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Studies on the Intracerebral Toxicity of Ammonia *

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Summary. **Interference with cerebral energy metabolism due to excess ammonia has been postulated as a cause of hepatic encephalopathy. Furthermore, consideration of the neurologic basis of such features of hepatic encephalopathy as asterixis, decerebrate rigidity, hyperpnea, and coma suggests a malfunction of structures in the base of the brain and their cortical connections.**

The three major sources of intracerebral energy, adenosine triphosphate (ATP), phosphocreatine, and glucose, as well as glycogen, were assayed in brain cortex and base of rats given ammonium acetate with resultant drowsiness at 5 minutes and subsequent coma lasting at least 30 minutes.

Cortical ATP and phosphocreatine remained unaltered during induction of coma. By contrast, basilar ATP, initially 1.28 ± 0.15 μmoles per g, was unchanged at 2.5 minutes but fell by 28.1, 27.3, and 26.6% ($p < 0.001$) at 5, 15, and 30 minutes after NH_4Ac. At comparable times, basilar phosphocreatine fell more strikingly by 62.2, 96, 77.1, and 71.6% ($p < 0.001$) from a control level of 1.02 ± 0.38 μmoles per g. These basilar changes could not be induced by anesthesia, psychomotor stimulation, or moderate hypoxia and were not due to increased accumulation of ammonia in the base. Glucose and glycogen concentrations in both cortex and base fell significantly but comparably during development of stupor, and prevention of the cerebral glucose decline by pretreatment with glucose did not obviate ammonia–induced coma or the basilar ATP fall.

These findings represent the first direct evidence that toxic doses of ammonia *in vivo* acutely affect cerebral energy metabolism and that this effect is preferentially localized to the base of the brain.

Introduction

Although the mechanisms involved in the pathogenesis of hepatic encephalopathy [1] are still undetermined, the observations that this state may be fully reversible and unaccompanied by structural changes in the brain suggest that the cerebral dysfunction is of metabolic origin. Moreover, although a variety of substances have been conjectured as possible causes of hepatic coma, excess ammonia [2] is generally accepted as one likely factor in the development of this disorder in susceptible individuals (1–3). Of the various

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[1] Hepatic encephalopathy connotes disturbance of cerebral function (including precoma and coma) associated with liver disease.

[2] The term "ammonia" refers to the sum of the ionized (NH_4^+) and un-ionized (NH_3) moieties.

hypotheses concerning the mechanism of ammonia intoxication, and possibly of hepatic encephalopathy, the major ones have postulated a depletion of α-ketoglutarate from the tricarboxylic acid cycle (4), impaired decarboxylation of pyruvate and α-ketoglutarate (5), decrease in available NADH (6), or increased consumption of ATP during synthesis of glutamine from ammonia (7). Each of the above mechanisms might result in interference with cerebral energy metabolism, and such a cerebral disturbance is now theorized as the ultimate basis of ammonia intoxication (8). Although many indirect observations supporting this thesis have been compiled (9–12), no comprehensive study of intracerebral energy metabolism in ammonia intoxication has been reported.

Consideration of the neurologic basis of such features of hepatic encephalopathy as asterixis, decerebrate rigidity, hyperpnea, and coma suggests a malfunction of specific structures in the base [3] of the brain (and possibly their cortical connections). Thus, in brief, asterixis and decerebrate rigidity are abnormalities of posture, which normally is integrated in the base of the brain; hyperpnea may result from dysfunction of the respiratory center, which is located in the pons; and most importantly according to current concepts, coma may result from impaired transmission of endogenous and exogenous stimuli via the reticular formation in the base of the brain to the cerebral cortex (13, 14). It was the purpose of this study to investigate directly the effect of ammonia on cerebral energy metabolism and to attempt to localize the site or sites of ammonia action. Precoma and coma were induced in rats with ammonium acetate (NH_4Ac), and the major cerebral energy sources, ATP, phosphocreatine, and glucose, as well as glycogen, were measured in rapidly frozen base and cortex removed from these animals. ATP and phosphocreatine concentrations were found to be substantially decreased in the base, but not the cortex, of the ammonia–intoxicated rats.

Methods

Experimental design

Female Sprague–Dawley rats provided with food (Purina Chow) and water ad libitum and weighing 60 to 80 g were used. Small animals were chosen to assure rapid freezing of labile brain constituents and yet to provide sufficient tissue for assay. Ammonium acetate was dissolved in 0.9% sodium chloride solution (60 mg per ml) and was administered to rats intraperitoneally in a dose of 60 mg per 100 g body weight. After injection of NH_4Ac, rats became hyperexcitable in 2 to 3 minutes, drowsy in 4 to 5 minutes, developed coma about 3 minutes later, and remained unresponsive to touch and light for approximately another 20 to 30 minutes, moving convulsively to sound stimuli. During the comatose state, some of the animals developed spontaneous periodic clonic–tonic convulsions, and these almost always died. Rats without ictal episodes recovered fully. As convulsions per se may affect cerebral energy metabolism (15), only those animals without convulsions were studied. Groups of rats given NH_4Ac were sacrificed for the appropriate studies at 2½, 5, 10, 15, and 30 minutes after injection. In addition, some animals were studied 5 minutes after partial recovery from coma of 25 minutes' duration. These still exhibited some drowsiness at the time of assay.

Three groups of animals served as controls: uninjected rats, those given an amount of 0.9% saline intraperitoneally equal to the average volume of the NH_4Ac solution, and those injected with sodium acetate in amounts equimolar and equal in volume to the administered NH_4Ac. The control rats exhibited no alteration from normal behavior. Animals given control solutions were sacrificed concomitantly with and after time intervals corresponding to those of the experimental animals. Rats in each control group and those sacrificed at various times after injection manifested no differences in most assays and in those instances have been pooled into one control group.

For assay of labile brain constituents (ATP, phosphocreatine, glucose, glycogen, ammonia, lactate, pyruvate) the unanesthetized animals were sacrificed by rapid submersion in a container with dry ice and acetone. The frozen brains were shelled out on dry ice and the cortex and base isolated. Unfrozen brain was obtained for studies of oxygen consumption, ATPase activity, protein concentration, water content, and electrolyte concentration. Blood samples for glucose, pH, and P_{CO_2} determinations were obtained by aortic puncture of animals anesthetized with ether for 15 to 30 seconds just before bleeding. Blood samples for pyruvate and lactate measurements were obtained by decapitation, this procedure having been found to give consistently the lowest lactate levels.

Methods of analysis

ATP was measured in frozen 30– to 100–mg brain sections by the luciferin–luciferase–luminescence reaction as described in detail previously (16). This procedure in our hands yields approximately 100% recovery of ATP added to brain homogenates of known ATP concentration, is accurate to within ± 1.8% when measured in contiguous samples obtained from the same brain site, and is unaffected by *in vitro* addition of NH_4Ac equal in quantity

[3] Base of the brain refers to the medullo-pontine and caudal midbrain areas.

to that determined to be present in brain *in vivo* after injection of NH_4Ac.

Phosphocreatine was determined in triplicate in 250- to 400-mg frozen brain sections by the method of Ennor (17), which estimates the difference between total and free creatine. This procedure in five experiments gave from 90.8 to 100% recovery with weighed quantities of phosphocreatine and creatine and was unaffected by *in vitro* addition of NH_4Ac.

Brain and blood glucose concentrations were measured by the glucose oxidase method (18), the frozen brain sections having been first homogenized in iced distilled water. Brain glucose concentration was corrected for blood glucose content by subtracting the product of blood content of the brain specimens (determined with albumin-^{131}I as described below) and blood glucose concentration from the total brain glucose concentration. Brain glycogen was determined by the glucose oxidase method after precipitating the glycogen with absolute alcohol and hydrolyzing the precipitate with sulfuric acid (19).

Brain ammonia was measured in quintuplicate in pooled 800- to 1,000-mg frozen brain specimens, employing the method of Nathan and Warren (20) except that sodium phenolate was used for color development. Equal amounts of base and cortex from the same animals were assayed, allowing meaningful comparison of ammonia concentration at both these sites in the same animals. Recoveries of weighed amounts of ammonia were between 80 and 90%.

Lactate and pyruvate in blood and brain were determined enzymatically (21, 22), the frozen 300- to 400-mg brain specimens having been first triturated in liquid nitrogen and extracted in the cold with 3 ml of 4% perchloric acid. Recoveries of weighed amounts of lactate and pyruvate were between 90 and 107%. All lactates were done in duplicate and the results averaged. Correction for brain blood content of lactate and pyruvate was calculated as described for brain glucose concentration.

Oxygen consumption of the base was measured by Warburg manometry as described previously (23), with either succinate or pyruvate as the substrate. All incubations were carried out in triplicate at 37° C and 50 strokes per minute; the results were averaged and expressed in microliters of oxygen consumed per milligram tissue (wet weight) per hour.

Brain ATPase activity was measured by determining the quantity of phosphorus (P) released from exogenous ATP by the tissue enzyme or enzymes. Phosphorus was measured by the method of Fiske-Subbarow, which was unaffected by addition of ammonium acetate. All results were expressed as micromoles of P released per milligram tissue protein (determined for each homogenate separately) per 10 minutes of incubation. The details of the ATPase procedure have been given elsewhere (23); the precise incubation systems employed for each assay are presented in Tables IV and V.

Cortical and basilar protein concentrations were determined by the method of Lowry, Rosebrough, Farr, and Randall (24), and the water content was obtained by weighing the tissue initially and then after drying under nitrogen to constant dry weight.

Blood pH was measured at 38° C on a Beckman expanded scale pH meter with an anaerobic electrode and constant temperature block; the plasma carbon dioxide content was determined with a Natelson microgasometer, and P_{CO_2} was calculated. Brain Na and K concentrations were measured in tissue homogenates by flame photometry.

Ancillary procedures

Brain blood volume. To determine the quantity of blood trapped in the frozen brain tissues, experimental and control animals were given a tracer dose of albumin-^{131}I [4] intravenously (under brief ether anesthesia). Ten minutes later, the rats were decapitated, the heads were frozen immediately by submersion in dry ice and acetone, and blood was collected from the torso. The quantity of blood in the frozen brain tissues was calculated from the isotope content in plasma and in weighed brain specimens, taking 45% as the mean blood hematocrit (25).

Control studies. a) To assess the specificity of NH_4Ac effect on decreasing basilar ATP and phosphocreatine, we also measured ATP and phosphocreatine concentrations in the base of control rats anesthetized with ether for 5 minutes and ATP in those rendered hyperexcitable by rotation in a revolving drum for 5 minutes. *b*) To determine whether hypoxia per se may have lowered the basilar ATP in the NH_4Ac-injected animals, we placed normal rats in an airtight chamber with 5.5% oxygen and a carbon dioxide trap. The rats were sacrificed after 5 or 30 minutes by rapid freezing for measurement of basilar and cortical ATP. Oxygen in the chamber was monitored throughout the experiments by a Beckman D oxygen analyzer. The degree of hypoxia in the animals was estimated from measurement of cortical, basilar, and blood lactate and pyruvate levels. Identical control studies with rats maintained in room air were carried out concomitantly.

Results

High energy phosphates in brain

Adenosine triphosphate. As shown in Figure 1, the means ± standard deviations of basilar ATP concentration in the control animals and those studied at 2½, 5, 10, and 30 minutes after NH_4Ac, as well as 5 minutes after recovery from coma of 25 minutes' duration, were, respectively, 1.28 ± 0.15, 1.36 ± 0.12, 0.92 ± 0.16, 0.93 ± 0.17, 0.94 ± 0.09, and 1.08 ± 0.03 μmoles per g (wet weight). The basilar ATP concentration at 2½ minutes after NH_4Ac, with the animals hyper-

[4] Lot X-589A-60 radioiodinated serum albumin, Abbott Laboratories, North Chicago, Ill.

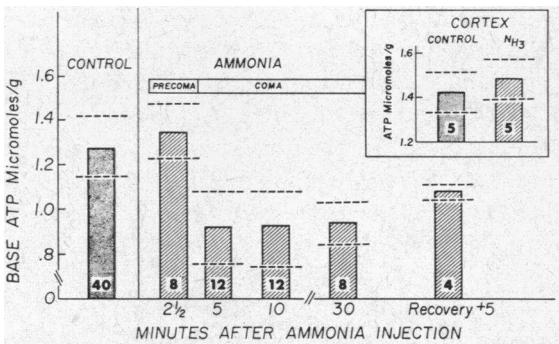

FIG. 1. EFFECT OF AMMONIA INTOXICATION ON BASILAR AND CORTICAL ATP CONCENTRATION. The bars represent mean levels of ATP, and the extended broken lines refer to standard deviations. The number of experiments performed is given within each bar. Statistical analysis is presented in the text.

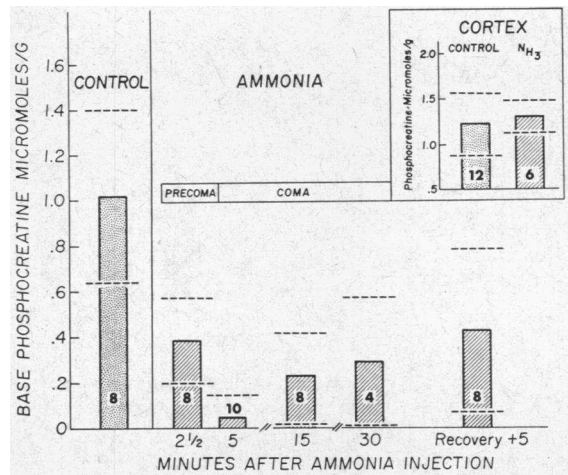

FIG. 2. EFFECT OF AMMONIA INTOXICATION ON BASILAR AND CORTICAL PHOSPHOCREATINE CONCENTRATION. The bars represent mean levels of phosphocreatine, and the extended broken lines refer to standard deviations. The number of experiments performed is given within each bar. Statistical analysis is presented in the text.

irritable but alert, was not significantly different from normal. Subsequently, however, during stupor and coma, mean basilar ATP was 28.1, 27.3, and 26.6% below normal ($p < 0.001$) at 5, 10, and 30 minutes, respectively, after ammonia injection. Five minutes after recovery from coma, with the animals still somewhat drowsy, basilar ATP rose toward normal but was still 15.3% below the control level ($p < 0.01$). By contrast, as shown in the insert at the top of Figure 1, cortical tissue, obtained at 5 and 10 minutes after NH_4Ac and from the same animals that manifested the lower basilar ATP values, had an ATP concentration comparable to the controls, 1.47 ± 0.09 and 1.42 ± 0.09 μmoles per g, respectively.

Phosphocreatine. As shown in Figure 2, the means ± standard deviations of basilar phosphocreatine concentration in the control animals and those studied at 2½, 5, 15, and 30 minutes after NH_4Ac, as well as 5 minutes after recovery from coma of 25 minutes' duration, were, respectively, 1.02 ± 0.38, 0.39 ± 0.11, 0.05 ± 0.10, 0.23 ± 0.22, 0.29 ± 0.38, and 0.43 ± 0.36 μmoles per g. These mean basilar phosphocreatine concentrations in the experimental animals represented, respectively, a 62.2, 96.0, 77.1, and 71.6% decrease from normal ($p < 0.001$) and reached a value of only 43% of normal ($p < 0.001$) shortly after recovery from coma. By contrast, as shown in the insert at the top of Figure 2, in the same comatose ammonia–injected animals with the lowest basilar phosphocreatine concentration, cortical phosphocreatine was similar to the normal value, 1.31 ± 0.17 and 1.21 ± 0.36 μmoles per g, respectively.

Brain ammonia

The selective depression of ATP and phosphocreatine in the base of the brain could be explained by increased accumulation of ammonia at that site or by sensitivity of the base to the toxic effect of ammonia. As shown in Figure 3, the preinjection ammonia concentration in the base and cortex of five sets of the same rats was similar, 1.89 ± 0.72 and 1.61 ± 0.23 μg per g wet weight, respectively. Furthermore, at 2½, 5, and 15 minutes after NH_4Ac injection the brain ammonia concentration increased about 3-, 11-, and 13-fold, respectively, over base line (Figure 3), but in each instance the values in the base and cortex remained almost identical.

Mechanisms of high-energy phosphate depletion in base

The ammonia–induced decrease in basilar (but not cortical) ATP and phosphocreatine may be the result of either decreased synthesis or increased utilization of these substances at that site or a combination of both effects. Three possible mechanisms of decreased synthesis were assessed: *a*) diminished basilar glucose and glycogen as

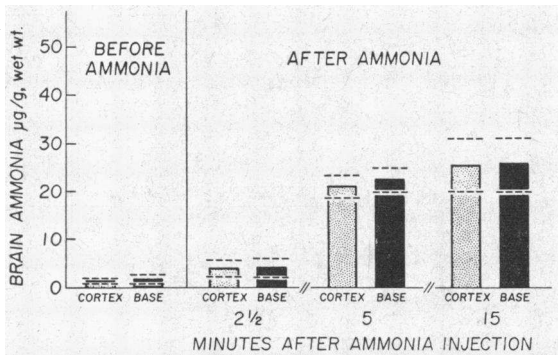

FIG. 3. AMMONIA CONCENTRATION IN BASE AND CORTEX OF RATS BEFORE AND AFTER ADMINISTRATION OF NH_4Ac. The bars represent mean values of five experiments, each carried out in quintuplicate. The extended broken lines are standard deviations. To assess the relative increment in ammonia in both base and cortex, we have given the data after NH_4Ac administration after subtraction of the base-line values listed on the left of Figure 3. Statistical analysis showed no difference between ammonia concentrations of base and cortex at any one time ($p > 0.05$).

substrates, *b*) selective hypoxia of the base, and *c*) impairment of the basilar mechanism [5] involved in oxidative phosphorylation. Increased utilization of ATP and phosphocreatine in the base due directly to ammonia-augmented ATPase activity was also considered.

a) *Brain glucose and glycogen.* As shown in Table I, rats developing ammonia-induced stupor manifested a significant decrease in both cortical and basilar glucose and glycogen concentration, whereas blood glucose was unaltered. At the approximate onset of stupor (5 minutes after NH_4Ac injection) brain glucose and glycogen concentrations in the experimental animals fell to about 38 and 73% of the normal values, respectively, but the decrease of each substrate was virtually identical in both cortex and base (Table I).

The role of cerebral glucose decrease in ammonia-induced stupor and basilar ATP decrease was further assessed in five sets of rats lightly anesthetized for 30 seconds with ethyl ether, infused with 50 mg glucose (1 ml 5% dextrose in water), and then immediately on regaining consciousness (1 to 2 minutes) injected with either a control solution or NH_4Ac (as described above). Five minutes later mean blood glucose concentration in the experimental animals was high, 219.6 mg per 100 ml, and glucose concentrations in the cortex and base (27.6 and 26.5 mg per 100 g brain, respectively) were maintained at levels comparable to those in controls. Yet in the same rats, stupor was not prevented, and basilar ATP concentration was again decreased by 28% from the control mean value of 1.53 μmoles per g in the glucose-injected controls ($p < 0.03$).

b) *Brain hypoxia.* Measurements of lactate and pyruvate concentrations in cortex, base, and blood were carried out to determine the presence of any regional accumulation of excess lactate, as an indication of selective basilar hypoxia in the ex-

[5] The term "mechanism" refers only to the integrity of the respiratory electron chain and phosphorylation coupled to it.

TABLE I

Effect of ammonia on brain glucose and glycogen concentrations

		Control		Ammonia*			
				Minutes after ammonia			
				2½		5	
		Cortex	Base	Cortex	Base	Cortex	Base
Glucose	Mean ± SD,† *mg/100 g wet wt*	27.74 ± 5.42	25.13 ± 5.01	20.62 ± 4.47	19.52 ± 3.58	12.51 ± 9.64	11.24 ± 9.51
	% decrease from control‡			28.6	26.1	61.9	62.3
Glycogen	Mean ± SD,† *mg/100 g wet wt*	68.38 ± 8.25	69.69 ± 14.14	57.77 ± 6.49	54.21 ± 13.70	50.57 ± 8.06	50.97 ± 13.38
	% decrease from control‡			15.5	22.2	26.1	26.9

* The decrease in glucose and glycogen concentrations at both 2½ and 5 minutes after ammonia injection was significantly ($p < 0.025$ or less below control values. Glucose and glycogen concentrations in base and cortex, both in control and in ammonia-injected animals, at both sampling times were comparable ($p > 0.05$).

† Mean of 7 to 20 assays each; in the case of base, specimens from several animals usually had to be pooled for one assay.

‡ These values have been adjusted for brain blood glucose content (see methods of analysis). Blood glucose concentrations in controls (103.8) ±12.8 mg per 100 ml) and after 5 minutes of ammonia (108.8 ± 18.1 mg per 100 ml) were comparable ($p > 0.05$).

TABLE II

Effect of ammonia on brain and blood lactate and pyruvate concentrations

	Minutes after ammonia	Lactate*			Pyruvate*		
		Cortex	Base	Blood	Cortex	Base	Blood
		mg/100 g brain wet wt		*mg/100 ml*	*mg/100 g brain wet wt*		*mg/100 ml*
Control		17.5 ± 2.6	26.8 ± 5.4	18.7 ± 4.0	1.68 ± 0.40	1.39 ± 0.33	0.59 ± 0.09
Ammonia-injected	2½	21.8 ± 5.5 (NS)	26.8 ± 5.0 (NS)		1.79 ± 0.24 (NS)	1.25 ± 0.15 (NS)	
	5	29.5 ± 8.7 (69.1%)	42.0 ± 5.6 (57.0%)	24.3 ± 7.1 (NS)	1.92 ± 0.52 (NS)	1.29 ± 0.22 (NS)	0.60 ± 0.08 (NS)
	10	33.1 ± 4.3 (91.4%)	49.0 ± 6.5 (84.1%)	19.1 ± 1.1 (NS)	1.90 ± 0.28 (NS)	1.32 ± 0.07 (NS)	
	30	38.3 ± 7.6 (116.6%)	53.8 ± 11.2 (99.3%)	53.8 ± 19.7 (187.7%)	2.34 ± 0.35 (39.8%)	1.80 ± 0.44 (29.8%)	0.64 ± 0.03 (NS)

* Each concentration is the mean ± standard deviation of 5 to 20 assays. The per cent increase of each experimental lactate or pyruvate concentration over the corresponding control value is given in parentheses below the appropriate experimental number. Statistics are given in the text. NS refers to lack of statistical significance. The per cent changes are adjusted for brain blood lactate and pyruvate content (see methods of analysis).

perimental animals (Table II). Blood pyruvate was not affected by ammonia injection, and blood lactate rose significantly ($p < 0.001$) only after 30 minutes. Brain pyruvate (both base and cortex) in the experimental animals also remained at control values except at 30 minutes after NH_4Ac, when it rose in both cortex and base ($p < 0.05$). By contrast, beginning with the 5-minute values, ammonia induced a significant increase in excess lactate in both cortex and base ($p < 0.001$), but the degree of rise in excess lactate at both these sites was similar (Table II).

Rats rendered hypoxic by exposure to 5.5% oxygen for 5 or 30 minutes hyperventilated and became ataxic and drowsy, more so on prolonged exposure, but none showed ictal manifestations or died. The hypoxic animals showed a substantial increase in both blood and brain pyruvate and lactate, the latter rising greatly in excess of the pyruvate in both cortex and base ($p < 0.001$). However, despite the obvious cerebral hypoxia, of a duration equal to and of magnitude (as judged by excess lactate) probably exceeding that noted in the ammonia-injected rats, both the cortical and basilar ATP concentrations of the hypoxic animals remained within control values.

c) Impairment of oxidative phosphorylation. Oxidative phosphorylation depends on a functioning respiratory electron chain and on phosphorylation properly coupled to it. To determine the integrity of the respiratory chain in the base of the ammonia-injected rats, we compared the oxygen consumption of base removed from the experimental animals with that from controls. As shown in Table III, with either succinate or pyruvate as substrate, respiration of base from all animal groups was comparable ($p > 0.05$). These data are in agreement with those obtained on adding to rat cerebral cortical slices, ammonia in a concentration comparable to that measured in the brain of the current experimental animals (26). In addition, measurements of P:O ratios by McKhann and Tower (5) indicated no uncoupling of oxidative phosphorylation by large concentrations of ammonia added to rat brain mitochondria. These *in vitro* observations suggest, therefore, that the mechanism for cerebral oxidative ATP synthesis in the ammonia-intoxicated animals is not impaired.

TABLE III

Effect of ammonia on basilar oxygen consumption

Substrate*	Control	Ammonia-injected Minutes after ammonia	
		5	15
	μl O_2/mg/hour	*μl O_2/mg/hour*	
Succinate	1.34 ± 0.14† (10)	1.31 ± 0.23 (5)	1.31 ± 0.19 (5)
Pyruvate	0.72 ± 0.09 (15)	0.65 ± 0.03 (5)	0.73 ± 0.06 (5)

* The incubation medium using succinate as substrate has been described previously (23). Pyruvate was substituted in equimolar amounts for succinate.

† Mean ± standard deviation for the number of rats indicated within the parentheses.

d) *ATPase activity.* As shown in Table IV, similar ATPase activity was found in base and cortex removed from control and NH_4Ac–injected animals. To assess more critically the effect of ammonia in various concentrations on brain ATPase activity, we compared the ATPase activity of submicroscopic particles, shown to have the highest Na^+–K^+–activated ATPase activity (27), from base and cortex of normal rats, in incubation media containing no added NH_4^+, or 1.77 and 17.7 mM NH_4^+. The 1.77 mM NH_4^+ concentration corresponds to that measured in both base and cortex of experimental animals at 15 minutes after injection of NH_4Ac, and the electrolyte composition is similar to that employed by Skou (27) to demonstrate an NH_4^+–augmented ATPase activity in brain. As shown in Table V (1a, b), in the absence of K^+, 1.77 and 17.7 mM NH_4^+ increased ATPase activity significantly ($p < 0.001$), but similarly in base and cortex. A similar ammonia–induced increase in ATPase activity was also noted in K^+–free submicroscopic particles obtained from whole brain (Table V, 2a, b). By contrast, addition to the medium of 3.5 mM K^+, a concentration present in extracellular and cerebrospinal fluids but considerably below that present in cortex or whole brain (28, 29), completely masked the stimulatory effect of NH_4^+ on ATPase (Table V, 2c), K^+ by itself strikingly augmenting ATPase activity. This effect of K^+ is in agreement with the data of Skou (27). Na^+ and K^+ concentrations, which play a key role in regulating ATPase activity, were measured in cortex and base of two control rats and two rats given NH_4Ac 5 minutes before sacrifice. The mean Na^+ and K^+ concentrations in control cortex were 50.3 and 96.2 μEq per g and in control base 46.7 and 89.7 μEq per g wet weight. The Na^+ and K^+ values in the experimental cortex were 49.2 and 97.5 μEq per g and in base 50.3 and 87.2 μEq per g wet weight. These data agree closely with those obtained by others in normal brain of a variety of species (28, 29).

Ancillary studies

Brain composition. *1*) Protein concentrations of cortex and base measured in five to seven sets of control animals as well as those injected with NH_4Ac 5 and 30 minutes previously were similar ($p > 0.05$). *2*) Dry weight of base removed from ten control and ten ammonia–injected animals also was comparable ($p > 0.05$), averaging 29.4 and 30.4% of wet weight, respectively. *3*) The means ± standard deviations for plasma content of control and ammonia–injected cortex were 1.43 ± 0.20 and 1.20 ± 0.26% and for base 1.27 ± 0.29 and 1.17 ± 0.23% of tissue wet weight. These differences are not statistically significant ($p > 0.05$).

Control studies. *1*) Rats rendered excitable by rotation for 5 minutes in a revolving drum had a normal basilar ATP, whereas those anesthetized for 5 minutes with ethyl ether showed a 12.2% increase in basilar ATP ($p < 0.05$) and a 64.8% increase ($p < 0.001$) in basilar phosphocreatine. *2*) In groups of four rats given sodium acetate, physiologic saline, or NH_4Ac 5 minutes before sampling, mean values ± standard deviations for blood pH, bicarbonate (in milli-

TABLE IV

Effect of ammonia intoxication on basilar and cortical ATPase activity

	Control*		NH_3*	
	Base†	Cortex†	Base†	Cortex†
	μmoles P/mg protein/10 min			
No Mg^{++}	0.47 ± 0.13‡	0.47 ± 0.06	0.53 ± 0.08	0.46 ± 0.02
Mg^{++}	1.44 ± 0.06	1.18 ± 0.04	1.46 ± 0.07	1.20 ± 0.03

* In the NH_3 group brain was removed from animals 5 minutes after administration of NH_4Ac (see experimental design); in the control group brain was removed from asymptomatic rats given 0.9% saline (with or without sodium acetate equimolar to NH_4Ac).

† Pooled tissue was homogenized in 30 vol of 0.3 M Tris, pH 7.6. The homogenate was prepared and kept at 0° C until used 2 hours later. Each incubation vessel contained 0.9 ml of homogenate, ATP 7.5, and (where present) $MgCl_2$, 5 mmoles per L, the latter having been added in 0.4 ml of 0.3 M Tris. The final volume of each flask was 1.3 ml, pH 7.6. Incubation temperature was 37° C.

‡ Mean ± standard deviation of five determinations.

TABLE V

Effect of exogenous ammonia on brain ATPase activity

Incubation system no.*	Control†	1.77 mM KH_4^+†	17.7 mM NH_4^+†
	μmoles P/mg protein/10 min		
1. a) Base: submicroscopic particles Na^+ 100 mM, Mg^{++} 5 mM	1.84 ± 0.12‡	3.19 ± 0.09	8.84 ± 0.14
b) Cortex: submicroscopic particles Na^+ 100 mM, Mg^{++} 5 mM	1.36 ± 0.19	2.65 ± 0.21	6.89 ± 0.27
2. a) Whole brain: submicroscopic particles Na^+ 100 mM, Mg^{++} 5 mM	3.26 ± 0.21	5.67 ± 0.32	8.58 ± 0.09
b) Whole brain: submicroscopic particles Na^+ 10 mM, Mg^{++} 5 mM	2.05 ± 0.23	5.59 ± 0.26	9.30 ± 0.39
c) Whole brain: submicroscopic particles Na^+ 10 mM, Mg^{++} 5 mM, K^+ 3.5 mM	9.01 ± 0.15	8.58 ± 0.60	8.63 ± 0.57

* Submicroscopic particles were prepared by ultracentrifugation by the method of Skou (27). Na^+, K^+, and Mg^{++} were added to the final incubation medium in 0.3 M Tris solution (prepared in doubly distilled deionized water) so as to give the final concentration of each specified above under incubation systems. The rationale for these Na^+ and K^+ concentrations is presented under Results. ATP (Tris salt) was added in each instance to give 7.5 mmoles per L in the final incubate. Final volume, pH, and temperature were as described for Table IV.

† NH_4^+ concentration is that in the final incubation vessel. The 1.77 mM NH_4^+ concentration approximates that obtained in both base and cortex 15 minutes after injection of NH_4Ac (Figure 3). The NH_4Ac was freshly dissolved in 0.3 M Tris just before use and added in 0.1 ml Tris; control consisted of 0.3 M Tris without NH_4Ac.

‡ Mean ± standard deviation of five to seven determinations each.

equivalents per liter), and P_{CO_2} (in millimeters Hg), respectively, were 7.39 ± 0.02, 27.5 ± 0.7, and 42.0 ± 4.0 for the sodium acetate animals; 7.30 ± 0.02, 24.6 ± 1.3, and 49.0 ± 4.0 for the saline animals; and 7.27 ± 0.03, 22.4 ± 1.2, and 49.0 ± 5.9 for NH_4Ac animals. The difference in blood pH between the NH_4Ac and saline–injected animals is not statistically significant ($p > 0.05$). Blood pH in rats given sodium acetate is significantly higher ($p < 0.001$), however, than in the other two groups. Basilar ATP concentration is similar in animals given saline or sodium acetate ($p > 0.05$). *3*) No morphologic alteration in the base of the brain, which could alter the reference basis for the high energy phosphate determinations, was seen by light or electron microscopy in the ammonia–intoxicated rats.

Discussion

This study clearly demonstrates that acute ammonia intoxication in rats results in a significant and consistent depletion of both ATP and phosphocreatine in the base of the brain (Figures 1 and 2). In contrast to the findings in the base, cortical ATP and phosphocreatine concentrations in the same ammonia–intoxicated rats were normal (Figures 1 and 2). This latter observation is in agreement with more extensive data obtained previously in the cerebral cortex of rats (16). The normal cortical concentrations of ATP and phosphocreatine serve as an internal control for the low basilar values of these high energy phosphates and indicate that there was no diffuse cerebral depletion of ATP and phosphocreatine as a result of some possible, nonspecific, ammonia–induced metabolic disturbance. Other control studies, here presented, also indicate that features associated with ammonia intoxication such as loss of consciousness per se (ether anesthesia) and hyperkinesis alone (drum rotation) do not account for the decrease in ATP and phosphocreatine observed in the base of the experimental animals.

The preferential toxic effect of ammonia on the base cannot be attributed to increased accumulation of ammonia at that site since ammonia concentrations in base and cortex were equal both before and at various time intervals after injection of NH_4Ac (Figure 3). It appears, therefore, that the ammonia–induced interference with basilar energy metabolism must be due to greater sensitivity of that area to ammonia. This interpretation is predicated on the currently untestable assumption that ammonia is not concentrated in some minute but vital basilar structure which, during the assay of whole base ammonia, is "diluted" by inclusion of contiguous tissue.

The decreased concentrations of ATP and

phosphocreatine in the base of the ammonia–intoxicated animals may be due to impaired formation, increased utilization of these substances at that site, or to a combination of both effects. The factors assessed in this study—decreased availability of glucose and nonstructural glycogen, faulty oxygenation, impairment of the respiratory electron chain, and a direct stimulatory effect of ammonia on ATPase activity—do not appear alone to account for the selective depletion of ATP and phosphocreatine in the base. Thus, the striking decrease in brain glucose and glycogen observed in ammonia–injected rats was virtually identical in both base and cortex (Table I), whereas the fall in ATP and phosphocreatine was noted only in the base. In addition, maintenance of normal brain glucose concentration by pretreatment with glucose neither prevented ammonia–induced coma nor the basilar ATP depletion. As regards oxygenation, the moderate increase in brain excess lactate in the ammonia–injected rats (Table II) is indicative of cerebral hypoxia or possibly of intracerebral acidosis (30). Neither event per se, however, is likely to account for the changes in the basilar high energy phosphates since a comparable rise in excess lactate occurred in both base and cortex of the experimental animals (Table II). Furthermore, cerebral hypoxia of a duration equal to and magnitude probably exceeding that noted in the ammonia–injected rats failed to depress either cortical or basilar ATP concentration. The possibility that ammonia interfered with cerebral oxidative phosphorylation by impairing directly the respiratory electron chain is negated by observations that this concentration of ammonia interferes with neither respiration nor phosphorylation of brain *in vitro* (Table III) (5, 26). Finally, it is unlikely that ammonia–induced ATPase activity per se can explain the decrease in high energy phosphates observed in base alone, since *1*) ATPase activity of base and cortex removed from experimental animals (Table IV) and that induced in these tissues by ammonia *in vitro* (Table V) were comparable, *2*) the concentration of ammonia at both sites was equal *in vivo* (Figure 3), *3*) the presence of even small concentrations of K^+ *in vitro* prevented the ammonia effect on ATPase (Table IV), and *4*) the concentration of both K^+ and Na^+ in base and cortex of control and ammonia–injected animals was substantial and similar. The other postulated mechanisms for ammonia–induced decreased synthesis of ATP [depletion of cerebral α-ketoglutarate (9), decreased utilization of pyruvate and α-ketoglutarate (5), and diminished available NADH (6)] as well as those possibly responsible for excessive utilization of ATP [formation of glutamine (7) and ammonia–induced increased electrical activity (31)] are currently under study in this laboratory. Preliminary data, employing a specific enzymatic technique for measuring tissue α-ketoglutarate (32), suggest that this ketoacid is not depleted in the base of these ammonia–intoxicated rats (33) and, although a decrease of a critical α-ketoglutarate pool cannot be excluded, this mechanism does not appear to be responsible for the decreased ATP concentration in the base. The role of the other factors is as yet undetermined.

The functional significance of the degree of ATP and phosphocreatine depletion noted in the base of the ammonia–injected animals is difficult to evaluate. First, methodologic differences render difficult a comparison of cerebral high energy phosphate levels in different studies. No current method measures the actual *in vivo* level of these substances in the brain, due to their rapid depletion during freezing (34), and it is assumed that the extrapolation slope from the observed to the *in vivo* levels in any study is the same for both experimental and control animals. The control cortical ATP and phosphocreatine values in this study were somewhat lower than those obtained by others employing liquid nitrogen for freezing (35). However, in four sets of control and ammonia–intoxicated 20–g rats, which freeze faster, the decreased basilar ATP in the experimental group was again reproduced percentagewise, although both the control cortical and basilar ATP levels matched the higher ATP concentrations of other workers. Second, the current measurements of the high energy phosphates were carried out in whole base, and it is possible that in such specialized structures within that area as the reticular ascending formation, a more pronounced decrease in ATP may have been present. Furthermore, ammonia metabolism in brain appears to be compartmented (36), and current methods cannot assess any differences in energy metabolism within individual cerebral pools of

varying size. In spite of these qualifications, in the one available study wherein neurologic findings were compared with acute cerebral ATP changes in anoxic rats, a one-third depletion in brain ATP was associated with death (37). Furthermore, delay of the cerebral ATP decrease by conditioning to anoxia delayed the onset of death in anoxic rats until the brain ATP fell again by about one-third from normal (37), and decreased ATP utilization by hypothermia and anesthesia likewise protected the animals against the effects of anoxia (34). The decrease in phosphocreatine before the development of drowsiness and the fall in ATP before onset of overt stupor suggest that these changes may have not only chronologically but also causally preceded the onset of ammonia–induced coma. The precise relationship between ATP depletion and impairment of consciousness is unknown, although decreased available energy at a critical brain site theoretically could induce defective transmission and integration of nervous impulses vital for proper cerebral function (34, 38). Alternatively, both the decreased basilar high energy phosphates and the neurologic disturbance could be independent facets of some more basic cerebral metabolic impairment. The correlation between acute ammonia toxicity and impairment of cerebral energy metabolism is consistent with the previously demonstrated protective effect of hypothermia (39) and may explain the enhanced susceptibility to ammonia in hypoxia (40) and hyperthermia (39), these conditions, respectively, reducing and augmenting the requirements for cerebral high energy phosphates. The meaning of the quantitative changes of basilar ATP and phosphocreatine after the onset of coma is confused by the possible effects of coma itself on decreasing ATP utilization and on reducing psychomotor stimulation during freezing, thus preserving and "falsely" elevating the high energy phosphates. The above considerations, therefore, though not conclusive, suggest that the observed decrease in basilar high energy phosphates is of functional significance.

Extrapolation of these data to human hepatic encephalopathy must be cautious, but these findings represent the first direct *in vivo* evidence that toxic doses of ammonia acutely affect cerebral energy metabolism. Furthermore, it is evident that as was anticipated from neuroanatomic considerations of some of the clinical features of hepatic encephalopathy, the acute toxic effect of ammonia is primarily exerted on the base of the brain.

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