



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

008045

JUL 24 1990

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OFFICE OF  
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

Subject: Hexahydro 1,3,5 tris(2 hydroxy-ethyl)s-triazine;  
Grotan; Bioban GK. EPA I.D. # C 62372;  
Record No 253898

To: John Lee/James Wilson PM # 31                      Tox Chem No 481 C  
Disinfectants Branch                                      Proj. No 0-0058  
Registration Division (H7505C)

Thru: Marion Copley, D.V.M., Section Head *M. Copley*  
Section II, Toxicology Branch                                      *7/23/90*  
Health Effects Division (H7509C)

From: Joycelyn E. Stewart, Ph.D. *JES*  
Section II, Toxicology Branch                                      *7/20/90*  
Health Effects Division (H7509C)

Registrant: Triazine Joint Venture  
Montvale, New Jersey

Action Requested

Review Unscheduled DNA Synthesis assay submitted in response to the Anti-Microbial Data Call-In Notice.

Conclusion

Hexahydro-1,3,5, tris(2 hydroxyethyl)-s-triazine was tested for unscheduled DNA synthesis in rat primary hepatocytes at doses of 0, 0.001, 0.003, 0.01, 0.03, and 0.1 ug/mL and gave a positive response at 0.1 ug/mL only. A duplicate assay was not performed to confirm the genotoxic effect. The study was therefore classified Unacceptable for regulatory purposes. In addition, the test chemical was not characterized. These comments are detailed in the conclusion of the DER.

This study does not satisfy the requirement for 84-2 (other mechanisms of mutagenicity), thus a data gap exists for this requirement.

The DER is attached.

EPA No.: 68D80056  
DYNAMAC No.: 277-A  
TASK No.: 2-77A  
April 10, 1990

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DATA EVALUATION RECORD

HEXAHYDRO-1,3,5-TRIS(2-HYDROXYETHYL)-S-TRIAZINE

Mutagenicity--Unscheduled DNA Synthesis in Primary  
Rat Hepatocytes

APPROVED BY:

Robert J. Weir, Ph.D.  
Program Manager  
Dynamac Corporation

Signature: *Roman J. Penta*

Date: 4-10-90

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HEXAHYDRO-1,3,5-TRIS(2-HYDROXYETHYL)-S-TRIAZINE

Mutagenicity--Unscheduled DNA Synthesis in Primary  
Rat Hepatocytes

REVIEWED BY:

Nancy E. McCarroll, B.S.  
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Date: 4-10-90

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Signature: Roman J. Pienta for  
Date: 4-10-90

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Signature: Roman J. Pienta  
Date: 4-10-90

Joycelyn Stewart, Ph.D.  
EPA Reviewer  
Review Section II  
Toxicology Branch I  
(H-7509C)

Signature: Joycelyn Stewart  
Date: 7/4/90 JTW 7/19/90

Marion Copley, D.V.M.,  
D.A.B.T.  
EPA Section Head  
Review Section II  
Toxicology Branch I  
(H-7509C)

Signature: Marion Copley  
Date: 7/17/90

DATA EVALUATION RECORD

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CHEMICAL: Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine.

STUDY TYPE: Unscheduled DNA synthesis in primary rat hepatocytes.

MRID NUMBER: 412623-01.

TEST MATERIAL: Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine.

SYNONYMS: None listed.

SPONSOR: Triazine Joint Venture, Montvale, NJ.

TESTING FACILITY: Microbiological Associates, Inc., Rockville, MD.

TITLE OF REPORT: Unscheduled DNA Synthesis in Rat Primary Hepatocytes: Test Article: Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine.

AUTHOR: Curren, R. D.

STUDY NUMBER: T8102.380.

REPORT ISSUED: July 20, 1988.

**CONCLUSIONS:** Five doses of hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine ranging from 0.01 to 0.10  $\mu\text{L}/\text{mL}$  were tested in the primary rat hepatocyte unscheduled DNA synthesis (UDS) assay. Cytotoxicity was apparent at doses  $>0.10 \mu\text{L}/\text{mL}$ . The highest dose scored for UDS (0.10  $\mu\text{L}/\text{mL}$ ) induced a "significant" increase (i.e.,  $\geq 5$  net nuclear grains over the control) in net nuclear grains and in the percentage of cells with  $\geq 5$  net nuclear grains (92%). However, the positive response was not dose related, the response was limited to the 0.10- $\mu\text{L}/\text{mL}$  treatment group, and the results were not confirmed. In addition, the study author failed to provide information on test material purity, the batch number of the assayed sample, and analytical data to verify actual concentrations used in the assay. We conclude, therefore, that the study is unacceptable, but we classify the test material as presumptively genotoxic in this test system.

**Study Classification:** The study is unacceptable and should be repeated to determine if the presumed genotoxic effect observed at 0.10  $\mu\text{L}/\text{mL}$  hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine is reproducible. It is further recommended that the report for the repeat study contain the relevant test material information identified in our review as missing in the present study.

**A. MATERIALS:**

1. **Test Material:**

Name: Hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine  
 Description: Yellow viscous liquid  
 Batch No.: Not reported  
 Purity: Not reported  
 Contaminants: Not reported  
 Solvent used: Williams Medium E (WME)  
 Other comments: The test material was stored at room temperature. Solutions of the test material were prepared and diluted in WME medium immediately prior to use.

2. **Indicator Cells:** Primary rat hepatocytes were harvested from the livers of adult male Fischer 344 rats obtained from Charles River Laboratories, Inc. Animals were quarantined at least 1 week prior to study initiation.

3. **Cell Preparation:**

a. **Hepatocyte Isolation:** Each rat was anesthetized by inhalation of metofane, and the livers were perfused with 0.5 mM EGTA in Hanks' buffered salt solution, pH 7.3, and WME containing L-glutamine, collagenase (80 to 100 units/mL, type I), and antibiotics, buffered to pH 7.3. Livers were excised, cleaned of extraneous tissue, shaken

in the collagenase perfusion solution, and either combed to release the hepatocytes or passed through a stainless-steel sieve.

- b. Hepatocyte Harvest/Culture Preparation: Recovered cells were collected, counted, and seeded at a density of  $5 \times 10^5$  cells, either into preconditioned 35-mm tissue culture dishes for the cytotoxicity assay or onto coverslips in 35-mm tissue culture plates for the UDS assay. Cultures were placed in a humidified, 5% CO<sub>2</sub> incubator for 90 to 150 minutes, washed, and refed with serum-free WME prior to use.

B. STUDY DESIGN:

1. Preliminary Cytotoxicity Assay: Duplicate cultures of cells, initiated from primary cultures, were exposed to 10 doses of the test material ranging from 0.0003 to 10  $\mu$ L/mL or the solvent (WME) for 18 to 20 hours. Following exposure, aliquots of the treatment medium were removed, centrifuged, and measured for lactate dehydrogenase (LDH) activity. Relative cytotoxicity was assessed by subtracting the LDH activity of the solvent control from the LDH activity in the treated cultures and comparing the values to the amount of LDH released by exposure of high-dose cultures or solvent control cells to 1% Triton.
2. UDS Assay:
  - a. Treatment/Slide Preparation: Six prepared hepatocyte cultures (three cultures seeded into tissue dishes and three cultures seeded onto coverslips) were exposed for 18 to 20 hours to nine selected doses of the test material, the test material solvent (WME), the positive control solvent (dimethylsulfoxide), or the positive control (3 and 5  $\mu$ g/mL 7,12-dimethylbenz[a]anthracene, DMBA). Treatment medium contained 10  $\mu$ Ci/mL [<sup>3</sup>H]thymidine. Monolayers grown directly on dishes were used to assess LDH activity as described for the cytotoxicity assay. Treated hepatocytes attached to coverslips were washed, swollen with 1% sodium citrate, fixed (ethanol-glacial acetic acid), dried, and mounted.
  - b. Preparation of Autoradiographs/Grain Development: Slides were dipped into Kodak NTB emulsion, dried for at least 1.5 hours, and stored at 4°C in desiccated slide boxes for 5 days. Slides were developed in Kodak D-19, fixed, stained with hematoxylin-sodium acetate-eosin, coded, and counted.

- c. Grain Counting: The nuclear grains of 150 randomly selected cells with appropriate background counts and normal morphology (50/slide) from each test, solvent, and positive control group were scored for incorporation of tritiated thymidine into DNA. Net nuclear grain counts were determined by subtracting the nuclear grain count of each cell from the average cytoplasmic grain count of three nuclear-sized areas adjacent to each nucleus. Means and standard deviations were calculated for each treatment group.

3. Evaluation Criteria:

- a. Assay Validity: For the assay to be considered valid, the following criteria must be satisfied: (1) the proportion of cells in repair in the solvent control group must be <15% and the net nuclear grain count of the solvent control group must be <1.0, and (2) the positive control compound must induce a significant increase in the net nuclear grain count ( $\geq 5$  grains/nucleus over the negative control).
- b. Positive Response: The assay was considered positive if the test material induced a dose-related increase in mean net nuclear grains and one or more of the doses had an increase in the mean net nuclear grain count that was  $\geq 5$  grains/nucleus over the negative control. In the absence of a dose-related effect, a compound that showed nuclear grain counts that were  $\geq 5$  grains/nucleus over two successive doses was also considered positive.

C. REPORTED RESULTS:

1. Preliminary Cytotoxicity Assay: Ten doses (0.0003 to 10  $\mu\text{L}/\text{mL}$ ) of the test material were examined in the cytotoxicity assay. The study author stated that the pH of the stock concentration was adjusted to 7.4 with 1 N HCl prior to dilution. However, there was a basic shift in the pH of cultures treated with the two highest assayed levels (3.0 and 10  $\mu\text{L}/\text{mL}$ ) at initiation and termination of treatment. The study author further stated that the LDH value could not be obtained for the highest test dose, presumably because of test material interference with the LDH determination. As shown in Table 1, cytotoxicity, as indicated by increased leakage of LDH into the culture medium, did not proceed in a conventional dose-related manner. At the highest assayed dose for which the LDH level could be determined (3.0  $\mu\text{L}/\text{mL}$ ), the percent relative cytotoxicity was 1%; however, as the dose was reduced, cytotoxicity increased to 52% at 1.0  $\mu\text{L}/\text{mL}$  and 66% at 0.3  $\mu\text{L}/\text{mL}$ . Although the percent relative cytotoxicity at 0.1  $\mu\text{L}/\text{mL}$  was 3%, the microscopic evaluation of the hepatocyte cultures revealed a cytotoxic effect. Below this level, the test material was not cytotoxic.

TABLE 1. Representative Results of the Preliminary Cytotoxicity Assay with Hexahydro-1,3,5-Tris(2-Hydroxyethyl)-S-Triazine:Lactate Dehydrogenase (LDH) Activity

Treatment	Dose ( $\mu\text{L}/\text{mL}$ )	Average <sup>a</sup> LDH Activity (units/L)	Corrected <sup>b</sup> LDH Activity (units/L)	Percent <sup>c</sup> Cytotoxicity
<u>Solvent Control</u>				
Culture medium	--	162.0	0.0	0
Culture medium + 1% Triton	--	649.0	487.0	100
<u>Test Material Control</u>				
Hexahydro-1,3,5-tris (2-hydroxyethyl)-s- triazine + 1% Triton	10.00	453.5	291.5	60
<u>Test Material</u>				
Hexahydro-1,3,5-tris (2-hydroxyethyl)- s-triazine	0.03 <sup>d</sup>	178.0	16.0	3
	0.10	201.5	39.5	8 <sup>e</sup>
	0.30	482.0	320.0	66
	1.00	413.5	251.5	52
	3.00 <sup>f</sup>	168.5	6.5	1

<sup>a</sup>Average of two samples.

<sup>b</sup>Corrected LDH = Average LDH of Test Groups - Negative Control LDH.

<sup>c</sup>Percent cytotoxicity =  $\frac{\text{Corrected LDH of Test Groups}}{\text{Corrected LDH of Culture Medium} + 1\% \text{ Triton}} \times 100$ .

<sup>d</sup>Lower doses (0.01, 0.003, 0.001, and 0.0003  $\mu\text{L}/\text{mL}$ ) were not cytotoxic.

<sup>e</sup>Microscopic examination of the hepatocyte cultures revealed cytotoxic effects.

<sup>f</sup>LDH determination of the highest dose (10  $\mu\text{L}/\text{mL}$ ) was not possible owing to compound interference with the assay.



2. UDS Assay: Two UDS and parallel cytotoxicity assays were conducted with nine doses of the test material (0.0001 to 1.0  $\mu\text{L}/\text{mL}$ ). Because of unspecified technical difficulties, the first assay was terminated.

In the repeat assay, the two highest test doses (0.3 and 1.0  $\mu\text{g}/\text{mL}$ ) induced marked cytotoxicity ( $\geq 85\%$ ) and were, therefore, not scored for UDS activity. The five levels selected for the evaluation of UDS induction were 0.1, 0.03, 0.01, 0.003, and 0.001  $\mu\text{L}/\text{mL}$ . As shown in Table 2, the highest test material dose scored for UDS (0.10  $\mu\text{L}/\text{mL}$ ) induced a marked increase in the net nuclear grain count compared to the solvent control. Similarly, the percentage of cells with  $\geq 5$  net nuclear grains was markedly higher at this level compared to the solvent control value. However, the evidence for a genotoxic effect was confined to the 0.10- $\mu\text{g}/\text{mL}$  dose group. The mean net nuclear grain counts and percentage of cells with  $\geq 5$  net nuclear grains for hepatocytes exposed to 0.001, 0.003, 0.01, or 0.03  $\mu\text{L}/\text{mL}$  of the test material, although higher than the solvent control, did not indicate a positive or a dose-related response. The study author concluded that hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine induced an equivocal response in this test system.

- D. REVIEWERS' DISCUSSION AND INTERPRETATION OF STUDY RESULTS: In the absence of a dose-related effect, confirmation of a "significant" increase at a single dose is necessary to conclude a positive response in the UDS assay.<sup>1</sup> We assess, therefore, that the assay should have been repeated to determine whether the positive response induced by 0.10  $\mu\text{L}/\text{mL}$  of the test material was reproducible. In addition, the study author failed to provide information on test material purity, the lot number of the assayed sample, and analytical data to support actual concentrations of the test material used in the assay. We noted that the study protocol indicated that a "Test Article Characterization" form (see Appendix B, Protocol, CBI p. 17) was attached to the report; however, the form was not present in the report. Since no definitive conclusions can be reached, the study is considered unacceptable; however, we classify hexahydro-1,3,5-tris(2-hydroxyethyl)-s-triazine as presumptively genotoxic in this test system.

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<sup>1</sup>Mitchell, A. D., Casciano, D. A., Meltz, M. L., Robinson, D. E., San, R. H. C., Williams, G. M., and Von Halle, E. S. "Unscheduled DNA Synthesis Tests, A Report of the U.S. Environmental Protection Agency Gene-Tox Program." Mutat. Res. 123(1983): 363-410.

TABLE 2. Representative Results of the Unscheduled DNA Synthesis Rat Hepatocyte Assay with Hexahydro-1,3,5-Tris(2-Hydroxyethyl)-S-Triazine

Treatment	Dose/ mL	Cytotoxicity			UDS Activity		
		Average <sup>a</sup> Lactate Dehydro- genase Activity (units/L)	Corrected <sup>b</sup> LDH Activity (units/L)	Percent <sup>c</sup> Cyto- toxicity	Number of Cells Scored	Mean Net Nuclear Grain Count ± Standard Deviations	Percent Cells with ≥5 Net Nuclear Grains
<u>Solvent Control (Test Material)</u>							
Culture medium	--	172.3	0.0	0	150	-2.1 ± 3.4	2
Culture medium + 1% Triton	--	527.3	355.0	100	--	--	--
<u>Solvent Control (Positive Control)</u>							
Dimethylsulfoxide	10 µL	182.0	0.0	0	150	-2.2 ± 4.0	3
<u>Positive Control<sup>d</sup></u>							
7,12-Dimethyl- benz(a)anthracene	3 µg	220.7	38.7	11	150	23.8* ± 7.9	99
<u>Test Material</u>							
Hexahydro-1,3,5- tris(2-hydroxy- ethyl)-s-triazine	0.03 µL <sup>e</sup>	202.3	30.0	8	150	0.2 ± 3.5	4
	0.10 µL	233.3	61.0	17	150	11.4* ± 5.4	92

<sup>a</sup>Average of three samples.

<sup>b</sup>Corrected LDH = Average LDH of Test Group - Negative Control LDH.

<sup>c</sup>Percent Cytotoxicity =  $\frac{\text{Corrected LDH of Test Group}}{\text{Corrected LDH of Solvent Control Cultures Exposed to 1\% Triton}}$

<sup>d</sup>Two levels were assayed; the lowest level was selected as representative.

<sup>e</sup>Results for lower doses (0.01, 0.003, and 0.001 µL/mL) did not indicate a genotoxic effect.

\*Conforms to the reporting laboratory's criteria for a positive response (i.e., ≥5 net nuclear grains over the solvent control).

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- E. QUALITY ASSURANCE MEASURES: A quality assurance statement was signed and dated July 20, 1988.
  
- F. CBI APPENDIX: Appendix A, Materials and Methods, CBI pp. 8-10; Appendix B, Protocol, CBI pp. 17-27.

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**APPENDIX A**  
**Materials and Methods**  
**(CBI pp. 8-10)**

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