

Endogenous production of ammonia – charge question 1 – Arthur J. L. Cooper

On p. G-9 the ACC commented “The ACC noted that the discussion of endogenous production of ammonia was not adequate and considered the rationale used to justify setting an RfC, at a level equivalent to the human breath level to be unclear.” I concur with both statements.

My comments/discussion is as follows:

1). Endogenous production of ammonia: There are very many enzyme-catalyzed reactions by which ammonia can be generated *in vivo*. For example, Cooper and Plum (1987) list at least seventeen enzyme-catalyzed reactions that can generate ammonia from amino acids and nucleotides in the brain. Considerable ammonia is generated in the gut from the action of bacteria on nitrogenous substance. In humans, a large portion of this ammonia is derived from the hydrolysis of urea by urease-containing bacteria in the colon (Gibson et al. 1976). Tracer studies suggest that in human volunteers 15-30% of urea synthesized in the kidneys is converted to ammonia by intestinal bacteria (Walser and Bodenlos 1959). An important source of endogenous ammonia is derived from the metabolism of amino acids. A major route for conversion of amino acid nitrogen to ammonia involves coupling of an aminotransferase (transaminase) to the glutamate dehydrogenase reaction. The amino acid is transaminated with  $\alpha$ -ketoglutarate to the corresponding  $\alpha$ -keto acid and glutamate. The glutamate is then converted back to  $\alpha$ -ketoglutarate with the concomitant formation of ammonia in a reaction catalyzed by glutamate dehydrogenase. This ammonia is mainly incorporated into urea in the liver or into glutamine in extrahepatic tissues (see below). Nitrogen transferred from an amino acid to glutamate via a transaminase reaction can be further transferred to aspartate via the aspartate aminotransferase reaction. In the muscle this aspartate nitrogen is a source of ammonia via the purine nucleotide cycle. For a recent discussion of these pathways see Cooper (2012).

2). Endogenous removal of ammonia: The main route for removal of ammonia carried to the liver by the portal vein is incorporation into urea by enzymes of the urea cycle in the periportal hepatocytes. Glutamine synthetase is located in the perivenous hepatocytes downstream in the sinusoid. This enzyme acts as a backup system to remove ammonia that is not removed as urea by the periportal cells (Häussinger 1998). This two system backup arrangement is very effective. For example, Cooper et al. (1987) showed that ~93% of tracer quantities of [ $^{13}\text{N}$ ]ammonia ( $^{13}\text{N}$  is a positron-emitting isotope with a  $t_{1/2}$  of 9.96 min) injected into the portal vein of anesthetized rats is removed in a single pass through the liver. Of the [ $^{13}\text{N}$ ]ammonia taken up by the liver about 93% is incorporated into urea and about 7% is incorporated into the amide position of glutamine. Despite the fact that the urea cycle consists of five enzyme steps and two mitochondrial transport processes the process is remarkably effective. It was estimated that the  $t_{1/2}$  for conversion of ammonia to urea in the rat liver is about 11 sec (Cooper et al. 1987).

Because extrahepatic tissues do not contain a functioning urea cycle ammonia generated by the breakdown of nitrogenous substances in these tissues must be removed by another mechanism. In most tissues this removal is accomplished by incorporation of ammonia into the amide position of glutamine via a reaction catalyzed by glutamine

synthetase. For example, it has been shown, using an intracarotid bolus of [ $^{13}\text{N}$ ]ammonia, that >95% of blood-derived ammonia entering the rat brain (and also, presumably, endogenously derived ammonia) is very rapidly incorporated (in seconds) into glutamine (amide) in a distinct metabolic compartment (astrocytes) (Cooper et al. 1979). The major route for cerebral metabolism of blood-derived [ $^{13}\text{N}$ ]ammonia in hyperammonemic rats is also via the glutamine synthetase reaction (Cooper et al. 1985). Ammonia enters the brain mostly by diffusion as the free base ( $\text{NH}_3$ ) (Cooper et al. 1979; Lockwood et al. 1980) although a small portion may cross the blood-brain barrier as ammonium ion ( $\text{NH}_4^+$ ) (Raichle and Larson 1981).

Freed and Gelbard (1982) determined the disposition of label in 14 major organs of anesthetized rats following intravenous (femoral vein) bolus injection of [ $^{13}\text{N}$ ]ammonia. They found that most of the administered dose was extracted by the musculature, kidneys and lungs. It was noted that labeled metabolites were rapidly lost from the lungs and kidney. Whole body imaging after administration of [ $^{13}\text{N}$ ]ammonia was previously used to show that skeletal muscle in human volunteers is a major sink for removal of circulating ammonia (Lockwood et al. 1979). Cachexia is a major risk factor for patients with liver disease and hyperammonemic encephalopathy. Lockwood et al (1979) concluded that muscle atrophy may thereby contribute to the development of hyperammonemic encephalopathy with an associated increase in the brain ammonia utilization rate. However, in portacaval shunted rats (a model of chronic liver disease) muscle glutamine synthetase is upregulated presumably in an attempt to counteract the loss of liver enzymes responsible for removing ammonia (Girard and Butterworth 1992).

The finding of Freed and Gelbard (1982) that lungs may be important for the removal of circulating ammonia is interesting given the fact that high levels of inhaled ammonia are toxic to the lungs. It was shown by Cooper and Freed (2005), using [ $^{13}\text{N}$ ]ammonia, that rat lungs contain glutamine synthetase and that a considerable portion of [ $^{13}\text{N}$ ]ammonia passing through the lungs is removed as L-[amide- $^{13}\text{N}$ ]glutamine. Evidently, however, the presence of glutamine synthetase in the lungs is ineffective at preventing damage to these organs at high levels of inspired ammonia.

It is noted in the draft report that ammonia can be detected in the breath of humans. However, in the study of Cooper and Freed (2005) it was shown that very little  $^{13}\text{N}$  could be detected in the exhaled rat breath after intravenous administration of [ $^{13}\text{N}$ ]ammonia despite a considerable first pass extraction of [ $^{13}\text{N}$ ]ammonia (~30% of the administered dose) by the lungs. This finding supports the notion that the major source of ammonia in the breath does not originate from endogenous ammonia in the lung tissue *per se*, but rather is formed by bacterial action on nitrogenous substances in the oral and nasal cavities (see below).

3. Neurotoxicity of ammonia: While inhalation studies do not provide evidence for the neurotoxicity of ammonia in humans (p I-27) there is considerable evidence that systemic administration of ammonia produces a neurotoxic response in experimental animals. The draft report suggests that administration of ammonium salts can be problematic due to the confounding effects of the counter anion. For example, if ammonium chloride is administered systemically the chloride ion may have a deleterious effect on kidney that is not directly related to the ammonium ion. For this reason, researchers studying the neurotoxic effects of ammonia in experimental animals usually

administer ammonium acetate on the assumption that the acetate is rapidly metabolized to CO<sub>2</sub>. The ammonium acetate is most often administered by an intraperitoneal route (e.g. Hindfelt et al. 1977), but sometimes by arterial infusion (Voorheis et al. 1983).

It has long been known that acute ammonia intoxication in experimental animals is associated with stupor, seizures and coma (e.g. Navazio et al. 1961). By the 1970s it was realized that inborn errors of the urea cycle result in elevated levels of ammonia that are devastating to the infant human brain (Shih 1976). The longer the period of neonatal hyperammonemia in children with defects of the urea cycle the greater the neurological impairment in the survivors (Msall et al. 1984). Hyperammonemia is now considered a major factor contributing to the encephalopathy associated with both acute and chronic liver disease (hepatic encephalopathy, HE).

Why is hyperammonemia so deleterious to the brain? In the draft review (p. I-27) it was suggested that glutamate and  $\gamma$ -aminobutyrate play a role in ammonia-induced toxicity. However, no explanation of how these amino acids may function in ammonia-induced neurotoxicity was provided. The reference provided is to a review by EA Jones (2002). Jones has for many years emphasized a role for GABA in ammonia-induced HE. Jones has suggested that increased brain ammonia increases the GABA-induced chloride channel current and affects the benzodiazepine receptors in neurons and astrocytes. According to a more recent review by Jones and Mullen (2012) "Evidence of increased GABAergic tone in models of HE has accumulated; potential mechanisms include increased synaptic availability of GABA and accumulation of natural benzodiazepine receptor ligands with agonist properties. Pathophysiological concentrations of ammonia associated with HE, have the potential of enhancing GABAergic tone by mechanisms that involve its interactions with the GABA<sub>A</sub> receptor complex". Other studies have suggested that hyperammonemia associated with liver disease may compromise energy metabolism, but the changes appear to be subtle (reviewed by Ott and Vilstrup 2104). However, most recent studies of ammonia-induced neurotoxicity have focused on excessive production of glutamine in the brain.

A clue implicating excess glutamine production in hyperammonemic encephalopathy is the finding that, unlike most neurological diseases, hyperammonemia results in damage that is largely confined to the astrocytes and not to neurons (Norenberg 1976). Interestingly, in the brain, glutamine synthetase is confined almost exclusively to astrocytes (Martinez-Hernandez et al. 1977; Norenberg and Martinez-Hernandez 1979). Thus, astrocyte end feet are uniquely poised to "intercept" ammonia entering the brain by diffusion across the blood-brain barrier and to incorporate this ammonia into glutamine. However, this arrangement comes at a price. Most investigators now believe that a major contributor to the neurotoxicity of excess ammonia is the associated increased levels of glutamine in astrocytes. Increased glutamine in these cells results from stimulation of glutamine synthetase perhaps coupled to an ammonia-induced decrease in glutamine egress from astrocytes via the SNAT-5 transporter (Desjardins et al 2012). Persuasive evidence for a role of excess cerebral glutamine in ammonia-induced encephalopathy is the finding that methionine sulfoximine, a potent inhibitor of glutamine synthetase, protects rodents against neurotoxic doses of ammonia (reviewed by Brusilow et al. 2010). Brusilow and colleagues have argued that the major insult to the brain in hyperammonemic syndromes is excess production of glutamine producing an osmotic stress in astrocytes (the osmotic gliopathy theory) (Brusilow et al 2010 and references

cited therein). Certainly, neural swelling as a result of osmotic stress occurs during hyperammonemia and this swelling can be detected in the brains of hyperammonemic HE patients by magnetic resonance (MR) techniques. For example, Mardini et al. (2011) used MR to investigate the cerebral water content of 13 cirrhotic patients confronted with an ammonia challenge. The authors concluded that ammonia can directly drive changes in brain water distribution as a mechanism for cerebral edema development. Since cerebral astrocytes contain glutamine synthetase, the MR data suggest intracerebral formation of glutamine from ammonia. The authors also noted a rapid decrease in myo-inositol indicating that this organic osmolyte plays a protective role in HE by release from astrocytes in order to maintain cell volume. Other MR studies have suggested that not only does hyperammonemia induce low grade swelling in astrocytes but edema can also be detected in white matter (Keiding and Pavese 2013).

While there seems to be little doubt that cerebral edema, resulting from excessive accumulation of glutamine in astrocytes, is a major contributing factor to hyperammonemia-induced encephalopathy (especially in acute liver failure) other factors may also contribute. For example, there is considerable evidence for an ammonia-induced neuroinflammatory response in hyperammonemic liver disease. The evidence includes activation of microglia, together with increased synthesis in situ of the proinflammatory cytokines TNF, IL-1 $\beta$  and IL-6 (reviewed by Butterworth 2013). In addition, according to Häussinger and colleagues once the astrocytes lose their capacity to self regulate volume during hyperammonemia and excessive glutamine accumulation, low grade edema sets in, resulting in triggering of “a complex signaling cascade which relies on NMDA receptor activation, elevation of intracellular Ca<sup>2+</sup> concentration and prostanoid-driven glutamate exocytosis, which result in increased formation of reactive nitrogen and oxygen species (RNOS) through activation of NADPH oxidase and nitric oxide synthase. Since RNOS in turn promote astrocyte swelling, a self-amplifying signaling loop between osmotic- and oxidative stress ensues, which triggers a variety of downstream consequences” (Görg et al 2013).

4. Comments on the draft document section on ammonia in the exhaled air: It is not entirely clear what is the point of the discussion in the draft document regarding measurements of ammonia in exhaled air. What is the relevance to hyperammonemia, ingested ammonia or to long term exposure to gaseous ammonia? The discussion in the draft includes three references to Spaněl et al who have measured ammonia in the expired air of human volunteers. In the latest cited study by Spaněl et al. (2013) the authors measured exhaled ammonia after *acute* inhalation of ambient ammonia. About 70% of the inhaled ammonia was recovered in the exhaled air. However, endogenous ammonia is rapidly converted to glutamine in rat lungs (Cooper and Freed 2005) and presumably also in human lungs. Thus, the findings of Spaněl et al (2013) suggest that absorption of ammonia in lungs occurs in a compartment that does not readily mix with the metabolic pool of ammonia. This compartment is presumably mucous. In this context it has long been known that cave-dwelling bats can tolerate levels of ambient ammonia that would quickly overcome and kill most mammals. It is thought that mucous in the respiratory tract of bats affords protection (Studier 1966). The ammonia is absorbed by the mucous to be released later in “ammonia-less” air. Presumably, humans have less mucous in the

respiratory tract than do bats and protection against ambient ammonia by respiratory tract mucous is more limited.

Some comment here may be appropriate on the relationship between endogenous ammonia in the lungs and alveolar air. In 1959, two groups suggested that ammonia in alveolar air reflects the concentration of ammonia in the lungs (Robin et al. 1959; Jacquez et al. 1959). With the techniques available at the time it was not possible to measure ammonia in expired breath of normal experimental animals. However, Robins et al. (1959) were able to measure ammonia in alveolar air of anesthetized dogs administered ammonium acetate, and Jacquez et al. (1959) were able to measure ammonia in alveolar air of anesthetized dogs made chronically hyperammonemic by a portacaval shunt. In later studies, Reinyk et al. (2007) noted that comatogenic doses of sodium thiopental in rats produced hyperammonemia that was associated with an increased exhalation of ammonia. Breath ammonia analysis has also been carried out on patients with kidney disease as a potential estimator of the severity of the associated uremia (Davies et al. 2014). Thus, hyperammonemic syndromes (e.g. liver disease, kidney disease) result in increased lung ammonia that in turn is reflected in increased expiration of ammonia. However, there are caveats regarding interpretation of these studies that need to be discussed.

The amount of ammonia that equilibrates between the endogenous lung metabolic pool and alveolar air is likely to be quite small even under hyperammonemic conditions. For example, in the study by Cooper and Freed (2005) mentioned above, the authors measured the amount of label in exhaled air of anesthetized rats administered an intravenous dose of tracer quantities of [<sup>13</sup>N]ammonia. Despite the fact that at least 30% of the dose administered to anesthetized rats must have passed through the lungs within seconds very little label (~1 part in 1,000,000 of the administered dose) could be detected in the expired breath over a five minute period.

As pointed out in the draft document, ammonia measured in exhaled air can vary considerably depending on the route of exhalation. Ammonia exhaled from the mouth or oral cavity is largely attributed to the production of ammonia via bacterial degradation of food protein in the oral cavity or gastrointestinal tract and can be influenced by such factors as diet, oral hygiene and age. In contrast, ammonia concentrations in breath exhaled from the nose and trachea are lower and appear to better represent levels at the alveolar interface of the lung or tracheo-bronchal region and are thought to be more relevant to understanding systemic levels of ammonia in breath exhaled from the mouth. [In addition to the references quoted in the draft document (i.e. Schmidt et al. 2013; Smith et al. 2008; Larson et al. 1977) a recent article by Solga et al. (2013) should also be quoted. These authors found that the amount of ammonia in expired air depends heavily on temperature of the breath sample and breath analyzer, the pH of a mouth rinse and mode of breathing (mouth open versus mouth closed).]

In conclusion to this section, there is no doubt that ammonia in expired breath is increased in pathological conditions (such as liver disease and kidney disease) that give rise to hyperammonemia. However, because of confounding problems with “contaminating” ammonia in the expired air and difficulties associated with the actual measurement it may be difficult to correlate prior *chronic* exposure of individuals to ammonia with alveolar ammonia concentrations.

5. Comments on the draft document section on ingested ammonia. As noted in the draft document accidental or deliberate ingestion of ammonia-containing solutions/foods has occasionally been reported in the literature. These studies have described deleterious gastrointestinal effects. The draft document also describes several studies in which experimental animals have been subjected to ingestion of ammonia. These studies show damage to the intestinal mucosa. Because of the diffusibility of ammonia it is probable that ingested ammonia enters the circulation. Thus, in humans who have ingested ammonia solutions reported nausea, drooling and erythematous/edematous effects on the lips could be considered as systemic effects. However, the draft document does not consider the possibility that ingested ammonia may result in elevated blood ammonia levels and possible neurological consequences.

There are, in fact, several reports in the literature in which chronic gastrointestinal administration of ammonia to experimental animals results in elevated levels of circulating ammonia. For example, Pilbeam et al. (1983a,b) gavaged ammonia to normal rats and rats with a portacaval anastomosis (PCA; a model of chronic liver disease). The slow release of ammonia from the resin simulates chronic hyperammonemia. Marked hyperammonemia was noted in the animals administered the ammoniated resin, especially in the PCA rats. Severe neurological symptoms were noted in the PCA rats administered the ammoniated resin. Damage not only to astrocytes, but also to some oligodendrocytes and neurons, was noted with nuclear and cytoplasmic swelling (Pilbeam et al 1983a). Rats with a PCA fed ammoniated resin showed increased chloride content and  $\text{Na}^+:\text{K}^+$  ratio in the brainstem, and an increased chloride space in the brainstem (Pilbeam et al. 1983b). In other studies Grisolia and colleagues administered ammonium acetate (20% w/w) in the diet of rats to generate a simple model of chronic hyperammonemia (Azorin et al. 1989). The concentration of ammonia in the blood of these animals was increased threefold and there were marked increases of ammonia in brain, liver and muscle. Urea excretion increased two fold and brain glutamine increased twofold. In other studies from this group it was shown that chronic ammonium acetate in the diet of rats altered the mitochondrial ratio  $\text{NAD}^+/\text{NADH}$  in the brain (Kosenko et al. 1993).

Thus, there is strong evidence that following *chronic* oral/intestinal administration of ammonia to experimental animals (rats) blood ammonia and brain glutamine levels are greatly increased. Elevation of brain glutamine in humans via chronic exposure to oral ammonia is of potential concern, but there appear to be no published case histories where a patient has been subjected to chronic ammonia administration via the oral route. On the other hand, since there are many documented cases of acute ammonia exposure through the oral route it would be important to determine whether *acute* oral exposure to ammonia will result in elevated ammonia in the circulation that has the potential to deleteriously alter brain nitrogen homeostasis in humans. But there appear to be no relevant published animal studies, and the published human studies have been more concerned with the gastrointestinal effects than with possible neurological outcomes. There are studies described in the literature where acute liver failure results in elevated blood ammonia and encephalopathy. For example, acetaminophen overdose leads to rapid increases in blood ammonia, followed by coma (e.g. Brusilow and Cooper 2011). Valproate is a widely used, generally safe drug used in the treatment of epilepsy and some neuropsychiatric disorders. On rare occasions,

however, the drug can precipitate acute hyperammonemic encephalopathy (reviewed by Lewis et al. 2012). The point to be made here is that acute elevations of blood ammonia can induce coma in humans. Thus, if ingested ammonia is sufficiently concentrated the possibility exists that enough ammonia will enter the circulation to deleteriously affect the brain.

In conclusion to this section: Acute oral administration of ammonia is well documented to cause gastrointestinal effects in humans and experimental animals. However, there are no detailed studies of the effect of acute oral ammonia dosing on brain function. Nevertheless, it is likely that oral dosing will lead to increased blood ammonia. This is of concern because it is well documented that acute hyperammonemia from whatever cause can lead to brain dysfunction.

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