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

008622

OCT 1 1991

OFFICE OF
PESTICIDES AND TOXIC
SUBSTANCES

MEMORANDUM

Subject: Dicofol technical. Mutagenicity data review.
Tox Chem No. 093
HED Project No. 1-2117 84-2a 84-2b 84-2b 84-2c
MRID Nos. 400420-48, 400420-49, 400420-50, 400420-51, and 400420-52
84-2a

From: Alberto Protzel, Ph.D. 9/20/91
Review Section III
Toxicology Branch II
Health Effects Division (H7509C)

To: Mr. Dennis Edwards Jr./Ms. Eileen McGovern (PM-12)
Insecticide-Rodenticide Branch
Registration Division (H7505C)

Thru: James N. Rowe, Ph.D., Head James N. Rowe 9/20/91
Review Section III
Toxicology Branch II
Health Effects Division (H7509C)

and

Marcia van Gemert, Ph.D., Chief
Toxicology Branch II
Health Effects Division (H7509C)

ACTION: Review of the following five mutagenicity studies on the chemical Dicofol (Kelthane[R] Technical Miticide) submitted by Rohm and Haas Company:

1. Microbial Mutagen Assay (MRID 400420-48)
2. CHO/HGPRT Gene Mutagen Assay (MRID 400420-49)
3. In vivo Cytogenetic Study in Rats (MRID 400420-50)
4. In vitro Cytogenetic Assay in Chinese Hamster Ovary (CHO) cells (MRID 400420-51)
5. In vitro Unscheduled DNA Synthesis Assay (MRID 400420-52)

CONCLUSIONS:

1. Microbial mutagen assay. Salmonella/microsome mutagenicity assay. (§84-2a, Gene mutation test), (MRID 400420-48). Negative: No increase in mutant colonies at doses of 5-5000 µg/ml with or without rat S-9 extract. The study was classified as Provisionally Unacceptable pending submission of positive-control assays without activation.

2. CHO/HGPRT Gene Mutagen Assay. In vitro detection of forward gene mutations using the CHO/HGPRT gene mutation assay. (§84-2a, Gene mutation test), (MRID 400420-49). Negative: No increase in forward mutations at doses of 3.0-6.0 µg/ml (-S9 from rat liver) and 4.5-20 µg/ml (+S-9 from rat liver). The study was classified as Acceptable.

3. In vivo Cytogenetic Study in Rats. In vivo cytogenetics using rat bone marrow. (§84-2b, Structural chromosome aberrations), (MRID 400420-50). Single oral doses of Dicofol at 47.8, 191.2, and 478.0 mg a.i./kg were given to COBS-CD(SD) male rats. Negative: No significant increases of chromosomal aberrations were reported. The MTD may have not been reached and no mitotic index data were available. No data on females was submitted. The study was classified as Unacceptable.

4. In vitro Cytogenetic Assay in Chinese Hamster Ovary (CHO) cells. (§84-2b, Structural chromosome aberrations), (MRID 400420-51). Negative: No significant increase in chromosomal aberrations at 7.5-20 µg/ml (-S9 from rat liver) and 7.5-22.5 µg/ml (+S9 from rat liver). The study was classified as Acceptable.

5. In vitro Unscheduled DNA Synthesis Assay. DNA damage/repair: UDS in rat primary hepatocytes. (§84-2c, Other genotoxic effects), (MRID 400420-52). Negative: No significant increase in unscheduled DNA synthesis at 0.025-0.5 µg/ml. The study was classified as Unacceptable.

Guideline Series 84: **MUTAGENICITY**

Reviewed by: James N. Rowe, Ph.D. James N. Rowe 9/17/91
Section I, Tox Branch II(H7509C)
Secondary reviewer: Irving Mauer, Ph.D. 9-17-91
Tox Branch I(H7509C)
Date: 9/17/91

008622

DATA EVALUATION REPORT

CHEMICAL: Dicofol (Kelthane) Tox. Chem. No.: 093

EPA File Symbol:

STUDY TYPE: Salmonella/mammalian microsome mutagenicity assay

MRID NUMBER: 400420-48

SYNONYMS/CAS No.:

SPONSOR: Rohm and Haas Company, Springhouse, PA 19477

TESTING FACILITY: Rohm and Haas Company, Toxicology Department, 727 Norristown Road, Spring House, PA 19477

TITLE OF REPORT: Dicofol (Kelthane Technical Miticide): Microbial Mutagen Assay Protocol No. 85P-019, Report No. 85R-0042

AUTHOR(S): C.A. Higginbotham and M.J. Byers

STUDY NUMBER(S): 85R-0042

REPORT ISSUED: July 26, 1985

CONCLUSION(S) - Executive Summary:

Dicofol was assayed in four strains of Salmonella typhimurium (TA-1535, TA-1537, TA-98, TA-100) with a solvent control, positive controls (activation only) and concentrations ranging from 5 to 5000 ug/plate. The Ames test was responsive in the activated assay based upon significant induction of mutations after the treatment of various positive control compounds to the 4 tester strains. However, a non-activated (direct acting mutagen) positive control was not employed. No elevation in mutations was noted in the presence or absence of S9 liver mixture at any concentration of test material.

Classification: Provisionally Acceptable. The study fulfills the Guideline requirements for Category I, Gene Mutations for an activated assay system, except for the requirement to report

positive control assays under non-activation conditions, which should be submitted to upgrade the study to fully acceptable.

SALMONELLA

A. MATERIALS

1. Test Material: Name: dicofol
Description : dark brown liquid/solid
Batch #: material key 904350-9 PROD CODE 62541 TD NO 84-393; Lot MLO-0953 Purity: 95.6%
Contaminants: if reported, list in CBI appendix
Solvent used: DMSO
Other comments:

2. Control Materials:
Negative: DMSO
Solvent/final concentration: not stated
Positive: Non-activation:
Not tested.

Activation:
2-Aminoanthracene (2-anthramine) 10 ug/plate
TA1535, TA1537, TA100
Other (list):
2-Acetamidofluorene: 50 ug/plate TA98
(2AAF)

3. Activation: S9 derived from (sex not stated)

x	Aroclor 1254	x	induced	x	rat	x	liver
___	phenobarbital	___	non-induced	___	mouse	___	lung
___	none			___	hamster	___	other
___	other			___	other		

If other, describe below
Describe S9 mix composition (if purchased, give details):

The S9 liver homogenate was prepared from livers coarsely chopped with a Waring blender for 2 seconds followed by a homogenization with a Brinkman Polytron for 10 seconds at full speed. Livers from 6 to 24 Charles River COBS-CD Br, approximately 200 grams body weight, were processed.

4. Test organisms: S. typhimurium strains
___ TA97 x TA98 x TA100 ___ TA102 ___ TA104
x TA1535 x TA1537 __ TA1538 ; list any others:
Properly maintained? Yes
Checked for appropriate genetic markers (rfa mutation, R factor)? Yes

5. Test compound concentrations used:

Non-activated conditions: 1) 50, 200, 500, 2000, 5000 ug/plate all strains noted above; 2) 20, 50, 200, 500, 2000 ug/plate- TA1537, TA1535; and 3) 5, 20, 50, 200, 500 ug/plate- TA 100

Activated conditions: 1) 50, 200, 500, 2000, 5000 ug/plate- all strains noted above; 2) 20, 50, 200, 500, 2000 ug/plate- TA1537, TA1535; and 3) 5, 20, 50, 200, 500 ug/plate- TA100

B. TEST PERFORMANCE

1. Type of Salmonella assay:
- _x_ standard plate test
- ___ pre-incubation (___ minutes)
- ___ "Prival" modification (i.e. azo reduction method)
- ___ spot test
- ___ other (describe in a.)

a. Protocol : A copy of the material and methods is attached (Attachment 1).

2. Preliminary cytotoxicity assay

Dose rangefinding studies were not performed. Compounds were tested over a 3 or 4 log range, the upper limit being either 7500 (nl/plate or ug/plate) or a concentration sufficient to cause a toxic effect (growth inhibition). Significant growth inhibition was demonstrated in all strains at concentrations of 2000-5000 ug/plate of dicofol. TA100 was the most sensitive, with growth inhibition (+/-S9) at concentrations of 500-5000 ug/plate.

Ranges of revertants for historical solvent controls were not presented. However, a large number of concurrent controls were performed for each assay (see Attachment 2) and their ranges are presented below (counts/plate):

	+S9	-S9
TA-1535:	6-31	7-38
TA-1537:	4-18	1-19
TA-98:	29-70	27-43
TA-100:	43-128	39-132

3. Mutagenicity assay

A photocopy of summary data for the Ames assay is attached (Attachment 2). These data are based upon 3 replicates per concentration. Assays with TA1535, TA1537 and TA100 were performed in duplicate, separate assays. Concentrations of test-material

used in these assays are depicted in Attachment 2.

The positive controls indicated that the assays were responsive to known mutagens (2-anthramine, 2-acetamidofluorene) requiring activation based upon the significant elevation in mean revertants per plate as compared to the saline control (i.e., in TA1535 10-15X, in TA1537 13-33X, in TA98 18X and in TA100 7-8X (see Attachment 2). Direct acting (non-activation) positive controls were not tested; no explanation was given.

There was no evidence of an increase in mutagenic response to test material in either activated or nonactivated assays at concentrations up to 5000 ug/plate (limit dose).

4. Reviewer's discussion/conclusions

Dicofol was assayed in four strains of Salmonella typhimurium (TA-1535, TA-1537, TA-98, TA-100) with a solvent control, positive controls (activation only) and concentrations ranging from 5 to 5000 ug/plate. The Ames assay was responsive based upon significant induction of mutations in the activated assays after the treatment of various positive control compounds to the 4 tester strains. No elevation in mutations was noted in the presence of absence of S9 liver mixture at any concentration of test material.

5. Was test performed under GLPs (is a quality assurance statement present)? Yes

6. CBI appendix attached No

7. A signed and dated statement of No Confidentiality Claims was included.

Guideline Series 84-2: MUTAGENICITY

Reviewed by: Alberto Protzel, Ph.D.
Section III, Tox Branch II (7509C)
Secondary Reviewer: Irving Mauer Ph.D.
Tox Branch I (7509C)

9/17/91

09-17-91

008622

DATA EVALUATION REPORT

CHEMICAL: Dicofol (Kelthane)

STUDY TYPE: In vitro detection of forward gene mutations using Chinese hamster ovary (CHO) cells; EPA Guideline 84-2

MRID NUMBER: 400420-49

SYNONYMS: Kelthane^{R} Technical Miticide. 1,1-Bis(chlorophenyl)2,2,2-trichloroethanol.

SPONSOR: Rohm and Haas Company, Spring House, PA 19477.

TESTING FACILITY: Rohm and Haas Company, Toxicology Department, 727 Norristown Road, Spring House PA 19477.

TITLE OF REPORT: Dicofol(Kelthane^{R} Technical Miticide): CHO/HGPRT Gene Mutagen Assay. Protocol No. 85P-038. Report No. 86R-002.

AUTHORS: S. Foxall, D.J. Doolittle, and K.L. McCarthy.

STUDY NUMBER: Rohm and Haas Report No. 86R-002.

REPORT ISSUED: March 27, 1986

CONCLUSION(S) - Executive Summary:

Chinese hamster ovary cells (CHO-K_1-BH_4 strain) were exposed in culture to dicofol at concentrations of 3.0, 4.0, 4.5, 5.0, or 6.0 µg/ml without metabolic activation mix and to 4.5, 10, 12, 15, 17, or 20 µg/ml with S9 metabolic activation. No significant increases in mutant frequency (mutants/10^6 clonable cells) above solvent controls were observed with or without metabolic activation in the test article-treated cultures at cytotoxic doses. The CHO/HGPRT test was responsive with and without metabolic activation, based on a significant induction of mutations in the presence of appropriate positive controls.

Classification: This study is classified as acceptable.

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MAMMALIAN CELLS IN CULTURE GENE MUTATION

1. Test Material:

Name: Dicofol Technical
Description (e.g. technical, nature, color, stability): Technical, dark brown liquid/solid.
Batch #: TD-393, Lot No. MLO-0953 Purity: 95.6%
Contaminants: No data reported
Solvent used: Dimethyl sulfoxide (DMSO)
Other comments: None.

2. Control Materials:
Negative: DMSO (solvent)
Solvent/final concentration: DMSO/0.5%.
Positive: Non-activation: Ethyl methanesulfonate (EMS, at 100 nl/ml).
Activation: 7,12-dimethylbenzanthracene (DMBA, at 7 µl/ml).

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	X liver
___ phenobarbital	___ non-induced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

No details were given on the preparation of the S9 extract; cofactors added to the activation mix were NADH, NADP, glucose-6-phosphate, and $MgCl_2$.

4. Test Cells: mammalian cells in culture
___ mouse lymphoma L5178Y cells
X Chinese hamster ovary (CHO) cells
___ V79 cells (Chinese hamster lung fibroblasts)
___ other (list):

Properly maintained? Yes

Periodically checked for Mycoplasma contamination? Yes

Periodically checked for karyotype stab ility? Yes

Periodically "cleansed" against high spontaneous background? No data

5. Locus Examined:

___ thymidine kinase (TK)
selection agent: _____ bromodeoxyuridine (BrdU)
(give concentration) _____ fluorodeoxyuridine (FdU)
_____ trifluorothymidine (TFT)

_X_ hypoxanthine-guanine-phosphoribosyl transferase (HPRT)
Selection agent: 6-thioguanine (6TG), 1.67 µg/ml (10 µM).

___ Na^+/K^+ ATPase
Selection agent: _____ ouabain
(give concentration)

___ other

6. Test compound concentrations used:
Non-activated conditions: 3.0, 4.0, 4.5, 5.0, and 6.0 µg/ml.

Activated conditions: 10, 12, 17, 20 µg/ml.

B. TEST PERFORMANCE

1. Cell treatment:
a. Cells exposed to test compound for:
18-20 hours (non-activated) 5 hours (activated)

b. Cells exposed to positive controls for:
18-20 hours (non-activated) 5 hours (activated)

c. Cells exposed to negative (solvent) controls for:
18-20 hours (non-activated) 5 hours (activated)

d. After washing, cells cultured for 8 days
(expression period) before cell selection

e. After expression, cells cultured for 7 days
in selection medium to determine numbers of mutants
and for 7 days without selection medium to
determine cloning efficiency

2. Protocol

A photocopy of the Methods section is included as Attachment 1. Experiments with metabolic activation were conducted with three concentrations of S9 mix: the main experiment at 1 mg S9 protein/ml and two other experiments using S9 mix at respective concentrations of 0.3 and 2.0 mg S9 protein/ml.

For the main experiment, on the day after seeding at 0.5 x 10^6 cells/60 mm plate, chinese hamster ovary (CHO) cells (CHO-K_1-BH_4) were exposed in culture to the test compound at predetermined doses without S9 activation mix (3.0, 4.0, 4.5, 5.0, 6.01 µg/ml; for 18-20 hours) and with S9 activation mix (10, 12, 17, and 20 µg/ml; for 5 hours). S9 protein was added at a concentration of 1 mg/ml. In the test without metabolic activation, the cells were subcultured immediately after exposure. In the test with metabolic activation, fresh medium was added and the cells were incubated overnight before subculturing. To subculture, the treated cells were trypsinized and seeded at 1 x 10^6 cells/100 mm-plate. The cells were carried through 2 additional subcultures (seeded at 0.5 x 10^6/100 mm-plate) to allow in total for an 8-day period of phenotypic expression. For toxicity assessment, 200 cells were seeded into each of four 60 mm-plates and incubated for 7 days, stained and counted. At the end of the genome expression period, each of five 100mm-dishes was reseeded with 2 X 10^5 cells/dish, fortified with 6-thioguanine (1.67 µg/ml; 10 µM) and cultured for 7 days for mutant selection. In addition, four 60 mm-dishes were seeded with 200 cells each to assess cloning efficiency after incubation for 7 days. Negative controls (solvent control: DMSO) and positive controls (See above, #2) were run concurrently with the test compound. Details of culture medium preparation were included. The protocol that was included stated that the testing laboratory periodically analyzes growing cultures for mycoplasma contamination, checks for karyotype stability, and verifies 6-thioguanine sensitivity and aminopterin resistance. Details of the S9 mix preparation were limited and incomplete.

An additional series of experiments was done in which S9 activation mix was added at concentrations of 0.3 and 2.0 mg S9 protein/ml. Test material was added at final concentrations of 4.5 and 15.0 µg/ml in these supplementary assays. All other details were as described above for the main experiment.

Criteria for a positive response included:

- The mutant frequency must exhibit a reproducible, significant, increase; and that increase must be accompanied by an increase in the average number of colonies per selection plate compared to that of the solvent control.
- Increases in the mutant frequency should be reproducible in an independent test.
- Negative results (no adverse effects) must include evaluation of the test compound to its limits of solubility or to cytotoxicity levels of 20% or less survival.

3. Preliminary cytotoxicity assay:

Cytotoxicity was studied in the absence and in the presence of S9 mix. In the absence of S9 mix, concentrations in the range of 5-1000 µg/ml resulted in "extreme toxicity" leading to no survivors; at concentrations of 0.1 and 0.5 µg/ml relative viabilities were 99.0% and 95.5%, respectively. Based on these results, test concentrations of dicofol were 3.0, 4.0, 4.5, 5.0 and 6.0 µg/ml. In the presence of S9 mix (1.0 mg S9 protein/ml), concentrations in the range of 10-1000 µg/ml resulted in "extreme toxicity" leading to no survivors; at concentrations of 0.1, 0.5, and 5.0 µg/ml relative viabilities were 101%, 99%, and 55%, respectively. Based on these results, test concentrations of dicofol were 10, 12, 17 and 20 µg/ml in one set of replicates and 4.5 and 15 µg/ml in another set of replicates.

4. Mutagenicity assay:

Mutant frequencies without metabolic activation ($\leq$18.5 x 10^6 survivors) were within the reported range of historical controls (μ=8.7, S.D.=12.2) at all test-article treatment concentrations, 3.0-6.0 µg/ml (Attachment 2). A seemingly dose-related increase of mutant frequencies (which were in the historical control range) in one replicate was not observed in the other replicate. Survival was 22-23% and 3% at the two highest concentrations, 5.0 and 6.0 µg/ml, respectively.

Mutant frequencies with metabolic activation ($\leq$ 12.99 x 10^6 survivors) were also within the reported range of historical controls at all treatment concentrations, 10-20 µg/ml and 1 mg S-9 protein/ml (Attachment 3) or 4.5-15 µg/ml and 0.3-2.0 mg S-9 protein/ml (Attachment 4). Survival values at the highest dose with 1 mg S-9 protein/ml were 25-38%. Although 100% cytotoxicity was observed at 15 µg/ml and 0.3 mg S-9 protein/ml, no cytotoxicity (> 100%) survival was observed at 15 µg/ml and 0.3 mg S-9 protein/ml.

The CHO/HGPRT test was responsive with and without metabolic activation, based on a significant induction of mutations in the presence of appropriate positive controls.

5. Reviewer's discussion/conclusions

a. The positive controls, ethyl methanesulfonate (EMS, 100 nl/ml, without activation) and 7,12-dimethylbenzanthracene (DMBA, 7 µl/ml, with activation) adequately demonstrated the sensitivity of the CHO system to detect forward gene mutations in vitro.

b. In the experiments without metabolic activation:

i. The two highest dose levels of test material, 5.0 and 6.0 µg/ml,

respectively, demonstrated cytotoxicity by limiting cell survival to levels of 22-23% and 3%, respectively in the treated cultures.

ii. Mutation frequencies at all test material concentrations were within historical control levels. This result is considered acceptable.

c. In the experiments with metabolic activation:

i. In the main experiment (S-9 protein at 1 mg/ml), the highest dose level of test material (20.0 µg/ml) demonstrated cytotoxicity by limiting cell survival to levels of 25-38% in the treated cultures. In a pilot experiment, doses over 10 µg/ml left no survivors.

ii. In a supplementary assay (S-9 protein at 0.3 mg/ml), the highest dose tested (15.0 µg/ml) showed complete toxicity and a dose of 4.5 µg/ml limited cell survival to 76%.

iii. Mutation frequencies were within the reported range of historical controls at all test material concentrations in the presence of S-9 protein at 0.3 and 1 mg/ml (See i and ii, above). These results are considered acceptable. In an additional assay, conducted with S-9 protein 2.0 mg/ml and test material concentrations up to 15µg/ml, mutation frequencies were within the reported range of historical controls; however, no cytotoxicity was observed.

6. Was test performed under GLPs?
A signed statement of compliance with EPA GLP was included. In addition, a signed Quality Assurance statement was included.

7. CBI appendix attached?
No CBI appendix was attached.

Guideline Series 84-2. MUTAGENICITY

Reviewed by: Alberto Protzel, Ph.D.
Section III, Tox Branch II (7509C)
Secondary Reviewer: Irving Mauer Ph.D.
Tox Branch I (7509C)

9/17/91
09-17-91

008622

DATA EVALUATION REPORT

CHEMICAL: Dicofol (Kelthane^{R})

STUDY TYPE: In vivo cytogenetics using rat bone marrow cells; EPA Guideline 84-2

MRID NUMBER: 400420-50

SYNONYMS: Kelthane^{R} Technical Miticide. 1,1-Bis(chlorophenyl)2,2,2-trichloroethanol.

SPONSOR: Rohm and Haas Company, Spring House, PA 19477.

TESTING FACILITY: Rohm and Haas Company. Toxicology Department. 727 Norristown Rd. Spring House, PA 19477.

TITLE OF REPORT: Dicofol (Kelthane^{R} Technical Miticide): In vivo cytogenetic study in rats.

AUTHORS: J.L. Sames and D.J. Doolitle.

STUDY NUMBER: Rohm and Haas Report No. 85R-215.

REPORT ISSUED: March 26, 1986

CONCLUSION(S) - Executive Summary:

Oral doses of Kelthane Technical B (47.8, 191.2 and 478.0 mg ai/kg) did not induce a clastogenic response in the chromosomes of bone marrow cells of male CRL:COBS-CD(SD) rats. The results were judged inconclusive, however, because although some systemic toxicity was observed at the highest dose, no evidence was presented (e.g. mitotic index) to demonstrate that the test material had reached the target tissue. No data for female rats were included.

Classification: This study is classified as unacceptable, pending the following:

i. Repetition of the study in males, to include evidence (e.g. mitotic index measurements) for transport of effective concentrations of test material or its active metabolites to bone marrow.

ii. Submission of an acceptable justification for the omission of female rats in this study, or submission of an acceptable replica of this study using female rats.

1. Test Material:

Name: Kelthane Technical B
Description (e.g. technical, nature, color, stability): Technical.
Batch #: TD 84-393, Lot No. ML0-0953
Purity: 89.6% A.I. (actual analysis); specified: 95.6% A.I.
Contaminants: <0.1 % DDTr

2. Control Materials:
Vehicle: Corn oil. Route of administration: oral, 10 ml/kg.
Positive: Triethylene melamine (TEM). Route of administration: intraperitoneal, 0.3 and 0.5 mg/kg in distilled water.

3. Test Animals:
Male CRL:COBS-CD(SD) rats weighing 183-250 g on the first day of dosing.

B. TEST PERFORMANCE

A copy of the Methods section of the Final Report is attached (Attachment 1).

1. Treatment Groups

Following a preliminary range-finding study at oral doses of 250, 354, 500, 707, and 1000 mg/kg (Final Report Appendix III), male CRL:COBS-CD(SD) rats were dosed with single doses as summarized in Table 1. Bone marrow was sampled at 6, 24 and 48 in negative controls and in kelthane-treated animals, and at 5 hours in TEM-treated animals. All animals received colchicine (1 mg/kg in distilled water) three hours prior to sacrifice.

Table 1. Treatment groups for the in vivo cytogenetic assay of kelthane.

Group No.	Compound	Single Dose (mg/kg)	Total No. of rats	Number of rats killed (hours after dosing)			
				6	18	24	48
1	Corn oil[a]	0	30	10	-[b]	10	10
2	Kelthane[c]	478.0	30	10	-	10	10
3	Kelthane	191.2	30	10	-	10	10
4	Kelthane	47.8	30	10	-	10	10
5	TEM[d]	0.3	5	-	5	-	-
6	TEM	0.5	5	-	5	-	-

[a] Oral dose at 10 ml/kg, vehicle control.
[b] - = No data.
[c] Kelthane was diluted in corn oil and administered orally at a volume of 10 ml/kg. Doses for groups 2-4 were based on a preliminary oral acute toxicity study.
[d] Triethylene melamine (positive control), was dissolved in water and administered intraperitoneally.

2. Femoral bone marrow samples were prepared according to standard methods, slides coded, and if possible up to 50 metaphase spreads from each animal were examined for clastogenicity (breaks, gaps, fragments, translocations and rearrangements) as well as for changes in chromomosome number. No determinations of mitotic index were performed; no tests were performed to assess cytotoxicity.

   It is noted that initially tibial bone marrow samples were obtained due to nonadherance to the protocol (Protocol 84P-620) and SOPs. When this was discovered, prior to slide scoring, the study was restarted under protocol No. 84P-620 R.

3. Statistical analysis

   The ß-binomial model was used to analyze the data.

4. Evaluation criteria

   Although criteria for selection of metaphase spreads for scoring were included, no criteria to establish assay validity, a positive response, or the biological significance of the results were presented.

5. Quality assurance measures

   A signed statement of compliance with EPA GLP was included. In addition, a signed Quality Assurance statement was included.

C. RESULTS

1. Dose verification

   Initial analytical data (sample data for Analytical Report SC-85-0074, included in Appendix IV of the Study Report) indicated that actual low, middle and high doses of Kelthane were 69.0, 74.0, and 78.0% of theoretical, respectively. In addition, technical Kelthane analyzed as 89.6% AI (not 95.6% as specified).

   Subsequently, (Addendum to Analytical Report SC-85-0074, included in Appendix IV of the Study Report), it was stated that dosing solutions had been labelled incorrectly prior to analysis and that the correct actual low, middle, and high doses were 82.3, 88.9, and 93.4% of theoretical, respectively. No data or evidence supporting this assertion was included in the submission. It is noted that 82.3% should be 81.6%, as calculated by the reviewer.

2. Toxicologic signs

   Passiveness (55%) and ataxia (20%) were reported at the high dose (nominal

478 mg ai/kg). Passiveness (5%) but not ataxia was reported at the mid dose (nominal 191.2 mg ai/kg). Examination of the data in the range-finding study indicates that at 500 mg/kg kelthane (478 mg ai/kg) passiveness and ataxia had incidences of 30%, in addition to somnolence (50%) and abdominal breathing (10%), which were not reported for the main study.

3. Cytogenetic analysis

As shown in Table 2, a single oral dose of kelthane at 478 mg ai/kg did not induce a statistically significant increase in chromosomal aberrations in bone marrow cells of male rats. No data were available for female rats.

By contrast, 18-hour bone marrow samples from triethylene melamine (TEM) - treated rats had a significant ($p<0.05$) incidence of cells with chromosomal aberrations with respect to vehicle controls. The incidences of cells with cromosomal aberrations in TEM-treated rats were dose related and amounted to 24.4% and 36.4% at 0.2 and 0.5 mg TEM/kg, respectively.

Table 2. Effect of Kelthane® on chromosomal aberrations (CA) in rat bone marrow.

Effect	Vehicle Control (Corn oil)	Kelthane (478 mg ai/kg) oral	Positive Control (TEM in mg/kg) 0.3	0.9
	6-Hours			
Incidence/100 metaphases[a]				
- Breaks	0.4	0.22	-[b]	-
- Fragments	0	0.43	-	-
- Gaps[c]	0.2	0	-	-
- Other[d]	0	0	-	-
Percent cells with CA[e]	0.4	0.65	-	-
	18-Hours			
Incidence/100 metaphases				
- Breaks	-	-	41	62
- Fragments	-	-	75.5	130.8
- Gaps	-	-	0.46	0.4
- Other	-	-	11.5	23.6
Percent cells with CA	-	-	24.4	36.4
	24-Hours			
Incidence/100 metaphases				
- Breaks	0	0	-	-
- Fragments	0.2	0.4	-	-
- Gaps	0.2	0.2	-	-
- Other	0	0	-	-
Percent cells with CA	0.2	0.4	-	-
	48-Hours			
Incidence/100 metaphases				
- Breaks	0.24	0.23	-	-
- Fragments	0	0.23	-	-
- Gaps	0.24	0.68	-	-
- Other	0	0	-	-
Percent cells with CA	0.24	0.46	-	-

[a] Computed by the reviewer from data in Appendix II of the Final Report.
[b] - = No data.
[c] Gaps were not included in the computation of chromosomal aberrations.
[d] Includes pulverized chromosomes, and rearrangements.
[e] As reported in Table 2 of the Study Report for cytogenetics data for 6, 24 and 48 hours (Kelthane) and 18 hours (TEM).

5. Reviewer's discussion/conclusions

a. The positive control, triethylene melamine (TEM, 0.3 and 0.5 mg/kg, intraperitoneal) adequately demonstrated the sensitivity of the in vivo rat bone marrow assay to detect chromosomal aberrations in vivo.

b. In the main experiment:

i. Although some systemic toxicity was seen at the highest dose level, problems with actual dose verification and the absence of the wider range of CNS/CNS-related symptoms observed in the pilot raise some concern about the achievement of an MTD in this study. In addition, no evidence was presented (e.g. mitotic index) that the test material (and/or its metabolites) had reached the target tissue (bone marrow).

ii. The test material at the highest dose (at the toxic level) did not produce a significant elevation in chromosomal aberrations in vivo in bone marrow cells of male rats. This result is considered inconclusive for male rats in the absence of evidence that test material (and/or its metabolites) reached the bone marrow. Thus, the study is classified as unacceptable for assessing the clastogenic potential of the test material in male rats.

c. No data were available on the effect of the test chemical on chromosomal aberrations in vivo in bone marrow cells in female rats. No justification for this omission was presented in the Study Report, other than stating in the Protocol that: "Males only will be used in this study because there was no significant difference in LD_{50} values or toxicological signs observed during oral LD_{50} studies in male and female Charles River CRL:COBS-CD(SD)BR rats". This justification is not adequate because acute oral LD_{50} studies do not address the issue of clastogenicity. It is noted that it is recommended for in vivo bone marrow cytogenetics tests (chromosomal analysis) [Health Effects Testing Guidelines, EPA, FR 50:39445-39446 (1985)] that animals of both sexes be used. The use of a single sex should be justified. Thus, this study is considered unacceptable pending:

i. Repetition of the study in males, to include evidence (e.g. mitotic index measurements) for transport of effective concentrations of test material or its active metabolites to bone marrow.

ii. Submission of an acceptable justification for the omission of female rats in this study, or submission of an acceptable replica of this study using female rats.

Guideline Series 84-2: MUTAGENICITY

Reviewed by: Alberto Protzel, Ph.D.
Section III, Tox Branch II(7509C)
Secondary Reviewer: Irving Mauer Ph.D.
Tox Branch I (7509C)

9/6/91

09-6-91

008622

DATA EVALUATION REPORT

CHEMICAL: Dicofol (Kelthane)

STUDY TYPE: In vitro cytogenetics using Chinese hamster ovary (CHO) cells; EPA Guideline 84-2

MRID NUMBER: 400420-51

SYNONYMS: Kelthane^{R} Technical Miticide. 1,1-Bis(chlorophenyl)2,2,2-trichloroethanol.

SPONSOR: Rohm and Haas Company, Spring House, PA 19477.

TESTING FACILITY: Litton Bionetics, Inc.; 5516 Nicholson Lane; Kensington, Marylan; 20895.

TITLE OF REPORT: Dicofol (Kelthane^{R} Technical Miticide): In vitro cytogenetic assay in chinese hamster ovary (CHO) cells.

AUTHORS: J.L. Ivett and B.C. Myhr.

STUDY NUMBER: Rohm and Haas Report No. 85RC-0068.

REPORT ISSUED: July, 1986

CONCLUSION(S) - Executive Summary:

Doses of dicofol technical ranging from 7.5-20 μg/ml (without S9 activation) and 7.5-22.5 μg/ml (with S9 activation), did not induce a clastogenic response in the chromosomes of Chinese hamster ovary (CHO) harvested 20 hours after dosing. In both the activated and the non-activated systems, doses of 20-22.5 μg/ml were cytotoxic. The assay systems were sensitive to the clastogenic effects of Mitomycin C (-S9) and Cyclophosphamide (+S9). The study is acceptable.

Classification: This study is classified as acceptable.

1. Test Material:

Name: Kelthane Technical
Description (e.g. technical, nature, color, stability): Technical, reddish brown, semisolid paste.
Batch #: TD-393, Lot No. ML0-0953 Purity: 95.6% AI
Contaminants: <0.1% DDTr
Solvent used: Dimethyl sulfoxide (DMSO)
Other comments: The test material was negligibly soluble in water. The test material was found to be soluble in DMSO at 418 mg/ml. Upon the dilution in medium the test material continued to form visible precipitate at diluted concentration through 100 µg/ml.

2. Control Materials:
Negative: Culture medium McCoy's 5a.
Solvent control: Dimethyl sulfoxide (DMSO).
Solvent/final concentration: DMSO/10 µl per ml.
Positive: Non-activation: Mitomycin C (MMC, at 40 & 80 ng/ml or 500 & 1000 ng/ml). Results analyzed and reported for 80 ng/ml.
Activation: cyclophosphamide (CP, at 12.5 & 17.5 µg/ml 25 and 50 µg/ml). Results analyzed and reported for 17.5 µg/ml.

3. Activation: S9 derived from

X Aroclor 1254	X induced	X rat	X liver
___ phenobarbital	___ non-induced	___ mouse	___ lung
___ none		___ hamster	___ other
___ other		___ other	

The S9 fraction used was purchased from an unspecified commercial supplier and was reported to be prepared from the livers of male Fisher or Sprague Dawley rats. The activated assay mixture contained S9 mix a level of 15 µl S9-reaction mixture/ml.

4. Test Cells: mammalian cells in culture
___ mouse lymphoma L5178Y cells
X Chinese hamster ovary (CHO) cells
___ V79 cells (Chinese hamster lung fibroblasts)
___ other (list):

The Chinese hamster ovary cells (CHO-WBL) used in this assay were obtained from Dr. Sheldon Wolff, University of California, San Francisco, CA. The CHO cells were re-cloned to maintain karyotype stability. The protocol indicated that the cells were checked for mycoplasma contamination. The CHO cells were grown in McCoy's 5a supplemented with 10% FCS, l-glutamine and antibiotics, for 24 hours prior to use.

5. Test compound concentrations used:
Non-activated conditions: 7.5, 10.0, 15.0, 20.0 μg/ml.
Activated conditions: 7.5, 10.0, 15.0, 20.0 μg/ml (initial experiment);
17.5, 20.0, 22.5, 25.0 μg/ml (repeat experiment).

B. TEST PERFORMANCE

A copy of the Methods section of the Final Report is attached (Attachment 1).

1. Preliminary Cytotoxicity Assay

Cell cultures were seeded at 0.3 x 10^6 cells/flask, were exposed to half-log dilutions of the test material ranging from 8.3 ng/ml of the test article solution through 250 μg/ml, the solvent control (DMSO) or the positive controls [mitomycin C (MMC, at 250 and 500 ng/ml), without S9 activation; and cyclophosphamide (CP, at 20 and 25 μg/ml), with S9 activation]. It is noted that although the protocol specified 0.8 x 10^6 cells/flask, 0.3 x 10^6 cells/flask were actually used. This minor discrepancy, did not alter the outcome of the study.

In the nonactivated system, cells were exposed for 2 hours to the test material, BrdUrd (10 μM) was added to the cultures, and incubation was continued for an additionasl 23 hours. Cell monolayers were washed, refed with fresh medium containing BrdUrd, and reincubated in the presence of 0.1 μg/ml colcemid to arrest mitosis. In the S9-activated system, cultures were incubated for 2 hours in the presence of test article and S9 mixture without FCS. After exposure, the cells were washed with buffered saline, reincubated and treated with colcemid for 2.5 hours before harvest.

After incubation, cultures were visually evaluated for percent confluency of the monolayers and metaphase cells were collectyed by mitotic shake-off. Estimation of cell cycle-delay was done by staining the cells by the modified fluorescent-plus-Giemsa techniques of Perry and Wolff [Nature 251:156-158 (1974)] and Goto et al. [Chromosoma 66:351-359 (19780]. One hundred metaphase cells per culture were examined for the percentage of first (M_1), between first and second (M_{1+}), and second or more (M_2) division metaphase cells.

2. Cytogenetics assay

For the nonactivated assay, prepared cultures (in duplicate), seeded at 1.2-1.5 x 10^6 cells/flask, were exposed to test material in doses ranging from 1.0-5.0 μg/ml for 7.5 hours (10-hour harvest) or from 5.0-20 μg/ml for 17.5 hours (20-hour harvest). The cultures were washed free of test article and reincubated with complete McCoy's 5a medium containing 0.1 μg/ml Colcemid for an additional 2.5 hours. The cells were harvested, dried and stained for analysis of chromosomal aberrations.

For the activated assay, prepared cultures (as above) were exposed to test material in doses ranging from 1.0-7.5 μg/ml for 2 hours (for 10-hour

harvest) or from 7.5-20 μg/ml for 2 hours (for 20-hour harvest). The cells were then washed free of test material and incubated for an additional 8 or 18 hours with colcemid (0.1 μg/ml) for the last 2.5 hours of incubation. The cells were harvested, dried and stained for analysis of chromosomal aberrations.

One hundred cells from each duplicate culture at four dose levels of the test article and from each of the negative and solvent control cultures were analyzed for chromosomal aberrations. From the positive controls 25 cells were scored for chromosomal aberrations.

3. Statistical methods

Statistical analysis was done with Fisher's exact test using a significance level of 0.05.

4. Evaluation criteria

No criteria to establish assay validity, a positive response, or the biological significance of the results were presented.

5. Quality assurance measures

A signed statement of compliance with EPA GLP was included. In addition, a signed Quality Assurance statement was included.

C. RESULTS

1. Preliminary cytotoxicity assay

a. Without metabolic activation

There was complete toxicity at 25 μg/ml through 250 μg/ml. As shown in Table 1, at 8.3 μg/ml there was a 17% decrease in monolayer confluence and a significant cell cycle delay. At 2.5 μg/ml monolayer confluency was 100% and there was no cell cycle delay. Based on these results a dose range of 5-20 μg/ml was selected for a delayed 20-hour harvest in addition to a dose range of 1-5 μg/ml for a regular 10-hour harvest.

b. With metabolic activation

There was a precipitate at the two higher doses (83.3-250 μg/ml) and complete toxicity at 25 μg/ml through 250 μg/ml. As shown in Table 1, at 8.3 μg/ml there was a 50% decrease in monolayer confluence and a significant cell cycle delay. Although monolayer confluence was decreased at 833.3 ng/ml and at 2.5 μg/ml, no significant cell cycle delay was observed at these doses. Based on these results a dose range of 7.5-20 μg/ml was selected for a delayed 20-hour harvest in addition to a dose range of 1-7.5 μg/ml for a regular 10-hour harvest.

Table 1. Range finding assay for delay of cell cycle progression[a]

Treatment	% Cells at[b]			Confluence[c] (% of Control)
	M_1	M_{1+}	$\geq M_2$	
Without metabolic activation:				
Negative Control (Medium)	6	17	77	100
Solvent Control (DMSO, 10 µl/ml)	4	12	84	100
Positive Control (MMC, 250 ng/ml)	85	15	--	83
Test article:				
2.5 µg/ml	4	25	71	100
8.3 µg/ml	43	14	18	83
25 µg/ml	n[d]	n	n	
With metabolic activation:				
Negative Control (Medium)	2	4	94	100
Solvent Control (DMSO, 10 µl/ml)	2	6	92	100
Positive Control (CP, 20 µg/ml)	30	65	5	83
Test article:				
833.3 ng/ml	2	8	90	83
2.5 µg/ml	2	8	90	67
8.3 µg/ml	24	46	30	50
250.0 µg/ml [sic]	n	n	n	

[a] From Table 1, page 12 of the Final Report.
[b] Percent of cells that have completed one (M_1), between one and two (M_{1+}) or two or more ($\geq M_2$) cycles in BrdUrd.
[c] Visual observations.
[d] n = No mitotic cells were observed.

2. Cytogenetics assay:

a. Without metabolic activation

In the 20-hour delayed harvest there was approximately a 30% reduction in monolayer confluency at 15 and 20 µg/ml. Cells at 20 µg/ml had an unhealthy appearance, whereas those at 15 µg/ml had a more normal appearance. Cells at 7.5 and 10.0 µg/ml had many mitotic cells and only a 10% reduction in confluency. As shown in Table 2, cytogenetic analysis of cells exposed to test material at levels of 7.5, 10.0, 15.0, and 20.0 µg/ml did not show a significant increase in either the number or the percent of cells with aberrations. The positive control (MMC) showed a significant ($p<0.01$) increase in the percent of cells with aberrations. In the 10-hour harvest there was no toxicity at the doses tested; no cytogenetic analysis of these cells was performed because data was available from the 20-hour harvest.

b. With metabolic activation

In the 20-hour delayed harvest of the initial experiment, cultures at the highest dose (20 μg/ml) presented an unhealthy appearance and approximately a 40% reduction in monolayer confluency. At the other doses harvested at 20 hours (7.5, 10.0, and 15 μg/g/ml) the cultures were generally equivalent to the controls and had only 10% (at 15 μg/ml) or no decreases (at 10 and 7.5% μg/ml) in monolayer confluency. As shown in Table 2, for the initial experiment, there was a significant ($p < 0.01$) increase in the percent of cells with aberrations at 20 μg/ml (8.5%) compared to pooled negative controls (2%). No aberrations were observed at the lower doses. When the experiment was repeated at doses of 17.5, 20.0, 22.5, and 25.0 μg/ml (Table 2, repeat experiment) no significant increases in cromosomal aberrations were observed at any dose level. In the repeat assay, there was complete toxicity at 25 μg/ml (no mitotic cells were observed) and unhealthy monolayers with few mitotic cells at the other concentrations. The positive control (CP) showed a significant ($p<0.01$) increase in the percent of cells with aberrations in both the initial and the repeat experiments, indicating that the test was sensitive. In the 10-hour harvest there was no toxicity at the doses tested and no cytogenetic analysis of these cells was performed.

Table 2. Chromosome aberrations in Chinese hamster cells.[a]

Treatment	No. of cells scored	Aberrations per cell	% Cells with aberrations	% Cells with > 1 aberration
Without metabolic activation				
Pooled negative controls[b]	200	0.01	1.0	0.0
Positive control (MMC, 80 ng/ml)	25	0.92	52.0*	16.0
Test Material:				
7.5 μg/ml	200	0.05	4.5	0.0
10.0 μg/ml	200	0.04	3.0	0.5
15.0 μg/ml	200	0.04	2.5	0.5
20.0 μg/ml[c]	200	0.01	1.0	0.0
With metabolic activation				
Initial Experiment				
Pooled negative controls	200	0.02	2.0	0.0
Positive control (CP, 17.5 μg/ml)	25	1.12	52.0*	36.0
Test Material:				
7.5 μg/ml	200	0.03	2.0	0.5
10.0 μg/ml	200	0.05	5.0	0.0
15.0 μg/ml	200	0.06	4.5	0.5
20.0 μg/ml[d]	200	>0.10	8.5*	1.0
Repeat Experiment				
Pooled negative controls	200	0.04	4.0	0.0
Positive control (CP, 17.5 μg/ml)	25	0.76	56.0*	12.0
Test Material:				
17.5 μg/ml	200	0.03	2.0	0.5
20.0 μg/ml	200	0.04	3.5	0.0
22.5 μg/ml	200	>0.04	3.5	0.5
25.0 μg/ml[e]	0[f]			

* Significantly greater than pooled negative and solvent controls, $p < 0.01$.

[a] Only cells harvested at 20 hours were reported.

[b] Medium and DMSO data were pooled if the data if they were not significantly different.

[c] Cytotoxicity at this those was indicated by 30% decrease in monolayer confluency and the unhealthy appearance of the cultures.

[d] Cytotoxicity indicated by 40% decrease in monolayer confluencyand unhealthy culture appearance.

[e] Complete cytotoxicity at 25 μg/ml; unhealthy monolayers with few mitotic cells at 17.5-22.5 μg/ml.

[f] No mitotic cells were observed.

5. Reviewer's discussion/conclusions

a. The positive controls, Mitomycin C (MMC, 80 ng/ml, without activation) and Cyclophosphamide (CP, 17.5 μg/ml, with activation) adequately demonstrated the sensitivity of the CHO system to detect chromosomal aberrations in vitro.

b. In the experiments without metabolic activation (20-hour) harvest:

i. The highest dose level of test material (20.0 μg/ml) demonstrated cytotoxicity in the form of a 30% reduction in monolayer confluency and unhealthy appearance of the cells.

ii. The test material at the highest dose (at the toxic level) did not produce a significant elevation in chromosomal aberrations in CHO cells. This result is considered acceptable.

c. In the experiments with metabolic activation (20-hour) harvest:

i. In the initial experiment, the highest dose level of test material (20.0 μg/ml) demonstrated cytotoxicity in the form of a 40% reduction in monolayer confluency and unhealthy appearance of the cells.

ii. In the repeat assay, the highest dose tested (25.0 μg/ml) showed complete toxicity (no mitotic cells). At lower doses (17.5-22.5 μg/ml) cytotoxicity was demonstrated by unhealthy monolayers with few mitotic cells. Although no values were given for the percent reduction in monolayer confluency at 22.5 μg/ml, the following items taken together indicate that adequate cytotoxicity was reached at this dose:

- The observation of unhealthy monolayers with few mitotic cells at this dose.
- The complete toxicity observed at 25 μg/ml in this experiment.
- A 40% reduction in monolayer confluency at 20 μg/ml in the initial experiment.

iii. In an initial experiment, the test material at the highest dose (20 μg/ml, a toxic level) produced a significant elevation in chromosomal aberrations in CHO cells. In a repeat experiment, including doses of 20 and 22.5 μg/ml, a significant elevation in chromosomal aberrations in CHO cells was not observed. Thus, in the absence of a statistically significant and reproducible response, the test material is not considered to produce chromosomal aberrations in the CHO in vitro system. This result is considered acceptable.

d. Under the conditions of the assay, doses of dicofol technical ranging from 7.5-20 μg/ml (without S9 activation) and 7.5-22.5 μg/ml (with S9 activation), did not induce a clastogenic response in the chromosomes of Chinese hamster ovary (CHO) harvested 20 hours after dosing. In both systems, doses of 20-22.5 μg/ml were cytotoxic and the assay systems were sensitive to the clastogenic effects of Mitomycin C (-S9) and Cyclophosphamide (+S9).

Guideline Series 84: **MUTAGENICITY**

Reviewed by: James N. Rowe, Ph.D. 9/17/91
Section III, Tox Branch II(H7509C)
Secondary reviewer: Irving Mauer, Ph.D. 09/17/91
Tox Branch II(H7509C)
Date: 9/17/91

DATA EVALUATION REPORT

CHEMICAL: Dicofol (Kelthane) Tox. Chem. No.: 093

EPA File Symbol:

STUDY TYPE: DNA damage/repair, as measured by UDS in rat primary hepatocytes.

MRID NUMBER: 400420-52

SYNONYMS/CAS No.:

SPONSOR: Rohm and Haas Company, Spring House PA 19477

TESTING FACILITY: Rohm and Haas Company, Toxicology Department, 727 Norristown Road, Spring House PA 19477

TITLE OF REPORT: Kelthane Technical In Vitro Unscheduled DNA Synthesis Assay, Protocol No. 85P-129, Report No. 85R-202

AUTHOR(S): S. Foxall

STUDY NUMBER(S): 85R-202

REPORT ISSUED: January 31, 1986

CONCLUSION(S):

Concentrations of dicofol ranging from 0.025 to 0.5 ug/ml in rat hepatocyte cultures did not appear to produce any appreciable increase in unscheduled DNA synthesis based upon the average net nuclear count, average % nuclei with six or more grains or the average % nuclei with 20 or more grains. Cytotoxicity was not demonstrated but stated to be unacceptable at concentrations >0.5 ug/ml. The test system was responsive as evidenced by the significant increase in unscheduled DNA synthesis produced by the positive control, 2-acetyl aminofluorene. While no effect upon UDS in primary rat hepatocytes was noted for this test compound, the adequacy of the test concentrations with regard to cytotoxicity can not be verified.

Study: Unacceptable. The study does not satisfy the Guideline requirements for genetic effects Category III, other mutagenic mechanisms, since the validity of the cytotoxity data cannot be verified.

A. MATERIALS

Test Material: Name: dicofol; (1,1-Bis(chlorophenyl)-2,2,2-trichlorphenol)
Description : technical, dark brown semi-solid
Batch #: Lot No. MLO-0953, TD no. 84-393 Purity: 95.6%
Contaminants: if reported, list in CBI appendix
Solvent used: DMSO
Other comments:

B. TEST PERFORMANCE

1. Indicator cells

A photocopy (Attachment I) of the materials and methods (protocol) is attached.

Hepatocytes were obtained from an adult male Fischer 344 rat (approximately 200 g) purchased from Charles River Breeding Labs, Inc. The cell were obtained by perfusion of the liver in situ and subsequent culturing on plastic coverslips in culture dishes.

2. Controls

A negative control consisted of assay procedures performed on cells exposed only to the test material solvent (10 mg/ml DMSO) plus William's Medium E (WME). The positive control was 2-acetyl aminofluorene (2-AAF) at 5.0, 2.0, 1.0, 0.2 and 0.05 ug/ml of WME.

3. Dose selection

Cells were exposed to test compound over at least a 4 log range of test concentrations. On the basis of the toxicity assessment, the slides from at least 5 concentrations were selected for processing for autoradiography and UDS assessment. A viable cell count (trypan blue exclusion, microscopic observations of abnormal cell morphology) was obtained about 24 hours after initiation of treatments. The study authors stated (pgs 7, 8) that 12 treatments ranging from 100 ug/ml to 0.025 ug/ml were performed. The highest treatment level that had sufficient cell survival was stated as 0.5 ug/ml dicofol (no data on cytotoxicity was included in the report).

4. UDS assay

Six wells/dish (three coverslip replicates) of monolayer cultures were exposed to the selected doses of the test material,

negative (DMSO)(12 wells/2 dishes) or positive control for this study. The assay utilizes the unscheduled incorporation of 3H-thymidine (10uCi/ml; 47 Ci/mmole) into hepatocyte DNA during a 18.5-19.5 hour incubation period. The assay is terminated by washing the cell monolayer 2X with WME. Nuclei in the labeled cells are swollen by placement of the coverslips in 1% sodium citrate for 10 minutes, fixation in acetic acid:ethanol (1:4), repeating the process two addtional times and drying (time not specified). The coverslips were mounted cell-side up on glass slides using Permount and coded.

The dry, mounted coverslips were dipped into Kodak NTB-2 nuclear track emulsion (held in 42C water bath) in total darkness and suspended from the frosted end of the microscope slide such that a thin uniform layer of emulsion coating formed on drying. Once dry, the slides were stored at -20C for a 7 day exposure. Additional processing completed the cell fixation and labeling.

The cells are examined using a Biotran III colony counter in the object-area connected to a Leitz Dialux 20 microscope by a remote TV camera. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (background count). This value is referred to as the net nuclear grain count. The coverslips were coded to prevent bias in grain counting.

The net nuclear grain count was determined for 50 randomly selected cells on each coverslip. The mean net nuclear grain count was determined from triplicate coverslips (150 total nuclei).

5. Assay evaluation criteria

A positive is exhibited by a reproducible significant increase in net nuclear grains compared to the concurrent negative (untreated and solvent) controls.

Confirmation of the reproducibility of a positive requires:

1) If the positive result was at several test concentrations, then 2 or more consecutive concentrations must have exhibited a significant increase in net nuclear grains.

2) If the positive result was at a single test concentration, then the assay would have been repeated with test concentrations, selected around the unconfirmed positive effect level. The result would have been considered reproducible if the positive effect at a single concentration was confirmed in the second trial with a different hepatocyte preparation.

A conclusive negative must have a minimum of three test concentrations with at least two concentrations having greater than 75% cell survival at 24 hours (relative to the solvent

controls).

6. Results

A photocopy of summary findings for the UDS assay is attached (Attachment II).

The five doses selected for evaluation of UDS were 0.5, 0.25, 0.1, 0.05 and 0.025 ug/ml. Cell survival in the three lower doses was acceptable (80 to 89% of solvent controls). Survival could not be determined in the two highest concentrations since the cells detached from the coverslips during fixation. There was no dose-related increase in average net nuclear grains/nucleus, or in the average % of nuclei with six or twenty or more grains.

The positive control, 2-AAF (0.05 to 5.0 ug/ml), resulted in a significant increase in the number of net nuclear grains/nucleus (e.g., 29.3/5.0 ug/ml vs -5.3 or -7.7, resp.), in the average % of nuclei with ≥6 grains (98.7 vs 0.0 or 2.6, resp.) and average % nuclei with ≥20 grains (82.7 vs 0.0, resp.) in the assay as compared to the solvent controls.

7. Reviewer's discussion/conclusions :

Concentrations of dicofol ranging from 0.5 to 0.025 ug/ml in rat hepatocyte cultures did not appear to produce any appreciable increase in unscheduled DNA synthesis based upon the average net nuclear count, average % nuclei with six or more grains or the average % nuclei with 20 or more grains. Cytotoxicity was not demonstrated but stated to be unacceptable at concentrations >0.5 ug/ml. The test system was responsive as evidenced by the significant increase in unscheduled DNA synthesis produced by the positive control, 2-acetyl aminofluorene. While no effect upon UDS in primary rat hepatocytes was noted for this test compound, the adequacy of the test concentrations with regard to cytotoxicity can not be verified.

8. Was test performed under GLPs (is a quality assurance statement present)? Y / N

9. CBI appendix attached Y / N

10. A signed and dated statement of No Data Confidentiality Claims was included.