¹⁵N n.m.r. measurement of the *in vivo* rate of glutamine synthesis and utilization at steady state in the brain of the hyperammonaemic rat

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The rate of glutamine synthesis and utilization at steady state was measured *in vivo* in the brains of hyperammonaemic rats by ¹⁵N n.m.r. in combination with biochemical techniques. Rats were given an intravenous ¹⁵NH₄⁺ infusion at the rate of 4.8 ± 0.3 mmol/h per kg body wt. for 3.5 ± 0.2 h, followed by ¹⁴NH₄⁺ infusion at the same rate for an additional 5.1 h (chase period). During the chase period, blood ammonia (0.61 ± 0.015 μmol/g), brain ammonia (2.9 ± 0.3 μmol/g), glutamate (9.4 ± 0.8 μmol/g) and glutamine (¹⁵N + ¹⁴N; 14.4 ± 1.3 μmol/g) were at steady state. The rate of change in the cerebral [5-¹⁵N]glutamine concentration was measured *in vivo* by ¹⁵N n.m.r. at 20.27 MHz. To estimate ¹⁵N enrichment of precursor ammonia for glutamine synthetase (GS) in astrocytes which are interposed between cerebral capillaries and neurons, ¹⁵N enrichments of blood and brain ammonia were measured by gas chromatography–mass spectrometry. The *in vivo* rate of glutamine synthesis, which is equal to the rate of glutamine

utilization at steady state, was estimated, from the observed rate of change in [5-¹⁵N]glutamine concentration and ¹⁵N enrichment of brain glutamine, to be 4.8 ± 1.1 μmol/h per g of brain if ¹⁵N enrichment of ammonia at the site of GS in astrocytes is equal to that of blood-borne ammonia, and 13.0 ± 3.9 μmol/h per g if it is equal to that measured for the whole brain. The observed GS activity *in vivo* in the brain of the hyperammonaemic rat is 2–5% of the reported optimum activity *in vitro* measured at enzyme-saturating concentrations of all substrates. The result suggests that substrates and/or cofactors other than ammonia kinetically limit GS activity *in vivo*. The g.c. chromatogram and mass spectrum of ammonia-derived *N*-trifluoroacetyl-*n*-dibutylglutamate (TAB-glutamate) are shown in Supplementary Publication SUP 50170 (4 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1993) 289, 9.

INTRODUCTION

Glutamine synthetase (GS) catalyses the ATP-dependent formation of glutamine from ammonia and glutamate [1]. In the brain, the enzyme is localized primarily in astrocytes [2] and to a lesser extent in oligodendroglia [3]. A widely accepted theory is that glia and neurons participate in a glutamine/glutamate cycle [4–7]. Glutamine molecules synthesized by glia are taken up by the neurons, where some are cleaved by glutaminase to provide the neurotransmitter pool of glutamate. Glutamate released into the synaptic space is taken up by surrounding astroglia processes, and recycled as a substrate for GS. There is increasing evidence that glutamine is a major precursor of neurotransmitter pools of glutamate [8–10] and of γ-aminobutyrate [11,12]. Therefore, development of a method for measurement of the *in vivo* rate of glutamine synthesis in the brain is important.

The activity of cerebral GS *in vivo* under conditions of hyperammonaemia is of interest because it may shed light on the aetiology of hepatic encephalopathy (HE). In HE, a metabolic disease of the brain caused by dysfunction of the liver [13], decreased removal of ammonia in the liver results in hyperammonaemia and diffusion of neurotoxic levels of ammonia into the brain. Two leading hypotheses on the mechanism of ammonia toxicity in HE are (a) ammonia *per se* is neurotoxic [14], and (b) hyperammonaemia causes HE through depletion of the excitatory neurotransmitter glutamate. The latter hypothesis is based on the consideration that removal of ammonia by cerebral GS results in the loss of equimolar glutamate which, in combination with ammonia-mediated inhibition of glutaminase in

nerve terminals, may lead to depletion of the neurotransmitter pool of glutamate [15]. GS activity *in vivo* is a direct measure of the rate of ammonia and glutamate removal from astrocytes. Therefore measurement of the *in vivo* activity in the brain of the hyperammonaemic rat as an animal model of HE can contribute to a better understanding of the mechanism of ammonia toxicity. Other cerebral metabolic changes observed in hyperammonaemia include a transient increase in the rate of glycolysis in some brain regions within 1 h of acute hyperammonaemia [16–18], followed by depression of the glucose consumption rate after 6 h to 2 days of chronic hyperammonaemia [19].

While much is known about GS activity in rat brain homogenates as measured *in vitro* [20,21] and hormone-mediated induction of GS synthesis [22], GS activity *in vivo* that depends on *in situ* substrate concentrations has not been measured to date. Rates of glutamine synthesis measured in cultured rat and mouse brain astrocytes [23–26] are informative, but it is also important to measure the rate in the brain *in vivo*, because glial–neuronal interaction plays a crucial role in the glutamate/glutamine cycle [27] and in the regulation of expression of the GS gene [28]. In an *in vivo* study using the radioisotope ¹³N, the half-life (*t*_{1/2}) for conversion of [¹³N]ammonia to [5-¹³N]glutamine in a small compartment of the rat brain was shown to be short (< 1–3 s) at physiological blood ammonia concentrations [29,30]. However, the rate of glutamine synthesis was not calculated because the isotope enrichment of the precursor ammonia and the physical size of the compartment were not known.

N.m.r. is a useful technique for the continuous and non-invasive monitoring of metabolic flux *in vivo* in animals and

Abbreviations used: GS, glutamine synthetase; HE, hepatic encephalopathy; g.c.–m.s., gas chromatography–mass spectrometry; GDH, glutamate dehydrogenase; TAB-glutamate, *N*-trifluoroacetyl-*n*-dibutylglutamate.

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humans [31–33]. The ready determination of elevated glutamine concentrations in the human brain *in vivo* in patients with HE [34], hypoxic encephalopathy [35] and Reye's syndrome [36] raises further interesting questions regarding the relationship of the steady-state glutamine concentration to that of the potentially neurotoxic glutamate.

^{15}N n.m.r. is particularly useful for the study of the rate of glutamine synthesis in the brain, because ammonia-derived glutamine amide nitrogen is clearly identified [37–39] and can be quantified in the brain *in vivo* [40]. We have recently measured the rate of increase of the cerebral [5- ^{15}N]glutamine pool *in vivo* in rats that were given an intravenous $^{15}\text{NH}_4^+$ infusion to induce hyperammonaemia [40]. In that study, the rate of glutamine synthesis was estimated from the observed rate of increase in the glutamine pool and an assumed rate of glutamine utilization calculated from the reported decrease in the glutamine pool [41] on termination of the ammonia infusion. We now report the measurement of the *in vivo* rate of glutamine synthesis and utilization at steady-state concentrations of brain ammonia, glutamate and glutamine in the hyperammonaemic rat, using an isotope chase method.

EXPERIMENTAL

Animal preparation

Male Wistar rats (fed), weighing 310 ± 35 g, were used. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal; 40 mg/kg body wt.), and prepared for infusion of ammonia through the right jugular vein as described in detail previously [37]. For measurement of blood ammonia concentration, ammonia was infused through the right jugular vein and blood samples were withdrawn from the left jugular vein at 0.5 h intervals. For isotope chase experiments, $^{15}\text{NH}_4\text{Cl}$ (99% enriched in ^{15}N) in 2 M aqueous solution at pH 7.4 was administered, after a bolus injection of 0.3 mmol, at an infusion rate of 4.8 ± 0.3 mmol/h per kg body wt. for 3.5 ± 0.2 h, followed by $^{14}\text{NH}_4^+$ infusion at the same rate for an additional 5.1 h ($n = 5$).

In another series of experiments, $^{15}\text{NH}_4^+$ infusion, following a bolus injection of 0.3 mmol, was given at a rate of 4.8–5.5 mmol/h per kg body wt. for 2.1 h (Group I; $n = 3$) or for 3.5 h (Group II; $n = 5$), and terminated without a chase. $^{15}\text{NH}_4^+$ infusion was performed outside the magnet, except where indicated otherwise, because an increase in the cerebral [5- ^{15}N]glutamine pool during $^{15}\text{NH}_4^+$ infusion has been studied *in vivo* by n.m.r. in a previous publication [40]. In the present work the decrease in the cerebral [5- ^{15}N]glutamine pool after the end of $^{15}\text{NH}_4^+$ infusion, with or without chase, was monitored *in vivo* by n.m.r.

N.m.r.

In vivo ^{15}N n.m.r. spectra were obtained on a CSI-II spectrometer from General Electric Instruments (Fremont, CA, U.S.A.), equipped with a 4.7 T, 25-cm bore magnet manufactured by Oxford Instruments (Oxford, U.K.), as described in detail previously [40]. The probe consisted of a solenoidal coil (4 cm $\times$ 3.3 cm) tunable to 20.27 MHz for ^{15}N observation, and a Helmholtz coil tunable to 200.12 MHz for ^1H decoupling and shimming. For the isotope chase experiment, the rat was placed in the magnet with its head inside the observe coil 40 min before the end of $^{15}\text{NH}_4^+$ infusion, and infusion was continued during shimming (approx. 10 min). After acquiring one ^{15}N spectrum of the $^{15}\text{NH}_4^+$ -infused rat, the infusate was changed to $^{14}\text{NH}_4^+$ and

^{15}N spectra were acquired every 20–30 min to monitor the decrease in [5- ^{15}N]glutamine peak intensity. Body temperature was maintained by blowing warm air into the bore of the magnet. To keep the rat anaesthetized throughout the chase experiment, approx. 5 mg of Nembutal was injected every hour through polyethylene tubing (PE 50; Clay Adams) connected to the intraperitoneal cavity. In experiments where the $^{15}\text{NH}_4^+$ infusion was terminated without an isotope chase, the rat was placed in the magnet immediately after the end of infusion, and ^{15}N spectra were acquired every 20–30 min for 3.5–5 h. When the rat showed signs of waking, it was removed from the magnet and had access to food and water for about 20 min before it was re-anaesthetized and placed in the magnet.

N.m.r. operating conditions employed a $95\ \mu\text{s}$ (31° flip angle) pulse, 0.25 s acquisition time and 2.5 s relaxation delay. Proton decoupling was achieved by WALTZ-16 composite pulse sequence. Decoupling was gated to give a high power level (6 W) sufficient for ^1H decoupling during the acquisition time and a low power level (0.25 W) sufficient to maintain the nuclear Overhauser enhancement during relaxation delay. In a preliminary experiment with $^{15}\text{NH}_4^+$ -infused rats these conditions optimized the signal-to-noise ratio for cerebral [5- ^{15}N]glutamine. ^{15}N chemical shifts are reported in p.p.m. from nitromethane, with the negative sign indicating an upfield shift. For quantification of cerebral [5- ^{15}N]glutamine, the observed *in vivo* peak intensity was converted to the [5- ^{15}N]glutamine concentration ($\mu\text{mol/g}$) in the brain by using the mean *in vivo* peak intensity per μmol of cerebral [5- ^{15}N]glutamine per min of acquisition. This conversion factor was obtained by calibrating the *in vivo* peak intensity with the actual quantity of [5- ^{15}N]glutamine measured in an extract of the same brain for seven rats, as described in detail previously [40]. At the end of the *in vivo* experiment, the rat was killed and the brain frozen immediately in liquid nitrogen for preparation of brain extract.

In vitro metabolite assay

Brain ammonia, glutamate and total ($^{15}\text{N} + ^{14}\text{N}$) glutamine present at the end of *in vivo* experiments were assayed in perchloric acid extracts of brain prepared as described above. Blood ammonia, brain ammonia and total glutamine at earlier time points were assayed in brain extracts from rats given parallel $^{14}\text{NH}_4\text{Cl}$ infusions and killed at the times indicated. Enzymic assays for ammonia, glutamine and glutamate were performed according to published procedures [42–44].

Kinetic analysis

Pathways of glutamine synthesis and utilization can be represented as follows:

where k_1 is the rate constant for glutamine synthesis and k_2 is the overall rate constant for glutamine utilization. Glutamine utilization pathways include not only the catabolic glutaminase reaction but also a number of anabolic reactions such as purine, pyrimidine and protein synthesis.

Suppose that the concentration of ammonia at the site of GS is maintained constant by intravenous infusion of ammonia at a constant rate, and that, initially, ammonia 99% enriched in ^{15}N is used. Let $A = \text{NH}_4^+$ ($^{14}\text{N} + ^{15}\text{N}$) concentration at the site of GS; $A^* = ^{15}\text{NH}_4^+$ concentration at the site of GS; $s_A = A^*/A$;

B = glutamine ($^{14}\text{N} + ^{15}\text{N}$) concentration in the brain; B^* = [^{15}N]glutamine concentration in the brain; and $s_B = B^*/B$. When the concentrations of ammonia, glutamate and glutamine are at steady state, Scheme I can be simplified to:

where only metabolites that undergo ^{15}N enrichment are represented. k_1 is the rate constant for glutamine synthesis at the prevailing steady-state concentrations of ammonia and glutamate, and k_2 is the rate constant for glutamine utilization at its steady-state concentration. In rat brain, this state is attained after 3–4 h of ammonia infusion, when cerebral glutamine reaches its steady-state concentration [40,41]. Then the following relationships [45] obviously hold:

$$dB/dt = k_1 - k_2 = 0 \quad (1)$$

$$dB^*/dt = k_1(A^*/A) - k_2(B^*/B) = k_1s_A - k_2s_B \quad (2)$$

Hence

$$k_1 = k_2 = (dB^*/dt)/(s_A - s_B) \quad (3)$$

Suppose at $t = 0$, $^{15}\text{NH}_4^+$ infusion is replaced by $^{14}\text{NH}_4^+$ infusion at the same rate. When ^{15}N enrichment of ammonia (s_A) becomes negligibly small compared with that of glutamine (s_B), then:

$$k_1 = k_2 = -(dB^*/dt)/s_B = -(dB^*/dt)B/B^* \quad (4)$$

On integration,

$$B^* = B_0^* e^{-(k_1/B)t} \quad (5)$$

where B_0^* is the concentration of B^* at $t = 0$. Therefore B^* changes exponentially with time.

Gas chromatography–mass spectrometry (g.c.–m.s.) analyses of ^{15}N enrichment of brain and blood ammonia

To determine ^{15}N enrichment, the ammonia in brain and blood extracts was converted to glutamate by the method of Yudkoff et al. [25], derivatized to the *N*-trifluoroacetyl-*n*-dibutyl ester (TAB-glutamate) by the method of Gehrke and co-workers [46,47], and isotopic abundance was analysed by g.c.–m.s.

Ammonia in brain and blood extracts was separated from glutamate and purified by elution through cation-exchange resin (AG50X8; 100–200 mesh), then converted to glutamate by glutamate dehydrogenase (GDH) as described in detail by Yudkoff et al. [25], with the following minor modifications. Because rat brain has a high concentration of glutamate, it was important to ensure complete separation of ammonia from glutamate before the GDH reaction. In a preliminary experiment, ammonia and glutamate concentrations in brain extract were assayed enzymically before and after elution through the AG50 resin. The post-column ammonia fraction contained no detectable glutamate ($< 0.8 \mu\text{M}$) and ammonia recovery was $> 90\%$. This ensured that all glutamate formed by the GDH reaction was derived from brain or blood ammonia. For the GDH reaction, the ammonia fraction was eluted into 0.1 M sodium phosphate buffer (pH 7.0) containing 152 μM NADH, 20 mM 2-oxoglutarate, 1.875 mM ADP and 6 units/ml GDH, and incubated at 37 °C for 20 min. The ammonia-derived glutamate was purified by elution through a 0.5 cm $\times$ 7 cm column of AG50 resin [25].

The ammonia-derived glutamate (15–150 nmol) was converted to TAB-glutamate by esterification in *n*-butanol 3 M in HCl at 100 °C, followed by acylation with trifluoroacetic anhydride in methylene chloride at 150 °C as described by Gehrke et al. [46].

Conversion of glutamate to the TAB-derivative was confirmed in preliminary experiments using 15 μmol of [^{14}N]glutamate or [^{15}N]glutamate as the starting material and verifying the structure of the derivatized product by ^1H n.m.r.

G.c.–m.s. analyses were performed using a Hewlett-Packard 5989A GC/MS/IR instrument. Separation of TAB-glutamate was performed with a 30 m $\times$ 0.25 μm internal diam. capillary column. The g.c. injector temperature was 220 °C and the oven temperature was programmed from 60 °C to 220 °C at 10 °C/min, followed by a 30 °C/min temperature ramp to 280 °C. Under these conditions, a well-resolved g.c. peak for the TAB-glutamate (derived from brain or blood ammonia) was observed at about 16 min (results not shown). The mass spectrometer was operated at 20 eV electron energy and a source temperature of 200 °C. The mass spectrum of the ammonia-derived TAB-glutamate (results not shown) was virtually identical to published spectra [48]. [The g.c. chromatogram and mass spectrum of TAB-glutamate are shown in Supplementary Publication SUP 50170 (4 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1993) 289, 9.] After initial experiments to determine retention time and mass spectra, all quantitative measurements for ^{15}N incorporation were performed using the mass spectrometer in the selected ion monitor mode (SIM). Three pairs of ions were chosen to ensure specificity of the analysis: 198,199; 254,255; and 152,153. The dwell time for each was 100 ms. Enrichments of TAB-glutamate were measured using the most intense pair of ions, i.e. 198,199.

RESULTS

The rate of glutamine synthesis and utilization at steady state

Rats ($n = 5$) were given an intravenous infusion of $^{15}\text{NH}_4^+$ (99% ^{15}N) at a rate of 4.8 ± 0.3 mmol/h per kg body wt. for 3.5 ± 0.2 h to permit the cerebral [^{15}N]glutamine concentration to reach a steady state [40]. This was immediately followed by $^{14}\text{NH}_4^+$ infusion at the same rate for an additional 5.1 h (chase period). To examine whether cerebral ammonia, glutamate and glutamine were at steady state during the chase period, their concentrations were measured in brain extracts. The results are summarized in Table 1. The brain ammonia concentration at the end of the chase was $2.9 \pm 0.3 \mu\text{mol/g}$ ($n = 4$), in close agreement with that

Table 1 Tissue levels and ^{15}N enrichment of cerebral metabolites and blood ammonia in rats given $^{15}\text{NH}_4^+$ infusion followed by $^{14}\text{NH}_4^+$ infusion

The tissue levels of metabolites, measured in extracts after 3.5 ± 0.2 h of $^{15}\text{NH}_4^+$ infusion and after a subsequent 5.1 h of $^{14}\text{NH}_4^+$ chase, are expressed as $\mu\text{mol/g}$ of tissue (means $\pm$ S.E.M.), with the numbers of rats in parentheses. Fractional ^{15}N enrichment, defined as [^{15}N -metabolite concentration]/[Total $^{15}\text{N} + ^{14}\text{N}$ -metabolite concentration], was measured 1.5 h after the start of the $^{14}\text{NH}_4^+$ chase by n.m.r. *in vivo* (glutamine) or by g.c.–m.s. in extracts (ammonia). For $n = 2$, the S.E.M. is equal to the range.

Metabolite	Tissue level ($\mu\text{mol/g}$)		
	After $^{15}\text{NH}_4^+$ infusion	After $^{14}\text{NH}_4^+$ chase	^{15}N enrichment at $t = 1.5$ h
Brain ammonia	2.9 ± 0.4 (5)	2.9 ± 0.3 (4)	0.18 ± 0.02 (2)
Brain glutamate	9.3 ± 0.3 (4)	9.4 ± 0.8 (3)	
Brain glutamine	14.4 ± 1.3 (5)	14.4 ± 1.5 (4)	0.24 ± 0.02 (2)
Blood ammonia	0.64 ± 0.06 (2)	0.61 ± 0.015 (2)	0.077 ± 0.017 (2)

Figure 1 *In vivo* ^{15}N n.m.r. spectra of $[5\text{-}^{15}\text{N}]$ glutamine in rat brain

(a) Spectrum acquired in 20 min from the head of a living rat after 3.5 h of intravenous $^{15}\text{NH}_4^+$ infusion. The peaks for $[5\text{-}^{15}\text{N}]$ glutamine (-271 p.p.m.) and $[2\text{-}^{15}\text{N}]$ glutamate/glutamine (-342 p.p.m.) arise exclusively from the brain [40]. The peak at -306 p.p.m. is blood-borne $[^{15}\text{N}]$ urea synthesized in the liver and circulating in various tissues in the head, including the brain. The peaks are inverted because proton-decoupling results in a negative nuclear Overhauser effect for these ^{15}N nuclei. (b) Cerebral $[5\text{-}^{15}\text{N}]$ glutamine peaks observed *in vivo* before ($t = -0.25$ h) and after $^{15}\text{NH}_4^+$ infusion was replaced by $^{14}\text{NH}_4^+$ at $t = 0$. Each spectrum was acquired in 30 min.

(2.9 ± 0.4 $\mu\text{mol/g}$; $n = 5$) previously observed at the end of 3.7 h of $^{15}\text{NH}_4^+$ infusion [40]. The cerebral glutamate concentration was 9.3 ± 0.3 $\mu\text{mol/g}$ ($n = 4$) before and 9.4 ± 0.8 $\mu\text{mol/g}$ ($n = 3$) after the chase. This represents a slight decrease from the pre-infusion glutamate level, which was 10.2 ± 0.8 $\mu\text{mol/g}$ ($n = 5$). The cerebral glutamine concentration ($^{15}\text{N} + ^{14}\text{N}$) at the end of the chase was 14.4 ± 1.5 $\mu\text{mol/g}$ of brain ($n = 4$), also in close agreement with the value (14.4 ± 1.3 $\mu\text{mol/g}$; $n = 5$) previously observed at the end of $^{15}\text{NH}_4^+$ infusion [40]. The blood ammonia concentration during the chase was 0.61 ± 0.015 $\mu\text{mol/g}$ ($n = 2$), also in accord with that measured at the end of $^{15}\text{NH}_4^+$ infusion (0.8 mM; 0.64 $\mu\text{mol/g}$) [40]. The results show that cerebral ammonia, glutamate and glutamine, as well as blood ammonia, were at steady state from the end of 3.5 h of $^{15}\text{NH}_4^+$ infusion and throughout the 5.1 h chase period.

Figure 2 Decrease in the cerebral $[5\text{-}^{15}\text{N}]$ glutamine concentration during chase by $^{14}\text{NH}_4^+$

After 3.5 h of $^{15}\text{NH}_4^+$ infusion, chase by ^{14}N was started at $t = 0$. The cerebral $[^{15}\text{N}]$ glutamine concentration ($\mu\text{mol/g}$ of brain) was calculated from the observed *in vivo* peak intensity for each rat as described in the Experimental section. Each data point is the mean $\pm$ S.E.M. for five rats. The best-fit exponential curve for the entire chase period was $B^* = 5.5 e^{-0.34t}$ ($r = 0.98$).

Figure 1(a) shows an *in vivo* ^{15}N n.m.r. spectrum obtained from the head of a living rat after 3.5 h of $^{15}\text{NH}_4^+$ infusion. We have previously demonstrated that the peaks for $[5\text{-}^{15}\text{N}]$ glutamine (-271 p.p.m.) and $[2\text{-}^{15}\text{N}]$ glutamate/glutamine (-342 p.p.m.) arise exclusively from the brain [40]. Figure 1(b) shows the changes in cerebral $[5\text{-}^{15}\text{N}]$ glutamine peak intensity observed *in vivo* when the chase by $^{14}\text{NH}_4^+$ was started at $t = 0$. The top trace shows the cerebral $[5\text{-}^{15}\text{N}]$ glutamine peak observed 15 min before the start of the chase ($t = -0.25$ h), and the subsequent decrease in peak intensity was measured during the chase for 4.5 h. Figure 2 shows the change in cerebral $[5\text{-}^{15}\text{N}]$ glutamine concentration ($\mu\text{mol/g}$ of brain), calculated from the *in vivo* peak intensity, and averaged for five rats. The $[5\text{-}^{15}\text{N}]$ glutamine concentration (B^*) decreased exponentially with time (see Figure 2 legend). Because B^* decays exponentially when $s_A \ll s_B$ in eqn. (3), the result suggests that ^{15}N enrichment of the precursor ammonia (s_A) was much lower than that of 5-N of cerebral glutamine (s_B) during most of the chase period. To estimate s_A , ^{15}N enrichments of blood and brain ammonia were measured. Because GS is most abundant in pericapillary regions of astrocytes [2], which are interposed between cerebral capillaries and neurons [49], and thus acts as metabolic trap for blood-borne ammonia diffusing into the brain [6], it is reasonable to assume that ^{15}N enrichment of ammonia at the site of GS is similar to that of blood ammonia during infusion. After 1.5 h of chase the ^{15}N enrichment of blood ammonia was 0.077 ± 0.017 ($n = 2$), as determined by m.s. (Table 1).

At steady-state concentrations of ammonia and glutamine, the rate of glutamine synthesis is equal to the rate of glutamine utilization and is given by eqn. (3) (see the Experimental section). Observed values at $t = 1.5$ h were $s_A = 0.077 \pm 0.017$, $B^* = 3.4 \pm 0.1$ $\mu\text{mol/g}$ (Figure 2), and $B = 14.4 \pm 1.3$ $\mu\text{mol/g}$; hence $s_B = B^*/B = 0.24 \pm 0.02$. The value of dB^*/dt was obtained from the best-fit exponential curve for the period $t = 1.5\text{--}5.1$ h, when s_A is known to have decreased to < 0.077 . This was $B^* = 3.7 e^{-0.23t}$; hence $\text{dB}^*/\text{dt} = -0.23 \times B^* = -0.782$ $\mu\text{mol/h}$ per g of brain. Substituting these values into eqn. (3), we obtain $k_1 = k_2 = 4.8 \pm 1.1$ $\mu\text{mol/h}$ per g of brain for the rates of glutamine synthesis and utilization at steady state.

Figure 3 ^{15}N n.m.r. spectra of brain extracts of rats given 3.5 h of $^{15}\text{NH}_4^+$ infusion followed by 5.1 h of $^{14}\text{NH}_4^+$ infusion

(a) At the end of the $^{15}\text{NH}_4^+$ infusion; (b) after a 1.5 h chase by ^{14}N ; (c) at the end of the 5.1 h chase.

^{15}N enrichment of total brain ammonia at 1.5 h after the start of the chase was 0.18 ± 0.02 ($n = 2$), which is 2.3-fold higher than that of blood ammonia. This is probably due to residual $^{15}\text{NH}_4^+$ which escaped the action of GS during $^{15}\text{NH}_4^+$ infusion and diffused into neurons, where ammonia removal is expected to be slow because GDH, the only ammonia-assimilating enzyme present in neurons, has low affinity for ammonia (K_m of 10–18 mM [50,51]). Endogenous $^{15}\text{NH}_4^+$ production from $[5-^{15}\text{N}]\text{glutamine}$ by glutaminase and from $[^{15}\text{N}]\text{glutamate}$ [present at $2.8 \pm 0.4 \mu\text{mol/g}$ ($n = 3$) at the end of the $^{15}\text{NH}_4^+$ infusion] by GDH-catalysed oxidative deamination may also have contributed to the $^{15}\text{NH}_4^+$ pool. Glutaminase is predominantly a neuronal enzyme [52,53]. Glutamate dehydrogenase is present in both glia and neurons, but oxidative deamination of glutamate is thought to be more rapid in the neuronal compartment, where the glutamate concentration is substantially higher than in glia [54]. These considerations suggest that both unmetabolized

residual $^{15}\text{NH}_4^+$ and endogenous $^{15}\text{NH}_4^+$ produced during the chase were present mainly in the neuronal compartment. Thus ^{15}N enrichment of ammonia measured for the whole brain may reflect mainly that of the larger neuronal compartment, and could be an overestimate for glial ammonia at the site of GS where blood-borne ammonia diffusing across the blood–brain barrier is likely to predominate. However, if we tentatively assume that ^{15}N enrichment of ammonia at the site of GS is equal to that measured for the whole brain, we obtain, by substituting $s_A = 0.18 \pm 0.02$, $s_B = 0.24 \pm 0.02$ and $\text{d}B^*/\text{d}t = -0.782 \mu\text{mol/h}$ per g into eqn. (3), $k_1 = k_2 = 13.0 \pm 3.9 \mu\text{mol/h}$ per g of brain as the maximum rate of glutamine synthesis and utilization under these experimental conditions.

Flux of ^{15}N through $\alpha\text{-N}$ of glutamate and glutamine at steady state

Figure 3 shows representative ^{15}N spectra of brain extracts prepared from rats after 3.5 h of $^{15}\text{NH}_4^+$ infusion (Figure 3a), after 1.5 h of ^{14}N chase (Figure 3b) and at the end of a 5.1 h chase (Figure 3c). Comparison of the peak intensity of $[2-^{15}\text{N}]\text{-glutamate/glutamine}$ (–342 p.p.m.) with that of $[5-^{15}\text{N}]\text{-glutamine}$ (–271 p.p.m.) shows that the $[2-^{15}\text{N}]/[5-^{15}\text{N}]$ peak intensity ratio changed progressively with time. The mean $[2-^{15}\text{N}]/[5-^{15}\text{N}]$ peak intensity ratio was 0.53 ± 0.05 ($n = 4$) before and 1.65 ± 0.23 ($n = 4$) after the 5.1 h chase. The 3.1-fold change in the peak intensity ratio shows that the flux of ^{15}N through 2-N was 3.1 times slower than that through 5-N at steady state. The flux through 2-N is therefore estimated to be 1.5 or $4.2 \mu\text{mol/h}$ per g. This flux represents the rate of turnover of glutamate/glutamine 2-N due to GDH and transaminase reactions. The slow flux is probably due to the low affinity of GDH for ammonia [50,51] and some recycling of ^{15}N between glutamate and other amino acids such as aspartate and alanine as it is chased by ^{14}N through the reversible transaminase reactions.

Decrease in brain ammonia and glutamine during post-infusion recovery

In this series of experiments, rats were given $^{15}\text{NH}_4^+$ infusion at the rate of 4.8–5.4 mmol/h per kg body wt. for 2.1 h (Group I; $n = 3$) or for 3.5 h (Group II; $n = 6$). After infusion was terminated without chase by ^{14}N , changes in the concentrations of blood ammonia, cerebral ammonia, $[5-^{15}\text{N}]\text{glutamine}$ and total glutamine ($^{14}\text{N} + ^{15}\text{N}$) were measured. As shown in Figure 4(a), the blood ammonia concentration decreased rapidly from $0.61 \pm 0.015 \mu\text{mol/g}$ to $0.12 \mu\text{mol/g}$ ($n = 2$) in 1 h, and was at the physiological level of $0.06 \pm 0.01 \mu\text{mol/g}$ by 1.5 h. ^{15}N enrichment of blood ammonia was 0.08 at 2 h (Group I) and at 3.2 h (Group II). The brain ammonia concentration, also shown in Figure 4(a), decreased slowly from $0.9 \pm 0.1 \mu\text{mol/g}$ (Group I) or $2.9 \pm 0.4 \mu\text{mol/g}$ (Group II) to $0.65 \pm 0.2 \mu\text{mol/g}$ in 2 h. ^{15}N enrichment of brain ammonia was 0.29 at 2 h (Group I) and 0.16 at 3.2 h (Group II). Brain ammonia was probably partly assimilated by GDH in the neuronal compartment, and partly by GS after diffusing into astrocytes when influx of blood-borne ammonia decreased. The slow rates of removal of brain ammonia and of the decrease in its ^{15}N enrichment are consistent with the observed slow flux of nitrogen through the GDH pathway and the relatively low activity of GS *in vivo*.

The progressive decreases in cerebral $[5-^{15}\text{N}]\text{glutamine}$ and total ($^{14}\text{N} + ^{15}\text{N}$) glutamine are shown in Figures 4(b) (Group I) and 4(c) (Group II). The $[5-^{15}\text{N}]\text{glutamine}$ pool decreased more rapidly than the total glutamine pool, as expected when newly

Figure 4 Decrease in blood and brain ammonia and brain glutamine concentrations during post-infusion recovery

Rats were given an ammonia infusion at the rate of 4.8–5.4 mmol/h per kg body wt. for 2 h (Group I) or for 3.5 h (Group II). After infusion was terminated without chase ($t = 0$), changes in metabolite concentrations were measured. (a) Blood ammonia ($\blacktriangle$) and brain ammonia ($\blacksquare$, Group I; $\square$, Group II). (b) and (c) Cerebral glutamine ($^{14}\text{N} + ^{15}\text{N}$) ($\circ$) and [^{15}N]glutamine ($\bullet$) for Group I (b) and for Group II (c). For cerebral [^{15}N]glutamine concentrations, measured *in vivo* by n.m.r., individual data from 3–5 rats are shown. For total [$^{14}\text{N} + ^{15}\text{N}$]glutamine concentrations assayed enzymically in brain extracts, the values are means $\pm$ S.E.M. for 2–5 rats.

synthesized [^{14}N]glutamine enters the pool for glutamine utilization pathways. The total glutamine pool decreased at an average rate of 1.1 (Group I) or 1.4 (Group II) $\mu\text{mol/h}$ per g. The observed rate is in accordance with the estimated rate of glutamine utilization used in our previous calculation of GS activity, as discussed below.

DISCUSSION

GS activity *in vivo*

This work demonstrates that the rate of glutamine synthesis and utilization at steady-state concentrations of brain ammonia, glutamate and glutamine in the hyperammonaemic rat, measured *in vivo* by ^{15}N n.m.r., is $4.8 \pm 1.1 \mu\text{mol/h}$ per g if ^{15}N enrichment of precursor ammonia at the site of GS is equal to that of blood ammonia, and $13.0 \pm 3.9 \mu\text{mol/h}$ per g if it is equal to that of total brain ammonia. The actual rate is probably closer to the lower estimate because astrocytes are anatomically positioned to act as metabolic traps for blood-borne ammonia, with their endfeet (where GS is most abundant [2]) ensheathing the cerebral capillaries and their distal processes abutting into neurons [49].

The rate of glutamine synthesis *in vivo* of $4.8 \pm 1.1 \mu\text{mol/h}$ per g at steady state is in reasonable agreement with the rate of synthesis of $3.5 \mu\text{mol/h}$ per g estimated in our previous study from the observation of an increase in the cerebral glutamine pool before it reached a steady state. In that study, the rate of synthesis was estimated from the observed rate of increase of the glutamine pool during ammonia infusion ($2.6 \mu\text{mol/h}$ per g) and a rate of glutamine utilization ($0.96 \mu\text{mol/h}$ per g) calculated from the reported decrease in the glutamine pool [41] on termination of the ammonia infusion. We have now measured the rate of decrease of the total glutamine pool for 5 h after the end of infusion. The average rate, $1.4 \mu\text{mol/h}$ per g, is reasonably close to that observed by Mans et al. [41] and used in our earlier calculation. It is interesting to note that the time required for cerebral glutamine to reach steady state (3–4 h) during continuous ammonia infusion and the rate of decrease of cerebral glutamine after the end of infusion were both quite similar in the anaesthetized rats studied here and in awake unrestrained rats given long-term ammonia infusion at a very similar rate (5 mmol/h per kg body wt.) to study its effect on amino acid transport [41]. This result suggests that the pentobarbital anaesthesia used in our study had little or no effect on the rates of glutamine synthesis and utilization *in vivo*.

It is important to consider whether glutamine efflux from the brain contributed to the observed decrease in the cerebral [^{15}N]glutamine concentration during the chase period. Dejong et al. [55] reported that, in portacaval-shunted rats after 4 h of hepatic artery ligation, the blood ammonia concentration increased to 1.0 mM and the brain glutamine concentration to 14–17 $\mu\text{mol/g}$, but net glutamine flux across the blood–brain barrier between the circulation and the cerebral cortex (measured in nmol/min from the plasma flow rate and venous–arterial concentration difference) was not significantly different from zero. Because the hyperammonaemic rats in our study had comparable or lower blood ammonia (0.8 mM) and brain glutamine (14.4 $\mu\text{mol/g}$) concentrations, it is reasonable to assume that glutamine release from the brain was negligibly small and that the observed decrease of [^{15}N]glutamine during the chase reflects efflux of ^{15}N into glutamine-utilizing pathways within the brain.

The rate of glutamine synthesis reported here was calculated on the assumption that, if [^{15}N]glutamine is hydrolysed by glutaminase, recycling of the released $^{15}\text{NH}_4^+$ to the precursor ammonia pool for GS is negligibly small during the chase. The following considerations suggest that this assumption is reasonable under our experimental conditions. Glutaminase and GS are spatially separated at the subcellular and cellular level: glutaminase is mitochondrial [56], while GS is cytosolic [2]. Biochemical [52] and immunocytochemical [53] studies have shown that glutaminase is predominantly a neuronal enzyme, concentrated mainly in the nerve terminals of glutamatergic

pathways, while GS is localized exclusively in glia [2,3]. Glutaminase activity is strongly regulated by glutamate and ammonia; in the presence of 1 mM glutamate and 1 mM ammonia, the activity is inhibited by 92 % [57]. Therefore, at a brain ammonia concentration of 2.9 $\mu\text{mol/g}$, glutaminase activity, if present, is likely to be very low. Furthermore, during chase by $^{14}\text{NH}_4^+$, any neuronal $^{15}\text{NH}_4^+$ recycled to glia is likely to be extensively diluted by blood-borne ammonia diffusing into astrocytes. Therefore the rate of glutamine synthesis and utilization calculated on the assumption that $^{15}\text{NH}_4^+$ recycling from glutaminase to GS is negligibly small under hyperammonaemic conditions is expected to be a reasonably accurate estimate. At a physiological concentration of brain ammonia, glutaminase activity may be higher, and ammonia recycling may need to be taken into account.

The observed GS activity *in vivo* represents the rate of ammonia removal from the glial compartment under our experimental conditions. At an ammonia infusion rate of 4.8 ± 0.3 mmol/h per kg body wt. and an ammonia removal rate of $4.8\text{--}13.0$ $\mu\text{mol/h}$ per g in the brain, the anaesthetized rat showed occasional myoclonus and hyperventilation after 2 h of infusion, but was otherwise in good physiological condition for at least 8 h. Mans et al. [41] report that awake unrestrained rats tolerated ammonia infusion at a rate of 5 mmol/h per kg body wt. for 4 h followed by infusion at a rate of 2.5 mmol/h per kg for an additional 17 h. Dejong et al. [55] report that portacaval-shunted rats with acute liver ischaemia developed ataxia and loss of righting reflex characteristic of HE, but did not become comatose when blood and brain ammonia concentrations were 1.5 mM and 2.5 $\mu\text{mol/g}$ respectively. Taken together, the results suggest that at the observed ammonia removal rate of $4.8\text{--}13.0$ $\mu\text{mol/h}$ per g in the brain, mild symptoms of HE (stages I–III as defined by Norenberg [58]) occur, but coma (stage IV) can be avoided. To evaluate the hypothesis that ammonia is in itself neurotoxic, it will be informative to use these non-invasive techniques to examine a possible correlation of the *in vivo* rate of ammonia removal with the onset of stages I–IV of HE in hyperammonaemic rats.

Regulation of GS activity *in vivo*

The *in vivo* GS activity in the brain of hyperammonaemic rats reported here is 2–5 % of the optimum *in vitro* GS activity in rat brain homogenates measured at enzyme-saturating concentrations of all substrates and cofactors (275–350 $\mu\text{mol/h}$ per g) [20,21]. This suggests that the rate of glutamine synthesis is strongly regulated in the intact brain. Major factors regulating the rate can be inferred from the observed *in vivo* activity, the kinetic and regulatory properties of purified GS [59–61] and immunocytochemical data on *in situ* concentrations of some substrates and potential regulators in the relevant brain compartments [54]. Novel findings reported within the last year or two in these areas permit a clearer understanding of factors likely to be controlling the rate *in vivo*, as discussed below.

The kinetic properties of rat brain GS can reasonably be assumed to be virtually identical to those described for purified rat liver GS [59–61], as the nucleotide sequences of GS genes cloned from a rat liver cDNA library [62] and from a rat brain cDNA library [63] show that the amino acid sequences of the liver and brain GS are identical except for possible substitution of Arg (brain) for His (liver) at position 128. The possibility that the substitution, if it is real and occurs at the active site, affects the enzyme kinetics cannot be ruled out. However, the apparent discrepancy may have arisen in the cloning process [63], since an earlier determination of amino acid composition showed that the

rat brain and liver GS enzymes contain the same number of His residues per subunit [60]. A report that a single gene codes for the brain and liver GS in mouse with His at position 128 and showing 93 % identity in amino acid sequence to rat GS [64] suggests that the assumption of identical brain and liver GS enzymes in the rat is reasonable. The rat GS (liver) has a K_m for ammonia of 0.3 mM [61]. Therefore GS is likely to be saturated with ammonia under our experimental conditions. In cultured astrocytes, the rate of glutamine synthesis at an apparently enzyme-saturating concentration of ammonia (> 0.2 mM) was 29 % of the *in vitro* GS activity measured in the same culture for rat brain astrocytes [26] and 7–10 % for mouse brain astrocytes [23,24]. Taken together, the results suggest that substrates and/or cofactors other than ammonia kinetically limit GS activity in both intact brain and cultured astrocytes.

The K_m of GS for glutamate increases sharply as the concentration of metal ion cofactor is reduced, and is 5 mM at 10 mM Mg^{2+} or 2 mM Mn^{2+} [61]. Recent immunohistochemical evidence shows marked heterogeneity in the spatial distribution of glutamate and glutamine in rat cerebellum; the glutamate/glutamine ratio was estimated to be 0.2 in the glial compartment and 2–4.5 in excitatory nerve terminals [54]. Therefore the glial glutamate concentration may be as low as 3 ± 1 mM and may thus limit GS activity *in vivo*. This effect is likely to be more pronounced in hyperammonaemic rats, whose whole-brain glutamate concentration was slightly lower than in controls, a result that is consistent with previous reports [55,65]. The rate of glutamine synthesis reported here thus represents the rate that occurs *in vivo* at an ambient steady-state glial glutamate concentration.

GS has a K_m for ATP of 2.3 mM [60] and requires Mg^{2+} or Mn^{2+} as a cofactor. The ATP concentration in the whole rat brain is 2.5 mM [66], with no change observed under hyperammonaemia *in vivo* [31], while the concentration of free Mg^{2+} is 0.7 mM [67]. At 2.5 mM ATP and 1 mM Mg^{2+} (concentrations close to those in rat brain), the GS activity is only 5 % of that at 10 mM ATP and 10 mM Mg^{2+} (concentrations close to those used in *in vitro* assays) [68]. These considerations suggest that the *in vivo* GS activity is also limited by *in situ* concentrations of ATP and the metal ion cofactor. Glutamine inhibits GS only with Mn^{2+} (not Mg^{2+}) as cofactor [60,61]. Therefore glutamine is a potential inhibitor only if rat brain GS is an Mn^{2+} enzyme, as has been claimed for the ovine brain GS [68]. Thus partial inhibition of *in vivo* activity at high glial glutamine concentrations remains a possibility.

Studies of brain tissue preparations have provided increasing evidence that the synthesis of glutamine by astrocytes is required to maintain the neuronal transmitter supply of glutamate and γ -aminobutyric acid [8,11,12]. While immunocytochemical studies have mapped the spatial distribution of metabolites [54] and enzymes [2,3,53] involved in the glutamine/glutamate cycle on the cellular level, n.m.r. can contribute to a better understanding of the dynamic aspects of the cycle through measurement of the rate of glutamine synthesis as it occurs *in vivo*. A promising method for measurement of GS activity *in vivo* at physiological concentrations of brain ammonia and glutamine is the sensitive indirect detection of this metabolite through amide protons by $^1\text{H}\text{--}^{15}\text{N}$ heteronuclear multiple quantum coherence (HMQC) n.m.r. This method permitted us to observe cerebral $[5\text{--}^{15}\text{N}]\text{--}$ glutamine in isolated rat brain in 2 min of acquisition time [69]. Development of instrumentation for *in vivo* studies using $^1\text{H}\text{--}^{15}\text{N}$ HMQC is in progress.

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