The Influence of Ammonia on Purine and Pyrimidine Nucleotide Biosynthesis in Rat Liver and Brain *in vitro*

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1. The effect of ammonia on purine and pyrimidine nucleotide biosynthesis was studied in rat liver and brain *in vitro*. The incorporation of $\text{NaH}^{14}\text{CO}_3$ into acid-soluble uridine nucleotide (UMP) in liver homogenates and minces was increased 2.5–4-fold on incubation with 10mM- NH_4Cl plus *N*-acetyl-L-glutamate, but not with either compound alone. 2. The incorporation of $\text{NaH}^{14}\text{CO}_3$ into orotic acid was increased 3–4-fold in liver homogenate with NH_4Cl plus acetylglutamate. 3. The 5-phosphoribosyl 1-pyrophosphate content of liver homogenate was decreased by 50% after incubation for 10min with 10mM- NH_4Cl plus acetylglutamate. 4. Concomitant with this decrease in free phosphoribosyl pyrophosphate was a 40–50% decrease in the rates of purine nucleotide synthesis, both *de novo* and from the preformed base. 5. Subcellular fractionation of liver indicated that the effects of NH_4Cl plus acetylglutamate on pyrimidine and purine biosynthesis required a mitochondrial fraction. This effect of NH_4Cl plus acetylglutamate could be duplicated in a mitochondria-free liver fraction with carbamoyl phosphate. 6. A similar series of experiments carried out with rat brain demonstrated a significant, though considerably smaller, effect on UMP synthesis *de novo* and purine base reutilization. 7. These data indicate that excessive amounts of ammonia may interfere with purine nucleotide biosynthesis by stimulating production of carbamoyl phosphate through the mitochondrial synthetase, with the excess carbamoyl phosphate in turn increasing pyrimidine nucleotide synthesis *de novo* and diminishing the phosphoribosyl pyrophosphate available for purine biosynthesis.

Hyperammonaemia in infants and children has been associated with impaired growth and the increased urinary excretion of pyrimidines, especially orotic acid (Levin *et al.*, 1969; Shih, 1976). Decreased growth and urinary bladder stones composed mainly of orotic acid have been described in mice with a sparse-fur mutation, which show an abnormal liver ornithine transcarbamoylase (carbamoyl phosphate-L-ornithine carbamoyltransferase, EC 2.1.3.3) (DeMars *et al.*, 1976). In addition, orotic aciduria has been induced in association with hyperammonaemia in human female carriers of ornithine transcarbamoylase deficiency after protein loading (Goldstein *et al.*, 1974; Hokanson *et al.*, 1978) and in normal rats by NH_4Cl (Kesner, 1965; Statter *et al.*, 1974).

These data suggest that hyperammonaemia results in the increased synthesis of pyrimidines. There is no evidence that orotic acid itself is toxic (Tubergen *et al.*, 1969; Wood & O'Sullivan, 1973). However,

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Abbreviation used: Hepes, *N*-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

administration of orotic acid to rats has been shown to deplete liver 5-phosphoribosyl 1-pyrophosphate (Rajalakshmi & Handschumacher, 1968) with a decrease in adenine nucleotides (Marchetti *et al.*, 1962; Clifford *et al.*, 1969). Similar results have been reported in humans fed with orotic acid, who showed depletion of phosphoribosyl pyrophosphate in erythrocytes and decreased incorporation of [^{14}C]glycine into urinary uric acid (Kelley *et al.*, 1970). Orotate phosphoribosyltransferase (orotidine 5'-phosphate-pyrophosphate phosphoribosyltransferase, EC 2.4.2.10), which catalyses the conversion of orotic acid into UMP, utilizes phosphoribosyl pyrophosphate. Phosphoribosyl pyrophosphate is also a substrate for the first and presumed rate-limiting step of purine synthesis *de novo* and for purine-base reutilization.

Metabolic changes which increase pyrimidine nucleotide synthesis *de novo* could result in a shift of phosphoribosyl pyrophosphate availability away from the purine pathways, leading to an imbalance in purine/pyrimidine production. This mechanism could account for the impairment in growth and mental retardation seen in infants and children with chronic hyperammonaemia.

Recent evidence indicates that carbamoyl phosphate synthesized from ammonia in mitochondria may be available for extramitochondrial pyrimidine biosynthesis (Natale & Tremblay, 1969; Tremblay *et al.*, 1977). Presumably, hyperammonaemia results in an increased synthesis of intramitochondrial carbamoyl phosphate, which cannot be fully utilized by subsequent steps in the urea cycle. The excess carbamoyl phosphate leaks into the cytoplasm where it serves as a source of carbamoyl phosphate for pyrimidine biosynthesis.

In the present paper, we present data for rat liver which show that high concentrations of ammonia stimulate pyrimidine synthesis *de novo* through the action of mitochondrial carbamoyl phosphate synthetase I [ATP-carbamate phosphotransferase (dephosphorylating), EC 2.7.2.5] with concomitant decreases in the phosphoribosyl pyrophosphate concentration and in the rate of purine nucleotide biosynthesis.

Experimental

Materials

Sodium [^{14}C]formate (58–61 mCi/mmol), [8- ^{14}C]hypoxanthine (52 mCi/mmol), and [8- ^{14}C]adenine (58 mCi/mmol) were from Amersham/Searle, Chicago Heights, IL, U.S.A.; $\text{NaH}^{14}\text{CO}_3$ (2 mCi/mmol) and Aquasol were from New England Nuclear, Boston, MA, U.S.A.; non-radioactive purine and pyrimidine bases, nucleosides and nucleotides, *N*-acetyl-L-glutamic acid, Hepes and bovine serum albumin (fraction V) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; diethylaminoethyl filter circles (Whatman DE81) were from Reeve Angel, Clifton, NJ, U.S.A.; Dowex AG50W(X4; 200–400 mesh; H^+ form) was from Bio-Rad Laboratories, Rockville Center, NY, U.S.A. All other chemicals were of reagent grade.

Tissues

Male Sprague-Dawley rats (100–125 g) were killed by decapitation and the liver and brain quickly removed to ice-cold Hepes medium (see below).

Incorporation of $\text{NaH}^{14}\text{CO}_3$ into UMP

Liver and brain homogenates were prepared in Hepes medium [130 mM-NaCl/40 mM-Hepes/NaOH (pH 7.4) / 1 mM- MgCl_2 / 0.1 mM-KCl / 5 mM-glucose / 2 mM-L-glutamine/2 mM-glycine/5 mM-L-aspartic acid] (0–4°C) at 40–50 mg of tissue/ml and 250 mg of tissue/ml (wet wt.) respectively by using seven complete strokes in a glass tube with Teflon pestle. Samples (0.4 ml) of homogenate or 20–25 mg of minced liver in 0.4 ml of Hepes medium were incubated for 5 min at 37°C, after addition of 0.1 ml of one of the following

stock solutions prepared in the pH 7.4 Hepes medium: 50 mM-NaCl/50 mM- NH_4Cl /50 mM-*N*-acetyl-L-glutamic acid; or 50 mM- NH_4Cl /50 mM-acetylglutamate (10 mM final concn. in the reaction). Reactions were started by adding the $\text{NaH}^{14}\text{CO}_3$ to 15 mM final concentration (2 mCi/mmol). After 7–10 min at 37°C the reactions were stopped by adding 25 μl of 12 M- HClO_4 to the homogenates or liver minces. Liver minces were homogenized at this time and samples left on ice for 10 min. For zero-time blanks, acid was added before $\text{NaH}^{14}\text{CO}_3$. Samples were then centrifuged at 1500g for 5 min at 5°C and the pellet was discarded. ^{14}C -labelled nucleotides were isolated from the acid-soluble fraction by means of adsorption on acid-washed charcoal (Tremblay *et al.*, 1976). Greater than 95% of the radioactivity incorporated into this fraction was found in UMP, by use of the ion-exchange chromatography system described by Tremblay *et al.* (1976). Therefore samples from this charcoal step were taken for radioactivity measurement without further separation. Samples were placed in Aquasol for liquid-scintillation counting at an efficiency of 80% (internal-ratio method).

Incorporation of $\text{NaH}^{14}\text{CO}_3$ into orotic acid

Liver homogenates (70 mg wet wt./ml) were prepared in the Hepes medium, pH 7.4 (0–4°C), containing 6-azauridine at a reaction concentration of 5 mM to inhibit the conversion of orotic acid into UMP (Handschumacher & Pasternak, 1958). Incubation mixtures contained 90 μl of homogenate and 5 μl of 0.02 M- or 0.2 M-NaCl, *N*-acetyl-L-glutamate, or NH_4Cl plus *N*-acetyl-L-glutamate (pH adjusted to 7.4 where necessary). After a 5 min preincubation at 37°C, reactions were started by adding 5 μl of 0.3 M- $\text{NaH}^{14}\text{CO}_3$ (2 mCi/mmol). Incubations were continued for another 15 min. Reactions were stopped by adding 5 μl of 90% (v/v) formic acid. For zero-time blanks, formic acid was added before $\text{NaH}^{14}\text{CO}_3$. Samples were placed in a boiling-water bath for 5 min. The [^{14}C]orotic acid synthesized and accumulated during the incubation period was isolated from the acid-soluble fraction of the reaction mixture by ascending paper chromatography (DeMars *et al.*, 1976).

Measurement of newly synthesized purine

Homogenates of liver or brain were prepared at 35–40 mg of tissue/ml (0–4°C) in Hepes medium, pH 7.4, containing 115 mM-NaCl and 15 mM- NaHCO_3 . Samples (0.5 ml) of homogenate or 35–40 mg of liver minces in 1 ml of Hepes medium were adjusted to various concentrations of NH_4Cl and/or acetylglutamate by addition of 20-fold concentrated stock solutions at pH 7.4. After a 5 min preincubation at 37°C, the reactions were started by addition of sodium [^{14}C]formate (58–61 mCi/mmol, 1 mCi/ml).

After an additional 10min incubation, the reactions were stopped by adding 1.5ml of 0.6M-HClO₄ to the homogenates, or 1.5ml of 1.2M-HClO₄ to the minced liver incubation samples; these latter samples were subsequently homogenized at 0–4°C. Zero-time blanks had acid added before [¹⁴C]formate. Samples were placed in a boiling-water bath for 60min to convert nucleic acids and purine nucleotides and nucleosides into the free bases. The [¹⁴C]purine bases were isolated on columns of Dowex AG50W (X4; H⁺ form) as described by Hershfield & Seegmiller (1976).

Purine nucleotide biosynthesis from preformed base

Liver and brain homogenates were prepared in Hepes medium, pH7.4 (0–4°C), containing 115mM-NaCl and 15mM-NaHCO₃, at 40mg of tissue/ml. Samples (0.1ml) of homogenate or 20mg of liver minces in 0.5ml of Hepes medium were adjusted to various concentrations of NH₄Cl and/or *N*-acetyl-L-glutamate by addition of 20-fold-concentrated stock solutions at pH7.4. After preincubation for 5min at 37°C, 5 or 25μl of 1mM-[8-¹⁴C]hypoxanthine (52mCi/mmol) was added to homogenates or liver minces respectively. Incubations were continued for 15min more. Reactions were stopped by adding 5μl of 5.7M-HClO₄; tissue minces were homogenized at this time. The reaction blanks had acid added before substrate. Samples were left on ice for 10min, neutralized with KOH, and the KClO₄ precipitate and denatured protein removed by centrifugation at 1500g for 5min at 5°C. ¹⁴C-labelled nucleotide products were isolated on diethylaminoethyl filter-paper circles (DE81) as described previously (Skaper *et al.*, 1976).

Determination of 5-phosphoribosyl 1-pyrophosphate content

Tissue homogenates were prepared at 80mg/ml in ice-cold Hepes medium, pH7.4, containing 115mM-NaCl, 15mM-NaHCO₃, 1mM-EDTA and either 1–13mM-NH₄Cl, 1–13mM-acetylglutamate, 1–13mM-NaCl or 1–13mM-NH₄Cl/acetylglutamate. Samples were incubated at 37°C for 10min. The tubes were immediately placed in a boiling-water bath for 20s, then chilled on ice and centrifuged at 1500g for 3min at 5°C. The phosphoribosyl pyrophosphate in the supernatant fractions was measured by following the conversion of [¹⁴C]adenine into [¹⁴C]AMP with an excess of mouse liver adenine phosphoribosyl-transferase, as described by Skaper *et al.* (1976).

Subcellular fractionation of tissue

Liver homogenate was prepared at 35–70mg/ml in Hepes medium, pH7.4, supplemented with 2mM-dithiothreitol, and, additionally, 5mM-6-azauridine for the orotic acid-labelling experiment. Homogenate (fraction H) was centrifuged at 600g for 10min at

5°C and the pellet discarded. The 600g supernatant was divided into two equal volumes and centrifuged at 20000g for 25min at 5°C. One supernatant (fraction S) was set aside and the pellet suspended in a volume of the appropriate Hepes medium equal to that of its original supernatant (Hepes-suspended pellet-fraction P). The other 20000g pellet was suspended in the entire volume of its original supernatant (supernatant-suspended pellet-fraction R).

Protein measurement

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Evidence for the participation of ammonia in pyrimidine biosynthesis in liver

Incubation of rat liver homogenate with increasing concentrations of NH₄Cl plus 10mM-*N*-acetyl-L-glutamic acid resulted in an increased incorporation of NaH¹⁴CO₃ into UMP, up to 230% at 10mM-NH₄Cl (Fig. 1). Omission of acetylglutamate (a cofactor for mitochondrial carbamoyl phosphate synthetase) gave a smaller and inconsistent increase in UMP synthesis. Replacement of NH₄Cl with NaCl plus acetylglutamate gave no stimulation (results not shown).

Initially these experiments were carried out using liver minces, with the changeover to homogenate being made for the sake of ease in sample handling. The above phenomenon could also be demonstrated in minced liver (Table 1). In the presence of acetylglutamate, NH₄Cl at a concentration of 10mM-caused an approximate 2.5-fold increase (*P* < 0.01) in uridine nucleotide labelling.

The increase in labelling of UMP after addition of NH₄Cl plus acetylglutamate could be attributed to increased synthesis of UMP, and not a decreased rate of UMP degradation or incorporation into nucleic acid. As shown in Table 2, 10mM-NH₄Cl plus acetylglutamate increased the incorporation of NaH¹⁴CO₃ into orotic acid 4-fold (*P* < 0.001). Alone, 10mM-acetylglutamate had no effect, whereas 10mM-NH₄Cl produced a small (34%) but significant (*P* < 0.05) increase.

The increased labelling of UMP in the presence of NH₄Cl plus acetylglutamate may not represent a maximum rate for pyrimidine synthesis. Cellulose thin-layer chromatographic separation of a reaction mixture with rat liver homogenate incubated with 10mM-NH₄Cl plus 10mM-acetylglutamate showed a 2-fold increase in UMP synthesis and a 3.5-fold increase in orotic acid synthesis without addition of 6-azauridine. The absolute values increased from 5.86 to 13.57 nmol/h per mg of protein and from 2.10 to 6.93 nmol/h per mg of protein for UMP and orotic acid respectively.

Fig. 1. Effect of ammonia and *N*-acetyl-L-glutamic acid on the incorporation of $\text{NaH}^{14}\text{CO}_3$ in rat liver homogenate

Liver homogenate was prepared as described in the Experimental section, adjusted to the indicated concentrations of NH_4Cl (pH 7.4), 15mM- $\text{NaH}^{14}\text{CO}_3$ (2mCi/mmol) was added and incubated at 37°C. The acid-soluble [^{14}C]UMP synthesized was isolated as described in the Experimental section. Results are the average of two or three measurements from two experiments (bars indicate range where three values used) expressed as percentage of control (6.91 nmol of UMP/h per mg of protein). ○, With 10mM-acetylglutamate; ●, without acetylglutamate.

Table 1. Effect of ammonia on pyrimidine nucleotide synthesis *de novo* in rat liver minces

Rat liver minces were incubated at 37°C in Hepes medium (pH 7.4)/15mM- $\text{NaH}^{14}\text{CO}_3$ (2mCi/mmol), acid-extracted, and the acid-soluble [^{14}C]UMP was isolated as described in the Experimental section. Results are means $\pm$ range for two independent experiments run in triplicate. A significant difference (Student's *t* test) of $P < 0.01$ was found for the NH_4Cl plus *N*-acetyl-L-glutamic acid addition.

Additions	UMP formed (nmol/h per g of liver)
10mM-NaCl (control)	428 $\pm$ 27
10mM- NH_4Cl plus 10mM-acetylglutamate	1016 $\pm$ 83
10mM- NH_4Cl	419 $\pm$ 62
10mM-Acetylglutamate	456 $\pm$ 38

Table 2. Effect of ammonia on orotic acid biosynthesis in rat liver homogenate

Samples of liver homogenate (70mg/ml) in Hepes medium, pH 7.4, with 5mM-6-azauridine to which 15mM- $\text{NaH}^{14}\text{CO}_3$ (2mCi/mmol) was added, were incubated at 37°C for 15 min, and the [^{14}C]orotic acid synthesized during the incubation period was isolated from the acid-soluble fraction of the reaction mixture as described in the Experimental section. Results are means $\pm$ range of two independent experiments run in duplicate. Significant differences (Student's *t* test) between results with 10mM-NaCl and other additions are: * $P < 0.05$; † $P < 0.001$.

Additions	Orotic acid formed (nmol/h per mg of protein)
1 mM-NaCl (control)	6.44 $\pm$ 0.50
1 mM- NH_4Cl plus 10mM-acetylglutamate	6.42 $\pm$ 0.55
1 mM-NaCl plus 10mM-acetylglutamate	6.09 $\pm$ 0.29
10mM-NaCl (control)	5.77 $\pm$ 0.36
10mM- NH_4Cl plus 10mM-acetylglutamate	22.7 $\pm$ 2.8†
10mM- NH_4Cl	7.74 $\pm$ 0.70*
10mM-NaCl plus 10mM-acetylglutamate	6.02 $\pm$ 0.66

Table 3. Effect of ammonia on orotic acid synthesis in rat liver after subcellular fractionation

Samples of subcellular fractions, to which 15mM- $\text{NaH}^{14}\text{CO}_3$ (2mCi/mmol) was added, were incubated at 37°C with the indicated additions, and the [^{14}C]orotic acid was isolated as described in the Experimental section. Results are the average of duplicate measurements. Fraction H, homogenate; fraction S, 20000g supernatant; fraction P, 20000g pellet suspended in Hepes medium; fraction R, 20000g pellet suspended in 20000g supernatant.

Addition	Subcellular fraction	Orotic acid formed (nmol/h per mg of protein)			
		H	S	R	P
None (control)		4.66	5.66	4.80	0
10mM- NH_4Cl plus 10mM-acetylglutamate		14.0	3.34	13.4	—
10mM- NH_4Cl		6.52	5.60	5.40	—
10mM-Acetylglutamate		5.20	5.74	4.82	—

In addition to a requirement for acetylglutamate, evidence for involvement of the mitochondrial carbamoyl phosphate synthetase in the ammonia-stimulated synthesis of pyrimidines is given by the subcellular fractionation data in Table 3. Orotic acid synthesis in the homogenate (fraction H) was increased 3-fold with 10mM- NH_4Cl plus 10mM-

Table 4. *Ammonia-associated reduction in purine biosynthesis and phosphoribosyl pyrophosphate concentration in rat liver homogenate: dependence on N-acetyl-L-glutamic acid*

The preparation of tissue homogenate and measurement of purine nucleotide synthesis *de novo* from [^{14}C]formate and from [^{14}C]hypoxanthine incorporation into nucleotide, and free phosphoribosyl pyrophosphate are described in the Experimental section. Results are means $\pm$ range for two independent experiments run in duplicate. Significant differences (Student's *t* test) are: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

Addition	Phosphoribosyl pyrophosphate (pmol/mg of protein)	Synthesis <i>de novo</i> (pmol/h per mg of protein)	Nucleotide from hypoxanthine (nmol/h per mg of protein)
None (control)	64.9 $\pm$ 6.3	215 $\pm$ 9	3.48 $\pm$ 0.17
10mM-Acetylglutamate	66.6 $\pm$ 7.3	225 $\pm$ 9	3.37 $\pm$ 0.37
10mM-NH ₄ Cl	61.0 $\pm$ 5.6	183 $\pm$ 8	2.92 $\pm$ 0.37
1mM-NH ₄ Cl plus 10mM-acetylglutamate	49.6 $\pm$ 4.5*	—	—
5mM-NH ₄ Cl plus 10mM-acetylglutamate	43.2 $\pm$ 2.0†	—	—
10mM-NH ₄ Cl plus 10mM-acetylglutamate	32.9 $\pm$ 4.5†	92.8 $\pm$ 10.0‡	2.20 $\pm$ 0.31†
13mM-NH ₄ Cl plus 10mM-acetylglutamate	35.7 $\pm$ 3.0†	—	—
13mM-NaCl plus 10mM-acetylglutamate	63.6 $\pm$ 4.9	—	—

acetylglutamate (column 1); 10mM-NH₄Cl alone resulted in a 1.5-fold stimulation. A 20000g supernatant (fraction S) although still able to synthesize orotic acid did not respond to the addition of NH₄Cl plus acetylglutamate with an increased rate of biosynthesis (column 2). After resuspension of the 20000g pellet (mitochondrial) in the 20000g supernatant (fraction R), NH₄Cl plus acetylglutamate were again effective in stimulating pyrimidine biosynthesis (Table 3, column 3). When the 20000g pellet was resuspended in Hepes medium (fraction P) very little, if any, incorporation of NaH¹⁴CO₃ into orotic acid could be detected (column 4).

Effect of ammonia on purine biosynthesis and phosphoribosyl pyrophosphate concentrations in rat liver

Since phosphoribosyl pyrophosphate is a substrate for purine as well as pyrimidine nucleotide biosynthesis, any factor that enhances the rate of pyrimidine nucleotide synthesis *de novo* could conceivably result in less phosphoribosyl pyrophosphate being available for purine biosynthesis with a concomitant decrease in this latter biosynthetic activity. To test this possibility with respect to ammonia, rat liver homogenates were incubated with several concentrations of NH₄Cl and acetylglutamate (Table 4). A significant decrease in phosphoribosyl pyrophosphate was observed at every concentration of NH₄Cl tested (for 1mM-NH₄Cl, $P < 0.05$; for 5mM and 13mM-NH₄Cl, $P < 0.01$), and the effect appeared to be concentration-dependent. Neither NH₄Cl, NaCl nor acetylglutamate alone or in combination at these concentrations had any effect on the activity of phosphoribosyl pyrophosphate synthetase (ATP-D-ribose 5-phosphate pyrophosphotransferase, EC 2.7.6.1). Phosphoribosyl pyrophosphate synthetase has been reported to be activated by P_i (Hershko *et al.*,

1969). In experiments with P_i below 4mM, no effect on the phosphoribosyl pyrophosphate values was observed. However, when 20mM-P_i was included in the reaction mixtures the phosphoribosyl pyrophosphate value in the control increased 200%, whereas the reactions containing 13mM-NH₄Cl plus 20mM-acetylglutamate increased by only 30%. Hence, even when there is a large increase in the rate of synthesis of phosphoribosyl pyrophosphate, it is still rapidly consumed for pyrimidine nucleotide synthesis when high concentrations of ammonia are present.

The acetylglutamate-dependence of an ammonia-associated decrease in purine biosynthesis and phosphoribosyl pyrophosphate in rat liver homogenate is demonstrated in Table 4. A combination of 10mM-NH₄Cl plus 10mM-acetylglutamate produced highly significant decreases in the rates of purine nucleotide synthesis *de novo* and from the preformed base ($P < 0.01$ and $P < 0.001$ respectively), and in the phosphoribosyl pyrophosphate concentration ($P < 0.01$). NH₄Cl alone at a concentration of 10mM produced small (6–16%) non-significant decreases. Acetylglutamate has no effect whatsoever.

The effect of ammonia on purine synthesis *de novo* was also examined in liver minces. As indicated in Table 5, 5mM-NH₄Cl plus 10mM-acetylglutamate gave a 38% decrease in the rate of purine synthesis *de novo*, whereas 20mM-NH₄Cl plus 10mM-acetylglutamate gave a 57% decrease ($P < 0.01$, Student's *t* test) compared with control values. The corresponding osmolarity of sucrose produced only a 16% decrease. A similar effect with ammonia was observed for the incorporation of purine base into acid-soluble nucleotides (results not shown). Maximum inhibition (50%) of incorporation occurred at NH₄Cl concentrations of 5mM or greater with 10mM-acetylglutamate. NH₄Cl and/or acetylglutamate did not inhibit the enzymes of purine base phosphoribosylation.

Table 5. *Effect of ammonia on purine synthesis de novo in rat liver minces*

Liver minces (35–40mg) were incubated at 37°C in Hepes medium, pH 7.4, containing 10mM-*N*-acetyl-L-glutamic acid and the indicated additions. The incorporation of sodium [14 C]formate (58 mCi/mmol) into total purines was measured as described in the Experimental section. Results are averages of duplicate assays from one experiment, or the means $\pm$ range of duplicate measurements from two independent experiments.

Addition	[14 C]Formate incorporated (nmol/h per mg of liver)
None (control)	35.5
2 mM-Sucrose	36.1
1 mM-NH $_4$ Cl	30.4
10 mM-Sucrose	29.4
5 mM-NH $_4$ Cl	22.0
40 mM-Sucrose	29.8 $\pm$ 1.2
20 mM-NH $_4$ Cl	15.4 $\pm$ 0.5

In separate experiments with liver homogenate, the effect of addition of phosphoribosyl pyrophosphate to the reaction was studied. With added 10mM-NaCl alone the incorporation of [14 C]formate into purine was 268 $\pm$ 22 pmol/h per mg of protein (mean $\pm$ S.E.M. of four observations). With 10mM-NH $_4$ Cl plus 10mM-acetylglutamate, incorporation decreased to 183 $\pm$ 15 pmol/h per mg of protein (P = 0.02). Inclusion of 3mM-phosphoribosyl pyrophosphate in this latter reaction increased the [14 C]formate incorporated to 282 $\pm$ 13 pmol/h per mg of protein, a value not significantly different (P > 0.05) from that with NaCl addition alone. The difference in incorporation values with NH $_4$ Cl plus acetylglutamate before and after phosphoribosyl pyrophosphate addition was significant (P < 0.05).

In addition to the dependence on acetylglutamate, evidence supporting the hypothesis that the ammonia-associated decrease in purine biosynthesis and phosphoribosyl pyrophosphate is mediated through the action of mitochondrial carbamoyl phosphate synthetase is presented by the following subcellular fractionation data. The fractionation of liver homogenate and measurement of purine synthesis *de novo* were carried out as described in the Experimental section. Fraction designations were the same as in Table 3. In a preliminary experiment, addition of 10mM-NH $_4$ Cl resulted in a 40% decrease in purine synthesis in homogenate (H) when compared with the value with 10mM-NaCl (115.8 pmol of [14 C]-formate incorporated/h per mg of protein); 1mM-NH $_4$ Cl gave a 20% decrease compared with the value with 1mM-NaCl (109.2 pmol of [14 C]formate/h per mg of protein). By using the 20000g supernatant fraction (S), 1mM- and 10mM-NH $_4$ Cl gave decreases of

8 and 5% respectively in comparison with the values for equal concentrations of NaCl (10mM-NaCl, 112.4 pmol of [14 C]formate/h per mg of protein). Reconstitution of the 20000g pellet in fraction S restored the inhibitory effect of ammonia: 1mM- and 10mM-NH $_4$ Cl decreased purine synthesis *de novo* by about 20 and 40% respectively compared with the value with 10mM-NaCl (126.2 pmol of [14 C]formate/h per mg of protein). All incubation mixtures contained 10mM-acetylglutamate.

If the effect of ammonia on purine and pyrimidine biosynthesis is mediated through carbamoyl phosphate synthetase I as hypothesized, it should be possible to replace NH $_4$ Cl and acetylglutamate with carbamoyl phosphate in the inhibition of purine synthesis, with a mitochondria-free liver fraction. In a preliminary experiment, liver homogenate was prepared in the pH 7.4 Hepes medium described in the Experimental section for measuring UMP synthesis. Samples of a 20000g supernatant were incubated with [14 C]formate and various concentrations of carbamoyl phosphate or sucrose, and the 14 C-labelled purine bases isolated as described earlier. Carbamoyl phosphate at 2.5 and 5mM decreased purine synthesis *de novo* by 25 and 75% of the sucrose controls respectively (240 and 280 pmol/h per mg of protein versus 180 and 70 pmol/h per mg of protein respectively). Using the same tissue preparation before centrifugation, incubation with 5mM- or 10mM-NH $_4$ Cl plus 10mM-acetylglutamate resulted in 35 and 55% less incorporation of [14 C]formate into total purines respectively. No inhibition was observed with the 20000g supernatant fraction. A similar effect was seen when measuring the incorporation of 14 C-labelled purine base into acid-soluble nucleotide.

Effect of ammonia on pyrimidine and purine nucleotide biosynthesis in brain

A series of experiments were carried out with rat brain, to ascertain if ammonia would have an effect on purine and pyrimidine synthetic rates and phosphoribosyl pyrophosphate concentrations similar to that observed with liver. These data are summarized in Table 6. Uridine nucleotide synthesis *de novo* was increased significantly (P < 0.01) only with the addition of 10mM-NH $_4$ Cl plus 10mM-acetylglutamate; however, the effect was not as dramatic as with liver (increases of 155 and 250–350% respectively). Purine synthesis *de novo* and phosphoribosyl pyrophosphate concentrations were not affected by NH $_4$ Cl and acetylglutamate individually or together. Inclusion of 10mM-NH $_4$ Cl plus 10mM-acetylglutamate in the reaction for measurement of purine-base incorporation into acid-soluble nucleotide resulted in small (15%) but significant (P < 0.01) decreases in activity.

Table 6. *Effect of ammonia on the rate of purine and pyrimidine nucleotide synthesis and 5-phosphoribosyl 1-pyrophosphate concentration in rat brain homogenate*

Tissue homogenates were prepared, and incubations for the various assays carried out as described for liver in Table 5 and in the Experimental section. [^{14}C]Hypoxanthine was used to measure incorporation of purine base into acid-soluble nucleotide. Results are means $\pm$ S.E.M. of three independent experiments performed in triplicate.

*Significant difference (Student's *t* test) in comparison with the results for NaCl (control) of $P < 0.01$.

Experiment	Addition (10mm) ...	NaCl	NH ₄ Cl+acetyl-glutamate	NH ₄ Cl	Acetyl-glutamate
UMP synthesis (pmol/h per mg of protein)		957 $\pm$ 73	1490 $\pm$ 60*	819 $\pm$ 84	914 $\pm$ 72
Purine synthesis <i>de novo</i> (pmol of [^{14}C]formate incorporated/h per mg of protein)		60.5 $\pm$ 5.9	58.6 $\pm$ 6.0	55.3 $\pm$ 6.4	62.0 $\pm$ 5.9
Purine-base reutilization (pmol/h per mg of protein)		692 $\pm$ 28	577 $\pm$ 20*	657 $\pm$ 28	653 $\pm$ 27
5-Phosphoribosyl 1-pyrophosphate (pmol/mg of protein)		32.5 $\pm$ 1.0	33.6 $\pm$ 1.3	33.4 $\pm$ 2.6	33.7 $\pm$ 2.4

Discussion

Data herein demonstrate that high concentrations of ammonia stimulate the synthesis of pyrimidine bases and nucleotides *de novo* in rat liver. This increased synthesis is a result of the increased synthesis of carbamoyl phosphate within the mitochondria by carbamoyl phosphate synthetase I, which catalyses the reaction:

Normally, carbamoyl phosphate originating within the mitochondria is utilized in urea biosynthesis. However, under the proper conditions, i.e., high ammonia concentrations and limiting ornithine concentrations, carbamoyl phosphate thus formed leaves the mitochondria and is available for use in pyrimidine synthesis (Natale & Tremblay, 1969). The involvement of carbamoyl phosphate synthetase-I activity in the stimulation of pyrimidine synthesis was clearly shown by demonstrating the requirement for *N*-acetyl-L-glutamic acid and for the presence of the mitochondrial fraction.

In addition, high ammonia concentrations inhibited the synthesis *de novo* of purine nucleotides and also the reutilization of purine bases by means of the salvage pathways. This inhibition was the result of depletion of the pool of phosphoribosyl pyrophosphate in the extract. The inhibition of purine synthesis and depletion of phosphoribosyl pyrophosphate was also shown to be acetylglutamate-dependent and also to require the presence of a mitochondrial fraction.

Thus high concentrations of ammonia may result in increased synthesis of carbamoyl phosphate in the mitochondria by carbamoyl phosphate synthetase-I, which in turn results in stimulation of pyrimidine synthesis *de novo*. Since the synthesis of pyrimidine nucleotides requires phosphoribosyl pyrophosphate, this compound is rapidly depleted when pyrimidine

synthesis is stimulated. The first committed step in purine synthesis *de novo*, catalysed by amidophosphoribosyltransferase (ribosylamine 5-phosphate-pyrophosphate phosphoribosyltransferase, EC 2.4.2.14) utilizes phosphoribosyl pyrophosphate. Hence depletion of the phosphoribosyl pyrophosphate pool results in decreased rates of synthesis of these compounds. Purine-base reutilization, which also requires phosphoribosyl pyrophosphate, is likewise diminished.

The concentrations of NH₄Cl (1–10mm) used in these studies were within the range found in hyperammonaemic individuals. The normal concentration of ammonia in human brain has been reported to be 0.34mm (Rubio & Grisolia, 1976). At physiological pH, most (>95%) NH₄Cl will exist at NH₄⁺. Normal blood ammonia concentrations of less than 0.09mm may exceed 1.2mm in patients with urea-cycle disorders (Alexander *et al.*, 1973). Blood ammonia concentration is generally one-third of the intracellular value, because of the lower intracellular pH (Conn, 1966).

The present data agree with other reports, which demonstrate the stimulatory effect of ammonia on pyrimidine biosynthesis in liver (Tremblay *et al.*, 1977), although the present data differ qualitatively in several respects from that reported by Tremblay *et al.* (1977) for rat liver. The maximum stimulation of orotate synthesis reported was 20-fold, compared with a 4-fold increase here. Tremblay *et al.* (1977) observed maximum rates of orotate synthesis of about 160nmol/h per g of liver, whereas we found values approaching 1000nmol/h per g of liver for UMP synthesis. These differences may reflect variations in incubation and assay conditions. Our studies also required acetylglutamate for a stimulatory effect of ammonia on pyrimidine biosynthesis. Tremblay *et al.* (1977) reported an approximate 3-fold increase in the incorporation of NaH¹⁴CO₃ into orotic acid with 12mm-acetylglutamate in the absence of added ammonia, whereas an equal amount of acetate and glutamate stimulated incorporation 2-fold over the

value obtained without acetylglutamate or ammonia. The hydrolysis of acetylglutamate in liver, releasing glutamate to serve as an ammonia source, may explain their observations. Also their reaction medium had substrate available for acetylglutamate synthesis. The much shorter incubation times (≤ 10 min) used in the present experiments compared with the 3 h incubations used by Tremblay *et al.* (1977), and the difference in composition of incubation media used could account for the observed requirement for acetylglutamate in our work.

The experiments with rat brain showed that NH_4Cl plus acetylglutamate could stimulate UMP synthesis in this tissue also, although to a much smaller extent in comparison with liver. Measurement of carbamoyl phosphate synthetase I (Fahien & Cohen, 1964) in brain indicated an activity only 10% of that in liver on a mg of protein basis. The lesser effect of ammonia in brain can be understood in terms of a decreased capacity for intramitochondrial carbamoyl phosphate production. Purine synthesis *de novo* and phosphoribosyl pyrophosphate content were unchanged, although purine base phosphoribosylation decreased by only 15%. The potential exists in brain for ammonia to alter rates of pyrimidine and purine biosynthesis, but the lower concentrations of carbamoyl phosphate synthetase I in brain would probably prevent ammonia from having a major influence by this mechanism. It is noteworthy that all parameters studied were lower in brain: the rates of purine and pyrimidine biosynthesis in brain were 20–25% of the activities in liver, and brain phosphoribosyl pyrophosphate content was 50% of liver.

A decreased rate of purine nucleotide biosynthesis in liver could explain at least partly the retardation of growth seen in individuals with chronic hyperammonaemia, especially early in life when these nucleotides would be crucial for the high rates of nucleic acid synthesis. The effect of ammonia in brain may be considerably less, as a result of the much lower capacity of this organ to synthesize carbamoyl phosphate intramitochondrially. However, if liver were to provide a major portion of the purine nucleotides for brain (purine biosynthetic activities of rat liver were 4–5 times that in brain), a decrease in purine biosynthesis in liver would influence brain nucleic acid synthesis and perhaps normal mental development.

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