UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

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MAR – 9 1994

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: ALUMINUM PHOSPHIDE. ID #066501. Evaluation of an In Vivo/In Vitro Unscheduled DNA Synthesis Assay in Primary Hepatocytes from Male Rats Exposed to Phosphine Gas by Inhalation.

Tox. Chem. No.: 031
PC No.: 066501
Submission No.: S446688
Barcode No.: D194410

FROM: Linnea J. Hansen, Ph.D.
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Linnea J. Hansen 3/27/94

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THRU: Marion P. Copley, D.V.M., D.A.B.T., Section Head
Section IV, Tox. Branch I
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Marion Copley 3/8/94

CONCLUSIONS:

The study report entitled "Genotoxicity Test on Phosphine in the In Vivo/In Vitro Assay for Unscheduled DNA Synthesis in Rat Primary Hepatocyte Cultures at Two Timepoints" (MRID No. 428777-01) has been reviewed by TB-I (DER attached to this memo). Male rats were exposed by inhalation to 0, 4.8, 13, 18 or 23 ppm phosphine gas for 6 hrs (or to dimethylnitrosamine by IP injection) and primary hepatocyte cultures prepared at 2-3 and 12-14 hrs post-exposure. Under the conditions of this assay, there was no evidence of increased unscheduled DNA synthesis in primary hepatocyte cultures prepared from male rats exposed to phosphine. The dosing was considered adequate in this study based on evidence of clinical toxicity at high dose (labored breathing, slight decrease in body weight) and on results of previously conducted studies indicating that higher doses would be expected to produce excessive mortality (see DER for details). There was, however, no toxicity observed in the liver at any dose. This study therefore does not necessarily demonstrate that phosphine is not genotoxic since there was no evidence that it reached the target organ.

Classification: Acceptable. This study appeared to be have been properly conducted and is considered acceptable for regulatory purposes.

ACTION REQUESTED:

Degesch America, Inc. submitted for review an unscheduled DNA synthesis assay in primary hepatocytes prepared from male rats exposed in vivo by inhalation to phosphine gas. This study was submitted on July 8, 1993 on behalf of the Metal Phosphide Industry Panel to support reregistration of aluminum phosphide. The study was required in a data call-in in October, 1986 (Reregistration Standard for Aluminum/Magnesium Phosphide).

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Guideline Series 84: **MUTAGENICITY**

Reviewed by: Linnea J. Hansen, Ph.D. 1-27-94
Section IV, Tox. Branch I (H7509C)
Secondary reviewer: Irving Mauer, Ph.D. 01-28-94
Tox Branch I (H7509C)

DATA EVALUATION REPORT

CHEMICAL: Aluminum Phosphide Tox. Chem. No.: 031

PC No.: 066501

STUDY TYPE: Unscheduled DNA Synthesis in Primary Male Rat Hepatocytes (In Vivo/In Vitro)

MRID NUMBER: 428777-01

SYNONYM/CAS NO.: Phosphine, 7803-51-2

SPONSOR: Degesch America, Inc. for Metal Phosphide Industry Task Force, Weyers Cave, Virginia

TESTING FACILITY: Hazleton Washington, Inc., Vienna, Virginia (inhalation exposures conducted at Pharmaco LSR, East Millstone, NJ)

TITLE OF REPORT: Genotoxicity Test on Phosphine in the In Vivo/In Vitro Assay for Unscheduled DNA Synthesis in Rat Primary Hepatocyte Cultures at Two Timepoints; Acute Inhalation Exposures of Rats to Phosphine (appended to study report)

AUTHOR: Marie E. McKeon, M. Phil.
(Paul E. Newton, Ph.D., D.A.B.T., inhalation exposure report)

STUDY NUMBER: HWA Study No. A0040-0-494
(Pharmaco LSR Study No. 92-5107)

REPORT ISSUED: July 2, 1993
(Inhalation Exposure June 24, 1993)

EXECUTIVE SUMMARY:

In an unscheduled DNA synthesis assay in rat primary hepatocytes cultures, male rats were exposed by inhalation to phosphine gas at exposure levels of 0, 4.8, 13, 18 or 23 ppm for 6 hr. Negative controls were exposed to room air

only in exposure chambers. Exposures were equivalent to 0, 11.4, 30.8, 42.6 or 54.5 mg/m^3, respectively. Dimethylnitrosamine (DMN) was administered intraperitoneally in sterile saline at 10 mg/kg as a positive control. Cultures from each test group were prepared at 2-3 hr or 12-14 hr post-exposure.

Rats were exposed to phosphine gas at dose levels causing clinical evidence of toxicity (difficulty breathing, slight decrease in body weight at 18 and 23 ppm) and which approached exposure levels expected to result in excessive mortality based on results of previous studies. **No evidence of increased unscheduled DNA synthesis compared to negative controls was observed in primary cultures of male rat liver cells from rats exposed to phosphine gas by inhalation.** Although doses were adequate based on observed clinical signs of toxicity (labored breathing, slightly decreased body weight at 18 and 23 ppm) and results of previous acute toxicity studies, there was no evidence of toxicity in liver cells. The study therefore does not definitively show that phosphine does not induce unscheduled DNA synthesis since there was no evidence that it reached the target organ (liver).

This study is classified as an acceptable study and satisfies the guideline requirement for an unscheduled DNA synthesis study (84-4).

Signed Quality Assurance and Good Laboratory Practice Statements were present.

A. MATERIALS

1. Test Material: Phosphine (technical). Description: colorless gas. Batch #s: 864-2241-01 through -04 (Praxair). Purity: 99.8%.

2. Control Materials: Negative: none (chamber supply air)
   Positive: Dimethylnitrosamine (DMN)
   Sigma Chemical, lot #29F0679
   Dose: 10 mg/kg (2-3 hr culture)
   15 mg/kg (12-14 hr culture)
   Route: Intraperitoneal injection
   Vehicle: deionized water

3. Test Animals: Species: rat. Strain: Fischer CD (F344)/CrlBR (male only). Age: young adult. Weight: 210-240 g. Supplier: Charles River Breeding Laboratories, Kingston, NY.

4. Animal Care: Housing: 2 per cage in suspended stainless steel wire mesh cages during equilibration; individually

thereafter during non-exposure periods. Temperature range: Light: 12 hr on/off. Food: Purina Rodent Laboratory Chow #5002. Water: Tap (both ad libitum except during exposure). Acclimatization: at least 7 days.

5. Test Cells: Primary Male Rat Hepatocytes from negative control, positive (DMN) control and phosphine-exposed male rats. See "Test Performance" section for details on cell preparation and culture.

B. TEST PERFORMANCE

1. STUDY DESIGN AND RATIONALE FOR DOSE SELECTION: The assay measured unscheduled DNA synthesis in primary hepatocytes from male rats exposed by inhalation for 6 hr to phosphine gas. Male rats were randomly assigned to the following test groups for sacrifice at two times post-exposure:

TABLE 1: ANIMAL ASSIGNMENT

Test Group	Dose Level ppm	Number Assigned 2-3 hr	12-14 hr
Negative Control[1]	0	3	3
Positive Control[2]	10 mg/kg	3	3
Low Dose	5 (4.8)[3]	5	5
Mid Dose	15 (13)	5	5
High Dose A	20 (18)	5	5
High Dose B	25 (23)	5	5

1 Exposed to air only
2 Dimethylnitrosamine (DMN) administered intraperitoneally in saline
3 Numbers in parentheses represent mean actual exposure concentrations

Doses were selected based on results of previous studies on acute exposures to rats. This issue was discussed by TB-I in memos commenting on the protocol for this study (L. Hansen to L. Probst, 9-4-92 and 5-11-93). In an acute inhalation toxicity study on phosphine gas conducted by Bushy Run Research Center (project report no. 49-528, 8-22-86; part of Submission 8EHQ-0241-1188 to the Office of Toxic Substances), an LC_{50} of 57 ppm was determined for both sexes for a 4-hr exposure. In an in vivo/in vitro chromosomal aberrations test conducted by Hazleton Laboratories America, Inc. (HLA Study No. 12256-0-444, 8-8-91, same submission to OTS), rats exposed to 25 ppm phosphine gas for 6 hrs showed excessive mortality (3/5 males, 2/5 females). The highest doses chosen in this study were therefore expected to produce some signs of toxicity and the high dose was approximately half of the LC_{50} for phosphine.

Clinical signs were monitored immediately prior to exposure, hourly during exposure in the chambers and upon removal from chambers until sacrifice. Mortality was determined twice daily.

Body weights were taken at random sorting (Day 0), pretest immediately before exposure (Day 1) and Day 2 (term body weight prior to perfusion; actually Day 1 for 2-3 hr sacrifice animals).

The study was conducted by Hazleton Washington at several facilities. Inhalation exposures of rats were conducted at Pharmaco LSR (East Millstone, NJ; data contained in Appendix II of UDS study report). Perfusion of livers was conducted at Mobil Laboratories (Princeton, NJ). Processing of samples and evaluation of data was performed at Hazleton Washington.

2. INHALATION EXPOSURE

Exposure Chamber/Aerosol Generation: Whole-body exposures were conducted in Wahmann style glass/stainless steel chambers (1000L) with dynamic operation and airflow rates between 201 - 218 liters/minute (approximately 5 changes per minute). Chambers were supplied with room air mixed with phosphine from a gas cylinder, regulated by a metering valve and monitored by a mass flowmeter. The T_{99} was about 22 min. Chambers were allowed to clear for 30 minutes after exposure before removal of animals.

Analysis of Test Atmosphere: Nominal concentrations were determined by dividing the total volume of test material used by the total volume of air passing through the chamber during the exposure period. Analytical concentrations were determined by withdrawing 20 ml chamber air at 90-min intervals during the exposures and analyzing by gas chromatography. Temperature and humidity within the exposure chambers were monitored twice per hour. The study author did not report measurements from different regions of the chambers for homogeneity of gas distribution within the chambers.

Results - Mean analytical concentrations were 0, 4.8, 13, 18 and 23 ppm. Mean target, nominal and analytical concentrations at each dose level were within 1% of each other. Chamber temperature ranged from 19 - 23°C (mean 21°C) and relative humidity from 28 - 48% (mean 42%).

Particle Size Distribution: A TSI Aerodynamic Particle Sizer with a diluter was used to perform hourly particle size analyses. Mass mean aerodynamic diameter (MMAD), geometric standard deviation (GSD) and total mass concentration (TMC) were calculated.

Results - Average background particle size MMAD $\pm$ GSD ranged from 1.2 $\pm$ 2.2 to 2.0 $\pm$ 2.4 μm and TMC from 0.00071 - 0.00082 mg/m^3. The data indicated that the test material was not in aerosol form and dust levels were minimal.

3. CELL CULTURE AND UDS ASSAY

Animal Sacrifice and Cell Preparation: Rats were anesthetized, exsanguinated and livers perfused in situ for 4 minutes with Hanks' balanced salts (Ca^{++}/Mg^{++}-free, with 0.5 mM EGTA and Hepes-buffered to 7.2). Serum-free William's Medium E buffer with 2 mM L-glutamine, 100 µg/ml streptomycin and 150 µg/ml gentamicin (WMEI) containing 50-100 units/ml collagenase was then perfused through the liver for 10 minutes. Isolated hepatocytes were prepared by mechanical dispersion in WMEC (William's Medium E containing 10% serum) and removal of cell clumps and tissue debri. 0.5×10^6 viable cells in 3 ml WMEC were plated into 35 mm culture dishes containing 25 mm round plastic coverslips. Six dishes per animal were prepared for UDS assays (3 were used for the UDS assay and 3 reserved in case of technical problems) and 2 per animal for assessment of attachment efficiency. Cells were incubated for 1.5 - 2.2 hrs at 37°C, 5% CO_2 to allow attachment to culture dishes and coverslips. Attachment efficiency of refed cultures was determined by trypan blue dye exclusion.

Cytotoxicity Assay: A cytotoxicity assay to test cytotoxicity of the test compound on primary hepatocytes was not performed since exposures were conducted in vivo. Viability of hepatocyte preparations was assessed for each culture.

Radiolabelling of cultures: After cells attached to culture plates, cultures were radiolabelled with 10µCi ^{3}H-thymidine for 4 hrs. Labelled cell cultures were chased with media containing cold thymidine (0.25 mM) and incubated for 17.7 - 18.2 hrs (2-3 hr timepoint) or 17.7-18.4 hrs (12-14 hr timepoint).

Autoradiography: Sodium Citrate (1%) was added to the cover slips containing cell monolayers for 10-12 minutes to swell nuclei. Cells were fixed in acetic acid:ethanol (1:3), dried, coated with photographic emulsion and stored for 1 week at 4°C in the dark. Slides were fixed and stained with Williams' modification of hematoxylin and eosin staining.

Scoring: Cells were examined at 1500X under oil immersion. Nuclear and cytoplasmic grains were counted using an automatic counter with video screen. Net nuclear grain count for each cell was obtained by subtracting the average cytoplasmic count (average of 3 nucleus-size areas near each nucleus) from the nuclear grain count. Net nuclear grain count was determined from coded slides for 50 randomly chosen cells per coverslip and 3 coverslips per animal. S-phase nuclei were not scored. An average mean net nuclear grain count with standard deviation was calculated for each animals and for each test group.

Evaluation of Results: The study author listed the following criteria as requirements for acceptability of an assay:

- Although viability of hepatocytes following perfusion is usually greater than 70%, values less than this are not uncommon and no lower limit will be set for viability. Test compound toxicity may also lower viability.

- monolayer culture viability must be at least 70% (usually is around 85%)

- a positive control must be included

- Grain count data must minimally include 50 nuclei from each of 2 replicate cultures (100 nuclei) and from at least 3 animals per test group

- At least 3 dose levels should be examined at each timepoint; repeated trials may be performed to augment the dose levels to 3.

The following assay evaluation criteria for determining whether a test compound is active in the UDS assay were outlined:

- Test material is considered active if the group average of mean net nuclear grain count is at least 5 grains per nucleus above the concurrent negative control average and/or;

- the group average of percent nuclei with five or more net grains is increased at least 10% above average of concurrent negative controls.

- test material is considered active if positive response is seen in 2 of the 3 animals in a dose group.

- in cases of ambiguous response, presence of a dose response, frequency distribution of cellular responses and reproducibility of responses among animals are considered.

C. RESULTS:

1. In Vivo Exposure to Phosphine

Clinical Signs: At 18 and 23 ppm labored breathing was observed immediately following exposure in 3/10 and 5/10 animals, respectively (persistent in 1 - 3 animals on Day 2). Slight decrease in body weight was observed at 13, 18 and 23 ppm. No other treatment-related clinical signs were observed.

2. Unscheduled DNA Synthesis Assay

Summary tables from the study showing perfusion and culture viabilities and results of the unscheduled DNA synthesis assays at 2-3 and 12-14 hrs post-exposure are included in the Appendix.

Cell viability and test compound cytotoxicity: Except for cultures from one animal (#4003, 20 ppm) at the 2-3 hr timepoint which was not used due to reduced attachment efficiency, viability of all perfused cell preparations exceeded 63%; all cultures except 2 from showed better than 71% viability. Attachment efficiency of cultures with the previously mentioned exception ranged from 46.7% - 87.7%. Viability of attachment exceeded 90% for all cultures. There was no evidence of increased cytotoxicity to liver cells with increasing exposure levels of phosphine.

Unscheduled DNA Synthesis Assay: There was no evidence that phosphine caused increased unscheduled DNA synthesis in liver cells of male rats exposed by inhalation to levels up to 23 ppm, based on the criteria outlined above. Mean net nuclear grains and % cells with more than 5 grains were comparable among all treated animals and air-exposed controls. Positive control animals treated with DMN showed marked increases in percent cells with greater than 5 net nuclear grains (89 - 99% at 2-3 hr; 67 - 82% at 12-14 hr) and had mean net nuclear grains of 23 - 33 (2-3 hr and 7 - 12 (12-14 hr). Individual culture data was not provided in this report.

D. REVIEWER'S DISCUSSION/CONCLUSIONS

TB-I agreed with the conclusions of the study author that there was no evidence of unscheduled DNA synthesis in primary hepatocyte cultures from male rats exposed to phosphine gas for 6 hrs at levels up to 23 ppm (target concentration 25 ppm). Although more severe toxicity was expected at high dose, the test compound was administered at appropriate exposure levels and clinical signs of toxicity (labored breathing, slight weight loss) were observed following exposure to 18 and 23 ppm (decreased body weight also seen at 13 ppm). The highest dose was selected based on previous studies in which mortality was observed at 25 ppm and an LC_{50} (4 hr exposure) of 57 ppm was determined. However, this study did not provide conclusive evidence that phosphine gas does not induce DNA damage and repair since there was no evidence of cytotoxicity to the target organ, the liver.

This study appeared to have been adequately conducted and is considered adequate for regulatory purposes for satisfying Guideline 84-4 (Mutagenicity).

RIN 1108-94

ALUMINUM PHOSPHIDE REVIEW

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