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Organ distribution of ^{13}N following intravenous injection of [^{13}N]ammonia into portacaval-shunted rats

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Abstract

Ammonia is neurotoxic, and chronic hyperammonemia is thought to be a major contributing factor to hepatic encephalopathy in patients with liver disease. Portacaval shunting of rats is used as an animal model to study the detrimental metabolic effects of elevated ammonia levels on body tissues, particularly brain and testes that are deleteriously targeted by high blood ammonia. In normal adult rats, the initial uptake of label (expressed as relative concentration) in these organs was relatively low following a bolus intravenous injection of [^{13}N]ammonia compared with lungs, kidneys, liver, and some other organs. The objective of the present study was to determine the distribution of label following intravenous administration of [^{13}N]ammonia among 14 organs in portacaval-shunted rats at 12 weeks after shunt construction. At an early time point (12 sec) following administration of [^{13}N]ammonia the relative concentration of label was highest in lung with lower, but still appreciable relative concentrations in kidney and heart. Clearance of ^{13}N from blood and kidney tended to be slower in portacaval-shunted rats versus normal rats during the 2–10 min interval after the injection. At later times post injection, brain and testes tended to have higher-than-normal ^{13}N levels, whereas many other tissues had similar levels in both groups. Thus, reduced removal of ammonia from circulating blood by the liver diverts more ammonia to extrahepatic tissues, including brain and testes, and alters the nitrogen homeostasis in these tissues. These results emphasize the importance of treatment paradigms designed to reduce blood ammonia levels in patients with liver disease.

Keywords

[^{13}N]Ammonia; Brain; Hepatic encephalopathy; Hyperammonemia; Portacaval shunt

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Introduction

Excess circulating ammonia is neurotoxic, and hyperammonemia is thought to be a major factor in the encephalopathy associated with several diseases, such as liver cirrhosis, inborn errors of the urea cycle, and various acidemias (e.g. [1–5]). Portacaval-shunting (PCS) increases blood and tissue ammonia levels, and the PCS rat is often used as an animal model of human liver disease (e.g. [6–10]). The concentration of ammonia in the portal vein in normal rats is ~1 mM, and the hepatic extraction efficiency of ammonia from the portal vein is ~100% [11]. Thus, under normal conditions little if any portal vein ammonia enters the general circulation and the liver maintains the level of circulating ammonia at relatively low levels, typically 100 μ M in rats (see Table 6 in [8]) and 40 μ M in humans (reference value for clinical laboratories). Following portacaval shunting, blood draining from the gut bypasses the liver and directly enters the systemic circulation. Diversion of ammonia-laden blood from the portal vein into the systemic circulation compounded by liver shrinkage and decreased ability of muscle to remove circulating ammonia, results in a 2–3-fold elevated concentration of ammonia in systemic blood in PCS rats. This, in turn, is associated with chronically-elevated brain ammonia levels after shunt construction [8]. However, it is not known whether long-term hyperammonemia alters the distribution of blood-derived ammonia nitrogen among body organs, and the goal of the present study was to gain some insights into ammonia-derived nitrogen metabolism by assessing uptake of tracer amounts of [13 N]ammonia and retention of 13 N in body tissues in PCS rats.

13 N is a positron emitter with a half-life of 9.96 min. The emitted positron annihilates a nearby electron giving rise to two coincidental (or almost coincidental) γ -rays that are easily detected and quantified by an external device. Two advantages of 13 N over 15 N (a non-radioactive isotope of nitrogen with a natural abundance of ~0.4%) to assess ammonia metabolism in short-term studies are that 13 N is radioactive so that the tissue distribution of label is relatively easy to measure and it is used in tracer amounts that do not alter blood and tissue ammonia levels and ammonia homeostasis (reviewed in [12]). 13 N has been useful for elucidation of ammonia metabolism in normal [13, 14] and hyperammonemic [10] rat brain, rat liver [15], and rat lung [16], as well as in positron-emission tomographic (PET) studies of cerebral ammonia uptake in patients with hepatic encephalopathy (HE) (e.g. [17]). Freed and Gelbard [18] determined the organ distribution of label after a bolus intravenous injection of [13 N]ammonia into normal, anesthetized adult male rats, and reported that most of the administered 13 N is cleared from the circulation within seconds, with a high extraction efficiency for most organs in the range of 60–100% in a single pass compared with brain (~40%) and lungs (~25%). Ammonia ingress for many tissues is via transport of NH_4^+ , whereas its entry into brain is largely by diffusion of NH_3 (and possibly some NH_4^+) across the blood-brain barrier (BBB) [2, 10, 12, 14, 19–22]. Recently, the water channel aquaporin 4 (AQP4) that is highly expressed in astrocytic endfeet [23] and is found in many body organs, including kidney, lung, muscle, and testes [24, 25], was identified as an NH_3 -permeable channel [26]. In contrast, the ammonium ion (NH_4^+) is not AQP4 permeable, and its plasma membrane passage may involve various K^+ transporters and channels ([26, 27] and references cited therein).

Patients with liver disease are sensitive to high dietary amino acid and protein content that causes release of high concentrations of ammonia into the general circulation (e.g. [28] and references cited therein), but it is not known how chronic liver dysfunction may influence whole-body distribution of ammonia nitrogen. The present study, therefore, evaluated the organ distribution of ^{13}N in PCS rats at intervals after a bolus intravenous injection of tracer concentrations of [^{13}N]ammonia and compared the results with those previously obtained in normal rats [18]. The results show that in PCS rats two target organs of liver disease, namely brain and testes, tend to take up relatively more ^{13}N and retain more ^{13}N over an extended time course compared to normal rats. On the other hand, a select group of other organs (spleen, pancreas, small intestine, and kidney) take up more ^{13}N during only the early intervals after [^{13}N]ammonia administration to PCS rats, whereas in other organs distribution of ^{13}N is relatively unaffected by shunting.

Materials and Methods

End-to-side portacaval anastomoses were constructed using the continuous suture procedure in adult male Wistar rats weighing ~240 g (Charles River, Wilmington, MA, USA) according to the method of Lee and Fisher [29] as previously described [9]. The animals had free access to Purina rat chow and water until ~1 h before the [^{13}N]ammonia study, which was carried out in the afternoon at about 12 weeks after shunt construction. Each rat was injected with sodium pentobarbital (~50 mg/kg, i.p.) at least 10 min before the study was initiated and kept continuously under sedation until euthanized at a timed interval after the injection of a bolus containing [^{13}N]ammonia (see below). Patency of the portacaval shunt was verified in each animal at the time of death by inspection of the liver and testes, both of which are shrunk by ~50% in successfully-shunted rats [29]. All animal use procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and the ethical guidelines of the institution.

Synthesis of [^{13}N]ammonia, the technique for injection of a bolus of [^{13}N]ammonia into a femoral vein, organ dissection, and radioisotope quantification of ^{13}N in various specimens or organs following euthanization were carried out as described previously [18]. Typically 0.25–0.5 mL of [^{13}N]ammonia in saline (~500 μCi , ~2 nmol/kg) was injected into each animal. It should be noted that the procedure for generating [^{13}N]ammonia [30] will theoretically result in a carrier-free tracer, but ammonia is a ubiquitous contaminant. Nevertheless, the amount of carrier ammonia in the preparation is typically small [14] so that injection of the bolus of [^{13}N]ammonia into the rats is unlikely to disturb the steady state level of ammonia in the circulation.

Results are expressed as percent of injected dose per whole organ (where measured) and as relative concentration, i.e., the ratio of tracer concentration in a tissue sample to that which would result from uniform distribution of the label throughout the body [31]. Relative concentrations were calculated as the fraction of the administered dose recovered in the specimen or organ divided by the fraction of body weight contained in that specimen or organ [31]. As discussed by Woodard et al. [31] expressing radioactivity content of a tissue as relative concentration has the advantage that it avoids the artifactual influence of dilution by body mass that is inherent in percent dose per specimen weight, and it facilitates

comparisons among subjects of different sizes or between normal and pathophysiological conditions where body mass may vary, such as normal and PCS rats. (Relative concentration has also been termed ratio to mean body concentration [16, 32]). For convenience, the term ammonia, except where noted, is used throughout the text to denote the sum total of NH_3 (ammonia free base) plus NH_4^+ (ammonium ion). The pK_a of ammonia is 9.2 at 37°C and moderate ionic strength. Thus, 1–2% of ammonia will be in the form of NH_3 at physiological pH values and temperature. The rate of proton exchange between NH_3 and NH_4^+ is extremely rapid compared to the transit time of blood through the brain (seconds). Therefore, rate of exchange between NH_4^+ and NH_3 is not limiting for ammonia (NH_3) diffusion across the blood-brain barrier.

All data presented below for ^{13}N are decay corrected to an arbitrary time and are expressed in terms of relative concentration. Relative decay-corrected amounts/concentrations (mean $\pm$ SEM) of ^{13}N in organs after intravenous injection of [^{13}N]ammonia into PCS rats obtained in the current study were compared to published relative concentrations after similar injections in normal rats [18] using two-tailed *t*-tests for each time point for each organ. However, because all organs at each time point of analysis are from the same groups of animals, a Bonferroni correction for multiple comparisons was applied to adjust the significance level downward from $p = 0.05$ to account for the number of comparisons (see Table legends). Note that the normal adult male rats (Sprague-Dawley strain) used in the previous study [18] averaged 315 g (range 191 – 420 g) and are of comparable age to those in the present study; the experiments in both studies were carried out in the afternoon under similar conditions. Freed and Gelbard [18] did not identify the rat chow used in their studies, but it was probably Purina chow that was used in the present study and in those cited in Table 1. Unpublished pilot experiments by N. F. Cruz and T. E. Duffy in which PCS rats were fed either the standard or a high-protein rat chow after shunt construction revealed no significant difference in arterial blood ammonia levels when blood samples were drawn from awake, non-fasted rats at ~3–5h after catheter implantation.

Results

Previous studies consistently reported sustained, 2–3-fold increases in arterial blood and brain ammonia levels in both awake and N_2O -sedated (70–75% $\text{N}_2\text{O}/25\text{--}30\%\text{O}_2$), paralyzed-ventilated PCS rats, with higher ammonia levels in brain than in blood (e.g., [6, 8–10, 33, 34]). Awake PCS rats exhibit 2–3-fold higher blood and brain ammonia levels than do controls within one week after portacaval shunting, and they remain elevated for at least 14 weeks (Table 1). Ammonia levels were not measured in the present study, but blood and brain ammonia levels in the ~12 week PCS rats are anticipated to be elevated by 2.6–3.4-fold and ~3-fold, respectively (Table 1). Note, however, that blood and brain ammonia and glucose concentrations can vary considerably with the procedure used to construct the portacaval shunt, especially at short time intervals following the surgery [8]. Moreover, ammonia levels can have a large impact on physiological and metabolic changes, such as cerebral blood flow and glucose utilization that depend in part on the time after shunting [8]. Based, in part, on these previous studies 12 weeks was considered sufficient time post-surgery for the animals to adapt metabolically and physiologically to the shunt procedure

and to chronic hyperammonemia. Accordingly, 12 week post-surgery was chosen for injection of [^{13}N]ammonia into the PCS rats.

The ^{13}N label is removed from the blood within seconds after an intravenous bolus injection of [^{13}N]ammonia into anesthetized, normal or portacaval-shunted adult male rats [16, 18] (Tables 2, 3; Figs. 1,2). Twelve seconds was chosen as the first sampling time using the prior reasoning of Freed and Gelbard [18] that was based on the transit time of blood (i.e. average time for a blood corpuscle to make one complete circuit) in the normal, pentobarbital-anesthetized adult male rat of maximally ~7–8 sec [16]. Although a small fraction of the label in blood is present as ^{13}N -metabolites within 6 sec [16], the radioactivity in any given tissue specimens at 12 sec is an approximate function of the single pass extraction of [^{13}N]ammonia by that tissue [18]. By 12 sec, 22% of the injected dose was present in the blood of the PCS rats (assuming a blood mass of 6% that of the whole body [16]) compared with 11% in the normal rats (calculated from the data in Fig. 2A). This slower rate of ^{13}N removal from the blood in PCS rats is likely due, in large part, to the reduced mass of the liver and decreased capacity of muscle to remove ammonia.

When ^{13}N levels are expressed as decay-corrected percent dose per organ for selected organs at intervals after the bolus injection of [^{13}N]ammonia into PCS rats, the organs with highest and lowest quantities of label, fastest clearance, and gradual accumulation and retention of labeled metabolites are readily apparent (Table 2). At 0.2 min the highest fractions (8–17%) of the injected dose were in the lungs, kidneys, and small intestine, intermediate levels (2–4%) were in the large intestine, liver, and heart, and smaller amounts (<1%) were in other organs; together these 11 organs accounted for about half of the injected dose (Table 2). With increasing time after the injection, ^{13}N levels in the organs with highest initial uptake fell substantially, except for the liver where it increased by ~50% at 50 min; this is of interest because the PCS liver is about half the normal weight [29]. In general, the level of label in the other organs gradually increased as a function of time, and some ^{13}N (~7% of the injected dose by 50 min) accumulated in urine (Table 2).

Relative concentrations of ^{13}N in 14 tissues of PCS rats as a function of time after intravenous [^{13}N]ammonia injection are shown in Table 3 and compared in Figs. 1 and 2 to relative concentrations previously obtained in normal rats by Freed and Gelbard [18]. Note the large differences in the magnitudes of the values for % dose (Table 2) versus relative concentration (Table 3) in the current study. To emphasize the large organ- and time-dependent differences in ^{13}N accumulation, the rank order (high to low for the normal rats along with the corresponding values for PCS rats, plotted left to right) of relative concentrations in various tissues is illustrated in Fig. 1 at three time points after the bolus injection of [^{13}N]ammonia. At 0.2 min, which approximates the first pass of the injectant to the body organs, label uptake into lungs, kidneys, and heart was similar in normal and PCS rats and was very high, about 10–40 times greater than in most other organs (Fig. 1). By 5 min after the injection, however, ^{13}N levels in the high-uptake organs had fallen substantially, and significantly higher-than-normal relative concentrations were evident in spleen, pancreas, and testes of the PCS rats, with a tendency to be elevated above normal in other organs (Table 3). By 30 min, many organs had relative concentrations in the range of 2–4, some had intermediate levels in the range of 1–2, and only a minority had values <1

(Fig. 1). At later times most differences between PCS and normal rats had diminished or disappeared, with label in the brain of PCS rats eventually becoming significantly elevated at 50 min, whereas testes had significantly increased levels of ^{13}N at 5–10 min after the bolus injection (Table 3). Note that if the ^{13}N were uniformly distributed among all organs, the relative concentration would be 1 (horizontal line in all 3 panels in Fig. 1). Most of the organs investigated exhibited a relative concentration >1 by 30 min, whereas skin, calvarium, blood, and testes had a relative concentration <1 (Fig. 1).

Comparisons of the time courses of relative concentrations of ^{13}N in various specimens from normal and 12-week PCS rats are shown in Fig. 2 (also see Table 3 for p values). After high initial uptake, ^{13}N levels fell rapidly in lung, heart, and kidney in both normal and shunted rats (Fig. 2A). Clearance from blood tended to be somewhat slower in PCS rats compared with normal rats at 2 and 5 min and in kidney from 2 to 10 min, at which time the differences reached statistical significance. After 10 min the relative concentrations of ^{13}N in these four tissues reached relatively stable levels in both groups, with the highest relative concentration in the heart at 50 min (Fig. 2A).

Notably, relative concentrations of ^{13}N in the two primary organs pathologically targeted during chronic hyperammonemia (i.e. brain and testes) in PCS rats, tended to exceed those in normal rats at early times and remain higher than the values in normal rats throughout most of the time course. However, relative concentrations at only a few time points were statistically significantly different between the two groups (Fig. 2B; Table 3). On the other hand, spleen, pancreas, small intestine, and submaxillary salivary glands tended to have higher-than-normal relative concentrations of ^{13}N at early, but not later time points (Fig. 2B). Of the tissues listed in Fig. 2B, testes had the lowest relative concentration of ^{13}N by 50 min for both groups of rats. Time courses of relative concentrations of ^{13}N were generally comparable in normal and PCS rats for skin, abdominal muscle, liver, and calvarium; they increased with time, and then stabilized (Fig. 2C). Thus, two general, broad categories of ^{13}N uptake/distribution may be discerned, 1) initially very high uptake of label followed by rapid label release, and 2) intermediate uptake of label that increases with time. At pseudo steady state ($\sim 10 - 50$ min) post injection of [^{13}N]ammonia brain, testes, pancreas (Fig. 2B) and possibly kidneys (Fig. 2A) accumulate tracer levels of ^{13}N to a greater extent in PCS rats than in normal rats.

Discussion

Almost 60 years ago Duda and Handler showed that the major *whole body* metabolites of [^{15}N]ammonia in rats over a 15-min time frame are L-[*amide*- ^{15}N]glutamine and, to a lesser extent, [^{15}N]urea [35]. Moreover, the rate of urea synthesis was found to represent a fixed percentage of ammonia over a wide concentration range [35]. These earlier findings are consistent with the present findings that uptake of tracer amounts of [^{13}N]ammonia and relative concentrations of ^{13}N in many tissues are remarkably similar in normal rats and PCS rats. However, the temporal aspects of partitioning of ammonia into specific body organs differ substantially in normal and PCS rats, as revealed by the two [^{13}N]ammonia tissue distribution studies used to assess nitrogen homeostasis ([18]; Tables 2,3; Fig. 2). Presumably, the glutamine synthetase (glutamate ammonia ligase) activity of tissues (with

the notable exception of liver and skeletal muscle) is largely intact in the PCS rats, thereby accounting for similar metabolic “trapping” of [^{13}N]ammonia in normal and PCS rats in these tissues. Although the liver still functions effectively in removing and metabolizing ammonia in PCS rats, its greatly diminished size, together with disrupted skeletal muscle metabolism of ammonia, ensures that PCS rats are hyperammonemic (Table 1).

The present results are also consistent with the previous observations that lungs [16, 18], skeletal muscle [36], and kidney [37] are prominent sinks for removal of circulating ammonia and its conversion to glutamine that can be utilized by other organs. Although the uptake of [^{13}N]ammonia in several tissues may be similar in normal and PCS rats, the fact that the concentration of circulating ammonia in PCS rats is far greater than in normal rats ensures that long-term nitrogen homeostasis is disrupted in PCS rats, and many organs in PCS rats take up more unlabeled ammonia or its metabolites compared to normal rats. AQP4 may have a role in NH_3 uptake into astrocytes, kidney, and other organs/cells in both normal and PCS rats, since its expression can be influenced by ammonia exposure [26, 38, 39]. This is of special importance in the brain where increased ammonia ingress across the BBB is also reflected in an increase in ammonia ingress into the astrocytes due to the extraordinarily high permeability of the astrocyte cell membranes to NH_4^+ [40] and possibly to increased NH_3 uptake across the AQP4 channel. This increase entry of ammonia into the astrocytes leads to increased synthesis of glutamine. However, the activity of glutamine synthetase is insufficient to remove all the ammonia entering the astrocyte compartment and, as noted above, both glutamine and ammonia are increased in the brains of hyperammonemic rats (e.g., [2, 4], and references cited therein).

Rapid ^{13}N uptake and disposition in lung, kidney, and heart of PCS rats

As noted above, most of the [^{13}N]ammonia is rapidly cleared from blood after the intravenous injection in normal rats. This was also found to be the case in the PCS rats. In both groups of rats ^{13}N uptake into three organs, namely, lung, heart, and kidney, greatly exceeded that into other organs and was followed by rapid release of label (Fig. 2A), whereas most other tissues accumulated or retained ^{13}N -labeled metabolites over an extended period. Glutamine synthetase activity is widespread in mammalian tissues [41], and it is, therefore, most likely that [^{13}N]ammonia entering the tissues is rapidly metabolically trapped as glutamine in a single pass. This is reflected by the presence of little free [^{13}N]ammonia in blood (~14% of the total radioactivity) at 2 min following intravenous administration of [^{13}N]ammonia to normal male rats [16]. Thus, changes in relative concentration of ^{13}N after about a minute reflect 1) exchange of label between unmetabolized [^{13}N]ammonia in tissue and the greatly diminished blood [^{13}N]ammonia pool, and 2) the redistribution of labeled metabolites between blood and tissues.

Lungs takes up a very large fraction of circulating ammonia in normal rats, and quickly converts it to glutamine that is then released into the circulation [16]. Thus, the lung is suggested to be important in maintaining normal whole-body glutamine homeostasis [18] because the glutamine released is an important source of energy and nitrogen for rapidly dividing cells, such as intestinal cells [42], and, perhaps, for normal skin function (see below). In the present work, the lungs in the PCS rats were also shown to rapidly take up a

large fraction of circulating [^{13}N] ammonia, followed by rapid release of label (Fig. 1), presumably as L-[amide- ^{13}N]glutamine. [^{13}N]Ammonia administered to human volunteers is also rapidly taken up into lungs, followed by rapid clearance, a process that is delayed in smokers [43].

The relatively high concentration of ^{13}N in the PCS kidney, 11% of the injected dose at 12s, and 6.8% in urine at 50 min, is similar to the respective values of 13.6% and 7.6% in normal rats [18], indicating that turnover of the total pool of blood ammonia nitrogen into urea nitrogen is equivalent in both groups. The relatively large amount of label in the kidneys presumably reflects the high cardiac output to the kidneys; human kidneys represent 0.4% of the body weight, yet receive 25% of the cardiac output [44]. Evidence from isolated perfused rat kidneys suggests that kidney may also provide a substantial amount of glutamine to the circulation [37]. In humans with cirrhosis, reduced renal ammonia release and enhanced ammonia uptake into muscle are important mechanisms for maintaining ammonia homeostasis [45], perhaps contributing to greater retention of ^{13}N in the kidneys of the PCS rats during early time intervals after the bolus injection of [^{13}N]ammonia compared to that of normal rats.

The heart rapidly took up then released ^{13}N in the PCS rat, with a time course similar to that noted for normal rats (Fig. 1). Label release was presumably due to glutamine efflux, since ^{13}N trapping in heart is mainly via glutamine synthesis [43, 46]. Compared with heart, abdominal muscle accumulated label more slowly and to a lesser extent. Skeletal muscle was not assessed in the previous [18] or present studies, but glutamine synthetase specific activity increases in skeletal muscle of PCS rats [47]. In humans, skeletal muscle is a major sink for removing circulating ammonia [36], presumably via its metabolism to glutamine. Liver cirrhosis is often associated with cachexia, and, indeed, uptake of label into the thigh area following administration of [^{13}N]ammonia was considerably reduced in patients with severe liver disease [36]. Nevertheless, a large fraction of ammonia taken up by forearm muscle in patients with liver disease is released as glutamine [48].

Although [^{13}N]ammonia is rapidly removed from the blood in both normal and PCS rats the relative concentration of label does not approach zero. Instead, the relative concentration reaches a pseudo steady state level of ~0.6 within 10 minutes (Fig. 2A), indicating that most of the label in the blood at this time is in the form of metabolites, the most prominent of which is glutamine. Taken together, rapid metabolism of ammonia with label release, presumably as glutamine, by lung, kidney, and heart (Figs. 1, 2), and skeletal muscle [48, 49] probably account for most of the elevated glutamine levels in the blood of 4 and 8-week PCS rats [6]. Other tissues in the PCS rats that exhibit higher initial ^{13}N uptake compared to that of normal rats followed by efflux of label (e.g., spleen, and pancreas; Fig. 2) probably also contribute to circulating glutamine levels. In the case of the small intestine, there is most likely a net *uptake* of glutamine with release of label from glutamine nitrogen in the form of ammonia, citrulline, alanine and proline [42] during the first 10 min (Fig. 2). These nitrogenous compounds may contribute to a small extent to the ^{13}N -metabolite pool in the blood at 10 min post injection of the [^{13}N]ammonia bolus. Note that tracer amounts of ^{13}N will not alter the steady-state level of ammonia or glutamine in blood, and there will not be

transient changes in blood glutamine levels secondary to [^{13}N]glutamine release from high-uptake organs to blood.

^{13}N disposition in liver of PCS rats

Livers of PCS rats are half-normal size but are not overtly diseased as in cirrhotic patients with HE. The liver still has an important role in ammonia clearance because the hepatic relative ^{13}N concentrations in PCS rats are at least as great as in normal rats even though their urea cycle capacity is not high enough to prevent hyperammonemia. In normal rats about 12% of blood flow to the liver is via the hepatic artery [50] compared with a higher proportion in dogs, sheep, and humans (about 20–35%) [51–53], and the remainder is via the portal vein. However, in PCS rats at early times after [^{13}N]ammonia injection into a femoral vein, the label is expected to arrive at the liver exclusively via the hepatic artery, which is intact in the PCS rats and which maintains a 2.7-fold increase in blood flow per gram of liver compared to that of normal liver [54], perhaps explaining the tendency for a higher-than-normal ^{13}N relative concentration in the livers of PCS rats in the first 5 min after the bolus injection of [^{13}N]ammonia. In normal rats, label will eventually reach the liver via the portal vein, whereas in the PCS rats this is not possible because the portal vein is connected to the vena cava, thereby by-passing the liver. Thus, the tendency for higher circulating blood ^{13}N levels in the PCS rats at early time points (Fig 2A) leads to greater uptake of label into kidneys, spleen, pancreas, small intestine, and testes, and to a tendency for somewhat higher uptake into brain, salivary glands, and liver compared to the uptake values in normal rats. Extrahepatic tissues convert circulating ammonia, in part, to glutamine, which as noted above, is a major energy source for the gut, where its metabolism liberates ammonia that is normally delivered to the liver by the portal vein and incorporated into urea [55]. In this way, extrahepatic ammonia nitrogen is incorporated into urea nitrogen and eliminated from the body in the urine. However, at least a quarter of urea generated by the urea cycle in humans and other mammals is probably converted back to ammonia by the action of intestinal bacterial ureases [56], and changes in intestinal flora may occur in PCS rats that influence ammonia levels. Thus, in normal animals the label in liver rises steadily after an i.v. bolus of [^{13}N]ammonia, reaching a plateau at 5–20 min, with a % dose in liver of ~14% and relative concentration of ~3.5–4 [18], a level 2.7 times that at 12s (Fig. 2C). In contrast, the peak percent dose in the liver of PCS rats was 7.6% at 5–10 min, only 1.9 times the 12s value (Table 2). These lower values at later time points in PCS rats presumably reflect the fact that secondarily-derived [^{13}N]ammonia (or [^{13}N]ammonia-derived metabolites) generated in the gut is no longer directly accessible to the liver via the portal vein. The lower liver mass in the PCS rats contributes to a reduced ‘sink’ that is still a participant in whole-body nitrogen metabolism.

[^{13}N]Ammonia entering the liver via the portal vein in normal rats is rapidly metabolized mainly to glutamate, aspartate, alanine, urea cycle intermediates and, to a lesser extent, glutamine [15]. Liver ammonia metabolism is compartmentalized, with the urea cycle present in periportal cells and glutamine synthetase present in a small ring of perivenous hepatocytes [55]. The urea cycle in periportal cells is considered to be a low affinity, high capacity system for removing portal vein ammonia, whereas the perivenous glutamine synthetase is a lower capacity, but higher affinity backup system to trap ammonia that

escapes the urea cycle [55, 57]. Since the complete urea cycle is present only in liver, extrahepatic tissues rely on the glutamine synthetase reaction for removal of potentially-toxic ammonia, and the released glutamine is taken up by periportal liver cells wherein glutaminase hydrolyzes the glutamine amide to ammonia for urea synthesis [55]. In PCS rat, total liver glutamine synthetase activity is decreased by up to 90% [47] reflecting a large decrease in its activity in perivenous hepatocytes [58]. Lack of this backup system for removing portal vein ammonia undoubtedly contributes to systemic hyperammonemia in the PCS rats. But even in the normal rat the liver glutamine synthetase would trap, at most, 30% of portal vein ammonia [55], and perhaps even less in our hands [15]. Thus, decreased ammonia removal by glutamine synthetase in the livers of the PCS rats may not have much of an influence on trapping of ammonia in the livers of the PCS rats. Other workers have estimated that the urea cycle removes 80–87% of ammonia reaching the liver in the PCS dog [59]. This is reinforced by other studies in PCS dogs in which urea synthesis was similar in experimental and control liver slices [60]. To sum up, ammonia levels rise in PCS rats owing to 1) a shrunken liver, 2) shunting of portal blood to the general circulation, and 3) a probable decrease in removal of circulating ammonia by skeletal muscle, and 4) perhaps to a lesser extent due to decreased glutamine synthetase in perivenous hepatocytes.

¹³N disposition in skin of PCS rats

Skin accounts for ~15% of total body weight in normal humans [61], and although, in the present study, the relative concentration of ¹³N in skin is low, the sheer size of the skin “compartment” suggests that, along with skeletal muscle, skin may be a major organ for the removal of circulating ammonia in both normal and PCS rats. Skin has a high content of glutamine synthetase [62], and skin has an extraordinarily-high content of 5-oxoproline (2-pyrrolidone-5-carboxylate; pyroglutamate) [63]; glutamine is an important precursor of this compound [64]. A second possible contributor to the early-time increase in relative concentration of ¹³N in skin is uptake of [¹³N]glutamine formed in and released from other tissues (e.g., lung).

Ammonia entry and metabolism in brain of PCS rats

Ammonia enters the brain largely by diffusion across the BBB, and its *rate of entry* is directly proportional to blood ammonia concentration [14, 20]. The brain uptake index (BUI) (i.e., the uptake of a test compound relative to a freely-diffusible radiotracer with 100% influx into brain in a single passage) of [¹³N]ammonia in anesthetized rats is ~40% [20], whereas it is ~25% in awake rats [10, 14]. Studies of acutely-hyperammonemic dogs [19] and monkeys [21, 22] suggested that fractional cerebral extraction of [¹³N]ammonia is largely independent of ammonia concentration at constant blood pH and blood flow. Indeed, the BUI of [¹³N]ammonia was invariant over at least a 1000-fold concentration range within the bolus (at constant pH) [14], explaining the similar BUI values in awake 12-week PCS rats and normal, awake adult rats [10]. These findings are consistent with the similar relative concentrations of label in brain of PCS and normal rats at 12s after the injection, but they do not explain the increases over the next 10 min (Fig. 2). Since the BBB possesses a carrier for L-glutamine [65], the time-dependent increase of ¹³N in brain is most likely due to uptake of L-[amide-¹³N]glutamine released from extracerebral tissues. However, there is some net release of unlabeled glutamine from PCS brain [6], suggesting BBB exchange of labeled

glutamine with unlabeled glutamine. The larger relative levels of ^{13}N in brain and testes may be due to increased ammonia and glutamine uptake and larger amounts of circulating [^{13}N]glutamine in PCS rats.

Ammonia entering the brain is normally detoxified within seconds by its incorporation into glutamine amide [10, 14]. Hyperammonemia is associated with increased levels of brain glutamine, especially in astrocytes ([66] and references cited therein) resulting in part from 1) increased glutamine synthesis, and perhaps to 2) decreased egress of glutamine from the astrocytic compartment together with altered glutamine metabolism [4, 67]. However, the brain's capacity to increase glutamine synthesis in the face of increased ammonia uptake is somewhat limited [4, 10, 67, 68], causing brain levels of ammonia and glutamine to rise in association with a spectrum of abnormalities of brain function [1, 2], including potential disturbances of cerebral water balance, ion transport, neurotransmitter function, oxidative stress, and inflammatory responses (e.g. [68–70]), and time-dependent changes in local rates of glucose utilization ([9] and references cited therein).

Concluding comments

The present findings reveal temporal differences in ^{13}N uptake and [^{13}N]metabolite retention in brain, testes, kidney, spleen, pancreas, and small intestine in PCS rats compared to normal rats, indicating that chronic hyperammonemia has organ-specific effects on ammonia homeostasis. These data underscore the importance of treatment regimens in HE patients designed to lower blood ammonia and perhaps to stimulate the ammonia-removing mechanisms of skeletal muscle, kidneys, skin, and lungs. Patients with liver disease who ingest an amino acid-rich drink or meal would exhibit a slower but more prolonged rise in blood ammonia levels than in normal individuals, and its partitioning into the target organs is expected to be greater in magnitude and longer in duration, emphasizing the importance of dietary control of protein and amino acid ingestion.

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Abbreviations

BBB	blood-brain barrier
BUI	brain uptake index
HE	hepatic encephalopathy
PCS	portacaval shunt
PET	positron-emission tomography

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Fig. 1. Relative concentrations of ^{13}N in various tissues at intervals after intravenous injection of $[^{13}\text{N}]\text{ammonia}$

Relative concentrations for tissues of PCS rats are from Table 3, and values for normal rats are from Freed and Gelbard [18]. Values are means $\pm$ SEM. To emphasize the large differences in ^{13}N uptake among body organs and substantial changes within and among organs at intervals after the bolus injection, values are plotted according to rank order (high to low) for the normal values, along with the corresponding values for PCS rats. Note the change in scales for the relative concentrations and the changes in rank order of tissues at the three time points after the $[^{13}\text{N}]\text{ammonia}$ injection. *PCS is significantly different from

normal (see Table 3 for details). The horizontal lines identify a relative concentration of one, i.e. assuming a uniform distribution of label throughout the body.

Fig. 2. Time courses for label uptake, clearance, and accumulation in blood and various organs following a bolus injection of [^{13}N]ammonia

Data for relative concentrations of ^{13}N in tissues of portacaval-shunted rats (PCS) from Table 3 are compared to normal values from Freed and Gelbard [18]; values are means $\pm$ SEM. *Given the number of comparisons made, the Bonferroni correction rendered a significance level of $p < 0.004$ ($0.05/14$) (see Table 3). **A**, Organs that rapidly take up label. **B**, Organs with different time courses for label accumulation in normal versus PCS rats. **C**, Organs with similar time courses for label accumulation in both groups; note that mean values for abdominal muscle tended to be lower than those of normal rats between 10–30 min, but the differences were not statistically significant (see the Discussion).

Table 1

Ammonia concentrations in control and portacaval-shunted (PCS) rats

Treatment	Ammonia concentration ^d		
	Arterial Blood ^b	Arterial Blood ^c	Brain ^c
Control			
Fed	0.085 ± 0.003	0.067 ± 0.011	0.182 ± 0.023
Fasted		0.143 ± 0.015	0.229 ± 0.048
Portacaval Shunt			
2 days	0.120 ± 0.004 ^d		
1 week	0.174 ± 0.023 ^d	0.272 ± 0.056 ^d	0.375 ± 0.120
4 weeks	0.206 ± 0.011 ^d	0.249 ± 0.093 ^d	0.543 ± 0.262
8 weeks	0.213 ± 0.006 ^d	0.294 ± 0.103 ^d	0.670 ± 0.319 ^d
12 weeks	0.225 ± 0.022 ^d	0.230 ± 0.124 ^d	0.554 ± 0.235 ^d
14 weeks ^c		0.193 ± 0.066 ^{c, d}	0.551 ± 0.185 ^{c, d}
14 weeks ^e		0.158 ± 0.031 ^{d, e}	0.274 ± 0.046 ^{d, e}

^aAmmonia concentrations in whole arterial blood and brain are expressed as $\mu\text{mol/mL}$ and $\mu\text{mol/g}$, respectively. Arterial blood samples were drawn ~3–5h after insertion of femoral catheters from awake rats that had been fed ad libitum with Purina rat chow, and brains were obtained by freeze-blowing. Data from ^b [9], ^c [8] and ^e [10]. Values are means $\pm$ ^{b, e} SEM or $\pm$ ^c SD for 4–24 rats/group.

^dSignificantly different from fed controls (see original reports for details). Note that collateral vasculature becomes evident at later times after portacaval shunting, causing blood and tissue ammonia levels to fall compared with the 4–8 week interval.

Table 2

Decay-corrected ^{13}N distribution expressed as percent of injected dose in various tissues after intravenous injection of ^{13}N ammonia into portacaval-shunted rats

Tissue	Percent of injected dose per whole organ Time after intravenous injection (min)					
	0.2	2	5	10	30	50
Heart	2.69 ± 0.15 [0.72]	1.78 ± 0.14 [0.88]	2.14 ± 0.30 [0.21]	1.29 ± 0.04 [0.14]	1.40 ± 0.08 [0.54]	1.24 ± 0.07 [<0.05]
Lungs	17.1 ± 1.7 [0.42]	7.5 ± 1.1 [0.17]	3.53 ± 0.62 [<0.05]	1.20 ± 0.10 [<0.006]*	0.96 ± 0.13 [0.07]	1.02 ± 0.136 [0.07]
Liver	3.98 ± 0.36 [0.35]	7.0 ± 1.2 [0.25]	7.6 ± 1.2 [<0.01]	7.6 ± 2.2 [0.14]	6.8 ± 0.26 [<0.05]	6.30 ± 0.29 [<0.006]*
Spleen	0.93 ± 0.18 [0.39]	1.88 ± 0.42 [0.05]	1.68 ± 0.14 [<0.006]*	1.19 ± 0.11 [<0.01]	0.435 ± 0.034 [0.20]	0.522 ± 0.024 [0.02]
Kidneys	10.7 ± 1.2 [0.12]	10.5 ± 1.0 [0.14]	6.15 ± 0.68 [<0.05]	3.80 ± 0.45 [<0.05]	2.58 ± 0.24 [<0.05]	2.15 ± 0.22 [0.33]
Testes	0.214 ± 0.037 [<0.01]	0.311 ± 0.034 [0.62]	0.357 ± 0.027 [0.54]	0.420 ± 0.033 [0.69]	0.409 ± 0.045 [0.84]	0.367 ± 0.039 [0.78]
Submaxillary salivary glands	0.202 ± 0.045 [0.20]	0.328 ± 0.055 [0.83]	0.373 ± 0.061 [0.77]	0.409 ± 0.082 [0.23]	0.369 ± 0.079 [0.14]	0.430 ± 0.075 [0.17]
Brain	0.488 ± 0.018 [0.23]	0.685 ± 0.063 [0.97]	0.937 ± 0.078 [0.14]	1.02 ± 0.11 [0.43]	0.896 ± 0.044 [0.07]	0.914 ± 0.042 [<0.05]
Stomach [†]	0.88 ± 0.15					
Small intestine [†]	8.0 ± 1.6					
Large intestine [†]	2.05 ± 0.26					
Urine						6.78 ± 0.99 [‡]
Rats per group	5	4	4	3	4	4

Values are means ± SEM with n provided in the bottom row and the p value for comparison against normal rats provided in square brackets. Data are from the same rats described in Table 3.

[†]With contents.

* Significantly different from values in normal rats provided in the study of Freed and Gelbard [18]; given the number of comparisons made, the Bonferroni correction renders a significance level of $p < 0.006$ (0.05/8) (see the Methods section).

[‡]The percent of injected dose in the bladder at 50 min after a bolus administration of ^{13}N ammonia in a previous study with normal adult, male rats was 7.6 ± 1.2 [18].

Decay-corrected ^{13}N distribution expressed as relative concentration after intravenous injection of [^{13}N]ammonia into portacaval-shunted rats

Table 3

Tissue	Relative concentration Time after intravenous injection (min)					
	0.2	2	5	10	30	50
Blood	3.6 ± 1.1 [0.12]	1.48 ± 0.05 [0.02]	1.10 ± 0.08 [<0.01]	0.679 ± 0.037 [0.51]	0.541 ± 0.018 [0.06]	0.548 ± 0.029 [0.30]
Heart	8.46 ± 0.74 [0.43]	5.31 ± 0.70 [0.75]	5.72 ± 0.56 [0.75]	3.85 ± 0.53 [0.11]	4.19 ± 0.27 [0.95]	3.93 ± 0.36 [0.81]
Lungs	40.4 ± 5.8 [0.88]	18.6 ± 3.1 [0.53]	8.2 ± 1.7 [0.12]	2.76 ± 0.32 [0.09]	2.24 ± 0.21 [0.18]	2.24 ± 0.28 [0.17]
Liver	2.18 ± 0.36 [0.10]	3.73 ± 0.43 [<0.05]	4.63 ± 0.68 [0.16]	3.80 ± 0.66 [0.87]	3.38 ± 0.26 [0.40]	2.74 ± 0.29 [0.76]
Spleen	3.38 ± 0.51 [0.23]	6.60 ± 0.42 [<0.004]*	6.67 ± 0.70 [<0.004]*	3.51 ± 0.50 [0.08]	1.59 ± 0.05 [0.22]	1.52 ± 0.12 [0.35]
Pancreas	5.15 ± 0.63 [0.67]	5.18 ± 0.63 [<0.01]	5.39 ± 0.35 [<0.004]*	4.10 ± 0.18 [<0.01]	3.41 ± 0.10 [0.21]	3.84 ± 0.76 [0.21]
Small intestine (-contents)	5.6 ± 1.2 [0.06]	5.20 ± 0.36 [<0.004]*	4.16 ± 0.35 [<0.01]	2.55 ± 0.22 [0.11]	1.95 ± 0.11 [0.89]	2.18 ± 0.19 [0.57]
Kidneys	15.6 ± 2.0 [0.28]	14.8 ± 0.8 [<0.01]	8.37 ± 0.85 [<0.01]	5.23 ± 0.38 [<0.004]*	3.56 ± 0.27 [0.06]	3.24 ± 0.21 [0.07]
Testes	0.325 ± 0.053 [0.22]	0.522 ± 0.032 [0.54]	0.642 ± 0.054 [<0.004]*	1.599 ± 0.016 [<0.004]*	0.605 ± 0.065 [0.03]	0.626 ± 0.069 [0.05]
Abdominal muscle	0.396 ± 0.054 [0.08]	0.55 ± 0.12 [0.11]	0.74 ± 0.23 [0.97]	0.64 ± 0.13 [0.12]	0.897 ± 0.016 [0.03]	1.054 ± 0.073 [0.68]
Submaxillary salivary glands	1.63 ± 0.41 [0.82]	2.81 ± 0.51 [0.03]	2.84 ± 0.39 [<0.05]	3.38 ± 0.55 [0.58]	3.20 ± 0.49 [0.74]	3.70 ± 0.58 [0.66]
Skin	0.206 ± 0.041 [0.02]	0.454 ± 0.020 [0.37]	0.548 ± 0.037 [0.17]	0.748 ± 0.095 [0.70]	0.681 ± 0.018 [0.71]	0.687 ± 0.019 [0.57]
Calvarium	0.59 ± 0.19 [0.20]	0.691 ± 0.087 [0.25]	0.732 ± 0.039 [<0.01]	0.627 ± 0.037 [0.22]	0.501 ± 0.062 [0.26]	0.463 ± 0.049 [0.14]
Brain	0.969 ± 0.030 [0.45]	1.51 ± 0.13 [<0.01]	1.78 ± 0.15 [<0.05]	1.92 ± 0.25 [0.46]	1.85 ± 0.04 [<0.05]	1.95 ± 0.09 [<0.004]*
Body Wt. (g)	409 ± 25	439 ± 23	404 ± 22	405 ± 27	431 ± 3.1	431 ± 19
Days after shunt	91.6 ± 2.1	86.3 ± 2.8	90 ± 1	84.7 ± 4.3	86.8 ± 3.1	81.3 ± 1.4
Rats/group	5	4	4	3	4	4

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Relative concentration is the fraction of the administered dose in the specimen divided by the fraction of body weight contained in the specimen [31, 32]. Values are means $\pm$ SEM with the p value for comparison against normal rats in square brackets. Data are from the same rats described in Table 2.

* Significantly different from the values in normal rats in the study of Freed and Gelbard [18]; given the number of comparisons made, the Bonferroni correction renders a significance level of $p < 0.004$ (0.05/14) (see the Methods section).