl} \) (Fig. 4, panel B). The production of \([^{15}\text{N}]\)glutamate (Fig. 5) showed immediate and very substantial labeling (about 35 MPE) when the livers were perfused with \( ^{15}\text{NH}_4\text{Cl} \) (100 MPE) and was similarly labeled when perfused with 100 MPE alanine alone (60–70 min). However, in perfusions with \([^{15}\text{N}]\)alanine and unlabeled ammonia, the glutamate enrichment was only about one-third that in the absence of ammonia.

The \( ^{15}\text{N} \) enrichments in citrulline and aspartate are crucial measurements, as we have already shown that these are good indicators of the \( ^{15}\text{N} \) enrichment in the two nitrogen precursor pools for urea synthesis, mitochondrial carbamyl phosphate and cytosolic aspartate, respectively (14). The perfusions with \( ^{15}\text{NH}_4\text{Cl} \) (100 MPE) resulted in very substantial labeling of citrulline (about 70 MPE) but much less labeling of aspartate (about 20 MPE) (Fig. 6, panel C). The metabolic response to \([^{15}\text{N}]\)alanine perfusions was very different in that aspartate was much more heavily labeled than was citrulline. The degree of labeling of both citrulline and of aspartate was more pronounced in the absence of ammonia (Fig. 6, panel A) than in its presence (Fig. 6, panel B). Enrichment data for nitrogen-containing metabolites in livers freeze-clamped at the end of the perfusions (70 min) are shown in Fig. 7. It is evident that, when \([^{15}\text{N}]\)alanine was the labeled substrate, \( ^{15}\text{N} \) was incorporated into both nitrogenous precursors of urea, but the enrichment in aspartate was 2–3-fold that in citrulline. The same relationship held with \([^{15}\text{N}]\) alanine in the presence of \( ^{15}\text{NH}_4\text{Cl} \) except that the incorporation into citrulline was very low (Fig. 7, panel B). With \( ^{15}\text{NH}_4\text{Cl} \) as precursor, citrulline became very heavily labeled, reaching an

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**Graphs:**
- **Graph A:** Total nitrogen balance and \( \text{O}_2 \) consumption in perfused liver.
- **Graph B:** Urea uptake during the course of liver perfusion.

**Legend:**
- \( \bullet \): \([^{15}\text{N}]\)alanine uptake during perfusion.
- \( \odot \): \([^{15}\text{N}]\)alanine uptake during perfusion.
- \( \square \): \([^{15}\text{N}]\)alanine uptake during perfusion.
- \( \triangle \): \([^{15}\text{N}]\)alanine uptake during perfusion.
- \( \bigodot \): \([^{15}\text{N}]\)alanine uptake during perfusion.

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**Table:**
- **Column 1:** Time (minutes)
- **Column 2:** \([^{15}\text{N}]\)alanine uptake (nanomoles/min/g)

**Bar Graph:**
- **X-axis:** Time (minutes)
- **Y-axis:** \([^{15}\text{N}]\)alanine uptake (nanomoles/min/g)
enrichment that was twice as much as aspartate (Fig. 7, panel C). Measurement of $^{15}$N-labeled metabolites in the liver at the end of 70 min of perfusion provides two important findings. First, the intra-hepatic $^{15}$N enrichment in glutamine, glutamate, aspartate, and citrulline is in excellent agreement with the $^{15}$N enrichment in the same metabolites in the effluent at the end of perfusion (Figs. 4–6), suggesting labeling in effluent faithfully reflects enrichment of the intra-hepatic compartment. This conclusion agrees with our previous investigation with $^{15}$N-labeled glutamine or ammonia (13, 14). The second observation is that $[15N]$alanine enrichment in the liver extract was about 70 MPE at the end of perfusion even though the $[15N]$alanine enrichment of the perfusate was 100 MPE (Fig. 7, panel A). Since $[15N]$alanine was the sole precursor provided to the liver, it follows that approximately one-third of hepatic alanine was derived from unlabeled sources. This calculation agrees with the estimated fraction of nitrogen, presumably derived from proteolysis, that was necessary to compensate the balance between nitrogen uptake and nitrogen output, as indicated above (Fig. 1, panel A). Similarly, the $[15N]$alanine enrichment in perfusion studies with unlabeled ammonia indicates that $\sim$65% of alanine was derived from unlabeled sources. This estimate is in agreement with the fraction of nitrogen derived from proteolysis in perfusion with $[15N]$alanine plus unlabeled ammonia, i.e. $\sim$60% (Fig. 1, panel B).

**DISCUSSION**

In this study we employed $[15N]$alanine as metabolic tracer in order to follow the metabolism of its nitrogen and, in particular, its contribution to urea and glutamine synthesis in the presence or absence of unlabeled NH$_4$Cl. We also examined the metabolic fate of nitrogen derived from $^{15}$NH$_4$Cl in the presence of unlabeled alanine. Alanine is well recognized as the
principal glucogenic amino acid (1), but its key role in nitrogen metabolism is less appreciated. Lopez et al. (4) have recently quantified the importance of alanine to hepatic nitrogen metabolism. It is, by far, the principal amino acid removed by the liver in the fed or postabsorptive state. Indeed, it contributes twice as much nitrogen to the liver as does pre-formed ammonia. Lopez et al. (4) emphasize the role of hepatic glutamine synthesis as an efficient nitrogen sparing mechanism. It is clear, therefore, that it is important to understand the hepatic disposition of alanine-N and its relationship to urea and glutamine synthesis.

Furthermore, alanine is well recognized as the principal amino acid released by skeletal muscle and is taken up by the liver during ingestion of a low protein diet or starvation (1). As such, alanine is a key amino acid precursor for hepatic gluconeogenesis (1, 3). Under these conditions, the liver does not receive via the portal blood equimolar aspartate-N for hepatic ureagenesis (3). Therefore, we used liver perfusion with physiological levels of alanine or alanine plus ammonia to explore the hypothesis that, when the nitrogen supply to the portal venous system differs from the 1:1 (NH₄⁺:aspartate-N) stoichiometry, there is increased hepatic proteolysis to provide cytosolic aspartate so that formation of cytosolic argininosuccinate can keep pace with the rapid incorporation of ammonia into mitochondrial carbamyl phosphate.

We employed the single-pass isolated perfused rat liver because this model preserves the normal lobular microcirculation of the liver and avoids problems of interpretation that may arise from recycling of substrates (such as products of perivenous hepatocytes being recycled to periportal hepatocytes) that occur in isolated hepatocytes or in a recirculating perfusion. The use of [15N]alanine allowed us to use an approach we had already introduced to define the degree to which a nitrogenous substrate provides nitrogen to urea via either aspartate or carbamyl phosphate (13, 14). We can similarly define the origins of the two nitrogen atoms of glutamine (14). These data are schematically summarized in Fig. 8, which represents the principal results of these experiments. (i) [15N]Alanine can provide both nitrogens of urea, but it is a much better precursor to urea nitrogen via aspartate than via citrulline. (ii) [15N]Alanine can provide both nitrogens of glutamine, but it is a much better substrate for the provision of the amino than the amide nitrogen. (iii) Addition of NH₄Cl to perfusions increases the uptake of alanine, both in terms of mass and of 15N, and increases the output of 15N products, such as urea and glutamine. (iv) The addition of NH₄Cl increases the net negative nitrogen balance over that seen with alanine alone. (v) The intra-hepatic 15N enrichment in glutamine, glutamate, aspartate, and citrulline is in excellent agreement with the 15N enrichment in the same metabolites in the effluent regardless of 15N precursor.

These observations are consistent with an inter-related metabolic pattern within the liver. We suggest that alanine is quite limited in its ability to provide ammonia to the mitochondrion for carbamyl phosphate synthesis, and ammonia is somewhat limited in its ability to provide nitrogen to cytoplasmic aspartate for incorporation into argininosuccinate. These proposals are supported by the very much lower 15N enrichment in citrulline than in aspartate with [15N]alanine as nitrogen donor. The converse is true with 15NH₄Cl as labeled precursor (Fig. 6). The low rate at which alanine gives rise to ammonia limits
alanine removal. Therefore, when unlabeled NH₄Cl is included in the [¹⁵N]alanine perfusions, we see an increased uptake of alanine (both alanine mass and [¹⁵N]) and increased production of [¹⁵N] products, principally urea.

The highest rates of urea synthesis are found with NH₄Cl. However, it is clear that the ability of ammonia to provide nitrogen to carbamyl phosphate synthetase is much greater than its ability to provide nitrogen to cytoplasmic aspartate.

Alanine can provide additional nitrogen to aspartate (by the combined action of alanine aminotransferase and aspartate aminotransferase), but this may be limited by the activity of alanine aminotransferase or, in these experiments, by the equilibrium poise of the enzyme, given that the perfusions are provided with physiological concentrations of pyruvate. Finally, there may be a failure of mitochondrial aspartate to equilibrate with cytoplasmic aspartate, as proposed by Yang et

FIG. 8. Schematic presentation of nitrogen balance across the liver and the primary [¹⁵N] flow from the labeled precursor into various metabolites in either the cytosolic or mitochondrial compartment of the hepatocytes. The rates of uptake of the external nitrogen sources are indicated (in parentheses), in experiments with [¹⁵N]alanine (I); [¹⁵N]alanine plus unlabeled ammonia (II); and unlabeled alanine plus [¹⁵N]NH₄Cl (III). The rates of the primary nitrogen output (urea and glutamine) are indicated in circles representing these metabolites along with % distribution of their mass isotopomers. The deficit between nitrogen uptake and nitrogen output is furnished by intra-hepatic proteolysis. For simplicity, this drawing does not differentiate between perivenous hepatocytes (glutamine synthesis) and the perportal hepatocytes (alanine uptake, glutamine metabolism, and urea synthesis). In parentheses are shown the percent enrichment (mol % excess) of [¹⁵N]-labeled isotopomers (taken from data in Figs. 3–7, at the end of 70 min of perfusion) of the indicated metabolite. Values for ammonia or alanine uptake and total glutamine or urea output (nmol nitrogen/min/g) are taken from data in Fig. 1, at the end of 70 min of perfusion. Bold arrows indicate primary input/direction of nitrogen metabolite. CP, carbamyl phosphate; GDH, glutamate dehydrogenase.
Thus, the liver must derive an alternative source of nitrogen if it is to detoxify the ammonia present in the perfusate. We suggest that this source is increased hepatic proteolysis that furnishes α-NH₂ groups. This interpretation is similar to that of Yang et al. (3) who infused [15N]NH₄Cl in fasted dogs and found that ammonia supplied nitrogen only to mitochondrial carbamyl phosphate synthetase, the other nitrogen of urea being derived from alanine provided by peripheral proteolysis.

These investigators interpreted this increased peripheral proteolysis as the “metabolic price” that must be paid for effective ammonia detoxification when the liver is presented with more ammonia than amino acids that can serve as precursors to aspartate. We speculate that a similar mechanism may be obtained in our experiments, except that in the current study the only possible source of aspartate-N would have to be hepatic rather than peripheral proteolysis. This conclusion is also supported by the agreement between the estimated fraction of nitrogen derived from proteolysis (Fig. 1) and the estimated fraction of [15N]alanine dilution in the intra-hepatic compartment (Fig. 7).

However, we must point out that the current studies were carried out with alanine or alanine plus ammonia as the only source of nitrogen in order to determine the relative contribution of alanine-N to urea and/or glutamine synthesis. This arrangement does not precisely reflect the amino acid milieu to which the liver is exposed. Indeed, the magnitude of hepatic proteolysis is smaller when glutamine is provided as the nitrogen source. In our previous studies (13), we found that the presence of glutamine (1 mM) and ammonia (0.3 mM) with either insulin or glucagon in the perfusate, nitrogen uptake (nanomoles of nitrogen/min/g) was ~1400 (control), 1450 (plus insulin), or 2000 (plus glucagon), respectively. The corresponding estimated nitrogen output was about 2100, 2250, or 3000 nanomoles of nitrogen/min/g, in experiments without or with addition of insulin or glucagon, respectively. Therefore, whether or not hormones were present, about 30% of nitrogen output was derived from an intra-hepatic source when a physiologic level of glutamine and ammonia was provided (13). In contrast to alanine, the metabolism of glutamine via phosphate-dependent glutaminase may provide sufficient mitochondrial and/or cytosolic glutamate to support ureagenesis, thereby curtailing the demand for intra-hepatic proteolysis. The glutamate so formed is rapidly transaminated to aspartate (Fig. 8), thereby permitting synthesis of argininosuccinate in the cytosol (13).

The origin of glutamine and its isotopomers is also of interest. Hepatic glutamine synthetase is restricted to the perivenous hepatocytes (22). Conceivably, glutamine may be entirely formed from alanine in these cells, as it is in the guinea pig kidney (23). Alternatively, glutamate produced in the periportal cells may be released and taken up by the high affinity portal cells (23). Alternatively, glutamate produced in the perivenous hepatocytes may be released and taken up by the high affinity portal cells (23). Alternatively, glutamate produced in the perivenous hepatocytes may be released and taken up by the high affinity portal cells (23). Alternatively, glutamate produced in the perivenous hepatocytes may be released and taken up by the high affinity portal cells (23). Alternatively, glutamate produced in the perivenous hepatocytes may be released and taken up by the high affinity portal cells (23). Alternatively, glutamate produced in the perivenous hepatocytes may be released and taken up by the high affinity portal cells (23). Alternatively, glutamate produced in the perivenous hepatocytes may be released and taken up by the high affinity portal cells (23).

A point of interest is that our ability to calculate the percentage of the different urea isotopomers attests to the robustness of our theoretical model for the incorporation of labeled nitrogen into urea. In our previous work we showed that, during perfusions with [15N]NH₄Cl, the 15N enrichment in perfusate citrulline and aspartate were reliable proxies for, respectively, mitochondrial ammonia and cytoplasmic aspartate (14). Figs. 6 and 7 show that the 15N enrichments in perfusate citrulline and aspartate were, respectively, faithful indices of the corresponding enrichment in liver. Calculation of the individual urea isotopomers after 70 min of perfusion (with either [15N]alanine or [15N]NH₄Cl) indicates an excellent agreement between predicted and observed isotopomer abundance (data not shown), as we had demonstrated previously with [15N]NH₄Cl alone (14) or [15N]-labeled glutamine (12, 13). The acinar location of alanine aminotransferase is not known as precisely as is glutaminase, as no in situ hybridization studies have been reported. It has, however, been reported to be predominantly periportal (25). Therefore, our theoretical model for the incorporation of labeled nitrogen into urea (14) is further substantiated, regardless of the 15N precursor.

In conclusion, the current investigation provides direct evidence to support the hypothesis that, when hepatic ammonia is present at a higher ratio than a 1:1 (NH₄⁺:aspartate-N) stoichiometry, there is increased hepatic proteolysis that provides cytoplasmic aspartate so that the formation of argininosuccinate can keep pace with the rapid incorporation of ammonia for mitochondrial carbamyl phosphate. This study may be useful in terms of identifying and preventing an excessive hepatic protein breakdown and liver damage in cases of end-stage renal disease, low protein diet, starvation, hepatic encephalopathy, and cancer (5–10). In these cases, the supply of NH₄⁺ in the portal vein may be higher than that of aspartate, thereby imposing a metabolic challenge that requires the liver to provide aspartate from internal hepatic proteolysis, thus exacerbating the clinical condition.

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Alanine Metabolism in the Perfused Rat Liver: STUDIES WITH 15N
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