Hyperammonaemia does not impair brain function in the absence of net glutamine synthesis

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1. It has been established that chronic hyperammonaemia, whether caused by portacaval shunting or other means, leads to a variety of metabolic changes, including a depression in the cerebral metabolic rate of glucose (CMR_{Glc}), increased permeability of the blood–brain barrier to neutral amino acids, and an increase in the brain content of aromatic amino acids. The preceding paper [Jessy, DeJoseph & Hawkins (1991) *Biochem. J.* 277, 693–696] showed that the depression in CMR_{Glc} caused by hyperammonaemia correlated more closely with glutamine, a metabolite of ammonia, than with ammonia itself. This suggested that ammonia (NH_3 and NH_4^+) was without effect. The present experiments address the question whether ammonia, in the absence of net glutamine synthesis, induces any of the metabolic symptoms of cerebral dysfunction associated with hyperammonaemia. 2. Small doses of methionine sulphoximine, an inhibitor of glutamine synthetase, were used to raise the plasma ammonia levels of normal rats without increasing the brain glutamine content. These hyperammonaemic rats, with plasma and brain ammonia levels equivalent to those known to depress brain function, behaved normally over 48 h. There was no depression of cerebral energy metabolism (i.e. the rate of glucose consumption). Contents of key intermediary metabolites and high-energy phosphates were normal. Neutral amino acid transport (tryptophan and leucine) and the brain contents of aromatic amino acids were unchanged. 3. The data suggest that ammonia is without effect at concentrations less than $1 \mu\text{mol/ml}$ if it is not converted into glutamine. The deleterious effect of chronic hyperammonaemia seems to begin with the synthesis of glutamine.

INTRODUCTION

Ammonia is believed to be an important cause of the cerebral dysfunction of hepatic encephalopathy and other diseases in which hyperammonaemia occurs (Plum & Hindfelt, 1976; Butterworth *et al.*, 1987a; Cooper & Plum, 1987). Portacaval shunting, which causes liver atrophy and hepatic insufficiency, raises the plasma ammonia levels ($0.2\text{--}0.8 \mu\text{mol/ml}$) and causes a variety of metabolic alterations (Hawkins *et al.*, 1987; Mans *et al.*, 1990). These changes include depressed brain function, increased transport of neutral amino acids across the blood/brain barrier, increased brain content of aromatic amino acids, and an increased rate of 5-hydroxytryptamine metabolism. Nearly identical changes occur when plasma ammonia is increased by artificial means (e.g. by urease injections) in otherwise healthy animals (Jessy *et al.*, 1990, 1991).

An important effect of portacaval shunting and hyperammonaemia is a decrease in CMR_{Glc} (between 20% and 30%) that occurs throughout the brain (Mans *et al.*, 1983b, 1986; Hawkins & Mans, 1989a). This decrease in CMR_{Glc} begins within 6 h after the onset of hyperammonaemia. This decreased rate of energy consumption, reflecting a decrease in the activity of brain cells, is maintained indefinitely thereafter (Hawkins & Mans, 1989a; Jessy *et al.*, 1990; Mans *et al.*, 1990; DeJoseph & Hawkins, 1991). The preceding paper (Jessy *et al.*, 1991) showed that the depression in CMR_{Glc} caused by hyperammonaemia correlates more closely with glutamine, a metabolite of ammonia, than with ammonia itself. This was puzzling, because ammonia is thought to be toxic. The synthesis of glutamine has long been considered to be a mechanism of ammonia detoxification (Krebs, 1936; Weil-Malherbe, 1950, 1962; Plum & Hindfelt, 1976; Cooper & Plum, 1987). The observations suggest that the incorporation of ammonia into glutamine is involved in the toxic response.

The present experiments were designed to clarify the issue by dissociating the effects of hyperammonaemia alone and hyperammonaemia accompanied by glutamine synthesis; in other words, to determine whether ammonia causes any metabolic disturbances in the absence of net glutamine synthesis. Low doses of methionine sulphoximine were used to produce a degree of hyperammonaemia that has been shown to cause cerebral dysfunction. However, the use of methionine sulphoximine prevented net glutamine synthesis and avoided the rise in brain glutamine content that normally accompanies hyperammonaemia. Several of the important variables known to be altered by hyperammonaemia (listed above) were measured after 24–48 h.

MATERIALS AND METHODS

Materials

Methionine sulphoximine and glutaminase were bought from Sigma Chemical Co., St. Louis, MO, U.S.A. The other enzymes and cofactors used for analyses were from Boehringer Mannheim G.m.b.H. Biochemica, Mannheim, Germany. L -[5- ^3H]Tryptophan (1070 GBq/mmol), L -[U- ^{14}C]leucine (11.5 GBq/mmol) and D -[6- ^{14}C]glucose (2.03 GBq/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Reagents for amino acid analyses (Dabs-Amino Acid Kit) were bought from Beckman Instruments, San Ramon, CA, U.S.A. All other reagents used were of the best available grade.

Rats

Adult male Long–Evans rats were bought from Charles River Laboratories, Wilmington, MA, U.S.A. All rats were acquired, cared for and handled in conformity with the U.S. Public Health Service's 'Guide for the Care and Use of Laboratory Animals'

Abbreviation used: CMR_{Glc} , cerebral metabolic rate of glucose.

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(NIH Publication No. 86-23, revised 1985) and the 'Guiding Principles for Research Involving Animals and Humans' (recommendations from the Declaration of Helsinki) approved by the Council of the American Physiological Society. They were maintained at 20 °C with 12 h-light/12 h-dark cycles. Food and water were freely available. The rats weighed 250–350 g at the time the experiments were conducted.

Experimental design

Three types of experiments were carried out. The first was to determine the response and its duration to different doses of methionine sulphoximine. The second was to study the permeability of the blood–brain barrier to representative neutral amino acids tryptophan and leucine during methionine sulphoximine-induced hyperammonaemia. The third was to measure CMR_{Glc} as well as intermediary metabolites in brain during methionine sulphoximine-induced hyperammonaemia.

Dose–response to methionine sulphoximine

Different doses of methionine sulphoximine, ranging from 5 to 200 mg/kg body wt., were injected into the peritoneal cavity. Control rats received an injection of 0.154 M-NaCl. There were two to four rats in each group, and they were killed 12 h after injection. A blood sample was withdrawn, centrifuged, and the plasma was used for ammonia measurement. The brain was removed and separated into two hemispheres. One hemisphere was used for the measurement of glutamine synthetase. Glutamine and glutamate were determined in the other hemisphere.

A second group of rats was given the optimum dose (50 mg/kg in a single intraperitoneal injection) and killed at different times after the injection to determine the time course of inhibition. Plasma ammonia, brain glutamine, glutamate and glutamine synthetase were measured.

Similar experiments on the dose–response relationship and the time course were also done on rats given a single dose of methionine sulphoximine by tail vein.

Measurement of tryptophan and leucine transport and monoamine levels

Experimental rats received a single intraperitoneal injection each of methionine sulphoximine (50 mg/kg) to produce hyperammonaemia. Control rats received equivalent volumes of 0.154 M-NaCl. Catheters were placed in a femoral artery and vein 47 h after the injection under halothane/N₂O anaesthesia [induction, 4% halothane in air; maintenance of anaesthesia, 1.5–2% halothane in N₂O/O₂ (7:3)]. An arterial blood sample was taken 1 h later and the plasma separated. An intravenous injection of 185 kBq of [U-¹⁴C]leucine with 3.7 MBq of [5-³H]tryptophan was given. (The radioisotopes were dissolved in fresh plasma obtained from a normal rat.) A continuous blood sample was withdrawn for 3 min into an ice-cold syringe. The rats were killed by a cardioplegic dose of pentobarbital at 3 min and decapitated. The brains were quickly removed and homogenized in 1.2 M-HClO₄. Ammonia was estimated in the non-radioactive plasma sample and amino acids were measured in plasma and brain. The permeability-to-surface area products, measure of transport, of tryptophan and leucine were calculated in accordance with Mans *et al.* (1982).

Determination of CMR_{Glc} and metabolites in plasma and brain

Hyperammonaemia was caused by a single intravenous injection of methionine sulphoximine (30 mg/kg body wt.) in this experiment. Catheters were placed in a femoral artery and vein under halothane anaesthesia (see above) 23 h after the injection.

The rats were left in restraining cages for 1 h to recover from the effects of surgery. Arterial blood was sampled and the plasma separated by centrifugation. [6-¹⁴C]Glucose was injected through the venous catheter, and blood samples (0.1 ml) were taken at 0.5, 1, 2, 3 and 4.5 min. Ketamine, a dissociative anaesthetic with minimal effect on brain metabolism (Davis *et al.*, 1988), was injected intravenously at 5.0 min, and the brain was removed by brain blowing at 6.0 min (Veech & Hawkins, 1974). Metabolites, high-energy phosphates, amino acids and ammonia were measured in the brain, and ammonia and glucose in the plasma. [6-¹⁴C]Glucose was measured in brain and blood. CMR_{Glc} was calculated as described by Hawkins & Mans (1989b).

Tissue preparation and assays

Plasma and brain samples were extracted with 0.5 M- and 1.2 M-HClO₄ respectively for the determination of metabolites (except as otherwise noted). The extracts were neutralized with 20% (w/v) KOH dissolved in 0.1 M-K₂HPO₄. The brain-blowing technique was used to obtain brain for the determination of intermediary metabolites and high-energy phosphates because it minimizes the changes *post mortem* (Veech & Hawkins, 1974). The deep-frozen brains were transferred into liquid nitrogen without thawing and stored at –80 °C until extraction. The extraction of these brains was as described by Veech & Hawkins (1974). All metabolites, high-energy phosphates, glutamate and glutamine were assayed by enzymic methods (Bergmeyer, 1984). Tryptophan was measured fluorimetrically (Eccleston, 1973). Other amino acids were treated with dimethylaminoazobenzene-sulphonyl chloride, by following the procedures established by Beckman Instruments, and separated by h.p.l.c. Glutamine synthetase activity was assayed in brain extracts as described by Butterworth *et al.* (1988).

Statistical analysis

The mean values for the experimental rats were compared with those for control rats by analysis of variance. A *P* value of 0.05 or lower was taken to be significant.

RESULTS

Response to a range of doses of methionine sulphoximine

The metabolic changes seen in hyperammonaemia, whether caused by injections of urease or portacaval shunting, occur within the first 2 days (DeJoseph & Hawkins, 1991; Jessy *et al.*, 1990; Mans *et al.*, 1990). The aim of the first experiments was to create comparable levels of hyperammonaemia for 24–48 h without net glutamine synthesis. Methionine sulphoximine, an inhibitor of glutamine synthetase, was chosen to raise the plasma ammonia levels. Methionine sulphoximine is phosphorylated by glutamine synthetase and γ -glutamylcysteine synthetase and binds tightly to the active sites of these enzymes. Glutamine synthetase is irreversibly inhibited (Meister, 1988), and γ -glutamylcysteine synthetase is inhibited to a lesser degree (Richman *et al.*, 1972). A single intraperitoneal injection of methionine sulphoximine raises the brain concentration of ammonia within 2 h. The new levels are maintained for at least 24 h owing to the prolonged inactivation of glutamine synthetase (Warren & Schenker, 1964).

The response to a range of doses injected into the intraperitoneal cavity is shown in Fig. 1. Glutamine synthetase activity was decreased in proportion to the dose. The decrease in enzyme activity was accompanied by a parallel decrease in the brain content of glutamine. The plasma ammonia values increased as a function of dose, as expected. There was no sign of

Fig. 1. Response to a range of doses of methionine sulphoximine

Rats were given a single intraperitoneal injection of methionine sulphoximine at the dose indicated and killed 12 h later. The results are the means of two to four individual determinations. The experimental values are normalized with control as 100. The control values are: ammonia, $0.075 \pm 0.008 \mu\text{mol/ml}$; glutamine, $6.51 \pm 0.21 \mu\text{mol/g}$; glutamine synthetase = $283 \pm 22 \mu\text{mol/h per g}$. Glutamine synthetase was not measured in the 200 mg/kg-body-wt. group. Key: ■, ammonia; ▨, glutamine; □, glutamine synthetase.

Fig. 2. Duration of response to a single intraperitoneal dose of methionine sulphoximine

A single intraperitoneal injection of methionine sulphoximine (50 mg/kg body wt.) was given and the rats killed at the times indicated. Measurements of glutamine synthetase were made in brain and liver. The results are the means of three or four individual determinations. The experimental values are normalized with control as 100. The control values were: liver glutamine synthetase, $248 \pm 17 \mu\text{mol/h per g}$; brain glutamine, $6.7 \pm 1.2 \mu\text{mol/g}$; brain glutamine synthetase, $303 \pm 31 \mu\text{mol/h per g}$. Key: ■, liver glutamine synthetase; ▨, brain glutamine synthetase; □, brain glutamine.

Fig. 3. Duration of response to a single intravenous dose of methionine sulphoximine

A single intravenous injection of methionine sulphoximine (30 mg/kg body wt.) was given, and the rats were killed at the times indicated. The results are the means of three or four individual determinations. The experimental values are normalized with control as 100. The control values were: ammonia, $0.081 \pm 0.018 \mu\text{mol/ml}$; glutamine, $5.83 \pm 0.95 \mu\text{mol/g}$; glutamine synthetase, $282 \pm 41 \mu\text{mol/h per g}$. Symbols as in Fig. 1.

Table 1. Transport of neutral amino acids and brain contents of aromatic amino acids are normal during methionine sulphoximine-induced hyperammonaemia

Results are given as means $\pm$ S.E.M. ($n = 6$) expressed in $\mu\text{mol/ml}$ or g, except where indicated otherwise: $**P < 0.01$. A single intraperitoneal injection of methionine sulphoximine (50 mg/kg body wt.) was given 48 h before the measurements were made.

Variable	Units	Control	Methionine sulphoximine-induced hyperammonaemia
Plasma			
Ammonia	nmol/ml	54 ± 3	$326 \pm 67^{**}$
Tryptophan	nmol/ml	176 ± 4	175 ± 3
Brain			
Glutamine synthetase	$\mu\text{mol/min per g}$	5.33 ± 0.18	$1.30 \pm 0.16^{**}$
Glutamine	$\mu\text{mol/g}$	5.66 ± 0.15	4.67 ± 0.36
Glutamate	$\mu\text{mol/g}$	9.66 ± 0.54	8.67 ± 0.77
Tryptophan	nmol/g	24 ± 1	23 ± 1
Tyrosine	nmol/g	63 ± 6	69 ± 10
Phenylalanine	nmol/g	88 ± 12	108 ± 18
Histidine	nmol/g	66 ± 56	79 ± 13
Blood-brain permeability-to-surface-area product			
Tryptophan	$\mu\text{l/min per g}$	12.7 ± 0.7	11.1 ± 1.0
Leucine	$\mu\text{l/min per g}$	18.6 ± 0.7	15.9 ± 1.1

hyperammonaemia (levels similar to those shown to affect brain metabolism) without changing brain glutamine very much. A single intraperitoneal dose of 50 mg/kg body wt. decreased brain and liver glutamine synthetase for at least 48 h (Fig. 2). This dose and route of administration were used to study the influence of hyperammonaemia on the transport of tryptophan and leucine, neutral amino acids transported by a common carrier.

It was later found that a smaller intravenous dose of methionine

obvious toxicity, except at the highest dose (200 mg/kg body wt.), which caused convulsions in some rats. A dose of 50 mg/kg body wt. was chosen for further study because it produced

Table 2. Intermediary metabolites, high-energy phosphates and glucose consumption are normal in methionine sulphoximine-induced hyperammonaemia

Results are given as means $\pm$ S.E.M. ($n = 6$) expressed in $\mu\text{mol/ml}$ or g, except where indicated otherwise: ** $P < 0.01$. A single intravenous injection of methionine sulphoximine (30 mg/kg body wt.) was given 24 h before the measurements were made.

Variable	Control	Methionine sulphoximine
Plasma		
Ammonia (nmol/ml)	80 ± 5	$246 \pm 16^{**}$
Glucose	8.04 ± 0.37	6.97 ± 0.48
Brain glucose consumption		
CMR _{Glc} ($\mu\text{mol/min per 100 g}$)	96 ± 12	112 ± 10
Brain metabolites		
Ammonia (nmol/g)	176 ± 10	$736 \pm 10^{**}$
Glucose	1.80 ± 0.15	2.18 ± 0.18
Lactate	2.03 ± 0.18	2.02 ± 0.21
Pyruvate	0.102 ± 0.009	0.109 ± 0.103
α -Oxoglutarate	0.179 ± 0.009	0.201 ± 0.022
Malate	0.796 ± 0.092	0.713 ± 0.073
ATP	3.05 ± 0.37	3.13 ± 0.26
ADP	0.59 ± 0.05	0.58 ± 0.05
AMP	0.04 ± 0.01	0.04 ± 0.01
Phosphocreatine	3.60 ± 0.25	4.05 ± 0.28
Glutamine	5.54 ± 0.30	4.38 ± 0.58
Glutamate	8.05 ± 0.87	7.76 ± 0.86
Glutamine synthetase ($\mu\text{mol/min per g}$)	3.83 ± 0.37	$1.22 \pm 0.12^{**}$

sulphoximine (30 mg/kg body wt.) decreased brain glutamine synthetase activity as much as the larger intraperitoneal dose (Fig. 3). However, the intravenous route had almost no effect on liver glutamine synthetase (results not shown). This dose and route of administration, which caused hyperammonaemia that reached a peak at 24 h, were used for experiments on CMR_{Glc}.

Amino acid transport and the brain content of tryptophan

In these experiments methionine sulphoximine (50 mg/kg body wt. given as a single intraperitoneal injection) decreased the activity of glutamine synthetase by 75% at 48 h (Table 1). Ammonia levels were raised to about 6 times normal, but brain glutamine content was decreased by only 17% ($P < 0.05$). Control and experimental rats were indistinguishable in all other respects. There were no changes in the rates of leucine and tryptophan transport from plasma to brain, nor were there any changes in the brain content of tryptophan or other aromatic amino acids (Table 2).

CMR_{Glc}, intermediary metabolites and brain energy state

Hyperammonaemia was induced by a single intravenous injection of methionine sulphoximine (30 mg/kg body wt.). CMR_{Glc} was measured at 24 h when the plasma ammonia concentration was at its highest. In these experiments glutamine synthetase was decreased by 70% and brain glutamine by 21% (not statistically significant, $P < 0.11$). The plasma and brain ammonia were raised, by 200% and 300% respectively, levels similar to those seen in portacaval-shunted rats in which there is a marked decrease in CMR_{Glc}. There were, however, no detectable

behavioral or metabolic differences between the control and experimental groups. Intermediary metabolites were indistinguishable, high-energy phosphates were nearly the same, and CMR_{Glc} was the same or slightly higher ($P < 0.3$) in the methionine sulphoximine-treated rats.

DISCUSSION

In this paper the discussion concerns chronic hyperammonaemia in which the ammonia concentrations are seldom greater than $1 \mu\text{mol/ml}$. Concentrations in this range are observed in various metabolic diseases such as portacaval shunting, chronic liver failure, inborn errors of the urea cycle etc. Acute ammonia toxicity, on the other hand, in which the circulating concentrations rapidly reach several $\mu\text{mol/ml}$, can cause convulsions and severe disturbances of brain function within minutes. The maintenance of such high concentrations leads to death in a short time (Cooper & Plum, 1987).

Cerebral dysfunction can be caused by chronic hyperammonaemia regardless of whether the increase in circulating ammonia is caused by portacaval shunting or by artificial means such as urease injections (see the Introduction). The present experiments were designed to determine whether the cerebral dysfunction is caused by ammonia itself or is a consequence of the metabolism of ammonia. Therefore a low dose of methionine sulphoximine was used to cause hyperammonaemia, with no increase in the net rate of ammonia metabolism to glutamine. The data show conclusively that hyperammonaemia can exist without causing detectable metabolic changes if net glutamine synthesis is near normal. It appears that ammonia itself is not toxic at these levels ($1 \mu\text{mol/ml}$ or less); rather, it is the metabolism of ammonia to glutamine that initiates an adverse response.

Methionine sulphoximine can inhibit other enzymes such as γ -glutamylcysteine synthetase (Richman *et al.*, 1972) and alanine aminotransferase (De Robertis *et al.*, 1967) when given at doses much higher than those used in the present study. The activities of these enzymes were not measured, and it is possible that they were inhibited. Nevertheless, it must be emphasized that, in spite of a large rise in the concentration of plasma and brain ammonia, there were no signs of any metabolic disturbances (Table 3).

The idea that ammonia must be metabolized to become toxic is contrary to the prevalent opinion that ammonia is detoxified by metabolism to glutamine. The detoxification hypothesis originated with the description of glutamine synthesis in brain (Krebs, 1936; Weil-Malherbe, 1936). It was known that ammonia could cause neurological disorders, and it was natural to assume that glutamine synthesis was a protective mechanism. Glutamine was taken to be an end-product of an ammonia-binding mechanism that is released by brain for metabolism or disposal by other organs (Krebs, 1936; Weil-Malherbe, 1950). This concept may be valid for some tissues, but it is now known that glutamine is not simply an end-product in brain. Glutamine, which is synthesized in astrocytes (Martinez *et al.*, 1977), is used by neurons as a precursor of neurotransmitters (i.e. glutamate, γ -aminobutyrate and aspartate). These neurotransmitters, after being released by neurons, are taken up by the astrocytes and reconverted into glutamine (Van den Berg & Garfinkel, 1971; Balazs *et al.*, 1973; Benjamin & Quastel, 1975; Bradford & Ward, 1975; Bradford *et al.*, 1978; Hamberger *et al.*, 1979b; Thanki *et al.*, 1983; Albrecht, 1989). Thus glutamine synthesis forms an important link in an intricate metabolic cycle between astrocytes and neurons. It seems unlikely that the primary role of glutamine synthetase in brain is to protect against ammonia in

Table 3. Summary of metabolic abnormalities in hyperammonaemia

Observations on portacaval-shunted rats are summarized from Mans *et al.* (1990). The data on hyperammonaemia are from Jessy *et al.* (1990) where hyperammonaemia was produced by intraperitoneal injections of urease. Data on methionine sulphoximine-induced hyperammonaemia are from this paper.

	Portacaval shunt (48 h)	Urease treatment (48 h)	Methionine sulphoximine (24–48 h)
Brain			
Ammonia	Increased	Increased	Increased
Energy consumption	Decreased	Decreased	Normal
Energy balance	Normal	Normal	Normal
Neutral amino acid transport across blood–brain barrier	Increased	Increased	Normal
Aromatic amino acid content	Increased	Increased	Normal
Glutamine content	Increased	Increased	Normal
Plasma			
Ammonia	Increased	Increased	Increased
Glutamine	Increased	Increased	Normal
Glucose	Normal	Normal	Normal

those rare circumstances when its concentrations rise. It seems more reasonable to suggest that high rates of glutamine synthesis disturb the normal metabolic balance between the astrocytes and neurons.

Chronic hyperammonaemia (0.2–0.8 $\mu\text{mol/ml}$ of plasma) leads, as mentioned, to a variety of metabolic changes as well as depression of cerebral function. Higher circulating concentrations ($> 1 \mu\text{mol/ml}$) can cause animals to become stuporous or even comatose, and to convulse within minutes (for review see Cooper & Plum, 1987). Warren & Schenker (1964) showed that mice could be protected against high ammonia concentrations by inhibiting glutamine synthetase with methionine sulphoximine. Hindfelt & Plum (1975) confirmed these results in rats. The most surprising aspect of Warren & Schenker's (1964) experiments was the fact that methionine sulphoximine-treated mice actually had brain ammonia concentrations much higher than the untreated mice. This led Warren & Schenker (1964) to the conclusion that 'ammonia intoxication does not depend upon the mere presence of high cerebral ammonia levels, but is related to the so-called mechanism of detoxification by which ammonia enters into the cerebral metabolic cycles.' Whereas acute and chronic ammonia toxicity have very different symptoms, glutamine synthesis seems to be a prerequisite for toxicity.

Methionine sulphoximine can cause convulsions at the doses used by Warren & Schenker (1964). The cause of these convulsions is not established, but very high NH_4^+ concentrations have been suspected. It should be noted, however, that the doses of methionine sulphoximine used by Warren & Schenker (1964) were very large and most probably depleted both glutamine and glutathione. This by itself may seriously interfere with cerebral function and cause convulsions.

If glutamine synthesis is the first step towards an adverse response to ammonia, the question arises, what is the mechanism? Various hypotheses regarding interference with brain energy production or ATP levels have been suggested to explain cerebral dysfunction in chronic hyperammonaemia (for a summary see Cooper & Plum, 1987). The evidence for a sustained interruption of energy production and consequent ATP depletion is, in our opinion, not strong (Hawkins & Mans, 1989a). Hyper-

ammonaemia caused by portacaval shunting increases the net synthesis of glutamine, but causes no detectable decrease in the concentration of tricarboxylic-acid-cycle metabolites, no decrease in the content of high-energy phosphates, and no change in the redox state (Holmin & Siesjö, 1974; Hindfelt *et al.*, 1977; Mans *et al.*, 1984). It appears that the activity of anaplerotic reactions is adequate over long periods of time to replenish glutamine precursors, and that energy-yielding mechanisms are sufficient to keep up with the demand.

Although the metabolic demand for glutamine synthesis in chronically hyperammonaemic rats (e.g. portacaval-shunted rats) is small, it falls entirely on astrocytes, which comprise only a small fraction of the rat brain. Astrocytes do not die, as might be expected in a situation of long-lasting energy failure, but they do show compensatory morphological changes. [Some of the early changes in astrocyte morphology may be related to ammonia accumulation rather than glutamine synthesis. Methionine sulphoximine-induced hyperammonaemia causes astrocyte swelling that is very much like that seen in ordinary hyperammonaemia when glutamine synthetase is not inhibited. This swelling has been suggested to be consequence of NH_4^+ accumulation (Gutierrez & Norenberg, 1975, 1977).] It is possible that the consequence of sustained hyperactivity may disrupt other activities of the astrocytes, for example neurotransmitter uptake, ion homeostasis, influence of blood–brain barrier transport etc.

It has been suggested that ammonia may disturb cerebral function by inhibiting glutaminase (Bradford & Ward, 1975; Matheson & Van den Berg, 1975; Benjamin, 1981), thereby disrupting the supply of glutamate for glutamatergic neurons (Hamberger *et al.*, 1979a; Butterworth *et al.*, 1987b). The present experiments do not support this hypothesis. Methionine sulphoximine caused hyperammonaemia, but the concentrations of glutamine and glutamate remained near normal. This could be explained if glutaminase was inhibited by ammonia to the same degree as glutamine synthetase was inhibited by methionine sulphoximine. In this circumstance, however, production of glutamate from glutamine would have been decreased well below normal rates. Thus it would have been expected that methionine sulphoximine-induced hyperammonaemia would have had an even greater effect on glutamate levels and glutamatergic neurotransmission than hyperammonaemia caused by other means. On the other hand, the unchanged level of brain glutamine could be explained if the glutamine synthetase remaining after methionine sulphoximine inhibition had a higher turnover of glutamine in response to hyperammonaemia. However, in this circumstance there would be no need to postulate ammonia-induced inhibition of glutaminase. In either case the fact is that experimental and control rats were normal and had normal brain glutamine and glutamate levels. This decreases the likelihood that ammonia disrupts the supply of glutamate by acting on glutamine synthetase.

There are at least two known effects of NH_4^+ on neuronal membranes that could disturb brain function. NH_4^+ can interfere with the generation of inhibitory post-synaptic potentials by inhibiting the Cl^- pump. This occurs at ammonia concentrations similar to those reported here and is maximal at a concentration of $1 \mu\text{mol/ml}$ (Raabe, 1989b, 1991). At even higher concentrations (greater than $2 \mu\text{mol/ml}$) NH_4^+ can depolarize neurons and interfere with synaptic transmission (Raabe, 1989a,b, 1991).

In the present experiments the brain concentrations of ammonia were high enough to inhibit the Cl^- pump and, at least in principle, cause neurons to become more easily excited (Raabe, 1982). Still there were no detectable changes in CMR_{Glc} or in the energy state. It is known that neurons adapt to the chronic presence of NH_4^+ , and it is possible that the Cl^- pump had recovered by 24 h (Raabe, 1989b). Therefore, although it remains

probable that NH_4^+ interferes with nerve-cell function in hyperammonaemic diseases, the effects may be too subtle to be detected by the techniques used.

It is well established that hyperammonaemia, whether caused by portacaval shunting or by artificial means, leads to a much greater activity of the carrier system that transports neutral amino acids across the blood-brain barrier, as well as to a rise in the brain content of aromatic amino acids (James *et al.*, 1976, 1978; Mans *et al.*, 1979, 1982, 1983a, 1984; Bachmann & Colombo, 1984; Jonung *et al.*, 1984; Jeppsson *et al.*, 1985; Bachmann *et al.*, 1986; Jessy *et al.*, 1990). The permeability of the blood-brain barrier to neutral amino acids and the accumulation of aromatic amino acids are both closely correlated with brain glutamine content (Jeppsson *et al.*, 1985; Jessy *et al.*, 1990). Moreover, the elevated rate of transport and the increased accumulation of neutral amino acids caused by hyperammonaemia or by portacaval shunting can be decreased by treatment with methionine sulfoximine (Bachmann, 1983; Jonung *et al.*, 1985; Rigotti *et al.*, 1985). The present experiments, in agreement with previous observations, show conclusively that sustained hyperammonaemia in the absence of net glutamine synthesis has no effect on either neutral amino acids or the accumulation of aromatic amino acids in brain. It may therefore be concluded that glutamine synthesis is linked to the stimulation of neutral amino acid transport caused by hyperammonaemia as well as to the decrease in cerebral energy metabolism (Jessy *et al.*, 1990). After portacaval shunting, the change in neutral amino acid transport occurs very early, beginning within 6 h, and is paralleled by substantial decrease in cerebral energy consumption (Mans *et al.*, 1990). The relationship between the changes in the permeability of the blood-brain barrier and cerebral dysfunction needs to be clarified.

Concluding comments

It is now evident that hyperammonaemia disturbs cerebral function and is an important, if not the primary, factor responsible for hepatic encephalopathy. The absence of a toxic response to chronic hyperammonaemia, when hyperammonaemia is created by inhibiting glutamine synthetase, indicates that ammonia itself is relatively innocuous at concentrations below $1 \mu\text{mol/ml}$. It is when ammonia is metabolized that an adverse response is initiated. This does not mean that glutamine itself is toxic; there are many other metabolites and cellular processes to be considered. Nevertheless, the observation that the metabolism of ammonia plays an essential role in causing cerebral dysfunction has important implications for diseases in which hyperammonaemia is a characteristic feature (e.g. hepatic encephalopathy and inborn errors of metabolism). Certainly, attention should be focused on the glutamine synthetase reaction and its products in the search for the mechanism of ammonia toxicity.

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