***In vivo* activity of glutaminase in the brain of hyperammonaemic rats measured by ¹⁵N nuclear magnetic resonance**

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The *in vivo* activity of phosphate-activated glutaminase (PAG) was measured in the brain of hyperammonaemic rat by ¹⁵N n.m.r. Brain glutamine was ¹⁵N-enriched by intravenous infusion of ¹⁵NH₄⁺ until the concentration of [5-¹⁵N]glutamine reached 6.1 μmol/g. Further glutamine synthesis was inhibited by intraperitoneal injection of methionine-DL-sulphoximine, an inhibitor of glutamine synthetase, and the infusate was changed to ¹⁴NH₄⁺ during observation of decrease in brain [5-¹⁵N]glutamine due to PAG and other glutamine utilization pathways. Progressive decrease in brain [5-¹⁵N]glutamine, PAG-catalysed production of ¹⁵NH₄⁺ and its subsequent assimilation into glutamate by glutamate dehydrogenase were monitored *in vivo* by ¹⁵N n.m.r. Brain [5-¹⁵N]glutamine (¹⁵N enrichment of 0.35–0.50) decreased at a rate of 1.2 μmol/h per g of brain. The *in vivo* PAG activity, determined from the observed rate and the quantity of ¹⁵NH₄⁺

produced and subsequently assimilated into glutamate and aspartate, was 0.9–1.3 μmol/h per g. This activity is less than 1.1 % of the reported activity *in vitro* measured in rat brain homogenate at a 10 mM concentration of the activator P_i. Inhibition by ammonia (brain level 1.4 μmol/g) alone does not account for the observed low activity *in vivo*. The result strongly suggests that, in intact brain, PAG activity is maintained at a low level by a suboptimal *in situ* concentration of P_i and the strong inhibitory effect of glutamate. The observed PAG activity *in vivo* is lower than the reported *in vivo* activity of glutamate decarboxylase which converts glutamate into γ-aminobutyrate (GABA). The result suggests that PAG-catalysed hydrolysis of glutamine is not the sole provider of glutamate used for GABA synthesis.

INTRODUCTION

Phosphate-activated glutaminase (PAG) catalyses the following reaction:

PAG (glutaminase I), localized on the inner mitochondrial membrane, is predominantly a neuronal enzyme, particularly enriched in glutamatergic nerve terminals [1,2]. Kinetic and regulatory properties of rat brain PAG have been extensively studied with the purified enzyme [3] and in brain homogenates and slices [4–7]. However, PAG activity *in vivo* in intact brain has not been reported. Measurement of the *in vivo* rate, when combined with the known kinetic and regulatory properties of purified PAG and immunocytochemical and biochemical evidence on the *in situ* concentrations of the substrate and regulators, will contribute to a better understanding of the factors that regulate the activity *in vivo*.

Regulation of PAG activity in intact brain has attracted considerable interest because PAG may play an important role in providing the neurotransmitter pools of glutamate (Glu) [1,8–10] and of γ-aminobutyrate (GABA) [10–13]. Studies with brain slices and intact animals using radioisotope-labelled precursors [8–11] or inhibitors of glutamine synthetase (GS) and of GABA transaminase [12,13] strongly suggested that glutamine (Gln) is an important, though by no means the sole, precursor of the neurotransmitter pools of glutamate, and of GABA which is formed from Glu by glutamate decarboxylase (GAD). On the other hand, a recent immunocytochemical study reports zero to low levels of detectable PAG immunoreactivity in many GABAergic neurons, suggesting that Glu for GABA synthesis is supplied by pathways other than PAG [2]. Thus, the role of PAG

in supplying Glu in intact brain and the quantitative importance of Gln as precursor of Glu and GABA remain to be clarified.

If Gln is indeed a major precursor of the neurotransmitter pools of Glu and GABA, the rate of Gln hydrolysis in the brain must be rapid enough to support GABA synthesis. Therefore, a new approach to studying this question is to measure PAG activity in intact brain for comparison with the GAD activity.

N.m.r. is a useful technique for non-invasive monitoring of metabolite fluxes *in vivo* in animals and man [14]. Our previous study showed that the rate of Gln synthesis and utilization at steady-state concentrations of ammonia, Glu and Gln in the brain of hyperammonaemic rats can be measured *in vivo* by ¹⁵N n.m.r. [15]. The total rate of Gln utilization in the brain includes (a) the rates of PAG (glutaminase I) and glutaminase II (glutamine aminotransferase + ω amidase) reactions; (b) the rates of anabolic amidotransferase reactions in which Gln 5-N is transferred to purine and pyrimidine nitrogens with concomitant release of Glu, and (c) the rate of Gln incorporation into proteins. Among these reactions, only the glutaminase reactions release free ammonia. In rat brain, PAG is the predominant glutaminase pathway, with glutaminase II playing a very minor role [16,17] (also see Discussion). ¹⁵N n.m.r. is useful for measuring the rate of PAG reaction in intact brain because changes in brain [5-¹⁵N]Gln concentration, as well as glutaminase-catalysed production of ¹⁵NH₄⁺, can be monitored *in vivo*. We report here measurement of *in vivo* PAG activity in the brain of the hyperammonaemic rat by ¹⁵N n.m.r. In addition, a new sensitive *in vitro* assay for ¹⁵NH₄⁺ by ¹H–¹⁵N heteronuclear multiple quantum coherence (HMQC)-transfer n.m.r. is described. Implications of the result for regulation of the activity *in vivo* and the role of Gln as a precursor of Glu for GABA synthesis are discussed.

Abbreviations used: PAG, phosphate-activated glutaminase; GS, glutamine synthetase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GABA, γ-aminobutyrate; MSO, methionine-DL-sulphoximine; HMQC, heteronuclear multiple quantum coherence.

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EXPERIMENTAL

Animal preparation and ammonia infusion

Male Wistar rats, weighing 360 ± 20 g and fasted overnight, 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 left femoral vein, as described in detail previously [18]. For ammonia infusion, $^{15}\text{NH}_4\text{Cl}$ (99% enriched in ^{15}N) in 1 M aqueous solution, or $^{14}\text{NH}_4\text{Cl}$ (0.35 M), at pH 7.4, were used. The rates and duration of $^{15}\text{NH}_4^+$ infusion to enrich brain Gln, followed by $^{14}\text{NH}_4^+$ infusion during observation of PAG-catalysed hydrolysis of $[5\text{-}^{15}\text{N}]\text{Gln}$, are shown schematically in Figure 1. Rationale for the infusion protocol is described in the Results section. When brain Gln was sufficiently ^{15}N -enriched, methionine-DL-sulphoximine (MSO), an inhibitor of GS, was injected intraperitoneally (150 mg/kg body wt.) through a length of polyethylene tubing (PE50, Clay Adams), to inhibit further Gln synthesis.

In vivo ^{15}N 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 [19]. The probe consisted of a solenoidal coil tunable to 20.27 MHz for ^{15}N observation, and a Helmholtz coil tunable to 200 MHz for ^1H decoupling and shimming. The rat was placed in the magnet with its head inside the observe coil in a specifically designed head holder to ensure immobility. After shimming on water protons, ^{15}N acquisition was started simultaneously with $^{15}\text{NH}_4\text{Cl}$ infusion.

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 the relaxation delay. In preliminary experiments with $^{15}\text{NH}_4^+$ -infused rats, these conditions optimized the signal-to-noise ratio for cerebral $[5\text{-}^{15}\text{N}]\text{Gln}$ and $^{15}\text{NH}_4^+$. ^{15}N chemical shifts are reported in p.p.m. from nitromethane with the negative sign indicating an upfield shift. ^{15}N spectra were acquired every 27.5 min to monitor changes in brain $[5\text{-}^{15}\text{N}]\text{Gln}$. For observation and quantification of brain $^{15}\text{NH}_4^+$ and $[^{15}\text{N}]\text{Glu}$, spectra acquired for 55 min were used. For quantification of cerebral $[5\text{-}^{15}\text{N}]\text{Gln}$, the observed *in vivo* peak intensity was converted into the $[5\text{-}^{15}\text{N}]\text{Gln}$ concentration ($\mu\text{mol/g}$) in the

brain by using the mean *in vivo* peak intensity per μmol of cerebral $[5\text{-}^{15}\text{N}]\text{Gln}$ per min of the acquisition. This conversion factor was obtained by calibrating the *in vivo* peak intensity with the actual quantity of $[5\text{-}^{15}\text{N}]\text{Gln}$ measured in an extract of the same brain for several rats, as described in detail previously [19]. For quantification of cerebral $[^{15}\text{N}]\text{Glu}$, the mean *in vivo* peak intensity per μmol of cerebral $[^{15}\text{N}]\text{Glu}$ per min of acquisition was determined by calibration of the *in vivo* peak intensity with the actual quantity of $[^{15}\text{N}]\text{Glu}$ found in the extract of the same brain for five MSO-treated rats.

At the end of the *in vivo* experiment (3.8 h after MSO injection), the rat was removed from the magnet and a blood sample was taken from the vena cava for analysis of blood ammonia. The rat was then killed and the brain frozen immediately in liquid nitrogen for preparation of brain extract (Group I rats; $n = 5$). Another group, Group II ($n = 5$), was given the same course of ammonia infusion but killed 2.4 h after MSO injection to quantify brain $^{15}\text{NH}_4^+$ observed *in vivo* by parallel *in vitro* analysis of brain extract.

In vitro assay for brain ammonia, Gln and Glu

Total ($^{14}\text{N} + ^{15}\text{N}$) concentrations of brain ammonia, Gln and Glu, and of blood ammonia were assayed in perchloric acid extracts by enzymic assay according to published procedures [20–22]. Assays were performed for rats in Groups I and II and for rats given ammonia infusion for 2.6 h and killed before MSO injection (Figure 1).

In vitro $^{15}\text{NH}_4^+$ assay by ^1H - ^{15}N HMQC-transfer n.m.r.

$^{15}\text{NH}_4^+$ in the brain extract of rats killed at $t = 2.4$ h (Group II) was quantified by selective observation of $^{15}\text{NH}_4^+$ protons by ^1H - ^{15}N HMQC-transfer n.m.r. This method [23], which permits selective observation of protons coupled to ^{15}N , is useful for detecting low-concentration ^{15}N -labelled metabolites in tissue extracts [24]. $^{15}\text{NH}_4^+$ in brain extract (2 ml sample volume) was examined at pH 3.4, where the rate of exchange of ammonium protons with water protons is slow. ^1H - ^{15}N HMQC spectra were obtained at 200 MHz for ^1H on the GE CSI-II spectrometer. The probe consisted of a solenoidal ^1H observe coil and a Helmholtz ^{15}N coil. ^1H and ^{15}N 90° pulses were $18\ \mu\text{s}$ and $300\ \mu\text{s}$ respectively at 100 W RF power. The pulse sequence for water-suppressed ^1H - ^{15}N HMQC with selection of zero and double quantum coherences by phase-cycling was

$\text{D1}-90^\circ(^1\text{H})-\text{D2}-90^\circ(^1\text{H})-\frac{1}{2}J_{\text{N-H}}-90^\circ(^{15}\text{N})-\text{D3}-90^\circ(^{15}\text{N})-\frac{1}{2}J_{\text{N-H}}-\text{acquire}$

Operating conditions employed a relaxation delay (D1) of 1000 ms and an acquisition time of 300 ms. The value of $\frac{1}{2}J_{\text{N-H}}$ was 6.85 ms. Water peak suppression was achieved by the $90^\circ(^1\text{H})-\text{D2}-90^\circ(^1\text{H})$ sequence with the ^1H transmitter frequency set at 906 Hz (Δf) downfield of the water peak for optimum water suppression and excitation of ammonium protons (^{15}N -coupled ^1H doublet), which resonate at 489 and 416 Hz [$\Delta f/2 + (J_{\text{N-H}})/2$] downfield of the water protons. D2, adjusted for optimum water suppression, was typically 0.52 ms. ^1H chemical shifts are reported in p.p.m. from 2,2-dimethyl-silapentane-5-sulphonic acid (DSS). The ^{15}N -coupled ^1H doublet of $^{15}\text{NH}_4^+$ was assigned by comparison of the observed chemical shift and ^{15}N - ^1H coupling constant with the published values [25,26]. For quantification of $^{15}\text{NH}_4^+$ from its observed ^1H peak intensity, ^1H peak intensity was measured for 200–1000 nmol of $^{15}\text{NH}_4\text{Cl}$ standard added to unlabelled brain extract at pH 3.4 to

Figure 1 Ammonia infusion protocol

provide the same environment as for the biologically ^{15}N -enriched brain extracts. The ^1H peak intensity (measured as the mean peak area of the ^{15}N -coupled ^1H doublet) of the $^{15}\text{NH}_4^+$ standards increased linearly with concentration. $^{15}\text{NH}_4^+$ in biologically ^{15}N -enriched brain extract was then quantified from its observed ^1H peak intensity by comparison with the ^1H peak intensities of the $^{15}\text{NH}_4^+$ standards acquired under identical n.m.r. operating conditions.

Stability of tissue Gln against chemical hydrolysis during perchloric acid extraction is well established [27]. All extracts were stored at -20°C until n.m.r. analysis. The stability of $[5\text{-}^{15}\text{N}]\text{Gln}$ in brain extract against chemical hydrolysis at pH 3.4 during the HMQC experiment was confirmed in a preliminary experiment as follows. ^{15}N peak area, which is proportional to the quantity of $[5\text{-}^{15}\text{N}]\text{Gln}$ in the sample, was measured by ^{15}N n.m.r. for $20\text{ }\mu\text{mol}$ of $[5\text{-}^{15}\text{N}]\text{Gln}$ added to brain extract at pH 7. The sample was then adjusted to pH 3.4 and kept at 20°C for 4 h, before measuring the peak area again. The peak areas agreed to within 0.6%, indicating that $[5\text{-}^{15}\text{N}]\text{Gln}$ was stable against chemical hydrolysis at pH 3.4 during the 1.4 h required for the HMQC experiment.

RESULTS

In vivo ^{15}N n.m.r.

Figure 1 shows the ammonia infusion protocol used to ^{15}N -enrich brain Gln and then monitor subsequent decrease in brain $[5\text{-}^{15}\text{N}]\text{Gln}$ due to PAG and other Gln-utilizing pathways. Figure 2(a) shows an *in vivo* ^{15}N -n.m.r. spectrum obtained from the head of a living rat after 1.1 h of $^{15}\text{NH}_4^+$ infusion at a rate of $4.5 \pm 0.1\text{ mmol/h}$ per kg body wt. $[5\text{-}^{15}\text{N}]\text{Gln}$ (-271 p.p.m.) formed by the GS pathway and $[^{15}\text{N}]\text{Glu}$ (-342 p.p.m.) by the glutamate dehydrogenase (GDH) pathway are observed. The peaks for these ^{15}N metabolites arise exclusively from the brain, as shown previously [19]. After 2.1 h of infusion (Figure 2b), brain $[5\text{-}^{15}\text{N}]\text{Gln}$ increased and brain $^{15}\text{NH}_4^+$ was just detectable at -362 p.p.m. The mean brain $[5\text{-}^{15}\text{N}]\text{Gln}$ concentration was $6.1 \pm 0.2\text{ }\mu\text{mol/g}$ ($n = 10$) after $2.6 \pm 0.2\text{ h}$ of $^{15}\text{NH}_4^+$ infusion. MSO was then injected to inhibit further Gln synthesis, and observation of decrease in brain $[5\text{-}^{15}\text{N}]\text{Gln}$, due to PAG and other Gln utilization pathways, was initiated. As shown in Figure 1, $^{15}\text{NH}_4^+$ infusion was continued for an additional 1 h but at a much lower rate of $0.85 \pm 0.06\text{ mmol/h}$ per kg to permit assimilation of residual brain $^{15}\text{NH}_4^+$ into Glu and Gln during progressive inhibition of GS, which peaks after 1–2 h [28,29]. Figure 2(c) shows an *in vivo* spectrum obtained at 0.6 h after MSO injection. [Each time period in the following description refers to the time after MSO injection (see Figure 1) and is centred at the middle of a 55-min acquisition period, unless indicated otherwise.] The $^{15}\text{NH}_4^+$ peak has disappeared, showing that residual brain $^{15}\text{NH}_4^+$ has been assimilated into Glu and Gln 5-N during this period. The infusate was then changed to $^{14}\text{NH}_4^+$ and infusion continued at the same rate for 2.8 h (Figure 1). $^{14}\text{NH}_4^+$ infusion was performed to maintain brain and blood ammonia concentrations close to steady state and to ensure that blood-borne ammonia diffusing into the brain thereafter is predominantly ^{14}N . Consequently, any brain $^{15}\text{NH}_4^+$ observed after assimilation of pre-existing $^{15}\text{NH}_4^+$ and onset of $^{14}\text{NH}_4^+$ infusion can reasonably be attributed to $^{15}\text{NH}_4^+$ released from ^{15}N -labelled brain metabolites. The *in vivo* spectrum obtained at 1.5 h (Figure 2d) shows decreased $[5\text{-}^{15}\text{N}]\text{Gln}$ but no $^{15}\text{NH}_4^+$. At 2.4 h (Figure 2e), a further decrease in brain $[5\text{-}^{15}\text{N}]\text{Gln}$ was accompanied by detection of $^{15}\text{NH}_4^+$ at -362 p.p.m. This $^{15}\text{NH}_4^+$ can reasonably be assigned to $^{15}\text{NH}_4^+$ released from $[5\text{-}^{15}\text{N}]\text{Gln}$ by the PAG reaction, because after 1.4 h of $^{14}\text{NH}_4^+$ infusion, any

Figure 2 *In vivo* ^{15}N -n.m.r. spectra of rat brain

Spectra acquired from the head of a living rat. The peaks for $[5\text{-}^{15}\text{N}]\text{Gln}$ (-271 p.p.m.) and $[^{15}\text{N}]\text{Glu}$ (-342 p.p.m.) arise exclusively from the brain [19]. The peak at -306 p.p.m. is $[^{15}\text{N}]\text{urea}$ synthesized in the liver and circulating in the blood. The peaks are inverted because proton-decoupling results in a negative nuclear Overhauser effect for these ^{15}N nuclei. Each spectrum was acquired in 55 min after (a) 1.1 h and (b) 2.1 h of $^{15}\text{NH}_4^+$ infusion to enrich brain $[5\text{-}^{15}\text{N}]\text{Gln}$. When its level reached $6.1\text{ }\mu\text{mol/g}$, MSO was injected. Post-MSO *in vivo* spectra at 0.6 h (c) and 1.5 h (d) show progressive decrease in brain $[5\text{-}^{15}\text{N}]\text{Gln}$. At 2.4 h (e), $^{15}\text{NH}_4^+$ released by PAG-catalysed hydrolysis of $[5\text{-}^{15}\text{N}]\text{Gln}$ is observed. At 3.3 h (f), $[^{15}\text{N}]\text{Glu}$ level increases due to assimilation of $^{15}\text{NH}_4^+$. (d'), (e') and (f') show corresponding spectra from a second rat, demonstrating reproducibility of the result ($n = 5$).

blood-borne ammonia diffusing into the brain is predominantly ^{14}N , and $[5\text{-}^{15}\text{N}]\text{Gln}$ is the only ^{15}N -labelled metabolite which showed substantial decrease in the preceding period. At 3.3 h (Figure 2f), $^{15}\text{NH}_4^+$ was no longer detectable, but a substantial increase in the peak intensity of $[^{15}\text{N}]\text{Glu}$ (-342 p.p.m.) was observed. This suggests that the $^{15}\text{NH}_4^+$ produced by the PAG-catalysed hydrolysis of $[5\text{-}^{15}\text{N}]\text{Gln}$ was assimilated into Glu by GDH. GDH, localized in mitochondrial matrix like PAG, is the only ammonia-assimilating pathway in the brain when GS is inhibited by MSO. Figure 3(a) shows the metabolic pathways responsible for the observed transfer of ^{15}N . To check reproducibility of the result, *in vivo* ^{15}N spectra were obtained for five rats. In all rats, brain $^{15}\text{NH}_4^+$ was absent at 1.5 h after MSO

Figure 3 PAG-catalysed hydrolysis of $[5-^{15}\text{N}]\text{glutamine}$, release of $^{15}\text{NH}_4^+$ and its assimilation into Glu

(a) Metabolic pathways (b) Progressive decrease in brain $[5-^{15}\text{N}]\text{Gln}$ concentration ($\mu\text{mol/g}$) during the PAG-catalysed hydrolysis, shown as the mean $\pm$ S.E.M. for five rats. Brain $[5-^{15}\text{N}]\text{Gln}$ concentration was determined from the observed *in vivo* ^{15}N peak intensity as described in the text. From the slope of the least-squares line through the plot ($r = 0.99$), the rate of decrease was determined to be $1.2 \mu\text{mol/h per g}$. (c) Gln-derived $^{15}\text{NH}_4^+$ ($\diamond$) and increase in brain $[^{15}\text{N}]\text{Glu}$ ($\blacklozenge$) due to $^{15}\text{NH}_4^+$ assimilation ($n = 5$). $^{15}\text{NH}_4^+ + [^{15}\text{N}]\text{Glu}$ is shown by $\circ$. The error bar shows $\pm$ S.E.M.

treatment, but was detected at 2.4 ± 0.12 h, followed by a substantial increase in $[^{15}\text{N}]\text{Glu}$ at 3.3 h. The spectra (Figures 2d', 2e' and 2f') obtained from a second rat demonstrate the reproducibility of the result.

Brain $[5-^{15}\text{N}]\text{Gln}$, $^{15}\text{NH}_4^+$ and $[^{15}\text{N}]\text{Glu}$ -quantification and concentration changes

Figure 3 shows changes in cerebral concentrations of $[5-^{15}\text{N}]\text{Gln}$ (Figure 3b) and $[^{15}\text{N}]\text{Glu}$ (Figure 3c), determined from the observed *in vivo* ^{15}N peak intensities by the quantification method described in the Experimental section and expressed as the mean $\pm$ S.E.M. for five rats. For accurate quantification of brain $^{15}\text{NH}_4^+$ whose *in vivo* ^{15}N peak has a low signal-to-noise ratio, parallel experiments were performed in which five rats, given the same course of ammonia infusion, were killed at 2.4 h after MSO injection when PAG-catalysed $^{15}\text{NH}_4^+$ release was observed in the *in vivo* ^{15}N spectra. Perchloric acid extracts of the brains were then analysed for $^{15}\text{NH}_4^+$ by the sensitive, selective observation of $^{15}\text{NH}_4^+$ protons by the ^1H - ^{15}N HMQC method (Experimental section). Figure 4 shows representative ^1H - ^{15}N HMQC spectra of $^{15}\text{NH}_4^+$ protons of $^{15}\text{NH}_4\text{Cl}$ standards (Figure 4a) and of brain

Figure 4 ^1H - ^{15}N -HMQC spectra of $^{15}\text{NH}_4^+$ in brain extracts

Each spectrum, acquired for 3840 scans (1.4 h), shows ^{15}N -coupled ^1H doublet of $^{15}\text{NH}_4^+$ protons (6.95 and 7.31 p.p.m.). The peak at 4.7 p.p.m. is residual water. Protons not coupled to ^{15}N are suppressed. (a) 400 nmol and 1000 nmol of $^{15}\text{NH}_4^+$ standards. Inset shows the ^1H peak intensity (measured as the mean peak area of the ^{15}N -coupled ^1H doublet) for 200–1000 nmol of the $^{15}\text{NH}_4^+$ standards. (b) Brain extract of a rat killed at 2.4 h after MSO injection. $^{15}\text{NH}_4^+$ released by PAG-catalysed hydrolysis of $[5-^{15}\text{N}]\text{Gln}$ *in vivo* is selectively observed in the extract at pH 3.4.

extracts of rats killed at 2.4 h after MSO injection (Figure 4b). The spectra were obtained at pH 3.4 where the rate of exchange of ammonium protons with water protons is low. The ^{15}N -coupled ^1H doublets of $^{15}\text{NH}_4^+$ protons at 6.95 and 7.31 p.p.m. ($^1J_{^{15}\text{N}-^1\text{H}}$ of 73 Hz) were selectively observed with suppression of non- ^{15}N -bound protons of other brain metabolites. The ^1H peak intensity of $^{15}\text{NH}_4^+$ standards increased linearly with concentration as shown in the inset to Figure 4(a). $^{15}\text{NH}_4^+$ concentration in the brain extracts of rats at 2.4 h, determined from the observed *in vivo* peak intensities by comparison with those of standards, was $0.46 \pm 0.08 \mu\text{mol/g}$ of brain ($n = 5$), as shown in Figure 3(c).

Rate of PAG reaction *in vivo*

Figure 3(b) shows that brain $[5-^{15}\text{N}]\text{Gln}$ decreased at a rate of $1.2 \mu\text{mol/h per g}$ of brain, with a total decrease of $4.1 \mu\text{mol/g}$

Figure 5 ^{15}N -n.m.r. spectra showing resolution of $2\text{-}^{15}\text{N}$ peaks of Gln, Glu and Asp

(a) Aqueous solution (pH 9.1) containing $[^{15}\text{N}]\text{Glu}$ ($10\text{ }\mu\text{mol}$; -343.6 p.p.m.), $[^{15}\text{N}]\text{Asp}$ ($7.5\text{ }\mu\text{mol}$; -344.3 p.p.m.) and $[2\text{-}^{15}\text{N}]\text{Gln}$ ($6.5\text{ }\mu\text{mol}$; -345.6 p.p.m.). The assignment was

over 3.6 h. This decrease is due to the combined effects of (a) $[5\text{-}^{15}\text{N}]\text{Gln}$ hydrolysis by glutaminase reactions; (b) anabolic amido-transferase reactions in which Gln N5 is transferred to asparagine N4, to N3 of pyrimidine and N3 and N9 of purines etc., with concomitant release of Glu; and (c) Gln incorporation into proteins. Among these pathways, only the glutaminase reactions (predominantly PAG) release free $^{15}\text{NH}_4^+$. Therefore the contribution of the PAG pathway to the observed $[5\text{-}^{15}\text{N}]\text{Gln}$ decrease can be estimated from the quantity of $^{15}\text{NH}_4^+$ released (Figures 2e and 3c) and subsequently assimilated into $[^{15}\text{N}]\text{Glu}$ by the GDH reaction (Figure 3a). As shown in Figure 3(c), at 2.4 h, free $^{15}\text{NH}_4^+$ in the brain was $0.46 \pm 0.08\text{ }\mu\text{mol/g}$ ($n = 5$) while $[^{15}\text{N}]\text{Glu}$ was $1.41 \pm 0.13\text{ }\mu\text{mol/g}$ ($n = 9$). By 3.3 h, $[^{15}\text{N}]\text{Glu}$ increased to $2.87 \pm 0.23\text{ }\mu\text{mol/g}$ ($n = 5$) as a result of $^{15}\text{NH}_4^+$ assimilation. This Glu level shows a net increase of $1.07 \pm 0.23\text{ }\mu\text{mol/g}$ over that observed at 0.6 h ($1.8\text{ }\mu\text{mol/g}$) before substantial decrease in brain $[5\text{-}^{15}\text{N}]\text{Gln}$ occurred. Taken together, the results strongly suggest that the minimum quantity of $^{15}\text{NH}_4^+$ released by the PAG reaction and subsequently assimilated into Glu is $1.07 \pm 0.23\text{ }\mu\text{mol/g}$.

The peak observed *in vivo* at -342 p.p.m. and assigned to $[^{15}\text{N}]\text{Glu}$ may contain a minor contribution from $[2\text{-}^{15}\text{N}]\text{Gln}$ [15,19] which, after MSO treatment, is expected to decrease due to progressive conversion of Gln into Glu by PAG and amido-transferase reactions. To determine the contribution of $[2\text{-}^{15}\text{N}]\text{Gln}$, ^{15}N spectra were taken of the perchloric acid extracts of the brains. A preliminary test with pure ^{15}N -labelled compounds in water (Figure 5a) showed that at pH 9.1, $[2\text{-}^{15}\text{N}]\text{Gln}$ (-345.6 p.p.m.) is well resolved from $[^{15}\text{N}]\text{Glu}$ (-343.6 p.p.m.) and $[^{15}\text{N}]\text{Asp}$ (-344.3 p.p.m.) because deprotonation of $-\text{NH}_3^+$ to $-\text{NH}_2$ causes an 8 p.p.m. upfield shift [30] and $\text{p}K_a$ values differ for Gln (8.9), Glu (9.6) and Asp (9.8). Figure 5(b) shows an ^{15}N spectrum of brain extracts of rats after 2.6 h of $^{15}\text{NH}_4^+$ infusion. From the relative peak intensities of the resolved $[^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$ (shown by arrows), the contribution of $[2\text{-}^{15}\text{N}]\text{Gln}$ to the -342 p.p.m. peak observed *in vivo* just before MSO treatment was estimated to be $18 \pm 4\%$. In representative ^{15}N spectra of brain extracts of rats killed at 2.4 h (Figure 5c) and 3.8 h (Figure 5d) after MSO injection, the $[2\text{-}^{15}\text{N}]\text{Gln}$ peak is undetectable or quite small compared with $[^{15}\text{N}]\text{Glu}$ and $[^{15}\text{N}]\text{Asp}$. From the relative peak intensities, the contribution of $[2\text{-}^{15}\text{N}]\text{Gln}$ to the -342 p.p.m. peak at $t = 3.8\text{ h}$ was estimated to be $8 \pm 3\%$ ($n = 3$). Hence, the contribution of $[2\text{-}^{15}\text{N}]\text{Gln}$ to the -342 p.p.m. peak observed *in vivo* was small and decreased from about 18% to 8% during measurement of PAG activity. This corresponds to an increase in the contribution of $[^{15}\text{N}]\text{Glu}$ from about 72 to 92%. Hence the net increase in brain $[^{15}\text{N}]\text{Glu}$ between $t = 0.6$ and 3.3 h is estimated to be approximately $(2.87 \times 0.92) - (1.8 \times 0.72) = 1.34\text{ }\mu\text{mol/g}$ (Table 1).

Table 1 lists the net concentration changes of brain metabolites due to PAG and related reactions shown in Figure 3(a). $[^{15}\text{N}]\text{Asp}$, derived by transamination from $[^{15}\text{N}]\text{Glu}$ (Figure 3a), was detectable in the perchloric extract of the brain at $t = 3.8\text{ h}$ at pH 7 (Figure 5e) and was present at a concentration of $0.39 \pm 0.09\text{ }\mu\text{mol/g}$ ($n = 4$). Hence, a reasonable estimate for the

made by taking separate spectra of mixtures of $[^{15}\text{N}]\text{Glu}$ and $[^{15}\text{N}]\text{Asp}$, and of $[^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$. The same chemical shifts were observed when the standards were dissolved in unlabelled brain extract. (b–d) ^{15}N spectra (expanded amine region) of the pooled brain extract at pH 9.1 from two rats. (b) After 2.6 h of $^{15}\text{NH}_4^+$ infusion; (c) at 2.4 h and (d) 3.8 h after subsequent MSO treatment. Relative peak intensities of $[^{15}\text{N}]\text{Glu}$ and $[2\text{-}^{15}\text{N}]\text{Gln}$ in (b) and (d) (shown by the arrows) were determined by measuring the half-peak area for each on the resolved side. (e) A representative ^{15}N spectrum of brain extract at pH 7.0 prepared from a rat killed 3.8 h after MSO treatment. $[^{15}\text{N}]\text{Asp}$ (-343.5 p.p.m.) is resolved from $[^{15}\text{N}]\text{Glu}/[2\text{-}^{15}\text{N}]\text{Gln}$ (-342.1 p.p.m.) at this pH.

Table 1 *In vivo* rate of PAG reaction calculated from the observed concentration changes in brain ^{15}N -labelled metabolites assimilating PAG-derived $^{15}\text{NH}_4^+$

Observed concentration changes in ^{15}N -labelled metabolites assimilating PAG-derived $^{15}\text{NH}_4^+$ and in brain glutamine ($n = 4-5$). The negative sign indicates decrease. From the data, the percentage contribution of PAG to the observed rate of $[5-^{15}\text{N}]\text{glutamine}$ decrease and the *in vivo* rate of PAG reaction were calculated, as described in the text.

Brain metabolites	Gln-utilizing pathway			<i>In vivo</i> rate ($\mu\text{mol/h per g}$)		
	Concn. change ($\mu\text{mol/g}$)					
	Total	PAG	% PAG	Total	PAG	% PAG
Assimilating PAG-derived $^{15}\text{NH}_4^+$						
$[^{15}\text{N}]\text{Glu}$	—	$+1.34 \pm 0.23$	—	—	—	—
$[^{15}\text{N}]\text{Asp}$	—	$+0.20 \pm 0.03$	—	—	—	—
Releasing PAG-derived ammonia						
$[5-^{15}\text{N}]\text{Gln}$	-4.1	$-1.54 \pm 0.26^*$	$37 \pm 6\%$	-1.2	-0.44 ± 0.07	$37 \pm 6\%$
Total Gln	-8.3	—	—	-2.4 to -3.6	-0.88 to -1.26	—

* Calculated as described in the text.

Table 2 Tissue levels of blood and brain ammonia and of brain glutamine and glutamate

The tissue levels of blood and brain ammonia and of brain glutamine and glutamate ($^{14}\text{N} + ^{15}\text{N}$), measured in extracts, and of brain $[5-^{15}\text{N}]\text{glutamine}$, measured *in vivo* by ^{15}N -n.m.r., are expressed as $\mu\text{mol/g}$ of tissue (mean $\pm$ S.E.M.), with the number of rats in parentheses. Fractional ^{15}N enrichment is defined as $([5-^{15}\text{N}]\text{glutamine concentration})/([^{14}\text{N} + ^{15}\text{N}]\text{glutamine concentration})$. For $n = 2$, individual values are shown.

Time (post MSO)(h)	Tissue levels ($\mu\text{mol/g}$)			Brain glutamine			Brain glutamate
	Ammonia ($^{14}\text{N} + ^{15}\text{N}$)						
	Blood	Brain	Brain/Blood ratio	Total ($^{15}\text{N} + ^{14}\text{N}$)	$[5-^{15}\text{N}]\text{Gln}$	^{15}N enrichment	Total ($^{15}\text{N} + ^{14}\text{N}$)
0	0.52 ± 0.1 (4)	1.1 ± 0.16 (3)	2.1	10.5, 14.1 (2)	6.14 ± 0.24 (10)	0.50	9.3, 10.6 (2)
2.4	0.44 ± 0.09 (5)	1.39 ± 0.12 (5)	3.2	7.45 ± 0.68 (5)	2.5 ± 0.16 (10)	0.35	7.0 ± 0.16 (5)
3.8	0.44 ± 0.07 (4)	1.49 ± 0.09 (4)	3.4	4.4, 3.6 (2)	1.04, 1.78 (2)	0.35	7.1 ± 0.59 (3)

quantity of $[^{15}\text{N}]\text{Asp}$ formed from PAG-derived $^{15}\text{NH}_4^+$ via $[^{15}\text{N}]\text{Glu}$ is $(0.39 \times 1.34)/2.64 = 0.20 \mu\text{mol/g}$ (Table 1). Therefore, the total quantity of $^{15}\text{NH}_4^+$ released by PAG reaction and incorporated into Glu and Asp is $1.54 \pm 0.26 \mu\text{mol/g}$. $[^{15}\text{N}]\text{GABA}$ was not detected in brain extracts. The total decrease in brain $[5-^{15}\text{N}]\text{Gln}$ over the same period is $4.1 \mu\text{mol/g}$. Therefore, the contribution of PAG to the observed $[5-^{15}\text{N}]\text{Gln}$ decrease is estimated to be $(1.54 \pm 0.26)/4.1 \times 100\% = 37 \pm 6\%$ (Table 1). Because $[5-^{15}\text{N}]\text{Gln}$ decreased at a rate of $1.2 \mu\text{mol/h per g}$, the rate of PAG-catalysed $[5-^{15}\text{N}]\text{Gln}$ hydrolysis is estimated to be $1.2 \times 0.37 = 0.44 \mu\text{mol/h per g}$. As shown in Table 2, ^{15}N enrichment of brain Gln was 0.5 at $t = 0$, and 0.35 at $t = 2.4-3.8$ h. Therefore, the rate of total $[^{14}\text{N} + ^{15}\text{N}]\text{Gln}$ hydrolysis by PAG in the brain is estimated to be $0.44/0.5 = 0.88 \mu\text{mol/h per g}$ to $0.44/0.35 = 1.26 \mu\text{mol/h per g}$ of brain (Table 1).

It is worth pointing out that if the observed brain $^{15}\text{NH}_4^+$ was produced by oxidative deamination of $[^{15}\text{N}]\text{Glu}$ by GDH acting in the catabolic direction and $^{15}\text{NH}_4^+$ was subsequently re-incorporated into Glu, the $[^{15}\text{N}]\text{Glu}$ concentration would have increased only to the pre- $^{14}\text{NH}_4^+$ infusion level of $1.8 \mu\text{mol/g}$. The observed net increase of $> 1 \mu\text{mol/g}$ over the pre- $^{14}\text{NH}_4^+$ -infusion level (Figure 3c) clearly shows that assimilated $^{15}\text{NH}_4^+$ originated from an ^{15}N -labelled metabolite other than $[^{15}\text{N}]\text{Glu}$. Because $[5-^{15}\text{N}]\text{Gln}$ is the only other ^{15}N -labelled brain metabolite (other than $[^{15}\text{N}]\text{urea}$ which is an end product) present in sufficient quantity to be detectable *in vivo*, and shows a substantial decrease during the observation period, the $^{15}\text{NH}_4^+$ released and sub-

sequently assimilated into Glu can most reasonably be ascribed to PAG-catalysed hydrolysis of $[5-^{15}\text{N}]\text{Gln}$.

As shown in Table 2, total $[^{15}\text{N} + ^{14}\text{N}]\text{Glu}$ in the brain was $9.3-10.6 \mu\text{mol/g}$ ($n = 2$) at $t = 0$ (i.e. after 2.6 h of $^{15}\text{NH}_4^+$ infusion), a result consistent with our previous observation of $9.3 \pm 0.3 \mu\text{mol/g}$ ($n = 4$) of Glu in hyperammonaemic rat brain [15]. Brain glutamate decreased to about $7 \mu\text{mol/g}$ ($n = 3-5$) at 2.4 and 3.8 h after MSO treatment. A similar decrease has been reported previously [29].

Brain and blood ammonia

It is important to consider whether $^{15}\text{NH}_4^+$, released by PAG-catalysed hydrolysis of $[5-^{15}\text{N}]\text{Gln}$ in the brain, could have partially diffused out of the brain into the blood during the observation period, thereby reducing the apparent rate. Because ammonia diffuses across the blood-brain barrier mainly as neutral NH_3 and brain pH (7.1) is lower than the blood pH (7.4), brain ammonia level at equilibrium is normally 1.5-3 times that of the blood [31]. Efflux of brain ammonia to blood is expected when the brain:blood ammonia concentration ratio becomes much higher than the equilibrium ratio. Table 2 shows blood and brain ammonia ($^{14}\text{N} + ^{15}\text{N}$) concentrations at $t = 0, 2.4$ and 3.8 h after MSO injection. At $t = 0$ (i.e. after 2.6 h of ammonia infusion, Figure 1), brain and blood ammonia levels were 1.1 ± 0.16 and $0.52 \pm 0.1 \mu\text{mol/g}$ respectively, with a brain/blood ammonia concn. ratio of 2.1:1. The observed ammonia levels are

in good agreement with those measured previously at a similar infusion rate [15]. After MSO injection and concomitant decrease in the ammonia infusion rate, the blood ammonia level decreased by 15% to $0.44 \pm 0.09 \mu\text{mol/g}$, but the brain ammonia level increased slightly to $1.39 \pm 0.12 \mu\text{mol/g}$, because of decreased ammonia removal on GS inhibition. During the 2.4–3.8 h period, blood and brain ammonia levels were stable at 0.44 ± 0.09 and $1.44 \pm 0.17 \mu\text{mol/g}$ respectively with a mean brain/blood ammonia ratio of 3.3:1. The results in Table 2 show that the brain/blood ammonia concentration ratios were within, or close to, the equilibrium ratio throughout the *in vivo* experiment. The results strongly suggest that the predominant fate of $^{15}\text{NH}_4^+$ released by the PAG reaction was retention in the brain and assimilation into Glu, rather than efflux into the blood.

DISCUSSION

Our results show that the *in vivo* rate of the PAG reaction in the brain of hyperammonaemic rat is 0.9 to $1.3 \mu\text{mol/h per g}$ of brain. The total rate of Gln utilization in the same brain is 2.4–3.6 $\mu\text{mol/h per g}$ of brain. This represents the maximum possible rate of PAG reaction, if contributions from other Gln-utilization pathways were negligibly small.

$^{15}\text{NH}_4^+$ released from [5- ^{15}N]Gln by PAG was present as free $^{15}\text{NH}_4^+$ in only moderate quantity ($0.46 \mu\text{mol/g}$) and was subsequently assimilated into Glu by GDH. Because PAG is localized on the inner mitochondrial membrane mainly of glutamatergic neurons, and GDH in the mitochondrial matrix of both neurons and astrocytes [32], ammonia released by PAG can be channelled into Glu in those mitochondria that contain both enzymes. The relatively low rate of $^{15}\text{NH}_4^+$ assimilation (about $1.4 \mu\text{mol/h per g}$, Figure 3c) is due to the low affinity of GDH for NH_4^+ [33], and is consistent with our previous finding that the flux of ^{15}N through Glu N is only about $1.5 \mu\text{mol/h per g}$ [15].

If GS inhibition by MSO was incomplete, a small fraction of PAG-derived $^{15}\text{NH}_4^+$ may have been reassimilated into 5-N of Gln by residual GS activity. Such recycling is less likely than assimilation by GDH because both PAG and GDH occur in neuronal mitochondria while GS is cytosolic and localized in astrocytes [34]. Inhibition of brain GS by MSO (150 mg/kg body wt.) is reported to be 86% *in vitro* [35]. Raabe et al. report that, *in vivo*, ammonia infusion caused a 70% increase of brain Gln in controls; however, when infusion was started 1 h or 2 h after MSO treatment, brain Gln increased only by 17% and 0% respectively [28]. In rat brain, the Gln level observed *in vivo* by ^1H n.m.r. decreased by about 50% 1 h after MSO treatment and remained at that level for 3–4 h [29]. These considerations suggest that GS was extensively inhibited by MSO under our experimental conditions. Therefore, recycling of PAG-derived $^{15}\text{NH}_4^+$ by residual GS activity, resulting in underestimation of the PAG activity, is likely to be small.

The observed decrease in brain [5- ^{15}N]Gln reflects efflux of ^{15}N into Gln-utilizing pathways within the brain. Efflux into the bloodstream is expected to be negligibly small because, in hyperammonaemic rat brain, no net flux of Gln between the cerebral cortex and the bloodstream was observed at brain Gln levels of $< 14\text{--}17 \mu\text{mol/g}$ [36].

An alternative route for $^{15}\text{NH}_3$ production from [5- ^{15}N]Gln is the glutaminase II pathway (glutamine transaminase + ω -amidase) [37]. The pathway is likely to be quite minor [16,17] compared with PAG for the following reasons. (1) The optimum *in vitro* activity of glutamine transaminase [16,37] is only 12% of that of PAG. Its *in vivo* activity is likely to be very limited due to the low availability (3.3 nmol/g) of the α -keto acid substrate, phenylpyruvate, in rat brain [38]. (2) The open-chain form of α -

oxoglutarate (the product of transaminase and the substrate of ω -amidase) represents only 0.3% of the total (open-chain + cyclic forms) at physiological pH [39], severely limiting the rate of NH_3 production from α -oxoglutarate [40]. These considerations strongly suggest that PAG is predominantly responsible for the observed $^{15}\text{NH}_4^+$ production from [5- ^{15}N]Gln.

Regulation of PAG activity *in vivo*

The *in vivo* PAG activity in the brain of the hyperammonaemic rat, 0.9–1.3 $\mu\text{mol/h per g}$, is less than 1.1% of the *in vitro* activity measured in rat brain homogenate, which is reported to be $126 \mu\text{mol/h per g}$ at 10 mM Gln and 10 mM concentration of the activator P_i at pH 8.0 [5]. The result suggests that PAG activity 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 PAG [3,4], and estimated intramitochondrial concentrations of the substrate, activator and inhibitors, as discussed below.

Studies with purified [3] and particulate [4] forms of rat brain PAG show that PAG activity depends on the levels of Gln, of the activator P_i and of inhibitors ammonia, Glu and $[\text{H}^+]$. In particulate form, PAG has a K_m of 1.6 mM for Gln [4]. In the present study, brain Gln decreased from 12.3 to $4 \mu\text{mol/g}$ (Table 2). Therefore, PAG was probably saturated with the substrate. The observed linear decrease of [5- ^{15}N]Gln (Figure 3b), suggesting zero-order reaction, is consistent with this idea. The effects of pH and P_i on PAG activity are well established [4]. At the estimated intramitochondrial pH of approximately 7.6 [41], the activity is expected to be about 60% of the optimum (pH 8–9). The phosphate activation curve of PAG is sigmoidal with the activity increasing sharply over the physiological P_i range of 1–10 mM [4]. If mitochondrial $[\text{P}_i]$ is close to the level of 1.3–2.1 $\mu\text{mol/g}$ reported for the whole brain [42,43], the activity would be 10–20% of that measured at 10 mM P_i . In brain homogenates, PAG activity decreased to 63–69% of the control on addition of 1–2 mM NH_4Cl , and to 20% on addition of 1 mM Glu ($K_i = 0.45 \text{ mM}$) [4]. At a brain ammonia concentration of $1.4 \mu\text{mol/g}$ and Glu level of 7–10 $\mu\text{mol/g}$ (Table 2), similar reduction in activity is expected.

It is informative to compare the observed *in vivo* activity with the activity expected when the effects of all the regulators considered above are taken into account. The reported *in vitro* activity of $126 \mu\text{mol/h per g}$ [5], when adjusted for the expected differences in pH, P_i and the effects of 1–2 mM NH_4^+ and Glu, reduces to $126 \times 0.6 (\text{pH}) \times 0.15 (\text{P}_i) \times 0.63 (\text{NH}_3) \times 0.2 (\text{Glu}) = 1.4 \mu\text{mol/h per g}$ of brain. This calculated activity is very close to the observed *in vivo* activity of 0.9–1.3 $\mu\text{mol/h per g}$. Clearly, ammonia inhibition alone does not account for the low activity observed in the brain of the hyperammonaemic rat. Our results strongly suggest that, in intact brain, PAG activity is maintained at a low level by other regulators, the most likely ones being the suboptimal mitochondrial level of P_i and the strong inhibitory effect of Glu.

Glutamine as precursor of GABA

If Gln is indeed a major precursor not only of the neurotransmitter pool of Glu but also of GABA, Gln synthesized in astrocytes and transported to neurons must be converted into Glu at a rate sufficient to support GABA synthesis. The rate of synthesis in rat brain, determined from the rate of accumulation on inhibition of GABA transaminase, is $2.6 \pm 1.5 \mu\text{mol/h per g}$ [12,44–46]. *In vivo* rate of PAG in intact brain of hyperammonaemic rat was 0.9–1.3 $\mu\text{mol/h per g}$, and is expected to

be 30–40% higher in normal brain. Comparison with the GAD activity suggests that PAG activity is too low for it to be a major provider of Glu for GABA synthesis, especially if the level of PAG in GABAergic neurons is much lower than in glutamatergic neurons, as suggested by immunocytochemical studies [2,47]. Thus, comparison of *in vivo* activities measured in intact brain suggests, as do studies from other laboratories using different approaches [10–13], that PAG-catalysed hydrolysis of Gln cannot be the sole provider of Glu used for GABA synthesis.

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REFERENCES

- Bradford, H. F. and Ward, H. K. (1976) *Brain Res.* **110**, 115–125
- Aoki, C., Kaneko, T., Starr, A. and Pickel, V. M. (1991) *J. Neurosci. Res.* **28**, 531–548
- Haser, W. G., Shapiro, R. A. and Curthoys, N. P. (1985) *Biochem. J.* **229**, 399–408
- Benjamin, A. M. (1981) *Brain Res.* **208**, 363–377
- Ward, H. K. and Bradford, H. F. (1979) *J. Neurochem.* **33**, 339–342
- Kvamme, E. and Lenda, K. (1982) *Neurochem. Res.* **7**, 667–678
- Erecińska, M., Zaleska, M. M., Nelson, D., Nissim, I. and Yudkoff, M. (1990) *J. Neurochem.* **54**, 2057–2069
- Thanki, C. M., Sugden, D., Thomas, A. J. and Bradford, H. F. (1983) *J. Neurochem.* **41**, 611–617
- Hamberger, A. C., Chiang, G. H., Nylén, E. S., Scheff, S. W. and Cotman, C. W. (1979) *Brain Res.* **168**, 513–530
- Ward, H. K., Thanki, C. M. and Bradford, H. F. (1983) *J. Neurochem.* **40**, 855–860
- Szerb, J. C. (1984) *Brain Res.* **293**, 293–303
- Paulsen, R. E., Odden, E. and Fonnum, F. (1988) *J. Neurochem.* **51**, 1294–1299
- Battaglioli, G. and Martin, D. L. (1991) *Neurochem. Res.* **16**, 151–156
- Bachelard, H. and Badar-Goffer, R. (1993) *J. Neurochem.* **61**, 412–429
- Kanamori, K. and Ross, B. D. (1993) *Biochem. J.* **293**, 461–468
- Makar, T. K., Nedergaard, M., Preuss, A., Hertz, L. and Cooper, A. J. L. (1994) *J. Neurochem.* **62**, 1983–1988
- Kvamme, E., Svenneby, G. and Torgner, I. A. (1988) in *Glutamine and Glutamate in Mammals* (Kvamme, E., ed.), pp. 53–67, CRC Press, Boca Raton
- Farrow, N. A., Kanamori, K., Ross, B. D. and Parivar, F. (1990) *Biochem. J.* **270**, 473–481
- Kanamori, K., Parivar, F. and Ross, B. D. (1993) *NMR Biomed.* **6**, 21–26
- Kun, E. and Kearney, E. B. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. and Gewehn, K., eds.), pp. 1802–1806, Academic Press, New York
- Lund, P. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. and Gewehn, K., eds.), pp. 1719–1722, Academic Press, New York
- Bernt, E. and Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U. and Gewehn, K., eds.), pp. 1704–1708, Academic Press, New York
- Bax, A., Griffey, R. H. and Hawkins, B. L. (1983) *J. Magn. Reson.* **55**, 301–315
- Kanamori, K., Ross, B. D. and Parivar, F. (1991) *J. Magn. Reson.* **93**, 319–328
- Baldeschwieler, J. D. (1962) *J. Chem. Phys.* **36**, 152–158
- Binsch, G., Lambert, J. B., Roberts, B. W. and Roberts, J. D. (1964) *J. Am. Chem. Soc.* **86**, 5564–5570
- Hindfelt, B., Plum, F. and Duffy, T. E. (1977) *J. Clin. Invest.* **59**, 386–396
- Raabe, W. A. and Onstad, G. R. (1982) *Brain Res.* **242**, 291–298
- Chamuleau, R. A. F. M., Bosman, D. K., Bovée, W. M. M. J., Luyten, P. R. and den Hollander, J. A. (1991) *NMR Biomed.* **4**, 103–108
- Witanowski, M., Stefaniak, L. and Webb, G. A. (1992) *Annu. Rep. NMR Spectrosc.* **25**, 1–468
- Cooper, A. J. L. and Plum, F. (1987) *Physiol. Rev.* **67**, 440–519
- Aoki, C., Milner, T. A., Sheu, K.-F. R., Blass, J. P. and Pickel, V. M. (1987) *J. Neurosci.* **7**, 2214–2231
- Colon, A. D., Plaitakis, A., Perakis, A., Berl, S. and Clarke, D. D. (1986) *J. Neurochem.* **46**, 1811–1819
- Norenberg, M. D. and Martinez-Hernandez, A. (1979) *Brain Res.* **161**, 303–310
- Cooper, A. J. L., McDonald, J. M., Gelbard, A. S., Gledhill, R. F. and Duffy, T. E. (1979) *J. Biol. Chem.* **254**, 4982–4992
- Dejong, C. H. C., Kampman, M. T., Deutz, N. E. P. and Soeters, P. B. (1992) *J. Neurochem.* **59**, 1071–1079
- Cooper, A. J. L., Abraham, D. G., Gelbard, A. S., Lai, J. C. K. and Petit, C. K. (1993) *J. Neurochem.* **61**, 1731–1741
- Cooper, A. J. L., Leung, L. K. H. and Asano, Y. (1989) *Anal. Biochem.* **183**, 210–214
- Duffy, T. E., Cooper, A. J. L. and Meister, A. (1974) *J. Biol. Chem.* **249**, 7603–7606
- Meister, A. (1953) *J. Biol. Chem.* **200**, 571–589
- Ogawa, S., Boens, C. C. and Lee, T. M. (1981) *Arch. Biochem. Biophys.* **210**, 740–747
- Veech, R. L., Harris, R. L., Veloso, D. and Veech, E. H. (1973) *J. Neurochem.* **20**, 183–188
- Behar, K. L., den Hollander, J. A., Petroff, O. A. C., Hetherington, H. P., Prichard, J. W. and Shulman, R. G. (1985) *J. Neurochem.* **44**, 1045–1055
- Chapman, A. G. and Evans, M. C. (1983) *J. Neurochem.* **41**, 886–889
- Casu, M. and Gale, K. (1981) *Life Sci.* **29**, 681–688
- Löscher, W., Hönack, D. and Gramer, M. (1989) *J. Neurochem.* **53**, 1737–1750
- Kaneko, T., Urade, Y., Watanabe, Y. and Mizuno, N. (1987) *J. Neurosci.* **7**, 302–309