[¹⁵N]Alanine and [¹⁵N]ammonia metabolism *in vivo*

In a recent paper, Farrow *et al.* [1] reported ¹⁵N disposition in brain, liver and kidney of rats administered massive doses of [¹⁵N]ammonia and [¹⁵N]alanine to induce hyperammonaemia. ¹⁵N-labelled metabolites were quantified in perchlorate extracts of freeze-clamped tissues by ¹⁵N-n.m.r. The authors pointed out that the wide chemical-shift range of ¹⁵N (400 p.p.m.) allows resolution of key metabolites in a single determination. However, the limit of detection of an ¹⁵N-labelled metabolite by this technique is 0.3–0.6 mM in the original tissues, and many key ¹⁵N-labelled metabolites were not detected. Indeed, the authors were not even able to measure [¹⁵N]ammonia in brain and liver. It is doubtful whether the study could have been carried out in normal rats with tracer doses of ¹⁵N-labelled ammonia and alanine. The authors downplay the problem of recirculating [¹⁵N]metabolites, e.g., in their interpretation of [¹⁵N]glutamine formation in the kidney. However, the circulation time in the rat is about 6 s. Thus, considerable recirculation and interorgan transfer of labelled metabolites may have occurred, complicating interpretation of the biochemical pathways involved in [¹⁵N]metabolite formation in the tissues. We believe that beyond demonstrating the feasibility of measuring ¹⁵N incorporation into a few key compounds present in *high* concentration in extracts of rat tissues by a relatively new technique, the study of Farrow *et al.* [1] provided limited new information.

In 1961, Berl *et al.* [2] showed that after continuous infusion of massive, toxicological doses of [¹⁵N]ammonia into cats, most of the label in the brain was in the amide group of glutamine. However, the relative enrichment (note that Farrow *et al.* [1] refer to ¹⁵N isotope incorporation into metabolites in terms of specific activity; however, this term should only be used for compounds labelled with radioactive isotopes and not for compounds labelled with a stable isotope) of ¹⁵N in glutamine amine was greater than that in glutamate, a finding not consistent with a simple product–precursor relationship between these two compounds. Since, as far as is known, glutamine is only made from glutamate, the authors postulated that ammonia entering the brain is rapidly incorporated into glutamine (amide) in a small pool of rapidly turning-over glutamate that is distinct from a larger pool of more slowly turning-over glutamate. This study and others from this group at about the same time established the important concept of metabolic compartmentation in brain. It is now known that the small pool is represented largely by astrocytes (which contain the bulk of brain glutamine synthase) (see refs. in [3]). In 1979 Cooper *et al.* [4] used ¹³N (positron-emitter; $t_{1/2}$ 9.96 min) to show that metabolic compartmentation can be detected in rat brain with truly tracer quantities of labelled ammonia. Thus the specific activity of L-[amine-¹³N]glutamine was found to be considerably greater than that of L-[¹³N]glutamate in rat brain after infusion of [¹³N]ammonia via the carotid artery [4,5] or cerebral ventricles [4]. The sensitivity of the present ¹⁵N-n.m.r. technique is so poor that Farrow *et al.* [1] were unable to compare ¹⁵N enrichment in brain glutamine (amine) and glutamate; in a sense, a backward step. Farrow *et al.* [1] suggest that “no major alternative pathway for ammonia detoxification, other than glutamine synthetase, exists in rat brain”. However, we have repeatedly pointed this out [3–6]. Farrow *et al.* [1] used methionine sulfoximine (MSO) to inhibit brain glutamine synthetase and noted that, despite pronounced inhibition of this enzyme, [¹⁵N]ammonia was still metabolized predominantly in the brain by incorporation into glutamine. We carried out a similar experiment in 1979; we noted predominant incorporation of label derived from [¹³N]ammonia into brain glutamine (amide), despite an 86% inhibition of brain

glutamine synthetase in MSO-treated rats [4]. Relatively more label was found to be present in glutamate than in glutamine (amine), suggesting a breakdown in the kinetically-defined compartmentation of brain ammonia/glutamate metabolism in MSO-treated rats [4]. Farrow *et al.* [1] pointed out that glutamine amide in the brain contained only 53% ¹⁵N after 20–30 min infusion of [¹⁵N]ammonia (99% enrichment) into the rats. To explain this phenomenon the authors suggested that the label may be diluted before entering the brain. It seems obvious that the glutamine synthetase of the astrocytic pool will convert both exogenous (blood-borne, CSF-borne) and endogenous (metabolic) ammonia to glutamine (e.g. [3]). Thus, dilution of label in [¹⁵N]ammonia may occur within the brain as well as prior to arriving at the brain. Moreover, some unlabelled glutamine present in the brain at the start of the experiment may still have been present at the end of the infusion.

Except for one sentence commenting on the ratio of label incorporation into urea versus glutamine in the liver, none of our extensive studies of [¹³N]ammonia and [¹³N]amino acid metabolism in the rat liver were mentioned by Farrow *et al.* [1]. Obviously, for long-term studies, ¹³N cannot be used as a tracer for studying nitrogen metabolism. However, we have pointed out that for short-term studies (≤ 1 h), ¹³N has many advantages over ¹⁵N as a tracer for nitrogen (e.g. [7]). ¹³N is radioactive and therefore easy to quantify and can be administered in truly tracer quantities. ¹³N can be easily quantified in whole tissue specimens without prior separation of isotope. Moreover, there is no natural background of ¹³N, so that, unlike ¹⁵N, minute quantities of tracer entering a large nitrogen pool are easy to quantify [7]. As we have demonstrated, many of the ammonia/amino acid pools turn over in seconds to minutes in the rat and are thus amenable to study with ¹³N. Farrow *et al.* [1] noted that after infusion of [¹⁵N]ammonia, label was detected in glutamine, glutamate and, occasionally, alanine in the liver. They also noted that after infusion of [¹⁵N]alanine, label was detected in liver glutamine, glutamate and aspartate. These findings are in accord with the rapidly reversible glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase reactions. However, these experiments mirror (albeit in much less detail) the previous work of Cooper *et al.*, who investigated the short-term (5 s–1 min) metabolism of [¹³N]ammonia [8] and [¹³N]alanine (and glutamate and glutamine) [9] in normal rat liver *in vivo*.

We wish to summarize our ¹³N findings as follows.

Brain (refs. [3–6]). (1) Ammonia enters the brain mostly by passive diffusion of the free base (NH₃). (2) Blood-borne [¹³N]ammonia on entering the rat brain is rapidly ($t_{1/2} \leq 3$ s) metabolized. (3) Almost all the label ($\sim 98\%$) is incorporated into glutamine (amide). (4) [¹³N]Ammonia entering the brain from the blood and CSF is metabolized to [amide-¹³N]glutamine in the small compartment ($\equiv$ astrocytes). (5) Endogenous ammonia produced in the neurons is also detoxified by incorporation into glutamine in the small, astrocytic pool. (6) Metabolic compartmentation of ammonia metabolism in the brain is disrupted by MSO, an inhibitor of glutamine synthetase. (7) In the chronically or acutely hyperammonaemic rat brain, the glutamine synthetase reaction is still the overwhelming route used to detoxify ammonia. (8) Since the enzyme may be turning over at a rate near capacity under normal conditions, the ability of the brain enzyme to ‘handle’ an excess of ammonia may become limited.

Liver (refs. [8–10]). (1) Despite the need for five enzymic steps and two mitochondrial transport steps, the conversion of ammonia nitrogen to urea nitrogen in the rat liver is very fast ($t_{1/2} \sim 10$ –11 s). (2) After bolus administration of [¹³N]ammonia via the portal vein, label is rapidly (in seconds) detected in all components of the urea cycle in rat liver except carbamyl

phosphate, suggesting rapid channelling of carbamyl phosphate to citrulline and normally very low levels of mitochondrial carbamyl phosphate in liver. (3) Despite high activity of glutamine synthetase, little portal-vein [^{15}N]ammonia is incorporated into glutamine (7%) compared with urea (93%). (This is a consequence of the enrichment of urea cycle enzymes in the periportal hepatocytes and the downstream location of glutamine synthetase in a small ring of perivenous hepatocytes. Under altered conditions (e.g., acidosis) the relative importance of hepatic glutamine synthetase in removing portal vein ammonia may increase; see the discussion in [12].) (4) Metabolic compartmentation of nitrogen metabolism in rat liver *in vivo* was demonstrated directly; thus, analysis of labelled metabolites in liver showed that [^{15}N]alanine administered via the portal vein was preferentially (~95%) taken up in the perivenous hepatocytes where the label was readily incorporated into urea; in contrast, portal vein [^{15}N]glutamate was preferentially (~67%) taken up by the periportal hepatocytes where a considerable fraction was converted to labelled glutamine. (5) Equilibration of label among ammonia and mitochondrial pools of aspartate and glutamate is very rapid ($t_{1/2} < 2$ s), attesting to the extreme rapidity with which nitrogen is exchanged among the components of the mitochondrial aspartate aminotransferase and glutamate dehydrogenase reactions in rat liver. Thus, it becomes a moot point to argue which of the two reactions (at least in rat liver) is more important for the metabolism of glutamate. Both are important and both will act as conduits for the channelling of nitrogen to urea as dictated by the requirements for urea synthesis. (6) Nitrogen exchange among components of the cytosolic aspartate aminotransferase and alanine aminotransferase reactions is also very rapid (order of seconds) in the rat liver. (7) Despite occasional reports to the contrary, glutamine (amide) is a source of hepatic urea nitrogen *in vivo*.

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[^{15}N]Alanine and [^{15}N]ammonia metabolism: a reply

Cooper and Gelbard [1] provide a very useful reiteration of their extensive studies with the short-lived isotope ^{15}N , and use this to criticize our study with ^{15}N -n.m.r. We do cite two major reviews of their work, and adequately acknowledge their contributions in this field. There are no significant differences between the conclusions drawn by them, and the necessarily much more limited conclusions of our study.

Two of the limitations of ^{15}N -n.m.r. indicated by Cooper and Gelbard have now been resolved. Sensitivity is much improved (up to 6-fold) by avoidance of the dilutions inherent in tissue extraction [2]. Using this 'whole-tissue' preparation and longer infusions of $^{15}\text{NH}_4\text{Cl}$, we have been able to detect [γ - ^{15}N]glutamine in 1 min of n.m.r. acquisition (our unpublished work). The inability to detect tracer amounts of ^{15}N will, we believe, be overcome by the use of another technical improvement; the use of ^1H - ^{15}N heteronuclear multiple quantum coherence (HMQC) n.m.r. By this means, more than 50-fold increase in sensitivity for detection of ^{15}N metabolites in rat brain and liver has been achieved, albeit only for ^{15}N coupled to non-exchanging protons [3]. However, this includes some of the most interesting intermediary metabolites, glutamine, urea, *N*-acetylaspartate, tryptophan and serotonin. In principle, HMQC n.m.r. would lend itself either to short-term, substrate-level infusions of ^{15}N , or to longer term infusions of 'tracer' amounts of precursors.

The full potential of ^{15}N -n.m.r. will be achieved when non-invasive and repeated assays *in vivo*, in both animals and man, can be performed. ^{15}N -n.m.r. will then join ^{31}P , ^{13}C and ^1H n.m.r. spectroscopy as tools for the re-examination of metabolic homeostasis in the brain *in vivo*. We are close to realizing the first aim in our laboratory.

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