Real-time study of the urea cycle using ^{15}N n.m.r. in the isolated perfused rat liver*

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1. Isolated rat liver was perfused with 10 mM- $^{15}\text{NH}_4\text{Cl}$, 5 mM-lactate and 1 mM-ornithine, or with 3 mM- ^{15}N -alanine and 1 mM-ornithine, in haemoglobin-free medium. The liver was physiologically stable for over 3 h and synthesized urea at the rate of $1.15 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}$ of liver $^{-1}$ ($^{15}\text{NH}_4^+$ -perfused) or $0.41 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ (^{15}N -alanine-perfused). 2. The perfused liver was continuously monitored by ^{15}N n.m.r. spectroscopy at 20.27 MHz for ^{15}N . Well-resolved ^{15}N resonances of precursors and intermediates of the urea cycle, present at tissue concentrations of 0.2–3.0 $\mu\text{mol/g}$, were observed from the intact liver in 5–40 min of acquisition. Key metabolites in liver extract and the final perfusion medium were analysed by n.m.r. and by biochemical assays to determine fractional ^{15}N enrichment and the total ^{15}N recovery. 3. In $^{15}\text{NH}_4^+$ -perfused liver ($n = 6$), ^{15}N incorporation into glutamate and alanine (1.0–1.3 $\mu\text{mol/g}$), as well as progressive formation of $^{15}\text{N}_2$ urea, was observed during the first 2 h of perfusion. In the second and third hour, hepatic concentrations of $[\omega\text{-}^{15}\text{N}]$ citrulline and $[\omega,\omega'\text{-}^{15}\text{N}]$ argininosuccinate increased to n.m.r.-detectable levels (0.3–0.9 $\mu\text{mol/g}$). The ^{15}N aspartate pool was large in the absence of added ornithine, but on its addition was rapidly incorporated into argininosuccinate ($n = 3$). 4. In ^{15}N -alanine-perfused liver, major metabolites were ^{15}N glutamate, $[\gamma\text{-}^{15}\text{N}]$ glutamine and ^{15}N urea. Urea-cycle intermediates were undetectable. 5. The results suggest that, in intact liver provided with excess ammonia, low concentrations of cytosolic argininosuccinate synthetase and argininosuccinate lyase limited the rate of metabolite flux in the urea cycle. By contrast, in alanine-perfused liver at a physiological rate of urea synthesis, mitochondrial carbamoylphosphate synthetase was rate-limiting. 6. The potential utility of ^{15}N n.m.r. for study of metabolite channelling through urea-cycle enzymes in intact liver is discussed.

INTRODUCTION

The enzymology, physiology and metabolic regulation of the urea cycle in mammalian liver has been exhaustively studied. Because the process is divided between cytoplasmic and mitochondrial compartments, and is dependent upon energy supply, an isolated liver is an appropriate system in which to study ureogenesis.

N.m.r. permits continuous non-destructive analysis of major metabolites in tissues, organs and *in vivo*, in animal and man, and has made significant contributions to our knowledge of biochemistry *in vivo* [1–4]. Most studies *in vivo* have used ^{13}C , ^1H and ^{31}P n.m.r. ^{15}N n.m.r., which has recently been successfully introduced into mammalian metabolite studies *in vivo*, in isolated tissues and in extracts, after enrichment of the relevant nitrogen pools [5–7], permits metabolites to be identified and often quantified without separation. The technique of ^{15}N n.m.r. seems particularly suited for analysis of the pathways of nitrogen in the liver *in vivo* and to synchronous detection of intracellular urea-cycle intermediates and other nitrogen metabolites.

We undertook this study to define the limits of resolution of relevant metabolites and, in particular, the sensitivity of ^{15}N n.m.r. in an intact tissue. The results provide some new insights into the regulation of the urea cycle, and indicate that dynamic studies with a time resolution of 10 min will be attainable with this technique. These studies were necessarily performed with labelled precursors, but because substrate levels rather than tracer amounts were used, and because total metabolite con-

centration as well as fractional enrichment was generally determined, we can be confident that we observe net synthesis of the compounds described, and not simply isotope exchange. ^{15}N n.m.r. will amplify the finding with ^{15}N m.s., for which the main limitation is the destructive nature of the assays. In comparison with ^{13}N , which only permits studies within the short half-life of the isotope, ^{15}N allows studies of longer duration.

A preliminary account of these findings has been presented [8].

MATERIAL AND METHODS

Perfusion

Male Wistar rats (Harlan Sprague-Dawley, Indianapolis, IN, U.S.A.), weighing 230 ± 59 g, were fasted overnight ($n = 15$) or fed on normal laboratory chow until used ($n = 5$). Liver perfusion was performed with a recirculating circuit as described previously [9], with the exception that the liver was excised and placed in a glass chamber for n.m.r. evaluation [10]. The animals were anaesthetized with Nembutal (Abbott Laboratories, Chicago, IL, U.S.A.; 50 mg/kg). After mid-abdominal incision, 100 units of heparin (Elkins-Sinn, Cherry Hill, NH, U.S.A.) were injected into the inferior vena cava. After placing three loose ligatures around the portal vein, the vein was cannulated with an Angiocath (1.7 mm diameter, length 3.2 cm; Deseret Medical, Sandy, UT, U.S.A.). Directly after cannulation the infusion of Krebs–Henseleit buffer was started and the cannula secured. The liver was rapidly removed, transferred to a stoppered glass container that fitted into the n.m.r. probe and connected to the

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Fig. 1. Proton-decoupled ^{15}N n.m.r. spectra of rat liver perfused with $^{15}\text{NH}_4^+$ under standard conditions

(a) Perfused liver after 1 h of perfusion; (b) 2 h; (c) 3 h. Each spectrum was acquired in 1 h. ^{15}N peaks are inverted because the negative gyromagnetic ratio of ^{15}N results in a negative nuclear Overhauser effect on proton decoupling. The $\omega\text{-N}$ of argininosuccinate (-311 p.p.m.) and citrulline (-308 p.p.m.) refers to the terminal N derived from ammonia. The $\omega'\text{-N}$ in argininosuccinate (-292 p.p.m.) refers to the nitrogen derived from aspartate. Peaks are assigned (left to right) as: Arg(s (ω')) (argininosuccinate), urea, Cit (citrulline), Arg(s (ω')) (argininosuccinate), Ala (alanine), Glu (glutamate), Asp (aspartate) and NH_4^+ (ammonia).

recirculating perfusion system. The liver was partially submerged in the perfusion medium. The medium was haemoglobin-free, consisting of Krebs–Henseleit buffer containing 2.5% (w/v) BSA (fraction V; ICN Immuno Biologicals, Lisle, IL, U.S.A.) dialysed against low-calcium Krebs–Henseleit saline [9,11]

and stored frozen at -20°C [11]. The perfusion flow was constant throughout at a level that allowed full oxygenation of the liver, typically $5.8\text{--}8.5\text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$. To achieve this, and minimize losses through elongated silastic perfusion tubing, the glass oxygenator was placed as close to the liver as the design of the magnet allowed. The oxygenation, pH, CO_2 and bicarbonate concentrations in inflowing and outflowing medium were determined with a blood gas analyser (ABL2; Radiometer Copenhagen, Cleveland, OH, U.S.A.).

The pH was kept at 7.4 by gassing with CO_2/O_2 (1:19) and by periodic addition of 0.5 M-NaHCO_3 , and the temperature of the inflowing medium and liver was maintained at 37°C by water-jacketing the oxygenator.

In some experiments ($n=10$) designed to examine the effects of varying rates of urea synthesis, a lower perfusion rate ($2.9\text{--}4.75\text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) and oxygen uptake, ($<2\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) were used.

Experimental design

After preliminary experiments to establish feasibility, studies were performed in five groups as follows.

Group 1. (Standard perfusion conditions) fasted rats ($n=6$), substrates lactate (5 mM), ornithine (1 mM), $^{15}\text{NH}_4\text{Cl}$ (10 mM).

Group 2. Fasted rats ($n=2$); substrates ornithine (1 mM) and [^{15}N]alanine (3 mM).

For Groups 3–5, low perfusion flow and oxygen uptake below $2\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ was used:

Group 3. Fed rats ($n=4$); substrates lactate (5 mM), ornithine (1 mM) and $^{15}\text{NH}_4\text{Cl}$ (10 mM).

Group 4. Fasted rats ($n=3$); substrates lactate (5 mM) and $^{15}\text{NH}_4\text{Cl}$ (5 mM) (no ornithine).

Group 5. Fasted rats ($n=3$); substrates lactate (5 mM) and $^{15}\text{NH}_4\text{Cl}$ (10 mM). Ornithine was added to a final concentration of 1 mM after 1 h of perfusion.

^{15}N -labelled compounds, and ornithine where indicated, were added to the perfusion medium, which also contained lactate, after a period of equilibration of 30–40 min [9]. Further additions of the same amounts of substrates were made every hour with the aim of maintaining near-constant concentrations of substrates. This was done to maintain nearly constant rates of urea synthesis over long periods of perfusion.

To determine overall rates of urea synthesis, and determine recovery of added ^{15}N , aliquots (3 ml) of medium were taken each hour and stored at -20°C . After a mean perfusion time of 3 h, the liver was rapidly frozen and stored in liquid N_2 until extraction. The final volume of perfusion medium was determined and an aliquot was taken for n.m.r. analysis.

Livers were extracted and deproteinized, using either HClO_4 [7] or trichloroacetic acid [12]. Liver extracts and untreated or HClO_4 -treated medium samples were used for ^{15}N n.m.r. and enzymic analysis.

N.m.r.

^{15}N n.m.r. spectra were obtained on a CSI-II spectrometer from General Electric NMR Instruments (Fremont, CA, U.S.A.), equipped with a 4.7 T, 25 cm-long horizontal-bore magnet manufactured by Oxford Instruments (Oxford, U.K.). The probe consisted of an 11-turn solenoidal coil (length 3.3 cm; diameter 4 cm) tunable to 20.27 MHz for ^{15}N observation and a Helmholtz coil tunable to 200.12 MHz for proton-decoupling and shimming. The probe was constructed by Robert L. Domenick (Magnetic Resonance Plus, Santa Clara, CA, U.S.A.).

Operating conditions employed a $95\text{ }\mu\text{s}$ (31° flip angle) pulse width, 0.25 s acquisition time and 2.5 s relaxation delay. Proton decoupling was achieved by WALTZ-16 composite pulse sequence with a decoupler power of 6 W during acquisition and

Fig. 2. Time course of ^{15}N incorporation into intra-hepatic glutamate and urea

Intrahepatic concentrations of [^{15}N]glutamate ($\square$) and [$^{15}\text{N}_2$]urea ($\blacktriangle$) for six livers perfused under standard conditions. Points, which are means $\pm$ 1 S.E.M., describe a linear rate of urea synthesis ($R = 0.99$). The [^{15}N]glutamate concentration, which is described by an exponential curve ($R = 0.93$), reached a near steady-state after 30 min of perfusion.

0.25 W during relaxation delay. These conditions optimized the signal-to-noise ratio for [^{15}N]glutamic acid, [^{15}N]alanine, [γ - ^{15}N]glutamine and [$^{15}\text{N}_2$]urea in aqueous solution, with ^{15}N signal intensity enhanced 3.3–3.9 fold due to nuclear Overhauser effect.

For perfused liver samples, shimming was performed until the water proton signal had a linewidth of 40–70 Hz. ^{15}N linewidths in the perfused liver were then generally 20 ± 15 Hz. ^{15}N acquisition was started simultaneously with the addition of ^{15}N -enriched precursor. Acquisition was restarted every hour ($^{15}\text{NH}_4^+$ perfusions) or every 20 min ([^{15}N]alanine perfusions).

^{15}N chemical shifts are reported in p.p.m. from nitromethane, the negative sign indicating upfield shift. ^{15}N -enriched metabolites were identified by comparison of their chemical shifts with reported values [7,13,14].

Quantification

^{15}N -enriched metabolites in the liver extracts and final perfusion media were quantified by comparison of their observed ^{15}N peak intensities with those of standards. A standard sample was prepared by dissolving known quantities of L-[^{15}N]glutamic acid, L-[^{15}N]alanine, L-[γ - ^{15}N]glutamine, $^{15}\text{NH}_4\text{Cl}$ and [$^{15}\text{N}_2$]urea (all 99 % enriched in ^{15}N) and [^{15}N]aspartic acid (95 % enriched) in unlabelled liver extracts. This ensured that the effects of T_1 and nuclear Overhauser enhancement on the ^{15}N peak intensity were the same for the standard and the biologically ^{15}N -enriched liver extract [7]. For citrulline, the standard compound at the natural abundance of ^{15}N (0.365 %) was used.

The peak intensity/scan per μmol of each ^{15}N -enriched standard (normalized to 99 % ^{15}N enrichment), measured over 3 months, was reproducible with an S.E.M. of 5.3 %. Peak intensities of standards dissolved in perfusion medium were not appreciably different from those in liver extract. The quantity (μmol) of each ^{15}N -metabolite in liver extract and final medium was calculated from its observed peak intensity by calibration with the peak intensity/ μmol of the corresponding standard.

For [ω, ω' - ^{15}N]argininosuccinate, for which a standard was not readily available, the peak intensity/ μmol of [ω - ^{15}N]citrulline, a structurally similar compound, was used to estimate the concentration in liver extract from observed peak intensity.

For direct quantification of spectra obtained from the intact perfused liver, the peak intensity per scan of a [^{15}N]metabolite

Fig. 3. Comparison of ^{15}N n.m.r. spectra of $^{15}\text{NH}_4^+$ -perfused liver, liver extract and medium

The principal spectra were obtained from the same experiment in which the liver was perfused under standard conditions for 3 h. The spectra are: (a) perfused liver; (b) liver extract; (c) final medium. Assignments are those given in the legend to Fig. 1. The peak at -223 p.p.m. (*) in (b) was not assigned. Insets to (b) and (c) show well-resolved [γ - ^{15}N]glutamine [Gln(γ)] in the extract and final medium respectively of another liver perfused under the same conditions.

and its intrahepatic concentration at the end of perfusion were determined. Changes in concentration during perfusion could then be estimated from the observed changes in the peak intensity.

Contribution of extra-hepatic metabolites to the observed ^{15}N signal during perfusion

For [$^{15}\text{N}_2$]urea, the contribution of intra- and extra-hepatic [$^{15}\text{N}_2$]urea to the observed ^{15}N peak intensity of perfused liver was calculated from its measured concentrations in the final liver and medium and the weight of liver (average 10.7 ± 1 g) and medium volume (average $4 \text{ ml} \pm 1 \text{ ml}$) in the coil region at the end of the experiment. The concentration of intrahepatic [$^{15}\text{N}_2$]urea during perfusion was then estimated from the observed peak intensity on the assumption that the relative

Table 1. Distribution of [^{15}N]metabolites in liver and perfusion medium of fasted rats

Concentrations of [^{15}N]metabolites in the final liver extract and medium were determined after 3 h of perfusion with $^{15}\text{NH}_4^+$ ($n = 6$) or [^{15}N]alanine ($n = 2$) under standard conditions. Results are given as $\mu\text{mol/g}$ of liver or $\mu\text{mol/ml}$ of medium (means $\pm$ S.E.M.) (n.d., not detected). For $n = 2$, S.E.M. is equivalent to the range. P values refer to the differences in metabolite concentrations between liver (L) and medium (M). The ratios for total metabolite(s) can be readily calculated by referring to the fractional enrichment given in Table 2.

Substrate...	$^{15}\text{NH}_4\text{Cl}$ (10 mM) ($n = 6$)				^{15}N]Alanine (3 mM) ($n = 2$)	
	Liver	Medium	P	L/M	Liver	Medium
Urea	4.0 ± 0.5	7.2 ± 1.0	0.01	0.6	0.5 ± 0.1	1.4 ± 0.4
Alanine	1.0 ± 0.2	0.4 ± 0.1	0.04	2.5	0.8 ± 0.3	1.9 ± 0.4
Glutamate	1.3 ± 0.2	0.2 ± 0.03	0.001	6.7	1.1 ± 0.2	0.2 ± 0.05
Glutamine	0.6 ± 0.2	0.2 ± 0.02	0.05	3.4	1.0 ± 0.7	0.4 ± 0.1
Aspartate	0.2 ± 0.03	0.04 ± 0.02	0.01	5.5	0.3 ($n = 1$)	n.d.
Citrulline	0.9 ± 0.2	n.d.	—	—	n.d.	n.d.
Ammonia	6.9 ± 2.3	10.3 ± 2.6	0.06	0.7	n.d.	n.d.

contribution of intra- and extra-hepatic [$^{15}\text{N}_2$]urea to signal intensity was constant during perfusion.

Metabolite assays

We assayed the total concentration of the relevant compounds to establish their fractional ^{15}N enrichment. Urea was assayed by the colorimetric method with diacetyl monoxime [15] and citrulline by that described by Archibald [16]. L-Glutamate [17], L-glutamine [18], L-aspartate [19] and L-alanine [20] were determined enzymically in specific assays.

Statistical analysis

Data are expressed as means $\pm$ S.E.M. To establish statistical differences in normally distributed data, a two-tailed paired t test was used. Otherwise, the two-tailed Wilcoxon test was used.

RESULTS

Fate of $^{15}\text{NH}_4^+$ in the perfused liver

The present preparation of isolated haemoglobin-free perfused rat liver proved to be physiologically stable for over 3 h, on the basis of a constant perfusate flow rate of $5.8\text{--}8.5\text{ ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($n = 9$) and oxygen consumption of $2.7 \pm 0.07\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($n = 2$), which increased as anticipated to $4.44 \pm 0.1\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ ($n = 6$) upon addition of biosynthetic precursors. Furthermore, net urea synthesis was linear over 3 h at a rate of $1.15 \pm 0.1\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ [9,21–23]. Well-resolved ^{15}N spectra were readily obtained directly from the isolated perfused rat liver and could be acquired every 15–30 min. ^{15}N incorporation increased progressively with time of perfusion, reflecting the continuous biosynthetic activity of the liver.

During perfusion with $^{15}\text{NH}_4\text{Cl}$ several distinct patterns of ^{15}N incorporation were observed. Alanine and glutamate were first formed, followed by the progressive appearance of [^{15}N]urea and related metabolites.

In Fig. 1, three ^{15}N spectra are presented to illustrate the labelling of hepatic metabolites after 1, 2 and 3 h in a representative liver perfused under standard conditions. During the first hour of perfusion, label was incorporated into the α -nitrogen atoms of alanine (-340 p.p.m.) and glutamate (-342 p.p.m.), but was barely detectable in urea (-306 p.p.m.) (Fig. 1a). During the second hour, the signal intensity of urea rapidly surpassed that of the α -amino acids (Fig. 1b). In the final hour, further incorporation of ^{15}N label was observed, the signal intensity of urea increased and the following peaks could be readily assigned: ^{15}N in α -nitrogen atoms of aspartate (-343 p.p.m.) and the urea-cycle intermediates citrulline

($\omega\text{-N} = -308\text{ p.p.m.}$) and argininosuccinate ($\omega\text{-N} = -311\text{ p.p.m.}$, $\omega'\text{-N} = -292\text{ p.p.m.}$) (Fig. 1c). Unmetabolized $^{15}\text{NH}_4^+$ (-362 p.p.m.), which was present in relatively large amounts, was nevertheless close to the limits of detection, because of its long T_1 (spin-lattice relaxation time [7]).

The time course of ^{15}N incorporation into glutamate and urea of the perfused liver is illustrated for all livers in this Group in Fig. 2. [^{15}N]glutamate was measurable at 15 min, reaching a plateau at 30 min. [^{15}N]urea was not detected during the first 15 min, but subsequently its concentration surpassed that of glutamate. The rate of [^{15}N]urea accumulation within the liver ($0.03\text{ }\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) was approx. 3% of the total [$^{15}\text{N}_2$]urea synthesized, the remaining 97% being recovered in the perfusion medium.

Incorporation of label into alanine paralleled that of glutamate, but [^{15}N]aspartate, visible in five out of six experiments, remained low. Glutamine ($\gamma\text{-N} = -271\text{ p.p.m.}$) was invariably formed from $^{15}\text{NH}_4\text{Cl}$ in this experiment, and was seen in the final liver extracts and perfusion media. However, [$\gamma\text{-}^{15}\text{N}$]glutamine was less readily detected in intact liver, being present after 1 h of perfusion in only one liver and in five of six experiments after 3 h of perfusion.

Citrulline ($n = 5$) and argininosuccinate ($n = 4$), two important intermediates of the urea cycle, could regularly be demonstrated in liver spectra.

Each of the metabolites assigned in the intact perfused liver (Fig. 3a) was also identified in the well-resolved spectrum of the liver extract (Fig. 3b and inset). The peak at -311 p.p.m. arises predominantly from $\omega\text{-}^{15}\text{N}$ of argininosuccinate, since its peak intensity is comparable ($\pm 10\%$) with that of the -292 p.p.m. peak ($\omega'\text{-}^{15}\text{N}$ of argininosuccinate; Fig. 3b). Any contribution from [$\omega, \omega'\text{-}^{15}\text{N}$]arginine, which has a chemical shift identical with that of $\omega\text{-}^{15}\text{N}$ argininosuccinate ($\delta^{15}\text{N} = -311\text{ p.p.m.}$), could not therefore have exceeded 10%. Large differences between liver and medium were seen. The medium contained mainly urea and ammonia (Fig. 3c). Glutamine (inset to Fig. 3c), alanine and glutamate were also present in the medium, but at concentrations too low to contribute to the perfused-liver spectrum (Fig. 3a).

Table 1 summarizes the distribution of ^{15}N into various metabolites for all livers of group 1. When considered together with the values obtained for the fractional ^{15}N enrichment (Table 2), the flux into each metabolite pool can be calculated.

In keeping with the results already shown for individual livers, the major metabolic fate of $^{15}\text{NH}_4^+$ was incorporation into doubly enriched urea, with significant accumulation in glutamate, alanine, citrulline and argininosuccinate. The latter is not shown, but is estimated to be $0.50 \pm 0.05\text{ }\mu\text{mol/g}$. Incorporation into

Fig. 4. ^{15}N n.m.r. spectra of a liver perfused with ^{15}N alanine

For peak assignment, see the legend to Fig. 1. (a) Perfused liver after 20 min of acquisition; (b) liver extract; (c) final medium.

$[\gamma\text{-}^{15}\text{N}]$ glutamine and ^{15}N aspartate was significantly less, and other intermediates of the urea cycle were not detected.

^{15}N metabolite distribution in the liver of the single fed rat (results not shown) was not different from that described in fasted animals (Table 1).

Fractional ^{15}N enrichment of metabolites in the liver

The fractional enrichment of intrahepatic urea (expressed as a percentage) was 55%, and that of alanine, glutamate and glutamine was close to 70% (Table 2). Citrulline (and argininosuccinate), intermediates of the urea cycle, were highly enriched.

Table 2. Fractional ^{15}N enrichment of metabolites in perfused livers of fasted rats

Fractional ^{15}N enrichment (expressed as a percentage) was calculated as $[(\text{mean concentration by n.m.r.})/(\text{mean concentration by enzymic analysis})] \times 100\%$. Enrichment of different metabolites ($n = 6$) is given. The fractional enrichment of intra-hepatic urea, alanine, glutamate and glutamine was statistically similar in $^{15}\text{NH}_4^+$ -perfused livers (column 2). Where indicated (*) the enrichment of individual metabolites in the liver was significantly different ($P \leq 0.01$) from that in the perfusion medium (column 3); n.d., not detected.

Substrate ... [^{15}N]metabolite	Fractional enrichment (%)			
	$^{15}\text{NH}_4\text{Cl}$ (10 mM)		^{15}N Alanine (3 mM)	
	Liver	Medium	Liver	Medium
Urea	55 ± 6	$90 \pm 7^*$	18 ± 4	61 ± 13
Alanine	67 ± 7	80 ± 6	50 ± 15	100 ± 5
Glutamate	67 ± 6	$91 \pm 6^*$	54 ± 13	96 ± 8
Glutamine	71 ± 9	58 ± 6	30 ± 9	38 ± 3
Aspartate	15 ± 3	33 ± 8	10 ± 10	n.d.
Citrulline	90 ± 8	n.d.	n.d.	n.d.

The poor detection of aspartate was accounted for by the relatively low fractional enrichment in this amino nitrogen (15%).

Significant concentration gradients for ^{15}N metabolites were demonstrated between perfused liver and medium (Table 1). A concentration gradient of between 2 and 10 (liver/medium) was established for all ^{15}N -enriched amino acids, as well as for total amount of metabolites. $[\omega\text{-}^{15}\text{N}]$ Citrulline was present in the liver, but not in the medium. The liver/medium gradient for $^{15}\text{N}_2$ urea was 0.55, but when the total amount of urea was taken into account, no gradient existed (urea in liver/urea in medium = 1.04).

^{15}N Alanine metabolism

Oxygen uptake in livers perfused with ^{15}N alanine (Group 2), was $3.59 \pm 0.3 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, and urea synthesis (total) was linear at a rate of $0.41 \pm 0.02 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$. Added ^{15}N alanine was visible after only 5 min of acquisition and, after 20 min of perfusion, glutamate reached half the signal intensity of alanine. α -Glutamate, γ -glutamine and urea appeared progressively (Figs. 4a and 4b), but no appreciable amounts of labelled aspartate, citrulline, argininosuccinate or ammonia could be detected.

$^{15}\text{N}_2$ Urea synthesis from alanine proceeded at a rate of $0.19 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$, and the fractional enrichment of urea and glutamine was only 18 and 30% respective (Table 2), indicating a considerable rate of formation of endogenous unlabelled urea.

Finally, because their relative concentrations in the perfusion medium were high, extrahepatic alanine and urea contributed significantly to the signal observed during perfusion (Fig. 4c). This has been taken into account in the calculations of synthesis rates.

^{15}N incorporation under modified conditions of perfusion

In order to establish factors influencing the routes of ^{15}N incorporation into urea, a small number of experiments was conducted under slightly modified conditions (Groups 3–5). As expected, when O_2 consumption was limited, by reducing the flow rate, reducing the NH_4Cl concentration or by limiting the availability of ornithine, ^{15}N urea synthesis was reduced in proportion to oxygen uptake ($R = 0.87$) (Fig. 5). More ^{15}N was observed in the α -nitrogen atoms of alanine and glutamate in general, and rather less appeared in urea, compared with the

Fig. 5. O_2 consumption versus $[^{15}N_2]$ urea synthesis

Rates of $[^{15}N_2]$ urea synthesis and oxygen consumption were compared in livers perfused with $^{15}NH_4^+$ (10 mM unless otherwise stated) under various conditions. +, Fasted rat, with ornithine; Δ , fed rat, with ornithine; $\square$, fasted rat, perfused with 5 mM- $^{15}NH_4^+$, without ornithine; $\blacksquare$, fasted rat, with ornithine added later.

livers perfused under standard conditions. The lowest rate of urea synthesis was found in the absence of ornithine (Group 4). Adding ornithine late in the course of the experiment (Group 5) slightly increased ^{15}N incorporation into urea.

In the liver perfused in the absence of ornithine (Fig. 6a), the $[^{15}N]$ aspartate concentration, $1.1 \pm 0.3 \mu\text{mol/g}$ (enrichment $31\% \pm 12\%$), reached five times that in livers perfused under standard conditions (see Table 1). Less $[^{15}N]$ urea was observed (Figs. 6a and 6b) and more residual ammonia was detected in medium (Fig. 6c).

The effect of addition of ornithine upon the distribution of ^{15}N metabolites was demonstrated in three experiments, one of which is illustrated in Fig. 7. When ornithine was added, the signal intensity of aspartate fell, and citrulline and argininosuccinate were detected (compare Figs. 7a and 7b).

DISCUSSION

This work demonstrates, for the first time, the feasibility of using ^{15}N n.m.r. to monitor the pathways of $[^{15}N]$ ammonia incorporation into precursors and intermediates of the urea cycle and to measure the rates of formation of some $[^{15}N]$ metabolites in isolated perfused liver. Well-resolved ^{15}N resonances of precursors and intermediates of the urea cycle, present at tissue concentrations of $0.2\text{--}3.0 \mu\text{mol/g}$, were observed from the intact liver in 5–40 min of acquisition. Although the use of radioactive tracer ^{13}N has provided valuable information on short-term (< 1 min) metabolic fates of ammonia and amino acids in rat liver [24,25], ^{15}N n.m.r. is useful for monitoring hepatic nitrogen metabolism on a longer time scale *in vivo*. Our finding that $^{15}NH_4^+$ -perfused liver contains high concentrations of the urea-cycle intermediates $[\omega\text{-}^{15}N]$ citrulline and $[\omega,\omega'\text{-}^{15}N]$ argininosuccinate has interesting implications for the efficiency of transfer of these metabolites from mitochondrial ornithine transcarbamoylase to cytosolic argininosuccinate synthetase and argininosuccinate lyase in intact hepatocytes [26].

Recovery of ^{15}N

Of the $^{15}NH_4Cl$ metabolized, 70 % appeared in urea, mostly labelled in both nitrogen atoms. All of the remaining metabolites together accounted for only 4.3 % of the missing ^{15}N (Table 3).

Fig. 6. ^{15}N n.m.r. spectra of a liver perfused without ornithine

The medium contained 5 mM- $^{15}NH_4^+$ and 5 mM-lactate. (a) Perfused liver; (b) liver extract; (c) final medium. The peaks were assigned as in Fig. 1. The peak (*) at -287 p.p.m. seen here and in Fig. 7(b) is unassigned.

The label not accounted for was probably incorporated into unidentified metabolites (e.g. the -223 p.p.m. peak in Fig. 3b and the -287 p.p.m. peak in Figs. 6 and 7), substances with concentrations below our limits of detection, and macromolecules. Among the latter, we could anticipate synthesis of albumin and other proteins, which would be lost from samples treated with $HClO_4$ and trichloroacetic acid.

Synthesis of glutamine

Glutamine and urea synthesis occurs in separate hepatocyte populations [27]. The partition of nitrogen between them is an

for glutamine and urea release (in $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$) without allowance for that retained within the liver [28].

By contrast in livers perfused with [¹⁵N]alanine, as well as in livers of animal infused with [¹⁵N]alanine *in vivo* [7], [γ -¹⁵N]-glutamine was prominent. Chamalaun & Tager [21] and Farrow *et al.* [7] pointed out that intracellular glutamate is high in the presence of excess alanine. This is simply a reflection of the high activity of near-equilibrium enzymes, alanine aminotransferase and glutamate dehydrogenase, in the liver [29,30].

Regulation of urea-cycle enzymes

In liver perfused with ¹⁵NH₄⁺ in the presence of ornithine, accumulation of [ω -¹⁵N]citrulline (0.92 $\mu\text{mol/g}$) and [ω,ω' -¹⁵N]-argininosuccinate (estimated to be 0.5 $\mu\text{mol/g}$) was observed. The result is consistent with very low activities of argininosuccinate synthetase (175 $\mu\text{mol/h per g}$) and argininosuccinate lyase (269 $\mu\text{mol/h per g}$) compared with ornithine transcarbamoylase (13400 $\mu\text{mol/h per g}$) and arginase 35000 $\mu\text{mol/h per g}$ in rat liver [31]. The singular absence of ¹⁵N resonances from carbamoyl phosphate and arginine can be explained on the basis of high activities of ornithine transcarbamoylase and arginase respectively and the relatively low steady-state concentrations of these metabolites in rat liver. The absence of a detectable level of [¹⁵N]acetylglutamate, the allosteric activator of carbamoyl-phosphate synthetase, is probably due to low fractional ¹⁵N enrichment and/or low intracellular concentration.

In livers perfused with [¹⁵N]alanine, transamination to [¹⁵N]-glutamate, followed by deamination by glutamate dehydrogenase, provided ¹⁵NH₄⁺ for synthesis of [¹⁵N]urea and [γ -¹⁵N]glutamine (Fig. 4a). With [¹⁵N]alanine provided at only 3 mM concentration in the medium, intramitochondrial ammonia concentration was probably much lower than in liver perfused with 10 mM-¹⁵NH₄⁺. Thus, at more physiological rates of urea synthesis, carbamoylphosphate synthetase (present at the level of only 340 $\mu\text{mol/h per g}$ [31]) and with a relatively high *K_m* for ammonia of 0.54–0.7 mM at intramitochondrial pH [32,33] may well have been the rate-limiting step in this pathway. Absence of detectable levels of [ω -¹⁵N]citrulline and [ω,ω' -¹⁵N]argininosuccinate in the alanine-perfused liver (Figs. 4a and 4b) is consistent with this interpretation. Wanders and colleagues [34], working with isolated liver mitochondria, which lack argininosuccinate synthetase and argininosuccinate lyase, concluded that carbamoylphosphate synthetase limited the rate of citrulline synthesis from 5 mM-NH₄Cl.

Many metabolically related enzymes are organized in multi-enzyme complexes *in vivo* [35]. For cytosolic urea-cycle enzymes, a study with permeabilized hepatocytes showed that there is demonstrable, though not very effective, channelling of citrulline from ornithine transcarbamoylase to argininosuccinate synthetase and of argininosuccinate from argininosuccinate synthetase to argininosuccinate lyase, whereas channelling of arginine to arginase is very efficient [26]. The observed accumulation of citrulline and argininosuccinate in perfused liver suggests that they were not efficiently channelled from ornithine transcarbamoylase $\rightarrow$ argininosuccinate synthetase $\rightarrow$ argininosuccinate lyase in intact hepatocytes under our experimental conditions. ¹⁵N n.m.r., which permits observation of [¹⁵N]-metabolites in intact liver, is expected to be useful for study of metabolite channelling through urea-cycle enzymes *in vivo*.

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Fig. 7. Effect of ornithine on the ¹⁵N n.m.r. spectra of the perfused liver

The spectrum of a liver perfused with 10 mM-¹⁵NH₄⁺ is shown (a) before and (b) after addition of 1 mM-ornithine to the perfusion medium.

Table 3. Recovery of ¹⁵N in metabolites of liver and perfusion medium

To calculate recovery, concentrations of [¹⁵N]metabolites were determined in final liver and perfusion medium and multiplied by liver weight and volume of perfusion medium respectively.

	Recovery of ¹⁵ N (mmol of ¹⁵ N) (mean $\pm$ S.E.M.)	¹⁵ N infused (%)	¹⁵ N metabolized recovered (%)
Ammonia infused	8.40 $\pm$ 0.68	100	
Ammonia remaining	2.41 $\pm$ 0.68	28.7	
Ammonia metabolized	5.99 $\pm$ 0.68	71.3	100
Metabolites			
Urea	4.24 $\pm$ 0.49		70.1
Alanine	0.11 $\pm$ 0.02		1.8
Glutamate	0.07 $\pm$ 0.01		1.2
Glutamine	0.05 $\pm$ 0.005		0.9
Aspartate	0.01 $\pm$ 0.005		0.2
Citrulline	0.009 $\pm$ 0.001		0.2
Ammonia unaccounted for	1.50 $\pm$ 0.12		25.6

interesting question. In ¹⁵NH₄-perfused liver extract, a ratio of 1:7 for [γ -¹⁵N]glutamine ($\mu\text{mol/g}$) versus [¹⁵N₂]urea ($\mu\text{mol/g}$), without correction for re-uptake and metabolism of released glutamine, was found. Our values therefore fall in between Cooper *et al.*'s value of 1:14 for ¹³N-labelled intra-hepatic glutamine and urea after 60 s [25] and Häussinger's value of 1:2

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