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

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OFFICE OF  
PESTICIDES AND TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Zineb Unscheduled DNA Synthesis (UDS) Study

TO: Ms. Joanna Dizikes  
Review Manager 64

FROM: Byron T. Backus, Toxicologist  
Toxicology Branch, HED (TS-769C)

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*06/24/87*

THROUGH: Marcia van Gemert, Ph.D.  
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*M. van Gemert 6/25/87*  
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and

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Branch Chief  
Toxicology Branch, HED (TS-769C)

Project No. 7-0759

Tox. Chem. No. 930

Action Requested:

Review of an unscheduled DNA synthesis (UDS) study on Zineb.

Conclusions and Recommendations:

1. There was no evidence of UDS induction at non-cytotoxic exposure levels of Zineb (0.1, 0.3, 1.0, 3.0 and 10 ug/ml). At levels above 10 ug/ml (20, 30 and 40 ug/ml) an adequate degree of cytotoxicity was demonstrated.
2. While the study was conducted according to an acceptable protocol, no information is provided regarding the purity of the technical Zineb used in this study. In order for the study to be upgraded to acceptable, an analysis - preferably on the sample or same batch of technical Zineb as was used in this study - should be submitted.

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Reviewed by: Byron T. Backus  
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DATA EVALUATION REPORT I

STUDY TYPE: Mutagenicity - Unscheduled DNA Synthesis in rat hepatocytes TOX. CHEM. NO.: 930

ACCESSION NUMBER: not given

MRID NO.: not given

TEST MATERIAL: Zineb

SYNONYMS:

STUDY NUMBER(S): T5412.380

SPONSOR: Micro Flo Company  
Sparks, GA 31647

TESTING FACILITY: Microbiological Associates, Inc.  
5221 River Road  
Bethesda, MD 20816

TITLE OF REPORT: Unscheduled DNA Synthesis in Rat Primary Hepatocytes

AUTHOR(S): Curren, R. D., Wallace, K., Head, B., Hall, A., & Hammond, V.

REPORT ISSUED: June 1, 1987

Classification: Not acceptable

Special Review Criteria (40 CFR 154.7)

CONCLUSIONS:

1. There was no evidence of UDS induction at non-cytotoxic exposure levels of Zineb (0.1, 0.3, 1.0, 3.0 and 10 ug/ml). At levels above 10 ug/ml (20, 30 and 40 ug/ml) an adequate degree of cytotoxicity was demonstrated.
2. While the study was conducted according to an acceptable protocol, no information is provided regarding the purity of the technical Zineb used in this study. In order for the study to be upgraded to acceptable, an analysis - preferably on the sample or same batch of Zineb as was used in this study - should be submitted.

A. MATERIALS:

1. Test compound: Zineb technical; description: light yellow powder; no information provided as to batch no. or purity.
2. Positive controls: 7,12-Dimethylbenzanthracene (DMBA) at 10 ug/ml.

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3. Test cells: from adult male Sprague-Dawley rats obtained from Charles River Laboratories, Inc.

B. STUDY DESIGN:

1. Isolation of hepatocytes:

Rats were sacrificed by inhalation of metofane, followed by dissection and perfusion with 1) 0.5 mM EGTA and 2) collagenase solution. The liver was removed, cells were dissociated, counted and seeded into 35 mm dishes containing coverslips ( $5 \times 10^5$  viable cells/dish). Cells were seeded in Williams Medium E (WME) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin and 100 ug of streptomycin/ml or 50 ug/ml gentamycin.

2. Cell culture:

"Cultures were incubated at  $37 \pm 1^\circ\text{C}$  in a humidified  $5 \pm 1\%$   $\text{CO}_2$  incubator for 90-120 minutes, washed and refed with serum-free medium and used in the test."

3. Dosage selection:

"A preliminary cytotoxicity test was performed to establish an appropriate dose range for the test article. Ten doses ranging from 0.125 ug/ml to 4167 ug/ml were tested. The test article was tested by treating replicate cultures... 90-120 minutes after seeding. Eighteen to twenty hours later, an aliquot of culture fluid was removed, centrifuged, and the level of lactic acid dehydrogenase (LDH) activity determined. Two replicate plates were used for LDH measurement at each dose level. The relative toxicities were obtained by comparing the treated to untreated control cultures."

4. Test procedure:

Based on the results of the preliminary cytotoxicity test, the Zineb was tested at dose levels of 0.03, 0.1, 0.3, 1.0, 3.0, 10, 20, 30 and 40 ug/ml.

At each dose level there were three replicate plates each seeded at  $5 \times 10^5$  hepatocytes/plate. Dimethylsulfoxide (DMSO) was used to dissolve the Zineb and was also used as solvent (negative) control. DMBA at 3 and 10 ug/ml, also dissolved in DMSO, was used as the positive control.

"Each test article and control dish received  $^3\text{H}$ -thymidine at a final concentration of 10 uCi/ml. In parallel with the test plates, two cultures per dilution were treated with the same compound for a parallel toxicity test."

"The cells were treated for 18-20 hours... The parallel toxicity plates were harvested by removal of a portion of of the medium for LDH determinations...to obtain the relative survivals and relative toxicities."

"After eighteen to twenty hours of exposure, the cells in the...assay plates were washed in serum-free WME, swelled in 1% sodium citrate and fixed in ethanol-acetic acid fixative. The coverslips were air-dried, mounted cell side up on glass slides, and allowed to dry. The slides were coated with Kodak N1L emulsion and stored for ten days at 4°C in light tight boxes with desiccant. The slides were then developed in Kodak D-19 developer, fixed in Kodak fixer and stained in haematoxylin-sodium acetate-eosin stain."

5. Scoring:

"The slides were read "blind" on an Artek Colony Counter. Nuclear grains were counted in 25 cells in random areas on each of three coverslips per treatment... The net nuclear counts were determined by counting three nucleus-sized areas adjacent to each nucleus and subtracting the average cytoplasmic count from the nuclear count. Replicative synthesis was identified by nuclei completely blackened with grains and such cells were not counted. Nuclei exhibiting toxic effects of treatment, such as dark staining, disrupted membranes or irregular shape, were not counted."

6. Criteria for a positive response:

"If the mean net nuclear count was increased by at least five counts over the control, the results for a particular dose level were considered significant. A test article was judged positive if it induced a dose-related response and at least one dose produced a significant increase in the average net nuclear grains when compared to that of the control. In the absence of the dose response, a test article which showed a significant increase in the mean net nuclear grain count in at least two successive doses was considered positive..."

7. Quality assurance:

A signed statement is provided on p. 4 of the report.

**C. RESULTS:****1. Cytotoxicity:**

The following representative values are from table 1, p. 12:

Material	Dose level	Average LDH	Corrected LDH	Relative Toxicity
Zineb	1250 ug/ml	297.5	174.0	47%
"	417 ug/ml	278.5	155.0	42%
"	125 ug/ml	451.5	328.0	88%
"	42 ug/ml	505.0	381.5	102%
"	12.5 ug/ml	152.5	29.0	8%
"	4.2 ug/ml	124.5	1.0	0%
"	1.25 ug/ml	121.0	-2.5	-1%
DMSO - solvent control		123.5	0.0	0%
WME - media control		83.5	-40.0	-11%
DMSO + 1% Triton		496.0	372.5	100%

Corrected LDH = Average LDH - solvent control LDH

Relative Toxicity = Corrected LDH/100% corrected LDH control.

100% LDH control = the amount of corrected LDH activity released by exposure of control cells to 1% Triton (100% lysis).

**2. Parallel cytotoxicity:**

The following is from table 2, p. 13:

Material	Dose level	Average LDH	Corrected LDH	Relative Toxicity
Zineb	40 ug/ml	523.0	456.0	115%
"	30 ug/ml	488.0	421.0	106%
"	20 ug/ml	573.5	506.5	128%
"	10 ug/ml	206.0	139.0	35%
"	3 ug/ml	105.5	38.5	10%
"	1 ug/ml	92.0	25.0	6%
"	0.3 ug/ml	92.0	25.0	6%
"	0.1 ug/ml	137.5	70.5	18%
"	0.03 ug/ml	103.5	36.5	9%
DMBA	10 ug/ml	135.5	68.5	17%
"	3 ug/ml	110.0	43.0	11%
DMSO (solvent control)		67.0	0.0	0%
WME (media control)		81.0	14.0	4%
DMSO + 1% Triton		463.5	396.5	100%

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3. Grain count data:

The following is taken from page 14:

Material	Dose level	# nuclei counted	Average net grains/nucleus + S.D.	% cells with 5 or more net nuclear grains
Zineb	10 ug/ml	75	-1.1 + 1.6	0
"	3 ug/ml	75	-1.3 + 2.3	0
"	1 ug/ml	75	-1.1 + 1.6	0
"	0.3 ug/ml	75	-0.9 + 1.8	0
"	0.1 ug/ml	75	-0.7 + 1.8	0
DMBA	10 ug/ml	75	8.8 + 2.8	91
DMSO (solvent control)		75	-1.2 + 1.0	0
WME (media control)		75	-1.1 + 1.2	0

According to the text (on p. 11) "The lowest dose of positive control (3.0 ug/ml DMBA) caused an elevation of 4.6 grains above the solvent control." These results are not reported in Table 3.

There was 65% relative survival at 10 ug/ml; for all other dose levels of Zineb and for controls relative survival was above 80%.

D. DISCUSSION:

While grain counts for individual nuclei are not presented, there is enough information available indicating that the test material, under the experimental conditions, did not induce UDS, as measured by an increase in mean net grains/nuclei. The cytotoxicity data indicate that the highest dosage level (10 ug/ml) at which grain counts were made was adequate.

Among other criteria which can be used in evaluating data from this type of study as to occurrence or lack of UDS are numbers of nuclei with 1) 5 or more net nuclear grains and 2) 20 or more net nuclear grains. While an increase in mean net nuclear grain count is usually accompanied by an increase in number of nuclei with 5 (and/or 20) grains, this is not an invariable occurrence and there may be cases where an increase in numbers of nuclei with 5 (and/or 20) grains is not accompanied by a statistically significant increase in mean net nuclear grains. However, in this case the information in Table 3 indicates that there were no cells with 5 or more net nuclear grains among those counted in this study.

Therefore it is concluded that there is no evidence that UDS occurred in this study. However, while the study was conducted according to an acceptable protocol, no information is provided regarding the purity of the technical Zineb used in this study. In order for the study to be upgraded to acceptable, an analysis - preferably on the sample or same batch of technical Zineb as was used in this study - should be submitted.

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