

**UNITED STATES AIR FORCE  
ARMSTRONG LABORATORY**

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**Developmental Toxicity Screen of  
Ammonium Dinitramide Using *Hydra  
attenuata***

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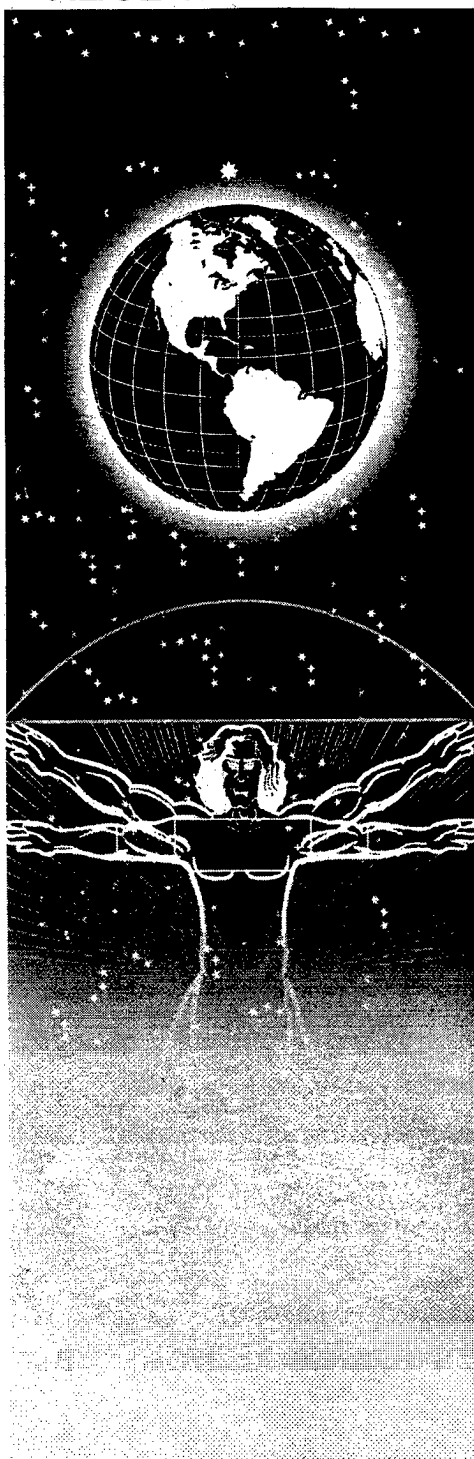
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The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996, and the Animal Welfare Act of 1966, as amended.

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FOR THE COMMANDER

  
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## PREFACE

This is one of a series of technical reports describing results of the experimental laboratory programs conducted at Armstrong Laboratory, Toxicology Division (AL/OET), Wright-Patterson Air Force Base, Ohio by the ManTech/Geo-Centers Joint Venture contract. This document serves as a final report on the hydra assay for developmental toxicity of ammonium dinitramide. The research described in this report began in June 1995 and was completed in August 1996 under Department of the Air Force Contract Nos. F33615-90-C-0532 and F41624-96-C-9010. Lt Col Terry A. Childress served as Contract Technical Monitor for the U.S. Air Force, AL/OET. Darol E. Dodd, Ph.D., served as Program Manager for the ManTech/Geo-Centers Joint Venture contract.

This work was initiated under the direction of Daniel J. Caldwell, Ph.D. The authors gratefully acknowledge Dr. E. Marshall Johnson, Dr. Lois D. Newman, and Navid Haghdoost for hydra assay training and supplying the *Hydra attenuata*. The authors also gratefully acknowledge the technical assistance of David L. Courson, SrA Frank M. Dessauer, Richard J. Godfrey, Willie J. Malcomb, Jerry W. Nicholson, and William B. Sonntag. The support of Four Seasons Environmental, Inc. in maintaining the hydra laboratory's heating and air conditioning systems was also greatly appreciated.

## ABBREVIATIONS

|                   |   |
|-------------------|---|
| A/D ratio         | Adult toxic (A) to developmentally toxic (D) ratio            |
| ADN               | Ammonium dinitramide  |
| AP                | Ammonium perchlorate  |
| °C                | Degrees Centigrade  |
| CaCl <sub>2</sub> | Calcium chloride  |
| cm                | Centimeter  |
| EDTA              | Ethylenediaminetetraacetic acid                               |
| Encl              | Hydra farm enclosure  |
| g                 | Gram(s)   |
| GD                | Gestation Day   |
| h                 | Hour(s)   |
| in.               | Inch(es)  |
| KCl               | Potassium chloride  |
| L                 | Liter   |
| MAC               | Minimal affective concentration                               |
| MgSO <sub>4</sub> | Magnesium sulfate anhydrous                                   |
| min               | Minute(s)   |
| mg                | Milligram   |
| mL                | Milliliter  |
| mm                | Millimeter  |
| mM                | Millimolar  |
| mOhm-cm           | MegaOhms per centimeter                                       |
| N                 | Normal  |
| NaOH              | Sodium hydroxide  |
| SD                | Standard deviation  |
| SIDS              | Screening Information Data Set                                |
| TES               | {2[Tris(hydroxymethyl)methylamine]-<br>1-ethanesulfonic acid} |
| μL                | Microliter  |
| ×g                | Times gravity   |



## SECTION I

### INTRODUCTION

The immediate military application for the propellant ammonium dinitramide (ADN) is as a clean burning replacement for ammonium perchlorate (AP) in a formulation that also gains in performance. AP leaves a heavy hydrochloric acid/smoke trail upon burning. The chlorine in the smoke trail has been identified as a major contributor to ozone depletion in the stratosphere and the smoke trail makes rockets more vulnerable to detection and tracking (Borman, 1994). Currently, Air Force personnel are working with ADN during field testing trials. It is expected that the production operation will soon be scaled-up to the production of bulk quantities resulting in decreased costs. Toxicity assessment of ADN by the Department of Defense is necessary for long-range program management issues such as reproductive effects.

ADN is a genotoxicant (Zhu et al., 1994). The reproductive toxicity potential of ADN was evaluated through use of a modified Screening Information Data Set (SIDS) study using Sprague-Dawley rats (Kinkead et al., 1995). Male and female rats received ADN-treated drinking water daily at concentrations of 2000, 1000, 200, or 0.0 mg ADN/L. No mortality occurred in any of the parental animals during this study. No adverse effects were noted on mating as 92 to 100% of the animals mated. Adverse effects were noted for litter parameters. Nine of the 11 control and 11 of 12 low-dose dams that mated produced litters. Only 3 of 12 mated mid-dose and 1 of 11 mated high-dose dams produced litters. The mid- and high-dose dams produced litter sizes smaller (mean of 7 pups) than the litters produced by the low-dose and control groups (mean of 16 pups). During the 21-day lactation phase, no significant differences in mean pup weights were noted between treated and control groups.

In a study of the effects of ADN on implantation, untreated male and female Sprague-Dawley rats were mated and the pregnant dams then received ADN-treated drinking water (2000 mg ADN/L) on a daily basis only during Gestation Days (GD) 1-3 (the preimplantation period) or GD 4-8 (the postimplantation period) (Kinkead et al., 1996). A group of untreated control dams were maintained on untreated drinking water during GD 1-9. All dams were necropsied on GD 9. Treatment with ADN during GD 1-3 resulted in complete abolition of implantations. ADN had no significant effect on implantations when administered during GD 4-8.

The Hydra Assay is performed using the fresh water coelenterate *Hydra attenuata* as the test species. *Hydra attenuata* is the most primitive invertebrate composed of complex tissues and organs, and it is the highest form that has the capability for whole body regeneration. The assay employs the use of both adult hydra and "artificial embryos," composed of disassociated hydra cells, to investigate the potential toxicity of test compounds. Artificial embryos (also called pellets) are made by disassociating adult hydra into their component cells (Gierer et al., 1972). These cells can be reaggregated randomly into the artificial embryo (Johnson, 1980) which will

regenerate into adult hydra under normal conditions and within a few days. In order for the pellets to regenerate into new adult hydra, the "embryo" must accomplish most, if not all, of the developmental events required during true embryogenesis (Johnson, 1990).

Test chemicals may affect one or more of these developmental events, causing abnormal development and/or death of the artificial embryo. The concentration of the test chemical which causes abnormal development in the embryo may or may not cause an effect in the adult hydra. For this reason, both adult hydra and the artificial embryos are tested concurrently. The lowest concentration of test chemical that causes death in the intact adult hydra is compared to the lowest concentration that produces death in the developing artificial embryo. The adult toxic (A) to developmentally toxic (D) ratio (A/D ratio) is calculated using these concentrations.

The A/D ratio, also referred to as the developmental hazard index, is predictive of a chemical's hazard potential in standard laboratory animals and man (Johnson and Gabel, 1982). A low A/D ratio ( $<3$ ) predicts a test chemical being toxic to an embryo only at levels which will also cause toxic signs in the adult animal. A high A/D ratio ( $\geq 3$ ) reveals a chemical's teratogenic hazard, displaying a toxic effect in the developing embryo while causing little to no toxicity in the adult (Johnson et al., 1988).

The Hydra Assay is a screening test used to detect potential developmental toxicity associated with exposure to chemical compounds. The assay was designed to be a rapid screen for determining whether more sophisticated and/or expensive tests (two-species vertebrate developmental toxicity testing) need to be performed. The hydra assay was performed to determine the developmental hazard index for ADN.

## SECTION II

### MATERIALS AND METHODS

#### Test Material

##### Ammonium dinitramide (ADN)

Source/Supplier: SRI International  
Menlo Park, CA

Empirical formula:  $\text{NH}_4\text{N}(\text{NO}_2)_2$

Because of the explosive nature of this compound, the U.S. Army assumed the responsibility of retaining an archive sample. The ADN sample, a water-soluble powder, was known to be contaminated with 1-2% ammonium nitrate and was maintained in an enclosed cabinet due to light sensitivity (Koppes, 1993). Stability of ADN in water was determined during the reproductive toxicity screen of ADN (Kinkead et al., 1995). Decomposition of ADN in drinking water solutions was determined to be caused by exposure to ultraviolet light and resulted in a change in solution pH. Water bottles that were covered with black polyethylene plastic showed no pH shift. During the hydra assay of ADN, all stock solutions and hydra test dishes were kept covered with aluminum foil or black polyethylene plastic during non-observation periods. During ADN test dish preparations, and hydra or pellet observations, the room lights were turned off and a distant, indirect light source was utilized. At the end of each observation period when adult hydra and pellets were examined, each test dish was briefly placed under the direct light of a dissecting microscope.

#### Test Species

*Hydra attenuata* were supplied *gratis* by the Thomas Jefferson Medical College, Philadelphia, PA. *Hydra attenuata* are the only hydroids with which a successful hydra assay may be performed. Polyps of the species *Hydra attenuata* live in fresh water, they are not complicated by algae associations, and they derive all nutrition by feeding (Johnson et al., 1988).

The hydra colonies were housed in shallow plexiglas aquaria which provided controlled aeration (referred to as farms). The hydra farms and test hydra were maintained within a plexiglas enclosure. The temperature within the laboratory and the enclosure was maintained near  $18\text{ }^\circ\text{C} \pm 2\text{ }^\circ\text{C}$  using an attached cooling system. This temperature is the condition under which hydra grow best. Temperatures of  $\geq 24\text{ }^\circ\text{C}$  or  $\leq 12\text{ }^\circ\text{C}$  will adversely affect normal growth of hydra. Hydra were maintained in a water-based medium (hydra medium) which contained 1.0 mM  $\text{CaCl}_2$  dihydrate, 0.458 mM TES (sodium salt), and 0.012 mM EDTA. Ultra-purified water (18 mOhm-cm) was used in the

preparation of all solutions throughout the study. The pH of the medium was adjusted to 6.90-6.95 using 0.5 N NaOH. The hydra were fed regularly and allowed to propagate naturally by budding. *Artemia nauplii* (brine shrimp), hatched in 1% saline solution, were provided as food for the hydra. Bowls of hatching brine shrimp were started on a daily rotation to provide a continuously fresh food supply for the hydra. These bowls were maintained in the laboratory under controlled temperature.

The test hydra were fed daily for three days prior to testing, but were not fed while on study. Test hydra were group housed in hydra aquaria (4-in. deep by 8-in. wide) containing hydra medium during the three-day pre-testing feedings. During exposure test hydra were in 35-mm × 12-mm glass hydra test dishes (three adult hydra or pellets per test dish).

Hydra not used for testing were group housed within the hydra farms. The group-housed hydra were fed six days/week minimally, more often if the numbers of hatched shrimp allowed. The hydra farms were cleaned on a regular schedule to deter any bacterial or algal growth.

### **Experimental Design**

The preparation of regenerating artificial hydra embryos and the performance of the hydra assays were accomplished following the methods in "The Hydra Assay Manual," Johnson et al., 1992.

The hydra assay for ADN consisted of four consecutive experiments, each experiment required testing of both adult hydra and regenerating artificial hydra embryos (pellets). Experiment 1 established the log concentrations of test material to produce the toxic endpoint and determine the minimal affective concentration (MAC). Experiment 1 was the range-finding study to designate the appropriate concentrations for the final experiments. Experiment 2 utilized a more narrow range of concentrations of test material for more precise resolution of the MAC. Experiments 3 and 4 were repetitions of Experiment 2 for confirmation of the MAC. If MACs from the final experiments were not in close agreement (within the same or adjacent concentration test dishes), the assay would have been repeated. All four experiments were run with test solutions adjusted (with 0.5 N NaOH) to the pH range of 6.90-6.95.

Control dishes were filled with 4 mL assay reaggregate media (pellets) or assay hydra medium (adults). Test dishes contained the appropriate medium and ascending concentrations of test material so that the total volume used in any test dish was 4 mL (4000  $\mu$ L). The volume of any solution added to the hydra test dish did not exceed 10% (400  $\mu$ L) of the total volume of the test dish because the hydra would not survive due to the medium being too dilute. The test material solutions were added directly to dishes already containing the appropriate volume of reaggregate or hydra medium. Doses

were selected following the procedures described in "The Hydra Assay Manual" (Johnson et al., 1992). Test and control solutions in each adult and developmental test dish were changed after each observation.

Assay test dishes were kept covered on the countertop during the period from Time Zero through the 4-h observations. Assay test dishes were also covered from 4 h through 90 h, but were housed within the hydra farm enclosure during non-observation periods. Temperatures were recorded for the laboratory and the hydra farm enclosure for each observation time point.

### **Assay of Developmental Hydra**

Artificial hydra embryos (pellets) were produced from disassociated cells of adult hydra. Pellet preparation began by placing 1.5-2.0 mL adult hydra into a clean 15-mL conical glass centrifuge tube containing 3.5 mL reaggregate media (3.9 mM KCl, 6.6 mM CaCl<sub>2</sub> dihydrate, 0.63 mM MgSO<sub>4</sub>, 6.6 mM sodium citrate, 6.6 mM sodium pyruvate, and 12.0 mM TES buffer). Phenol red was also included in the media as a pH indicator. The pH of the reaggregate media was adjusted to 6.90-6.95 with 0.5 N NaOH prior to use in the assay. Amikacin sulfate USP (Elkins-Sinn, Inc., Cherry Hill, NJ) was also added to the reaggregate media (2 µL/mL media) to inhibit microbial growth. After incubating in the reaggregate media for 30 minutes, the adult hydra were disassociated by repeated shearing (disassociation) with a glass fire-polished pipette. The resulting suspension was left undisturbed for 6 minutes and then the supernatant was collected. This procedure was repeated two to three times depending upon the amount of intact hydra left in the centrifuge tube.

The pooled supernatant from the disassociations was centrifuged at 300 ×g for 5 min and then decanted, leaving a volume of fluid approximately equal to the volume of the cell pellet. The cells were resuspended in the tube by gentle mixing, and the resulting slurry was drawn up into 3-cm pieces of polyethylene tubing. The pieces of tubing containing cells and media were placed into 2.5 mL microcentrifuge tubes and centrifuged at 200 ×g for 5 min. The pellets formed by the centrifugation consisted of randomly aggregated cells. These "pellets" or artificial hydra embryos were ejected into test dishes containing reaggregate media with or without test material. The time the last pellet was placed into a test dish was Time Zero for the developmental portion of the hydra assay experiment.

Three or more pellets were placed into each test dish. Pellets were observed visually at Time Zero to insure viability. Pellets were observed using a dissecting microscope and observations were recorded 4, 18, 26, 42, 66, and 90 h after the pellets were placed into the test dishes. Observations consisted of recording the stage of development for each pellet. At the

4-h observations, three viable pellets were kept in the dish. All other pellets were discarded. The three pellets remained in the test dishes for the remainder of each experiment. Normally developing hydra pellets are solid at Time Zero. Pellets begin to appear "hollow" at 4 h of development. At 18 h, normal pellets will have tentacle buds, which appear randomly across the pellet as small bumps. At 26 h, normal pellets' tentacle buds begin to elongate into true tentacles. By 66 h, hypostomes begin to appear on the pellet near the tentacles. Hypostomes are the openings in the top portion of the body of hydra through which they consume food. Ninety hours after beginning to develop, the pellets consist of hydra polyps. The area of the hypostomes will have elongated into the body of a hydra polyp. If allowed to continue to grow, the polyps will separate into individual adult hydra.

The toxic endpoint for the pellet was death. Once any two pellets in one test dish were dead, the concentration in that dish was considered an affective dose. If viable pellets remained within a test dish in which a pellet was found dead, the dead pellet was removed and the surviving pellets allowed to continue through the experiment. Once the 90-h observation was made, the experiment was complete. The MAC was then recorded for the artificial embryos.

### **Assay of Adult Hydra**

Hydra assay medium was prepared by adding Amikacin sulfate USP (Elkins-Sinn, Inc., Cherry Hill, NJ) to hydra medium (2  $\mu$ L/mL) to prevent bacterial growth. The pH was then adjusted to 6.90-6.95 using 0.5 N NaOH. Three normal adult hydra were placed into each test dish containing either 4 mL of hydra assay medium (control) or assay medium containing the appropriate volume of test material. Adult hydra were visually observed at Time Zero to ensure viability. Adult hydra observations were performed using a dissecting microscope. Microscopic observations were recorded 4, 18, 26, 42, 66, and 90 h after the hydra were placed into the test dishes. Once the 90-h observation was recorded, the experiment was complete.

Adult hydra react to a change in their environment (i.e., test material) by contracting their tentacles. Sometimes contraction of the body also occurs. This reaction is called "clubbing." Adult hydra undergoing stress will continue to contract their tentacles until they are very short. The contraction continues under stress until the tentacles are barely discernible, and the hydra physically appear to look like tulip flowers. Hydra will not survive once reaching the "tulip" stage. They will eventually disintegrate, which is recorded as death. The toxic endpoint for adult hydra is death or the "tulip" stage. When any two hydra in a test dish died or reached the tulip stage, the concentration in that test dish was considered an affective dose. The MAC was then recorded for the adult hydra in each experiment.

### **Test Material Stock Solutions**

One tenth of a gram of ADN was added to 10 mL hydra media to make Stock Solution A. Further dilution of Stock A were made to create the other stock solutions.

- 1 mL Stock A to 9 mL media = Stock B
- 1 mL Stock B to 9 mL media = Stock C
- 1 mL Stock C to 9 mL media = Stock D
- 1 mL Stock D to 9 mL media = Stock E
- 1 mL Stock E to 9 mL media = Stock F

### **Determination Of A/D Ratio**

The A/D ratio for the test material was calculated using the average MACs from Experiments 3 and 4 using the following equation:

$$\text{MAC adult hydra} \div \text{MAC artificial embryo} = \text{A/D ratio.}$$

### **Statistics**

Standard statistical analyses are inapplicable for results from the hydra assay due to the small number of invertebrates used per concentration. Hydra respond to environmental conditions in a stereotypical manner. The assay's endpoints take into account any differences by specifying that an effect equals the "observed effect" in two out of three animals per test concentration. Since experiments are performed using a wide range of test material concentrations, and the experiment which determines the affective concentration is repeated, a clear and obvious concentration-response relationship is evident for the assay. The endpoints for the hydra assay are specific and conclusive for both the adult and the developing artificial embryo, and statistical analysis would be unreasonable (Newman et al., 1990).

## SECTION III

### RESULTS

The observations recorded during each of the four experiments for the hydra assay of ADN are listed in Tables 1 through 8. Timepoints in which a test dish of adult hydra or pellets reached the toxic endpoint are typed in bold print on each table. Temperatures of the hydra laboratory and the hydra farm enclosure (Encl) at each of the observation timepoints are included on these tables. The average temperature ( $\pm$  SD) in the laboratory was  $19.7 \pm 0.9$  °C, and the average temperature ( $\pm$  SD) in the enclosure was  $18.8 \pm 1.0$  °C. During Experiment 1, hydra adults and pellets were exposed to concentrations of ADN from 1000 through 0.01 mg ADN/L, and to media only (control). All adult hydra survived through 26-h treatment (Table 1). All adults in the 1000 mg ADN/L test dish were either dead or had reached the tulip stage by 42-h exposure. All other adult hydra lived through the 90-h observations. The MAC for the adult hydra was 1000 mg ADN/L.

During Experiment 1, all pellets were solid at the 4-h observation timepoint. By 18-h treatment, the pellets in the 1000 mg ADN/L test dish were dead. All surviving pellets displayed advancing developmental stages through 90 h. All dishes except for those treated with 100 mg ADN/L contained normal hydra polyps at this timepoint (Table 2). The MAC for the developmental portion of Experiment 1 was 1000 mg ADN/L.

Since both the adult and developing pellets were observed to have a MAC at 1000 mg ADN/L, this concentration was chosen as the highest dosing level for both forms of hydra for the final experiments that would determine the A/D ratio for ADN. Experiment 2 was performed using ADN concentrations of 100 through 1000 mg ADN/L, plus a control. The adult hydra displayed dose-response reactions to the test chemical (Table 3). The first deaths and tulip stage observations were recorded at 42-h treatment in the 1000, 900, and 800 mg ADN/L test dishes. By 66-h treatment, the highest concentration of ADN in which all adult hydra were viable was 400 mg ADN/L. All adult hydra from 400 mg ADN/L through the controls survived throughout the 90-h observation. The MAC for adult hydra in this experiment was 500 mg ADN/L.

The developmental observations of Experiment 2 were not highly comparable to the observations recorded during Experiment 1. The most significant difference noted was the pellets in the 400 mg ADN/L test dish died at 26 and 42 h instead of surviving through the entire observation period (Table 4). Control pellets developed normally and survived through the 90-h observation. The MAC for the developmental portion of Experiment 2 could not be determined due to pellet death in the 100 mg ADN/L test dish.

Experiment 3 was a repeat of Experiment 2. In this experiment, all adult hydra survived through 26-h treatment (Table 5). The hydra in the 1000 mg ADN/L test dish displayed toxicity at 42 h; the hydra in the 900 mg ADN/L test dish reached the endpoint



at 66 h. At the 90-h observation timepoint, the hydra in the 800 mg ADN/L test dish exhibited toxicity. Two of three 700 mg ADN/L hydra, and all other hydra survived through the end of the experiment. The adult MAC for Experiment 3 was 800 mg ADN/L.

In the developmental portion of Experiment 3, pellets were exposed to doses of ADN from 600 to 100 mg ADN/L. A control dish was also included in this experiment. All pellets survived through the 26-h observation (Table 6). By 42 h, the 600 and 500 mg ADN/L test dishes had reached the toxic endpoint. At the 90-h observation, the 400 mg ADN/L test dish reached the toxic endpoint. The 300, 200, and 100 mg ADN/L test dishes and the control dish held viable pellets. The developmental MAC was 400 mg ADN/L for Experiment 3.

Experiment 4 was a repetition of Experiments 2 and 3. This procedure was done to assure that the MAC for both adult and developing hydra obtained in Experiment 3 was accurate. As in Experiment 3, all adult hydra survived through the 26-h observations (Table 7). At 66 h of exposure, the six lowest ADN concentration test dishes and the control test dish contained viable hydra. At the 90-h observation, live hydra remained in the 600 through 100 mg ADN/L dishes, and in the control dish. Two viable pellets per dish were observed in each of the three highest test levels of ADN remaining at the end of Experiment 4. The adult MAC determined in Experiment 4 was 700 mg ADN/L.

During the developmental portion of Experiment 4, pellets were tested at concentrations of 700 through 100 mg ADN/L, plus controls. The pellets in the 700, 600, and 500 mg ADN/L test dishes were dead by 26 h exposure (Table 8). Two or more of the 400 and 300 mg ADN/L test pellets were dead at 42 h. The three test dishes which held viable pellets at the 90-h observation were the 200 and 100 mg ADN/L and the control. The MAC for the developmental portion of Experiment 4 was 300 mg ADN/L.

### A/D Ratio

The MACs from Experiments 3 and 4 were used to determine the A/D ratio. The MACs determined in Experiment 2 were not usable when compared to the MACs determined in Experiments 3 and 4 (i.e., test dishes determining MAC did not reach the toxic endpoints at the same test material concentration or within adjacent test dishes). The A/D ratio determined by the hydra assay of ADN was 2.14 (average adult MAC of 750 mg ADN/L divided by the average developmental MAC of 350 mg ADN/L).

**TABLE 1. EXPERIMENT NUMBER 1 ADULT HYDRA OBSERVATIONS  
MAC 1000 mg ADN/L**

| Test Agent Concentration | 4 hours   | 18 hours              | 26 hours                      | 42 hours              | 66 hours                         | 90 hours                         |
|--------------------------|-----------|-----------------------|-------------------------------|-----------------------|----------------------------------|----------------------------------|
| 1000 mg ADN/L            | 3 clubbed | 3 short               | 2 short,<br>1 short<br>severe | 2 dead,<br>1 tulip    | 1 dead                           |                                  |
| 100 mg ADN/L             | 3 normal  | 3 normal              | 3 normal                      | 3 normal              | 1 clubbed,<br>2 normal           | 1 clubbed,<br>2 normal           |
| 10 mg ADN/L              | 3 normal  | 3 normal              | 3 normal                      | 3 normal              | 1 clubbed<br>slight,<br>2 normal | 1 clubbed<br>slight,<br>2 normal |
| 1 mg ADN/L               | 3 normal  | 3 normal              | 3 normal                      | 3 normal              | 3 normal                         | 1 clubbed<br>slight,<br>2 normal |
| 0.1 mg ADN/L             | 3 normal  | 3 normal              | 3 normal                      | 3 normal              | 3 normal                         | 3 normal                         |
| 0.01 mg ADN/L            | 3 normal  | 3 normal              | 3 normal                      | 3 normal              | 3 normal                         | 3 normal                         |
| CONTROL<br>0.0 mg ADN/L  | 3 normal  | 3 normal              | 3 normal                      | 3 normal              | 3 normal                         | 3 normal                         |
| Temp °C                  | Lab 21.6  | Lab 21.1<br>Encl 19.2 | Lab 20.6<br>Encl 19.6         | Lab 18.6<br>Encl 17.9 | Lab 18.8<br>Encl 18.4            | Lab 20.1<br>Encl 18.9            |

**TABLE 2. EXPERIMENT NUMBER 1 PELLETT OBSERVATIONS  
MAC 1000 mg ADN/L**

| Test Agent Concentration | 4 hours  | 18 hours              | 26 hours              | 42 hours   | 66 hours                           | 90 hours                    |
|--------------------------|----------|-----------------------|-----------------------|--|------------------------------------|-----------------------------|
| 1000 mg ADN/L            | 3 solid  | 3 dead                |                       |  |                                    |                             |
| 100 mg ADN/L             | 3 solid  | 3 hollow              | 3 tentacle buds       | 1 elongated,<br>1 elongated<br>slight,<br>1 tentacle bud | 1 elongated, 2<br>elongated slight | 2 elongated, 1<br>hypostome |
| 10 mg ADN/L              | 3 solid  | 3 hollow              | 3 tentacle buds       | 2 elongated, 1<br>elongated slight                       | 2 hypostomes,<br>1 elongated       | 3 polyps                    |
| 1 mg ADN/L               | 3 solid  | 3 hollow              | 3 tentacle buds       | 1 elongated, 2<br>elongated slight                       | 3 hypostomes                       | 2 polyps,<br>1 hypostome    |
| 0.1 mg ADN/L             | 3 solid  | 3 hollow              | 3 tentacle buds       | 3 elongated  | 3 hypostomes                       | 3 polyps                    |
| 0.01 mg ADN/L            | 3 solid  | 3 hollow              | 3 tentacle buds       | 2 elongated,<br>1 elongated<br>slight                    | 2 hypostomes, 1<br>elongated       | 3 polyps                    |
| CONTROL<br>0.0 mg ADN/L  | 3 solid  | 3 hollow              | 3 tentacle buds       | 2 elongated,<br>1 elongated<br>slight                    | 2 hypostomes, 1<br>elongated       | 3 polyps                    |
| Temp °C                  | Lab 21.6 | Lab 21.1<br>Encl 19.2 | Lab 20.6<br>Encl 19.6 | Lab 18.6<br>Encl 17.9                                    | Lab 18.8<br>Encl 18.4              | Lab 20.1<br>Encl 18.9       |

**TABLE 3. EXPERIMENT NUMBER 2 ADULT HYDRA OBSERVATIONS  
MAC 500 mg ADN/L**

| Test Agent Concentration | 4 hours                     | 18 hours                              | 26 hours                              | 42 hours                              | 66 hours                              | 90 hours                              |
|--------------------------|-----------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|---------------------------------------|
| 1000 mg ADN/L            | 3 short                     | 2 short severe, 1 short               | 3 short severe                        | 1 tulip, 2 short severe               | 2 dead, 1 tulip                       | 1 dead                                |
| 900 mg ADN/L             | 3 short                     | 1 short severe, 2 short               | 3 short severe                        | 1 dead, 2 tulip                       | 2 dead                                |                                       |
| 800 mg ADN/L             | 3 clubbed                   | 1 short severe, 2 short               | 2 short severe, 1 short               | 1 dead, 2 short severe                | 1 dead, 1 tulip                       | 1 tulip                               |
| 700 mg ADN/L             | 1 short, 2 clubbed          | 2 short, 1 clubbed severe             | 3 short severe                        | 3 short severe                        | 2 dead, 1 tulip                       | 1 tulip                               |
| 600 mg ADN/L             | 2 short, 1 clubbed          | 2 short, 1 clubbed                    | 2 short severe, 1 clubbed             | 2 short severe, 1 short               | 2 dead, 1 short severe                | 1 short severe                        |
| 500 mg ADN/L             | 2 short, 1 clubbed          | 2 short, 1 clubbed                    | 3 short                               | 3 short                               | 1 dead, 1 tulip, 1 short              | 1 tulip, 1 short                      |
| 400 mg ADN/L             | 1 clubbed, 2 clubbed slight | 2 clubbed, 1 clubbed slight           | 3 clubbed severe                      | 2 short, 1 clubbed severe             | 3 short                               | 3 short                               |
| 300 mg ADN/L             | 2 clubbed, 1 clubbed slight | 2 clubbed, 1 clubbed slight           | 3 clubbed                             | 1 clubbed severe, 2 clubbed           | 1 short, 2 clubbed severe             | 1 short, 2 clubbed severe             |
| 200 mg ADN/L             | 3 clubbed slight            | 1 clubbed severe, 2 clubbed slight    | 1 clubbed severe, 2 clubbed slight    | 1 clubbed severe, 2 clubbed slight    | 1 clubbed severe, 2 clubbed slight    | 1 clubbed severe, 2 clubbed slight    |
| 100 mg ADN/L             | 2 clubbed slight, 1 normal  | 2 clubbed slight, 1 normal            | 2 clubbed slight, 1 normal            | 2 clubbed slight, 1 normal            | 2 clubbed slight, 1 normal            | 2 clubbed slight, 1 normal            |
| CONTROL 0.0 mg ADN/L     | 2 clubbed, 1 normal         | 1 clubbed, 1 clubbed slight, 1 normal | 1 clubbed, 1 clubbed slight, 1 normal | 1 clubbed, 1 clubbed slight, 1 normal | 1 clubbed, 1 clubbed slight, 1 normal | 1 clubbed, 1 clubbed slight, 1 normal |
| Temp °C                  | Lab 19.6                    | Lab 19.3<br>Encl 18.7                 | Lab 19.9<br>Encl 18.9                 | Lab 19.5<br>Encl 18.7                 | Lab 18.3<br>Encl 17.9                 | Lab 19.5<br>Encl 18.6                 |

**TABLE 4. EXPERIMENT NUMBER 2 PELLETT OBSERVATIONS  
MAC <100 mg ADN/L**

| Test Agent Concentration | 4 hours      | 18 hours                    | 26 hours                                     | 42 hours                               | 66 hours                      | 90 hours              |
|--------------------------|--------------|-----------------------------|--|--|-------------------------------|-----------------------|
| 1000 mg ADN/L            | 3 semi-solid | 3 dead                      |  |  |                               |                       |
| 900 mg ADN/L             | 3 semi-solid | 3 dead                      |  |  |                               |                       |
| 800 mg ADN/L             | 3 solid      | 3 dead                      |  |  |                               |                       |
| 700 mg ADN/L             | 3 solid      | 3 dead                      |  |  |                               |                       |
| 600 mg ADN/L             | 3 solid      | 1 dead,<br>2 solid          | 2 dead                                       |  |                               |                       |
| 500 mg ADN/L             | 3 solid      | 3 solid                     | 3 dead                                       |  |                               |                       |
| 400 mg ADN/L             | 3 solid      | 3 solid                     | 2 dead,<br>1 solid                           | 1 dead                                 |                               |                       |
| 300 mg ADN/L             | 3 solid      | 1 hollow slight,<br>2 solid | 2 tentacle buds,<br>1 bud slight             | 3 dead                                 |                               |                       |
| 200 mg ADN/L             | 3 solid      | 2 hollow,<br>1 solid        | 3 tentacle buds                              | 2 dead,<br>1 elongated                 | 1 dead                        |                       |
| 100 mg ADN/L             | 3 solid      | 3 hollow                    | 2 tentacle buds,<br>1 tentacle bud<br>slight | 1 elongated slight,<br>2 tentacle buds | 1 dead,<br>2 elongated slight | 1 dead,<br>1 solid    |
| CONTROL<br>0.0 mg ADN/L  | 3 solid      | 3 hollow                    | 3 tentacle buds                              | 3 elongated                            | 3 hypostomes                  | 3 polyps              |
| Temp °C                  | Lab 19.6     | Lab 19.3<br>Encl 18.7       | Lab 19.9<br>Encl 18.9                        | Lab 19.5<br>Encl 18.7                  | Lab 18.3<br>Encl 17.9         | Lab 19.5<br>Encl 18.6 |

**TABLE 5. EXPERIMENT NUMBER 3 ADULT HYDRA OBSERVATIONS  
MAC 800 mg ADN/L**

| Test Agent Concentration | 4 hours                        | 18 hours                       | 26 hours                       | 42 hours                       | 66 hours                       | 90 hours                       |
|--------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| 1000 mg ADN/L            | 3 clubbed                      | 2 clubbed severe,<br>1 clubbed | 3 short severe                 | 1 dead,<br>2 tulip             | 2 dead                         |                                |
| 900 mg ADN/L             | 3 clubbed                      | 2 clubbed severe,<br>1 clubbed | 3 short                        | 1 tulip,<br>2 short severe     | 3 dead                         |                                |
| 800 mg ADN/L             | 1 clubbed,<br>2 clubbed slight | 1 clubbed severe,<br>2 clubbed | 1 short severe,<br>2 short     | 1 dead,<br>2 short severe      | 2 short severe                 | 1 dead,<br>1 tulip             |
| 700 mg ADN/L             | 3 clubbed slight               | 3 clubbed                      | 2 short,<br>1 clubbed severe   | 1 short severe,<br>2 short     | 3 short severe                 | 1 dead,<br>2 short severe      |
| 600 mg ADN/L             | 2 clubbed slight,<br>1 normal  | 3 clubbed                      | 1 short,<br>2 clubbed severe   | 3 short                        | 3 short                        | 3 short severe                 |
| 500 mg ADN/L             | 2 clubbed slight,<br>1 normal  | 3 clubbed                      | 1 clubbed severe,<br>2 clubbed | 2 short,<br>1 clubbed          | 2 short,<br>1 clubbed          | 2 short,<br>1 clubbed          |
| 400 mg ADN/L             | 2 clubbed slight,<br>1 normal  | 3 clubbed                      | 1 clubbed severe,<br>2 clubbed | 2 short,<br>1 clubbed          | 2 short,<br>1 clubbed          | 2 short severe,<br>1 clubbed   |
| 300 mg ADN/L             | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed,<br>2 clubbed slight | 1 clubbed,<br>2 clubbed slight | 1 clubbed,<br>2 clubbed slight |
| 200 mg ADN/L             | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  |
| 100 mg ADN/L             | 3 normal                       | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  | 1 clubbed slight,<br>2 normal  |
| CONTROL<br>0.0 mg ADN/L  | 3 normal                       | 2 normal,<br>1 clubbed slight  | 1 clubbed slight,<br>2 normal  | 3 normal                       | 2 clubbed slight,<br>1 normal  | 1 clubbed slight,<br>2 normal  |
| Temp °C                  | Lab 19.6                       | Lab 18.8<br>Encl 18.1          | Lab 19.3<br>Encl 18.0          | Lab 20.0<br>Encl 19.3          | Lab 20.8<br>Encl 20.8          | Lab 21.4<br>Encl 20.2          |

**TABLE 6. EXPERIMENT NUMBER 3 PELLETT OBSERVATIONS  
MAC 400 mg ADN/L**

| Test Agent Concentration | 4 hours  | 18 hours                     | 26 hours                         | 42 hours                           | 66 hours  | 90 hours                               |
|--------------------------|----------|------------------------------|----------------------------------|------------------------------------|---|--|
| 600 mg ADN/L             | 3 solid  | 3 solid                      | 2 tentacle buds,<br>1 semi-solid | 3 dead                             |   |  |
| 500 mg ADN/L             | 3 solid  | 3 solid                      | 3 tentacle buds                  | 2 dead,<br>1 tentacle bud          | 1 dead  |  |
| 400 mg ADN/L             | 3 solid  | 1 hollow slight,<br>2 solid  | 3 tentacle buds                  | 3 tentacle buds                    | 1 dead,<br>2 tentacle buds                          | 2 dead                                 |
| 300 mg ADN/L             | 3 solid  | 2 hollow,<br>1 hollow slight | 3 tentacle buds                  | 1 elongated,<br>2 elongated slight | 1 hypostome slight,<br>1 elongated,<br>1 semi-solid | 1 dead,<br>1 hypostome,<br>1 elongated |
| 200 mg ADN/L             | 3 solid  | 3 hollow                     | 3 tentacle buds                  | 2 elongated,<br>1 elongated slight | 2 hypostomes,<br>1 hypostome slight                 | 3 polyps                               |
| 100 mg ADN/L             | 3 solid  | 3 hollow                     | 2 tentacle buds,<br>1 bud slight | 2 elongated,<br>1 elongated slight | 3 hypostomes  | 3 polyps                               |
| CONTROL<br>0.0 mg ADN/L  | 3 solid  | 2 hollow,<br>1 hollow slight | 3 tentacle buds                  | 3 elongated                        | 3 hypostomes  | 3 polyps                               |
| Temp °C                  | Lab 19.6 | Lab 18.8<br>Encl 18.1        | Lab 19.3<br>Encl 18.0            | Lab 20.0<br>Encl 19.3              | Lab 20.8<br>Encl 21.2                               | Lab 21.4<br>Encl 20.2                  |

**TABLE 7. EXPERIMENT NUMBER 4 ADULT HYDRA OBSERVATIONS  
MAC 700 mg ADN/L**

| Test Agent Concentration | 4 hours                                   | 18 hours                                  | 26 hours                       | 42 hours  | 66 hours                      | 90 hours                           |
|--------------------------|---|---|--------------------------------|---|-------------------------------|------------------------------------|
| 1000 mg ADN/L            | 3 clubbed                                 | 1 short severe,<br>2 short                | 2 short severe,<br>1 short     | 2 tulip,<br>1 short severe                        | 3 dead                        |                                    |
| 900 mg ADN/L             | 2 clubbed severe,<br>1 clubbed            | 3 short                                   | 2 short severe,<br>1 short     | 3 short severe                                    | 2 dead,<br>1 tulip            | 1 dead                             |
| 800 mg ADN/L             | 2 clubbed,<br>1 clubbed slight            | 1 short severe,<br>2 short                | 1 short severe,<br>2 short     | 1 tulip,<br>2 short severe                        | 1 dead,<br>2 tulip            | 2 dead                             |
| 700 mg ADN/L             | 2 clubbed,<br>1 clubbed slight            | 3 short                                   | 3 short                        | 3 short severe                                    | 2 dead,<br>1 tulip            | 1 tulip                            |
| 600 mg ADN/L             | 3 clubbed                                 | 2 clubbed,<br>1 clubbed slight            | 2 clubbed severe,<br>1 short   | 3 short severe                                    | 1 tulip,<br>2 short severe    | 1 dead,<br>2 short severe          |
| 500 mg ADN/L             | 2 clubbed slight,<br>1 normal             | 1 clubbed,<br>2 clubbed slight            | 3 clubbed                      | 1 short severe,<br>1 short,<br>1 clubbed          | 2 short severe,<br>1 clubbed  | 1 tulip,<br>2 short                |
| 400 mg ADN/L             | 1 short,<br>1 clubbed slight,<br>1 normal | 1 short,<br>1 clubbed slight,<br>1 normal | 1 clubbed severe,<br>2 clubbed | 1 short severe,<br>1 clubbed severe,<br>1 clubbed | 1 short severe,<br>2 clubbed  | 1 tulip,<br>2 short                |
| 300 mg ADN/L             | 3 normal                                  | 2 clubbed slight,<br>1 normal             | 3 clubbed slight               | 3 clubbed   | 3 clubbed                     | 1 short,<br>2 clubbed              |
| 200 mg ADN/L             | 1 clubbed slight,<br>2 normal             | 1 clubbed slight,<br>2 normal             | 3 normal                       | 3 normal  | 1 clubbed slight,<br>2 normal | 1 short,<br>1 clubbed,<br>1 normal |
| 100 mg ADN/L             | 3 normal                                  | 3 normal                                  | 3 normal                       | 3 normal  | 3 normal                      | 1 clubbed slight,<br>2 normal      |
| CONTROL                  | 3 normal                                  | 3 normal                                  | 3 normal                       | 3 normal  | 1 clubbed slight,<br>2 normal | 1 clubbed slight,<br>2 normal      |
| 0.0 mg ADN/L             | Lab 19.8                                  | Lab 19.7<br>Encl 19.8                     | Lab 19.8<br>Encl 20.0          | Lab 18.6<br>Encl 16.7                             | Lab 18.1<br>Encl 18.0         | Lab 19.2<br>Encl 18.2              |
| Temp °C                  |   |   |                                |   |                               |                                    |



**TABLE 8. EXPERIMENT NUMBER 4 PELLET OBSERVATIONS  
MAC 300 mg ADN/L**

| Test Agent Concentration | 4 hours  | 18 hours                     | 26 hours                                     | 42 hours                                 | 66 hours   | 90 hours                      |
|--------------------------|----------|------------------------------|--|--|--|-------------------------------|
| 700 mg ADN/L             | 3 solid  | 1 dead,<br>2 solid           | 3 dead                                       |  |  |                               |
| 600 mg ADN/L             | 3 solid  | 3 solid                      | 3 dead                                       |  |  |                               |
| 500 mg ADN/L             | 3 solid  | 3 solid                      | 3 dead                                       |  |  |                               |
| 400 mg ADN/L             | 3 solid  | 3 solid                      | 3 tentacle buds                              | 3 dead                                   |  |                               |
| 300 mg ADN/L             | 3 solid  | 1 hollow,<br>2 hollow slight | 3 tentacle buds                              | 2 dead,<br>1 tentacle bud                | 1 dead   |                               |
| 200 mg ADN/L             | 3 solid  | 3 hollow                     | 3 tentacle buds                              | 2 elongated<br>slight,<br>1 tentacle bud | 1 elongated,<br>1 elongated<br>slight,<br>1 tentacle bud | 1 dead,<br>2 elongated slight |
| 100 mg ADN/L             | 3 solid  | 3 hollow                     | 2 tentacle buds,<br>1 tentacle bud<br>slight | 3 elongated slight                       | 2 elongated,<br>1 hypostome                              | 1 polyp,<br>2 hypostomes      |
| CONTROL<br>0.0 mg ADN/L  | 3 solid  | 3 hollow                     | 3 tentacle buds                              | 2 elongated,<br>1 elongated slight       | 3 hypostomes<br>slight                                   | 2 polyps,<br>1 hypostome      |
| Temp °C                  | Lab 20.0 | Lab 19.7<br>Encl 19.8        | Lab 19.8<br>Encl 20.0                        | Lab 18.6<br>Encl 16.7                    | Lab 18.1<br>Encl 18.0                                    | Lab 19.2<br>Encl 18.2         |

## SECTION IV

### DISCUSSION

A/D ratios calculated from hydra assays have been compared to the A/D ratios from standard Segment II-type studies (Johnson et al., 1988). A comparison was made of A/D ratios obtained for 61 chemicals in which both hydra assays and Segment II-type studies were performed. Of the 61 chemicals, 57 of them had A/D ratios for hydra and Segment II-type studies which were in agreement. The hydra assay is 90% accurate and has a low incidence of false negatives (Johnson et al., 1988).

The developmental hazard index, or A/D ratio, of 2.14 for ADN would predict that ADN would only be toxic to a developing embryo at levels which would also cause maternal toxicity. This result is comparable to the results obtained when pregnant Sprague-Dawley rats were treated with ADN during the pre- and postimplantation periods (Kinkead et al., 1996). Female Sprague-Dawley rats were orally dosed with 2.0 g ADN/L in the drinking water during the preimplantation period (Gestation Days 1-3) or during the postimplantation period (Gestation Days 4-8). Complete blockade of implantation occurred in animals receiving ADN during the preimplantation period. Animals treated during the postimplantation period had a mean of 16.4 implantations per dam, similar to the mean of 15.2 implantations per dam in the control group.

In the reproductive toxicity screen (Kinkead et al., 1995) the pregnant animals received ADN-treated drinking water continuously for 90 days. The results from this study included treatment-related adverse effects on reproductive and litter parameters for animals dosed with 2.0 and 1.0 g ADN/L drinking water. Treatment in this study occurred throughout mating, gestation, and lactation. The preimplantation exposure to ADN is the probable cause for these adverse effects.

A study was performed to investigate the cause of the preimplantation effects of ADN (Graeter et al., 1996). Female Sprague-Dawley rats received ADN-treated drinking water beginning at GD 0. A control and treated group of dams were necropsied on GD 1 through 4, and location and stage of development of the embryos were evaluated. This study determined that embryo lethality may be partially responsible for the implantation failure. The same study reported development of B6C3F1 mouse embryos *in vitro* was also affected in a dose-dependent manner by ADN treatment. The actual mechanism of the effect of ADN on embryo lethality is currently not known.

The postimplantation dosing regimen reported in Kinkead et al., 1996, would be comparable to that used in developmental toxicity studies, where pregnant animals are dosed during the period of organogenesis, GD 6-15 for mice and rats (OECD, 1993). Therefore the A/D ratio determined in this developmental toxicity screen of ADN using *Hydra attenuata* should be analogous to results obtained from ADN exposure using a vertebrate developmental toxicity dosing regimen. Ammonium dinitramide is not a

probable developmental toxin according to the results of the hydra assay developmental toxicity screen performed in this laboratory.

## SECTION V

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