Bull. Environ. Contam. Toxicol. (1985) 35:15–22

Bulletin of Environmental Contamination and Toxicology

Effects of Ammonia on Selected Hepatic Microsomal Enzyme Activity in Mice

John C. Kapeghian,[1] Alan B. Jones,[2] and I. Wade Waters[1]

[1]Department of Pharmacology and [2]Department of Pharmaceutics, School of Pharmacy, University of Mississippi, University, MS 38677

Atmospheric ammonia (NH_3) is produced by a number of industrial sources (National Academy of Sciences, 1979); however it is the biological action of urease-containing bacteria upon urine and fecal waste products that is responsible for producing NH_3 in laboratory animal facilities. Since NH_3 is a primary irritant (Braker et al. 1977) studies emphasizing the pulmonary toxicity of both acute (Boyd et al. 1944; Dalhamn and Sjoholm 1963; Niden 1968) and repeated (Coon et al. 1970; Doig and Willoughby 1971; Mayan and Merilan 1972; Stombaugh et al. 1969; Weatherby 1952; Weedon et al. 1940) exposures have appeared. It was not until Vesell and coworkers (1973) suggested that repeated exposure to NH_3 (from dirty cage environments) could produce hepatic microsomal enzyme inhibition in laboratory rats that the generation of NH_3 in animal rooms was regarded seriously. These investigators reported that rats kept in a dirty environment for 7 d had significantly lower levels of hepatic microsomal enzyme activity (ethylmorphine N-demethylase and aniline hydroxylase activity) as well as reduced levels of cytochrome P-450 compared to controls. The authors postulated that this phenomenon may be the result of hepatotoxicity due to the NH_3 generated from urease-containing bacteria in feces; however, NH_3 levels were not actually monitored. Recently Schaerdel et al. (1983) reported a significant drop in ethylmorphine N-demethylase activity as well as cytochrome P-450 levels in rats after 24 ppm NH_3 for 7 d. However, at 714 ppm NH_3 for 3 d there was no change in either parameter, while after 7 d ethylmorphine N-demethylase activity was significantly reduced. These workers could not produce a direct correlation between liver microsomal enzyme activity and environmental NH_3 concentration and therefore concluded that NH_3 was probably not responsible for the inhibition of microsomal enzyme activity in rats kept in a dirty environment.

Since the mouse has proven to be a sensitive animal model for use in NH_3 toxicity studies (National Academy of Sciences 1979), and work in the rat has produced only minimal microsomal changes (Schaerdel et al. 1983), the present study was undertaken to determine whether acute or repeated exposure to NH_3 gas inhibits selected microsomal enzyme activity in the mouse.

MATERIALS AND METHODS

Male albino ICR mice (25–30 g) purchased from Harlan Industries

(Cumberland, IN) were allowed to acclimate to our facilities at least 1 week prior to use in this study. Animals were fed laboratory chow (Purina #5001, Ralston Purina Co., St. Louis, MO) and water ad libitum except during NH_3 or air exposure studies. Sterilized ground corn-cob bedding (SAN-I-Cel®, Paxton Processing Co., Inc., Paxton, IL) was used throughout the study and replaced daily. A 12:12 h light:dark cycle and an ambient temperature of 23 ± 1°C were maintained continually.

Mice were exposed (12 per group) to either NH_3 or air for 4 h acutely or daily for 4 d in a dynamic-flow multichamber whole-body exposure system previously described (Kapeghian et al. 1980; 1982). Immediately following each exposure, animals were returned to their respective home cages (6 per cage) for observation. Body weights were recorded prior to each daily exposure session. All experiments were conducted 1 h following removal of the animal from the exposure chamber. NH_3 concentrations were determined by the method described by Kapeghian et al. (1981). Equilibration time for chamber NH_3 concentrations was 10-15 min.

In the acute exposure studies hexobarbital sleeping times were determined 1 h following exposure to either air, 1350 or 4380 ppm NH_3 for 4 h. Hexobarbital (85 mg/kg, i.p.) sleeping times and latency to hypnosis (time to loss of righting reflex) were determined for each animal in an environment that maintained normal temperature. In vitro hepatic microsomal enzyme activity was determined after a single 4 h exposure to air or 4700 ppm NH_3. Microsomal enzyme activity was also determined in isolated 12,500 g liver supernatant fractions incubated for 30 min in atmospheres of 341 and 673 ppm NH_3 in O_2, and compared to O_2 controls.

In repeated-exposure studies, in vitro microsomal assays were performed following exposure to NH_3 (115, 350 ppm) or air for 4 h per day, 4 d. An additional air control group was pair-fed to induce an overall reduction in mean body weight quantitatively equivalent to that observed after repeated NH_3 exposures (350 ppm).

For preparation of in vitro liver microsomal assays, all animals were sacrificed by cervical dislocation followed by decapitation, the livers (minus the gall bladder) were removed, blotted, weighed (g/kg), homogenized in ice-cold 0.05 M Tris HCl - 0.15 M KCl buffer and spun at 12,500 g for 10 minutes at 4°C. The supernatant fraction was then measured and used for all enzymatic assays. Metabolic procedures were conducted according to the methods described by Fouts (1970). Each assay employed a portion (0.5 ml) of the 12,500 g supernatant fraction equivalent to approximately 5.0 mg of microsomal protein. Aminopyrine N-demethylase activity was determined according to the method of Cochin and Axelrod (1959). Formaldehyde (HCHO) generation was followed after a 30 min incubation by measuring the absorbance of product at 415 nm. Aniline hydroxylase activity was determined by procedures outlined by Mazel (1971). Product formation (p-aminophenol, PAP) was measured at 640 nm. Twenty µmoles of aminopyrine (a type I cytochrome P-450 binding substrate) or 10 µmoles of aniline HCl (a type II binding substrate) were added to incubation mixtures at 37°C in a 100% O_2

atmosphere. The hepatic microsomal fraction was obtained by centrifuging 0.5 ml aliquots of the 12,500 g supernatant fraction at 105,000 g for 1 h at 4°C. Microsomal protein in the resulting resuspended pellet was determined by the method of Lowry et al. (1951).

Microsomal enzyme activity is expressed both as "total activity" (μmoles product/30 min/liver) and as "specific activity" (nmoles product/30 min/mg of microsomal protein) for most *in vitro* data. Total microsomal protein is expressed as mg/liver.

Statistical analyses were conducted using either the Student "t" test or the one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. The criterion for significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Exposure to NH_3 at 1350 ppm for 4 h had no effect on the duration of hexobarbital sleeping times (with respect to air controls) as indicated in Table 1. Following exposure to 4380 ppm NH_3, sleeping times were significantly increased compared to air controls; however, this concentration proved lethal to 3/12 mice during the exposure and to one animal during hexobarbital hypnosis. Latent periods were significantly reduced in both exposure groups.

Table 1. Effect of NH_3 Gas Exposure (4 Hour) on Hexobarbital Sleeping Times in Mice

Treatment (4-Hour)	Latency to Hypnosis (Min)	Hexobarbital Sleeping Time (Min)	N
Air	3.2 ± 0.2[a]	17 ± 1.4	12
NH_3 (1350 ppm)	2.6 ± 0.1*	19 ± 1.7	12
Air	3.5 ± 0.1	11 ± 1.0	12
NH_3 (4380 ppm)	2.8 ± 0.2*	19 ± 2.0*	8[b]

[a]Values represent mean ± 1S.E.
[b]Three mice died during exposure; one died during hypnosis
*Significantly different from air control at $p \leq 0.05$ by the Student t-test

The effect of exposure to a lethal concentration (4380 ppm) of NH_3 on the prolongation of hexobarbital hypnosis is not surprising when pathophysiological effects of this exposure are considered (Kapeghian et al. 1982). In fact, other atmospheric pollutants such as ozone (Gardner et al. 1974) and nitrogen dioxide (Miller et al. 1980) also prolong barbiturate-induced hypnosis in mice. Since all of these agents (NH_3, ozone, and nitrogen dioxide) have in common the capacity to induce severe pulmonary lesions following short-term exposures (Niden 1968; Amdur 1980) it may not be unlikely that *in vivo* markers of extrapulmonary function could be indirectly altered. The reduction in latency periods for both NH_3 treatment groups may be related to this phenomenon.

Since hexobarbital hypnosis was prolonged only after exposure to a potentially lethal concentration of NH_3, a similar regimen was employed to examine in vitro hepatic microsomal enzyme activity (Table 2). Contrary to sleeping time data, microsomal protein levels were significantly elevated compared to controls following the 4 h exposure.

Table 2. Effect of NH_3 Gas Exposure (4 Hour) on Hepatic Microsomal Enzyme Activity In Vitro

Treatment	Body Weight (g)	Liver Weight (g/kg)	Microsomal Protein (mg/liver)	Aminopyrine N-demethylase A	Aminopyrine N-demethylase B	Aniline Hydroxylase A'	Aniline Hydroxylase B'
Air	24.3[a] ± 0.2	48.5 ± 0.9	39.1 ± 2.9	1.74 ± 0.16	44.4 ± 2.56	1.53 ± 0.19	39.6 ± 2.2
NH_3[b] (4700 ppm)	25.2 ± 0.5	51.8 ± 1.6	51.2* ± 1.5	2.34* ± 0.89	46.2 ± 1.98	1.39 ± 0.08	27.2* ± 1.5

[a]Values represent mean ± 1S.E.
[b]5/12 mice died during exposure
A=μmoles HCHO/30 min/liver
B=nmoles HCHO/30 min/mg protein
A'=μmoles PAP/30 min/liver
B'=nmoles PAP/30 min/mg protein
*Significantly different from air control ($p \leq 0.05$) by the Student "t" test

Also total (per liver) aminopyrine N-demethylase activity was significantly increased while total aniline hydroxylase activity remained unchanged. There were no changes in the specific activity of aminopyrine N-demethylase (per mg of microsomal protein) while the specific activity of aniline hydroxylase declined significantly. Compositely these results indicate that the increased microsomal protein observed was predominantly associated with the metabolism of the type I substrate (aminopyrine) since the total activity of aminopyrine N-demethylase was elevated while that of aniline hydroxylase was unchanged. The apparent inhibition of the specific activity of aniline hydroxylase was created "on paper" by expressing its activity in terms of elevated microsomal protein. Since the standard measurement of microsomal protein (Lowry et al. 1951) does not separate enzymatic systems associated with the metabolism of a particular substrate, the term "specific activity" so widely used in microsomal studies is a misnomer. In this regard then, unless the enzyme studied is isolated and purified, the effect of treatment on its activity must include a comparison of its total activity and specific activity with respect to changes in microsomal protein. The data in Table 2 then would support the hypothesis that the observed increase in hexobarbital sleeping times (Table 1) was not due to hepatic microsomal inhibition but rather the result of an extrahepatic physiological alteration. The increase in total microsomal protein and aminopyrine N-demethylase activity (Table 2) may be the result of a corticosteroid-mediated event (Castro et al. 1970) during the stressful and potentially lethal NH_3 exposure.

Since it was not possible to demonstrate inhibition of liver microsomal enzymes by in vivo exposures, pooled microsomal supernatant fractions (12,500 g) were incubated in the presence of NH_3 in O_2. The results in Table 3 indicate that there is no inhibition of the metabolism of either the type I (aminopyrine) or type II (aniline) substrate after incubation with either 341 or 673 ppm NH_3.

Table 3. Microsomal Enzyme Activity Following Incubation of 12,500 g Supernatant Fraction in NH_3 Atmospheres In Vitro

Incubation Atmosphere	Aminopyrine N-demethylase (nmoles HCHO/30 min/mg protein)	Aniline hydroxylase (nmoles PAP/30 min/mg protein)
Control (100% O_2)	118 ± 7.22[a]	29.9 ± 0.37
NH_3 (341 ppm)[b]	129 ± 3.18	33.9 ± 0.63
NH_3 (673 ppm)[b]	130 ± 2.00	31.3 ± 0.14

[a]Mean activity ± 1S.E. for 3 determinations per group
[b]NH_3 concentration in O_2

Since there was no evidence of inhibition of hepatic microsomal enzyme activity following acute exposure to NH_3 either by in vivo or in vitro measures, mice were then exposed to NH_3 for 4 h daily for 4 days. Results of preliminary studies indicated that at concentrations over approximately 150 ppm NH_3, repeated exposures resulted in significant weight loss by Day 4. For this reason, a group of animals exposed to air only (4 h/d, 4 d) were pair-fed to produce an equivalent drop in body weight observed with repeated NH_3 treatment (350 ppm) under the same exposure conditions. In addition, a group of animals were exposed to a lower NH_3 concentration (115 ppm) to reduce the effect of NH_3-induced inanition on the results. An air-only control group (allowed food ad libitum) was also included as in previous studies. As seen in Table 4, body weights of animals exposed to 350 ppm NH_3 as well as the pair-fed controls were significantly lower than air-only controls as expected.

Body weights of animals receiving NH_3 at 115 ppm were not significantly different from air controls. Liver weights were significantly reduced in the pair-fed group only. Total microsomal protein was significantly decreased both in NH_3-treated groups and in pair-fed controls. Furthermore, total aminopyrine N-demethylase activity (per liver) was significantly depressed both in NH_3-treated groups and in the pair-fed controls. However, when the activity of this enzyme is expressed with respect to microsomal protein, there is no difference between any of the groups. Since there was no change in total aniline hydroxylase activity as a result of either NH_3 treatment or the pair-feeding regimen, we would conclude that the decreased microsomal protein in these groups most likely represents a reduction in enzymatic protein responsible for metabolism of the type I substrate (aminopyrine). This phenomenon is responsible for the significant elevation of the specific activity of aniline hydroxylase activity (created "on paper") after 350 ppm NH_3 or the pair-feeding regimen. There was no effect of the low level NH_3 exposure (115 ppm) on either total or specific aniline hydroxylase activity.

Table 4. Effect of Repeated NH_3 Gas Exposure or Pair-Feeding on Hepatic Microsomal Enzyme Activity In Vitro

Treatment (4 h/d, 4 d)	Body Weight (g)	Liver Weight (g/kg)	Microsomal Protein (mg/liver)	Aminopyrine N-demethylase A	Aminopyrine N-demethylase B	Aniline Hydroxylase A'	Aniline Hydroxylase B'
Air	29.2[a] ± 0.5	51.1 ± 0.9	53.8 ± 1.1	5.60 ± 0.14	104 ± 2.57	1.65 ± 0.05	30.7 ± 1.06
Air[b] (Pair-fed)	27.5* ± 0.2	44.7* ± 0.5	45.2* ± 1.6	3.89* ± 0.30	85.5 ± 5.00	1.69 ± 0.11	37.1* ± 1.50
NH_3 (115 ppm)	29.7 ± 0.4	52.7 ± 0.7	46.8* ± 1.9	4.74* ± 0.23	100 ± 4.06	1.52 ± 0.05	32.3 ± 0.66
NH_3 (350 ppm)	27.9* ± 0.4	49.3 ± 0.9	40.8* ± 1.5	4.59* ± 0.33	113 ± 8.36	1.50 ± 0.06	36.8* ± 0.96

[a]Values represent mean ± 1S.E; N=12 mice per group
[b]Fed to maintain body weight changes in 350 ppm NH_3 group
A=μmoles HCHO/30 min/liver
B=nmoles HCHO/30 min/mg protein
A'=μmoles PAP/30 min/liver
B'=nmoles PAP/30 min/mg protein
*Significantly different ($p \leq 0.05$) from air control by ANOVA followed by Duncan's Multiple Range Test

These results indicate that repeated NH_3 exposures at a concentration which does not significantly reduce body weights (115 ppm, 4 d) will reduce microsomal protein apparently associated with the metabolism of a type I substrate (aminopyrine) in the mouse. However, when mice were repeatedly exposed to a non-lethal NH_3 level (350 ppm, 4 d) which significantly reduced body weights, hepatic microsomal metabolism was altered in a manner indistinguishable from effects of restricted food intake alone (pair-feeding). Both pair-feeding and NH_3 treatment also affected microsomal protein apparently associated with the metabolism of aminopyrine and not aniline. In no instance (during non-lethal NH_3 exposures) was there any evidence of inhibition or reduction in the specific activity of either enzyme as is the case with known microsomal enzyme inhibitors such as SKF 525-A (Mannering 1971).

Since neither acute nor repeated exposures of NH_3 gas produced a reduction in the specific activity of selected hepatic microsomal enzymes, we conclude that NH_3 is not a microsomal enzyme inhibitor in the mouse. This study then supports the contention of Schaerdel et al. (1983) that NH_3 is probably not responsible for the inhibition of hepatic microsomal metabolism in animals housed under dirty environmental conditions as originally speculated by Vesell et al. (1973).

Acknowledgments. The authors wish to express their appreciation to Ms. Eileen Starks and Ms. Patricia Skaggs. This work was supported by NIH training grant 5 T32 GM07099-05, and by the Univ. of Mississippi Research Institute of Pharmaceut. Sciences, Univ., MS 38677.

REFERENCES

Amdur MO (1980) Air pollutants. In: Doull J, Klaassen CD, Amdur MO (ed) Casarett and Doull's Toxicology. Macmillan Publishing Co., New York, pp 608-631.

Boyd EM, Maclachlan ML, Perry WF (1944) Experimental ammonia gas poisoning in rabbits and cats. J Ind Hyg Toxicol 26:29-34.

Braker W, Mossman AL, Siegel D (1977) Effects of exposure to toxic gases/first aid and medical treatment. Matheson, Lyndhurst, New Jersey.

Castro SA, Green FE, Gigon P, Sasame H, Gillette JR (1970) Effect of adrenalectomy and cortisone administration on components of the liver microsomal mixed function oxygenase system of male rats which catalyze ethylmorphine metabolism. Biochem Pharmacol 19:2461-2467.

Cochin J, Axelrod J (1959) Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. J Pharmacol Exp Ther 125:105-110.

Coon RA, Jones RA, Jenkins LJ, Siegel J (1970) Animal inhalation studies on ammonia, ethylene glycol, formaldehyde, dimethylamine, and ethanol. Toxicol Appl Pharmacol 16:646-655.

Dalhamn T, Sjoholm J (1963) Studies of SO_2, NO_2 and NH_3: effect on ciliary activity in the rabbit trachea of single in vitro exposure and resorption in rabbit nasal cavity. Acta Physiol Scand 58:287-291.

Doig PA, Willoughby RA (1971) Response of swine to atmospheric ammonia and organic dust. J Amer Vet Med Assoc 159:1353-1361.

Fouts JR (1970) Some in vitro assay conditions that affect detection and quantitation of phenobarbital-induced increases in hepatic microsomal drug-metabolizing enzyme activity. Toxicol Appl Pharmacol 16:48-65.

Gardner DE, Illing JW, Miller FJ, Coffin DL (1974) The effect of ozone on pentobarbital sleeping time in mice. Res Commun Chem Pathol Pharmacol 9:689-700.

Kapeghian JC, Jones AB, Masten LW (1980) A multichamber system for exposure of small laboratory animals via the inhalation route. Pharmacologist 22:197.

Kapeghian JC, Mincer HH, Jones AB, Verlangieri AJ, Waters IW (1982) The acute inhalation toxicity of ammonia in mice. Bull Environ Contam Toxicol 29:371-378.

Kapeghian JC, Waters IW, Jones AB (1981) A simplified method for sampling atmospheric ammonia from inhalation chambers during exposure studies. Bull Environ Contam Toxicol 27:282-287.

Lowry O, Rosebrough N, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265-275.

Mannering GJ (1971) Microsomal enzyme systems which catalyze drug metabolism. In: LaDu BN, Mandel HG, Way EL (ed) Fundamentals of drug metabolism and drug disposition. Williams and Wilkins Co., Baltimore, Maryland, pp 206-252.

Mayan MH, Merilan CP (1972) Effects of ammonia inhalation on respiration rate of rabbits. J Anim Sci 34:448-452.

Mazel P (1971) Experiments illustrating drug metabolism in vitro IV. Determination of microsomal aniline hydroxylase. In: LaDu BN, Mandel NG, Way EL (ed) Fundamentals of drug metabolism and drug disposition. Williams and Wilkins Co., Baltimore, Maryland, pp 569-572.

Miller FJ, Graham JA, Illing JW, Gardner DE (1980) Extrapulmonary effects of NO_2 as reflected by pentobarbital-induced sleeping time in mice. Toxicol Letters 6:267-674.

National Academy of Sciences, National Research Council (1979) Ammonia. University Park Press, Baltimore, Maryland.

Niden AH (1968) Effects of ammonia inhalation on the terminal airways. Aspen Emphysema Conf 11:41-44.

Schaerdel AD, White WJ, Lang CM, Dvorchik BH, Bohner K (1983) Localized and systemic effects of environmental ammonia in rats. Lab Anim Sci 33:40-45.

Stombaugh DP, Teague HS, Roller WL (1969) Effects of atmospheric ammonia on the pig. J Anim Sci 28:844-847.

Vesell ES, Lang CM, White WJ, Passananti GT, Tripp SL (1973) Hepatic drug metabolism in rats: impairment in a dirty environment. Science 179:896-897.

Weatherby JH (1952) Chronic toxicity of ammonia fumes by inhalation. Proc Soc Exp Biol Med 81:300-301.

Weedon FR, Hartzell A, Setterstrom C (1940) Toxicity of ammonia, chlorine, hydrogen cyanide, hydrogen sulfide, and sulphur dioxide gases V. Contrib Boyce Thompson Inst 11:365-385.

Received September 4, 1984; accepted October 5, 1984