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Effect of Inhaled Azodicarbonamide on F344/N Rats and B6C3F₁ Mice with 2-Week and 13-Week Inhalation Exposures

M. A. MEDINSKY,* W. E. BECHTOLD,† L. S. BIRNBAUM,‡ J. A. BOND,* D. G. BURT,† Y. S. CHENG,† N. A. GILLET,† D. K. GULATI,§ C. H. HOBBS,† AND J. A. PICKRELL†

*Chemical Industry Institute of Toxicology, P.O. Box 12137, Research Triangle Park, North Carolina 27709; †Inhalation Toxicology Research Institute, Lovelace Biomedical and Environmental Research Institute, P.O. Box 5890, Albuquerque, New Mexico 87185; ‡National Toxicology Program, P.O. Box 12233, Research Triangle Park, North Carolina 27709; and §Environmental Health Research and Testing, Inc., 2514 Regency Road, Lexington, Kentucky 40503

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Effect of Inhaled Azodicarbonamide on F344/N Rats and B6C3F₁ Mice with 2-Week and 13-Week Inhalation Exposures. MEDINSKY, M. A., BECHTOLD, W. E., BIRNBAUM, L. S., BOND, J. A., BURT, D. G., CHENG, Y. S., GILLET, N. A., GULATI, D. K., HOBBS, C. H., AND PICKRELL, J. A. (1990). *Fundam. Appl. Toxicol.* 15, 308-319. Azodicarbonamide (ADA), a compound used in the baking and plastics industries, has been reported to cause pulmonary sensitization and dermatitis in people. Two-week repeated and 13-week subchronic inhalation exposures of F344/N rats and B6C3F₁ mice to ADA were conducted to determine the toxicity of inhaled ADA. The mean air concentrations of ADA in the 2-week studies were 207, 102, 52, 9.4, or 2.0 mg/m³. No exposure-related mortality nor abnormal clinical signs were observed in rats or mice during or after exposure. The terminal body weights were slightly depressed in the highest exposure group. Liver weights were lower in male rats exposed to 200 mg ADA/m³. No significant lesions were noted on either gross or histologic evaluation of rats or mice. In the 13-week subchronic study, the mean air concentrations of ADA were 204, 100, or 50 mg/m³. No mortality or clinical signs related to exposure were observed. The terminal body weights of exposed rats were not significantly different from those of control rats but were significantly depressed in mice exposed to 100 or 200 mg ADA/m³. No histopathological lesions were noted in mice. Lung weights were increased and enlarged mediastinal and/or tracheobronchial lymph nodes were noted in rats exposed to 50 mg ADA/m³. No exposure-related lesions were observed microscopically in rats exposed to 100 or 200 mg ADA/m³. All rats in the 50 mg ADA/m³ exposure group only had lung lesions that consisted of perivascular cuffing with lymphocytes and a multifocal type II cell hyperplasia, suggesting a possible immune reaction to an antigen in the lung. Viral titers for rats exposed to 50 mg ADA/m³ were negative for Sendai virus and pneumonia virus of mice, which produce similar lesions. The possibility of an unknown viral antigen causing this lesion cannot be eliminated. Lung tissue from male rats was analyzed for ADA and biurea, the major metabolite of ADA. No ADA was detected. The amount of biurea in the lungs increased nonlinearly with increasing exposure concentration, suggesting that clearance was somewhat impaired with repeated exposures. However, even at the highest exposure concentration, this amount of biurea was less than 1% of the estimated total ADA deposited over the exposure period. In summary, ADA is rapidly cleared from the lungs, even when inhaled at concentrations up to 200 mg/m³. Exposure to ADA for up to 13 weeks did not appear to be toxic to rodents. © 1990 Society of Toxicology.

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Azodicarbonamide (diazenedicarboxamide; CAS No. 123-77-3, H₂NOC—N=N—CONH₂, ADA) was nominated for toxicity testing by NCI. This nomination was sup-

ported by the FDA, NIOSH, OSHA, and CPSC, because of (1) the high level of occupational exposure (~232,000 workers) in the rubber, plastics, and baking industries; (2) levels of production as high as 10 million pounds per year; and (3) structural relationships to known carcinogens such as 3-amino-1,2,4-triazole, azoethane, and hydrazine. ADA is an orange crystalline compound that is produced as a condensation product of urea and hydrazine. ADA is an orange crystalline compound that is produced as a condensation product of urea and hydrazine. ADA is manufactured predominantly as a fine yellowish powder milled to particle sizes in the range 2 to 10 μm (Slovak, 1981). Principal worker exposure occurs in factories where it is ground and in bakeries where it is used as an additive in flour.

Because ADA is used as a flour-maturing agent, toxicity testing using oral exposures was originally recommended. However, in making dough, ADA is quantitatively reduced to biurea (1,2-hydrazinedicarboxylic acid diamide; $\text{H}_2\text{NOCHN-NHCONH}_2$). ADA decomposes at temperatures $> 180^\circ\text{C}$, releasing ammonia, nitrogen, and carbon monoxide and producing urazone, biurea, cyanuric acid, and cyanidine (Herweh and Fantazier, 1974). Subchronic toxicity testing of ADA in rats and mice by gavage revealed only a nonspecific nephrotoxicity resulting from precipitation of ADA (or biurea) in the kidneys of animals given 2.5 g/kg body wt or more. Similarly, nonspecific nephrotoxicity was noted in dogs fed 5 or 10% biurea in their diet for up to 1 year (Oser *et al.*, 1965). Methemoglobin levels measured by Oser *et al.* (1965) were below the limit of detection (0.2 mg/100 g).

ADA has been reported to exhibit an anti-thyroid effect (Gafford *et al.*, 1971) and an inhibitory effect on cholinesterase activity in blood and liver (Mel'nikova and Selikhova, 1965). Anorexia, weight loss, and gross hematuria have also been observed in rats given ADA intraperitoneally at a dose of 200 mg/kg body wt daily for 1 week (Gafford *et al.*,

1971). Five out of eight rats died from this dose of ADA. There were no deaths among rats given the same dose of ADA orally.

Reports of airway constriction in workers exposed to ADA suggest that ADA might be an airway sensitizer or irritant. Ferris *et al.* (1977) observed decreased forced vital capacity and forced expiratory volume in exposed workers. Slovak (1981) reported the occurrence of asthma in almost one-fifth of workers in a factory manufacturing ADA. Whitehead *et al.* (1987) found symptoms similar to chronic bronchitis in workers in ADA plants. One case of dermatitis, with a positive response to 1% ADA in a patch test, was noted (Bonsall, 1984).

Studies of the effects of an acute, 1-hr exposure of guinea pigs to ADA (19, 58, or 97 mg/m³) demonstrated minor changes in tidal volume and respiratory frequency at the two higher concentrations (Shopp *et al.*, 1987). No lesions were noted in respiratory tract tissue of animals euthanized immediately after exposure or 24 hr later. Repeated exposure of guinea pigs to 51 or 200 mg ADA/m³ for 4 weeks did not result in either specific or non-specific airway sensitization on inhalation challenge with ADA or aerosolized histamine, respectively (Gerlach *et al.*, 1989). The objective of the studies reported here was to describe the toxic effects in rats and mice of inhalation exposure for 2 or 13 weeks to airborne concentrations of ADA as high as 200 mg/m³.

MATERIALS AND METHODS

Chemical

Azodicarbonamide, obtained from Midwest Research Institute, was 98% pure, as determined by iodometric titration. The major impurity identified was biurea, which was from 0.4 to 0.7% of the bulk chemical. A compound stability study was also done to confirm that the chemical composition of ADA did not change upon aerosolization. Filter samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC), using the method of Bechtold *et al.* (1988). Contamination of ADA aerosols with biurea was less than 1%. This was not

different from the amount of contamination in the bulk samples.

Exposure Atmosphere Generation and Characterization

Stainless-steel, multitiered, whole-body exposure chambers (H1000, Hazleton Systems, Aberdeen, MD), with a total internal volume of 1.0 m³, were used in both the 2-week and the 13-week studies. The flow rate through the chambers was 7 ± 1 ft³/min, corresponding to 12 ± 2 air changes per hour. Chambers were maintained at a temperature between 21.2 and 25.4°C (mean 23.6°C). The average relative humidity ranged from 70 to 85%.

To reduce the spatial variation of aerosol concentration and to increase the uniformity of mixing, the aerosol was diluted in a radial diluter before entering the chamber. Animal cages were rotated on a regular basis to reduce any variation in the relative levels of ADA inhaled by animals over the course of the study. Rats and mice were housed in the same chambers, but in different cage units, for both studies.

ADA aerosol was generated by a Jet-O-Mizer/screw feed method (Cheng *et al.*, 1985). ADA powder was put into the hopper of the screw feeder (Model 300, Accu-Rate, Whitewater, WI) and delivered to the funnel of the Jet-O-Mizer (Model 0101, Fluid Energy Co., Hatfield, PA) for dispersion. One generator was provided for each exposure chamber.

The aerosol concentration in the exposure chamber was monitored by sampling at a flow rate of 0.5 liter/min for three, 2-hr periods during the 6-hr exposure. Samples were collected on 25-mm fiberglass filters (Type AE, Gelman, Ann Arbor, MI). Each exposure day, aerosol generation was started and sampling began after 12 min of rise time, when the chamber concentration had reached 90% of equilibrium concentration (T_{90}). Therefore, the total exposure was 6 hr plus a T_{90} of 12 min. The control chamber was also sampled daily.

A RAM-S continuous aerosol monitor (GCA, Bedford, MA) was used to monitor the stability of the aerosol concentration and to adjust the aerosol generator during exposure. It was operated on each chamber at the beginning, middle, and end of the filter sampling period as described previously (Cheng *et al.*, 1988).

Aerosol size distribution was obtained once per study by using a Lovelace multijet cascade impactor (Newton *et al.*, 1977), with a flow rate of 15 liters/min. The sampling period ranged from 1 to 6 hr, depending on the chamber concentration.

Animals

Two-week repeated study. Four-week-old F344/N rats and B6C3F₁ mice were received from Frederick Cancer

Research Facility (Frederick, MD). The animals were acclimated two per cage for the first week and then one per cage in cage units within two Hazleton 1000 chambers prior to exposure [total of 21 days (males) or 22 days (females)]. Exposures began when animals were 7 weeks old. Animals were exposed 5 days per week for 2 weeks, plus an additional 2 (males) or 3 (females) consecutive exposure days before terminal euthanasia for a total of 12 exposure days for both sexes. Exposures of females were initiated 1 day later than males.

13-week subchronic study. Four-week-old F344/N rats and B6C3F₁ mice were received from Simonsen Laboratories, Inc. (Gilroy, CA). Rats and mice were acclimated as described for the 2-week study, for a total of 18 days (males) or 22 days (females) prior to exposure. Animals were exposed by inhalation, 5 days per week for a total of 13 weeks, with 2 or 3 (males) or 4 or 5 (females) consecutive dose days before terminal euthanasia. The total number of exposure days was 65 or 66 for both males and females.

The feed used in both studies was Zeigler NIH-07 Open Formula Rat Ration (Zeigler Brothers, Inc., Gardeners, PA). Water was provided by an automatic watering system. The light cycle was automatically controlled to provide 12 hr of fluorescent light and 12 hr of darkness each 24 hr (lights on at 6 AM and off at 6 PM).

Prior to study start, the rats and mice in both studies were randomly assigned by weight to treatment groups, using a computer-based system (Path/Tox, Xybin Corp.). Animals within exposure groups were further randomized by use of computer-generated random numbers into groups for the basic studies or special studies and, for the 13-week study, into termination days (Day 1 or Day 2) for each group.

Disease Surveillance

In the 2-week study, serum (approximately 0.5 ml) was obtained from five male and five female rats and mice 3 days prior to the first day of exposure, and was analyzed for antibody titers to specific bacteria and viruses (*Mycoplasma pulmonis*, *M. arth.*, pneumonia virus of mice (PVM), Sendai virus, rat coronavirus (RCV)/SDA, and *Mycoplasma*—rats; Reo3, M. ad., *M. pulmonis*, *M. arth.*, PVM, Send, MHV, Ectro, and GDVII—mice). Titers were negative for these agents.

In the 13-week study, serum was also obtained from five male and five female rats and mice 3 days prior to study start. Analyses for the agents described above showed positive titers for RCV/SDA in the rats and negative titers for all agents in the mice. No gross lesions were noted in the rats on necropsy. Tissue sections were taken from all lung lobes, liver, both kidneys, spleen, Harderian gland, and mandibular salivary glands and processed for histology. Lesions were only noted in the salivary gland or Harderian gland tissue of 8 of the 10 animals. Ductal

squamous metaplasia was noted in both tissues and corresponded with those animals having positive titers to RCV/SDA. These lesions are consistent with lesions induced by sialodacryoadenitis virus.

At the end of the 13-week study, serum from 5 male and 5 female rats from all exposure groups and controls (40 rats total) were analyzed for titers to Sendai virus, PVM, RCV/SDA, and *Mycoplasma*. All 40 sera were negative for titers to Sendai, PVM, and *Mycoplasma*. Twenty-five of the 40 sera were positive for RCV/SDA (≥ 0.17 absorbance units by Elisa assay).

Experimental Design

Two-week repeated study. Rats and mice in the basic study group (Table 1) were weighed prior to study start, after 1 week of exposure, and at terminal euthanasia. Detailed clinical observations were made at these times. Morbidity/mortality checks were made twice daily on all animals. Evaluations of gross and microscopic pathology and organ weight changes were made on the basic study group (Table 1). Separate groups of rats and mice were used for evaluation of methemoglobin levels and cholinesterase levels in whole blood.

Thirteen-week subchronic study. Rats and mice in the basic study group (Table 1) were weighed weekly at the time of detailed clinical examinations. Mortality/morbidity checks were performed twice daily on all animals. Histopathology, hematology, organ weight, sperm morphology, and vaginal cytology evaluations were also made on this group of rats. Each animal assigned to the special study groups (Table 1) was used for the following determinations: levels of ADA and biurea in lungs and kidneys, acetylcholinesterase activity in whole blood, and T3 and T4 levels in serum.

Basic Study Endpoints

Necropsy and histopathology. All rats and mice in the basic study group, for both the 2-week and the 13-week studies, were given complete gross necropsy examinations. Rats and mice were killed by cardiac puncture exsanguination while under halothane anesthesia and were necropsied immediately. Weights of liver, thymus, right kidney, right testicle, brain, heart, and lungs (including trachea) were taken. Tissues were fixed in 10% neutral-buffered formalin. Tissues for microscopic examination were embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin.

The following tissues were trimmed and sectioned for histopathology: adrenals, bone (vertebra, with bone marrow and spinal cord; femur; rib), brain, epididymus or oviduct, esophagus, heart, small intestine (duodenum, jejunum, ileum), large intestine (cecum, colon, rectum), both kidneys, larynx, liver, lung (4 lobes), lymph nodes

(bronchial, mandibular, mediastinal, mesenteric), mammary glands, nose (3 levels), pancreas (including islets), parathyroid gland, pituitary gland, prostate or uterus, salivary glands, seminal vesicles, skin, spleen, stomach (including forestomach and glandular portions), testes or ovaries, thymus, thyroid gland, trachea, and urinary bladder.

All tissues from each rat and mouse exposed to filtered air or 200 mg ADA/m³, for both the 2-week and the 13-week studies, were examined microscopically. In addition, lungs of rats exposed to 50 or 100 mg ADA/m³ for 13 weeks were examined.

Hematology. For the 13-week study, blood was collected at necropsy from anesthetized rats and mice from the basic study group by cardiac puncture and placed in glass vials containing EDTA. A Coulter Electronics Model S-550 was used for analysis of erythrocyte count, mean corpuscular volume, hemoglobin concentration, hematocrit (calculated), and leukocyte count. Smears were made from the blood, stained with Wright's stain, and examined under a light microscope to obtain differential leukocyte counts and counts of nucleated erythrocytes. Additional blood smears were stained with new methylene blue and examined for the presence of reticulocytes.

Sperm morphology-vaginal cytology. In the 13-week study, vaginal cytology samples were taken on a daily basis from basic study females for 1 week before final termination. Samples were obtained with sterile saline, placed on duplicate Dakin slides, fixed with Spray Cyte (Clay Adams 7180), and stained with toluidine blue (0.5% in 20% ethanol).

Live sperm were obtained from the cauda of the right epididymis of male rats and mice at necropsy. Sperm were incubated at 37°C in Tyrode buffer, and viability was quantitated as the percentage of motile sperm in the sample. Sperm density (number of sperm per gram of caudal tissue) was quantified on a hemocytometer. The number of sperm in the sample was divided by the weight of the cauda of the epididymis to obtain the value for sperm density. Evaluations of sperm morphology were done using preparations of sperm fixed in ethanol and stained with eosin Y.

Urinary enzymes. In the 13-week study, 1 week prior to termination, all rats in the basic study group were placed in metabolism cages for overnight urine collection. Urine was collected on ice and analyzed for total amounts of lactate dehydrogenase (LDH), β -galactosidase (β -G), *N*-acetylglucosaminidase (NAG), and alkaline phosphatase (AP) using methods described (Medinsky *et al.*, 1989). Lactate dehydrogenase was quantitated by using pyruvate as a substrate; 4-nitrophenylphosphate was the substrate for alkaline phosphatase; *p*-nitrophenol was the substrate for both *N*-acetyl- β -D-glucosaminidase and β -galactosidase.

Special Study Endpoints

Determinations of methemoglobin in whole blood of rats and mice exposed to ADA for 2 weeks were made

TABLE 1
SUMMARY OF THE EXPERIMENTAL DESIGN AND SIGNIFICANT RESULTS OF INHALATION EXPOSURE OF RATS AND MICE TO AZODICARBONAMIDE

Exposure duration (target concentration)	Subgroup (number/concentration)	Endpoint	Species	Sex	Evaluation
2 weeks (0, 2, 10, 50, 100, 200 mg/m ³)	Basic (5)	Mortality	R, M	M, F	Not exposure related
		Growth rate	R, M	M, F	Decreased terminal body weight, 200 mg/m ³ , male R; male, female M
	Clinical signs	R, M	M, F	Normal	
		R, M	M, F	Decreased liver weight, 200 mg/m ³ , male R; male, female M	
	Special (5)	Gross pathology	R, M	M, F	Normal, NSL ^a
		Histopathology	R, M	M, F	NSL
		Methemoglobin	R, M	M, F	NSD ^b
		Acetylcholinesterase	R, M	M, F	NSD
	Basic (10)	Mortality	R, M	M, F	Not exposure related
		Growth rate	R, M	M, F	NSD, R; decreased terminal body weight, 100, 200 mg/m ³ , male M; 200 mg/m ³ , female M
Clinical signs		R, M	M, F	Normal	
Organ weights		R, M	M, F	Increased lung weights, 50 mg/m ³ , rats only	
(0, 50, 200 mg/m ³)	Basic (10)	Gross pathology	R, M	M, F	Enlarged lymph nodes, 50 mg/m ³ , rats only
		Histopathology	R, M	M, F	Lung lesions, 50 mg/m ³ , rats only
	Special (10)	Sperm morphology	R, M	M	NSD
		Vaginal cytology	R, M	F	NSD
	Basic (10)	Urinary enzymes	R	M, F	NSD
		Serum T3, T4	R	M, F	Increased T3, T4 at 200 mg/m ³ , males only
	Special (10)	Acetylcholinesterase	R	M, F	NSD
		ADA/biurea in tissues	R	M	Dose-related increase in biurea levels in lungs

^a NSL, no significant (exposure-related) lesions noted.

^b NSD, no significant difference from control.

using the method of Evelyn and Malloy (1938). Acetylcholinesterase concentrations in whole blood of rats only in the 13-week study and both rats and mice in the 2-week study were determined using the method of Ellman *et al.* (1961), in which acetylthiocholine is used as the substrate. Serum thyroxine (T4 or tetraiodothyronine) and triiodothyronine (T3) levels for rats exposed to ADA for 13 weeks were measured by radioimmunoassay (Tietz, 1986).²

Samples of lung and kidney from male rats exposed to ADA for 13 weeks were analyzed for ADA and biurea. This procedure involved derivatization of ADA with triphenylphosphine and quantitation of the resulting derivative by HPLC (Bechtold *et al.*, 1989). Biurea in the sample was oxidized to ADA using potassium permanganate in acid solution. The ADA produced was then quantitated by HPLC (Bechtold *et al.*, 1989).

Statistics

All data were analyzed separately for each sex. Organ and terminal body weights on animals found dead or euthanized because of a moribund condition were not included in the statistical analysis. Analysis of variance techniques were used for statistical evaluation. Provided Bartlett's test of homogeneity of variance was not significant, exposure groups were compared to controls by using Dunnett's multiple range test. When Bartlett's test was significant, comparisons with the control group were made by a modified Student's *t* test, making allowance for unequal variance. All statistical tests were conducted at a 5%, two-sided risk level.

RESULTS

Two-Week Study

The overall, mean ADA concentration for each chamber was within 8% or less of target concentration (Table 2). The relative standard deviation of daily means was within 16%. The aerosol had an average mass median aerodynamic diameter (MMAD) of 2.13 μm (range 1.89 to 2.45) with a mean geometric standard deviation (σ_g) of 1.9.

There were no abnormal clinical observations during the 2-week exposures for either rats or mice. No rats died. One male (50 mg/

m^3 exposure group) and one female (200 mg/ m^3 exposure group) mouse died of undetermined causes. The mean terminal body weights for the basic study male rats exposed to 200 mg/ m^3 ADA were significantly less than those of controls (95% of control value). The mean terminal body weights for basic study male and female mice were significantly lower (89 or 94% of controls, respectively) for the 200 mg/ m^3 exposure group.

The mean liver weights of male rats and of male and female mice were significantly less at the 200 mg/ m^3 exposure than those of controls (83, 79, and 79% of control values, respectively). Other organ weights of either mice or rats were not influenced by ADA exposure (data not shown).

A complete set of tissues from rats and mice exposed to 200 and 0 mg ADA/ m^3 was examined histologically. The two exposure groups could not be distinguished on the basis of histologic findings. Because no effect was seen at the highest exposure concentration, we did not examine tissues from rats exposed to lower concentrations.

For mice, the only change noted that may have been related to the ADA exposure was the degree of fine vesicular vacuolation of the hepatocyte cytoplasm, which is indicative of glycogen accumulation. All of the livers examined for both groups were considered to be within normal limits. However, the degree of cytoplasmic vacuolation varied among animals. The livers from the control and high-dose groups were examined blindly and sorted on the basis of cytoplasmic vacuolation. Seven of the high-dose animals and one control animal showed no cytoplasmic vacuolation, indicative of glycogen depletion. When the next lowest dose group (100 mg ADA/ m^3) was compared with the controls, four treated animals and one control showed no cytoplasmic vacuolation. It must be emphasized that none of the livers were regarded as being abnormal. However, there did appear to be a correlation between ADA exposure and loss of hepatocyte vacuolation. Possibly the ADA-exposed animals were eating

² Assays were conducted by Veterinary Diagnostics, Inc., Albuquerque, NM.

TABLE 2
SUMMARY OF AIR CONCENTRATIONS AND PARTICLE SIZES IN EXPOSURE CHAMBERS FOR RATS AND MICE
EXPOSED TO AZODICARBONAMIDE^a

Target exposure concentration (mg/m ³)	2-week repeated				13-week subchronic			
	Rats (mg/m ³)		Mice (mg/m ³)		Particle size ^b		Particle size ^b	
					MMAD	σ_g	MMAD	σ_g
Control	0	0	—	—	0	0	—	—
2	2.0 (13)	2.1 (12)	1.89	2.1	— ^c	—	—	—
10	9.4 (11)	9.6 (10)	1.95	1.8	—	—	—	—
50	52 (10)	52 (7)	2.15	1.8	50 (10)	50 (10)	2.33	1.8
100	102 (16)	102 (15)	2.22	1.7	100 (7)	100 (7)	2.45	1.7
200	207 (10)	217 (11)	2.43	1.9	204 (5)	204 (5)	2.37	1.8

^a Values represent means (percentage standard deviation) of daily measurements over the 2- or 13-week exposure period. Although rats and mice were housed in the same exposure chamber, the starts of exposure for each species were staggered by 1 week.

^b Particle size was measured once during the study when both rats and mice were being exposed to ADA. MMAD, mass median aerodynamic diameter; σ_g , geometric standard deviation.

^c No exposure at this target concentration.

less, resulting in depletion of glycogen stored in the liver. The mice in the 200 mg/m³ group had smaller body and liver weights than those of controls.

There were no significant differences in methemoglobin levels or acetylcholinesterase activities in whole blood of male and female rats or mice exposed to any concentration of ADA, when compared to controls.

Thirteen-Week Study

The overall mean ADA concentration for each chamber was within 3% or less of target concentration (Table 2). The relative standard deviations of daily means were within 10%. The aerosol had an average MMAD of 2.38 μ m (range 2.33 to 2.45), with a mean σ_g of 1.7.

There were no abnormal clinical observations during the 13-week exposures for either rats or mice that could be attributed to exposure to ADA. No deaths occurred that could be attributed to ADA. One female rat (50 mg/m³ exposure group) was euthanized because

of weight loss and dehydration. Three male mice died of unknown cause, and one female mouse was accidentally killed. The mean terminal body weights for the basic study rats exposed to ADA were not significantly different from those of the controls. The mean terminal body weights for basic study male mice were significantly depressed (93 and 91% of controls) at the 100 and 200 mg/m³ exposure levels, respectively. For female mice, terminal body weights were significantly depressed (94% of controls) only at the 200 mg/m³ level.

Lung weights of male and female rats were increased (111% of control) at the 50 mg/m³ exposure level. Mean liver weights for male and female mice exposed to 200 ADA mg/m³ were lower than those of controls (85 or 86% of controls, respectively). Lung weights of mice were not influenced by ADA exposure (data not shown). No other effects of exposure on organ weights were noted.

A complete set of tissues from rats and mice exposed to 200 and 0 mg ADA/m³ was examined histologically. Although several incidental lesions were present in both groups,

TABLE 3

INCIDENCE OF MICROSCOPIC OBSERVATIONS IN LUNGS AND LYMPH NODES OF RATS EXPOSED TO AZODICARBONAMIDE FOR 13 WEEKS

Microscopic observations	Males				Females			
	0	50	100	200	0	50	100	200
Exposure group (mg/m ³):	0	50	100	200	0	50	100	200
No. in group:	10	10	10	10	10	9	10	10
Lungs (number examined)	10	10	10	10	10	9	10	10
Inflammation; nonsuppurative; alveolus	0	10	0	0	0	8	0	0
Hyperplasia; alveolar epithelium	0	10	0	0	0	8	1	0
Infiltrating cell; lymphocytic plasmacytic; perivascular	0	10	0	1	0	9	1	0
Hemorrhage; multifocal, acute; alveolus, right apical	0	0	0	1	0	0	0	0
Tracheobronchial lymph nodes (number examined)	8	10	0	7	9	9	0	7
Hemorrhage; acute; sinus	0	0	0	0	1	0	0	0
Hyperplasia; lymphoid	0	7	0	0	0	7	0	0
Mandibular lymph nodes (number examined)	9	0	0	9	9	0	0	10
Hemorrhage; acute; sinus	0	0	0	0	1	0	0	0
Hyperplasia; lymphoid; follicular	0	0	0	0	1	0	0	0
Mediastinal LN (number examined)	7	10	0	7	7	9	0	7
Hemorrhage; acute; sinus	2	0	0	1	2	0	0	6
Hyperplasia; lymphoid	0	5	0	0	0	4	0	0

lesions attributable to the ADA exposure were not present. The decrease in hepatocyte vacuolation with increasing ADA exposure described for the 2-week study was not observed in mice exposed to ADA for 13 weeks.

Five male and six female rats exposed to 50 mg/m³ ADA were described as having enlarged bronchial or bronchial and mediastinal lymph nodes at gross necropsy. Histological examination of these nodes showed a moderate-to-severe lymphoid hyperplasia (Table 3). Subsequently, the lungs of all rats and mice in the 50 mg/m³ exposure group were examined histologically. All male and female rats in the 50 mg/m³ exposure group euthanized at the end of the 13-week exposure period had a spectrum of lung lesions that consisted of perivascular cuffing with lymphocytes and a multifocal type II cell hyperplasia that was associated with a moderate number of mixed inflammatory cells (Fig. 1). The extent of lung involvement varied from mild to moderate among animals. In some animals, the perivascular cuffs were predomi-

nant, while other animals showed both the perivascular cuffs and the epithelial hyperplasia (Table 3). The lungs from all rats and mice exposed to 100 mg ADA/m³, as well as the lungs of mice exposed to 50 mg/m³, were examined. There were no significant lesions on histologic examination.

Results obtained for hematology in rats and mice exposed to ADA for 13 weeks indicated that there were no exposure-related alterations in blood parameters. There were no significant, exposure-related changes, in either male or female rats, in the amounts of the four urinary enzymes (LDH, AP, β -G, NAG) excreted.

There were no adverse effects from inhalation exposure to ADA for 13 weeks, with respect to right caudal weight, right epididymal weight, right testicular weight, sperm motility, sperm count per gram caudal tissue, or incidence of abnormal sperm. However, there was a small, but significant, increase in sperm count in male rats exposed to 50 or 100 mg ADA/m³. No abnormal effects were noted in male mice.

There were no apparent adverse effects on estrual cyclicity or on estrous cycle length in any of the dose groups, except in 2 out of the 10 animals in the 200 mg/m³ dose group. For these animals, estrous cycle length was >7 days or was not precisely determined. No abnormal effects were noted in female mice.

There were no significant differences in acetylcholinesterase activities in whole blood of male and female rats exposed to any concentration of ADA when compared to controls. T3 and T4 levels in serum from male rats increased with increasing ADA exposure concentration in male rats. T3 and T4 levels in the highest exposure group were significantly elevated, relative to control. T3 was increased approximately 50%, while T4 was increased approximately 40%. T3 and T4 were unchanged in female rats exposed to ADA.

Samples of lung and kidney of male rats exposed to ADA for 13 weeks were analyzed for the presence of ADA and biurea. These tissues were chosen, because they are the most likely tissues to contain significant quantities of one or both compounds, lung being the organ of exposure and kidney, because biurea had been detected in kidney (Oser *et al.*, 1965).

The results of chemical analysis of ADA and biurea in lung and kidney of male rats are summarized in Table 4. No ADA was detected in either lung or kidney of male rats exposed to ADA for 13 weeks. Biurea, however, was detected in lungs, but not kidney, of rats exposed to 50, 100, and 200 mg/m³ ADA. The amount of biurea in the lungs increased nonlinearly with increasing exposure concentration. The percentage of biurea retained in lungs was calculated as a percentage of ADA deposited on the last day of exposure (Table 4). Although 66% of the amount of ADA expected to be deposited in rats exposed to 200 mg/m³ on the last exposure day is retained in the lungs as biurea, this is a small percentage (~1%) of the total amount of ADA deposited over the entire study.

DISCUSSION

After inhalation of ADA, the only apparently exposure-related lesions observed were

those in lung and lymph nodes of rats exposed to 50 mg/m³ for 13 weeks. The lung and lymph node lesions in rats exposed to 50 mg ADA/m³ for 13 weeks were striking and suggest an immune response to a virus or another antigen. Several viral agents have been reported to produce similar pulmonary lesions in rats (Jones *et al.*, 1985; Hamm, 1986). One such agent is Sendai virus. Factors suggesting that Sendai virus is not involved in this case are the following: (i) absence of bronchiolar epithelial involvement, including bronchiolar epithelial necrosis and hyperplasia; (ii) absence of similar lesions in mice housed in the same chamber as the rats (mice are even more susceptible to the virus than are rats); and (iii) negative viral titers for Sendai virus from animals within the same chamber terminated at the same time.

PVM has been reported to produce perivascular mononuclear cell infiltrates and a multifocal interstitial pneumonia in rats. However, as for Sendai, negative viral titers for PVM were reported from animals within the same chamber as the rats having the lung lesions. For both Sendai virus and PVM, serum antibody titers would be present at 8 to 14 days after exposure to the virus. It is unlikely that histologic lesions from such a viral infection would be present in the animals examined in the absence of positive antibody titers.

RCV infection produces mild lung lesions in young adult rats, consisting of patchy interstitial pneumonia and lymphocytic perivascular infiltrates. Mice are unaffected by the virus. Rat coronavirus is closely related antigenically to SDA, and serology cannot distinguish between exposure to either of these two coronaviruses. As previously indicated, rats in this study had positive titers to RCV/SDA. We, therefore, cannot determine if the titers reflect only exposure to SDA, or subsequent infection with RCV. Because antibody titers for RCV/SDA were equivalent across dose groups, any pulmonary lesions due to the viral infection would be expected to be present in all dose groups.

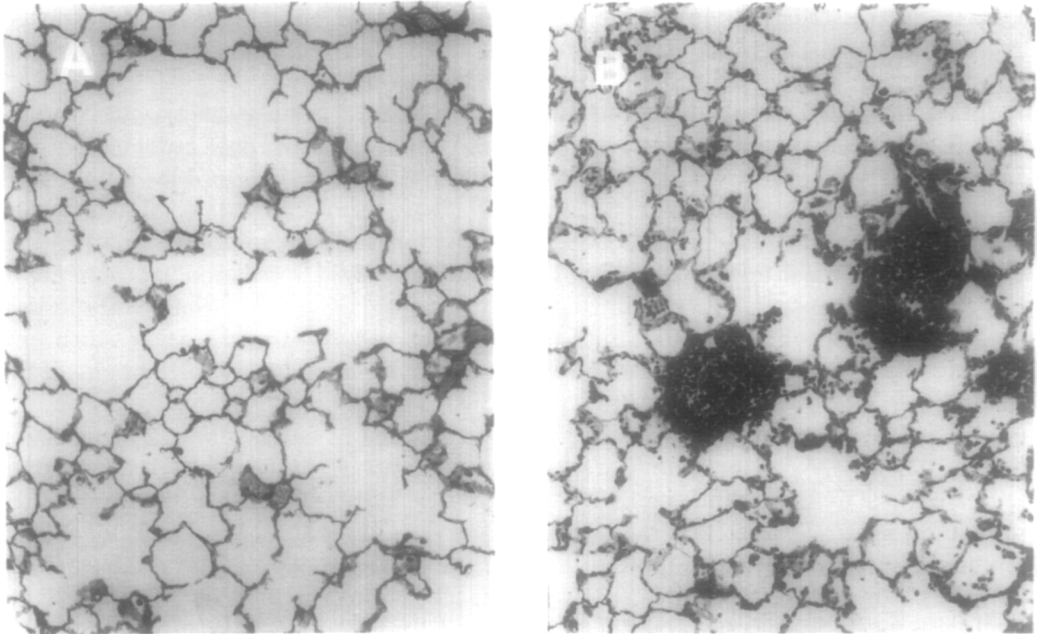


FIG. 1. (A) Lung section of a control rat exposed to filtered air. (B) Lung section of a rat exposed to 50 mg ADA/m³ for 13 weeks. Note prominent perivascular cuffs composed of lymphocytes.

Another possible etiology for the lung lesions we observed is that ADA acts as a hapten, inducing an immune response within the lung. Several reports in humans suggest that

ADA is capable of inducing an immune reaction and/or asthma-like responses (Slovak, 1981; Bonsall, 1984). The absence of a response to the two higher exposure concentra-

TABLE 4
LEVELS OF ADA AND BIUREA IN LUNGS AND KIDNEYS OF MALE RATS EXPOSED TO AZODICARBONAMIDE (ADA) FOR 13 WEEKS

Exposure level (mg/m ³)	ADA		Biurea		Biurea in lungs and bronchi expressed as the percentage of ADA deposited during 1 exposure day ^a
	Kidney	Lungs and bronchi	Kidney	Lungs and bronchi	
0	NP ^b	NP	NP	NP	—
50	ND ^c	ND	ND	79 ± 15 ^d	22
100	ND	ND	ND	303 ± 79	42
200	NP	NP	NP	948 ± 238	66

^a Assuming a rat minute volume of 0.2 liter/min, a 6-hr (360 min) exposure, and a 10% deposition efficiency in the pulmonary region (lungs and bronchi), the amount of ADA deposited in the lungs per day is estimated as (0.2 liter/min) × (200 mg/liter) × 0.1 × 360 min = 1440 μg/lungs and bronchi for the 200 mg/m³ group, 720 μg for the 100 mg/m³ group, and 360 μg for the 50 mg/m³ group.

^b NP, no peak was observed at the expected retention time. Limit of quantitation is 100 μg per sample.

^c ND, not determined.

^d Mean μg/g tissue ± standard deviation; n = 5.

tions used in the studies reported here (i.e., 100 and 200 mg/m³) suggests that the lung lesions in rats are not related to ADA exposure. In addition, two recent studies of acute and repeated inhalation exposure to ADA in guinea pigs did not report any evidence of airway irritation (Shopp, 1987) or a potential for sensitization to ADA (Gerlach *et al.*, 1989).

Pyelonephritis, renal tubular concretions, and intratubular renal crystals were noted after subchronic exposure of rats and mice to ADA by gavage, at levels of 2.5 g/kg body wt or greater per day. In the present study, no renal lesions were observed in animals exposed to 200 mg ADA/m³ for up to 13 weeks. The lack of renal toxicity in the present study is most likely related to differences in the total amount of ADA given to the animals in the two studies. For example, for the 2- to 3- μ m particle size used in this study, total deposition should be about 49% (Raabe *et al.*, 1988). For animals exposed to 200 mg ADA/m³, with a 200 ml/min minute volume, the total amount of ADA deposited should be about 20–40 mg/kg body wt per day for 338-g males or 190-g females (mean terminal body weights of 200 mg/m³ group). This dose is 100-fold lower than that resulting in nephrotoxicity.

The lack of an effect on whole blood cholinesterase activity in the inhalation studies, compared to those previously reported, is also most likely due to differences between the two studies in administered dose. Mel'nikova and Selikhova (1965) used oral exposures of up to 7000 mg/kg body wt for 5 days to achieve a 50% reduction in blood cholinesterase.

The increased levels of T3 and T4 found in male rats after 13 weeks of exposure to ADA differ from those observed by Gafford *et al.* (1971), who reported a decreased radioactive iodine uptake in rats 24 hr after being fed ADA at 5% of their diet. No changes in iodine uptake were noted at lower levels of ADA in the diet in studies of Gafford *et al.* The reason for the difference between the results reported by Gafford and those reported here is unclear,

but may include differences in exposure route, exposure concentration, and duration of exposure.

The findings in male rats exposed to ADA for 65 or 66 exposure days are generally consistent with the findings of Mewhinney *et al.* (1987), who showed that [¹⁴C]ADA deposited in the lungs of rats is rapidly absorbed into blood during the inhalation exposure and is cleared from tissues with a half-time of 1 day. The rats in the 13-week, ADA subchronic study were euthanized 16 to 18 hr after exposure to ADA was stopped. No ADA or biurea was detected in kidney tissue. The detection limit was at least 100 μ g/g tissue. Thus, in the 13-week study, ADA and biurea were cleared from the kidney at least as fast as for the rats in the previous studies. The nonlinearity of the amount of biurea in the lungs of rats exposed for 65 or 66 days to ADA aerosols would not be expected, based on the results of the study by Mewhinney *et al.*, in which the rats were exposed once for a period of 6 hr. This may, of course, be due to the much greater amount of ADA deposited in the lung over the 65- or 66-day period of exposure or to the higher exposure concentration in the 13-week study compared to that in the study involving ¹⁴C. The amount of biurea present in the lungs of rats exposed to ADA for 65 or 66 exposure days was small (~1%), compared to the total amount of ADA inhaled and deposited over the 13-week period.

In summary, ADA is rapidly cleared from the lungs, even when inhaled at concentrations up to 200 mg/m³. Exposure to ADA for up to 13 weeks did not appear to be toxic to rodents. However, the question as to the nature of the chemical sensitization noted in workers exposed to ADA still needs to be addressed.

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