1,3-DICHLOROPROPENE **SALMONELLA GENE MUTATION (84-2)**

Principal Reviewer: Nancy E. McCarroll
Toxicology Branch 1/HED (7509C)
Signature: _____
Date: _____

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Toxicology Branch 2/HED (7509C)
Signature: _____
Date: _____

DATA EVALUATION REPORT

STUDY TYPE: Mutagenicity: Salmonella typhimurium mammalian microsome (mouse lung S9 mutagenicity assay; OPPTS 870.5265/5100 [§84-2]

DP BARCODE: D242582 SUBMISSION NO.: S536506

PC CODE: 029001 TOX. CHEM. NO.: 324A MRID NO: 44460501

TEST MATERIAL (PURITY): 1,3-Dichloropropene (unspecified purity); test substance was purified by passage through a silicic acid column, did not contain an epoxidized soybean oil stabilizer and was stored at 5±3°C under nitrogen.

SYNONYM(S)/COMPOSITION: Telone II; 1,3-D

CITATION: Lawlor, T.E. (1996). Evaluation of 1,3-Dichloropropene for Mutagenic Potential in Salmonella in the Presence of Mouse Lung Homogenate (S9); Corning Hazleton Inc. (CHV), Vienna, VA; CHV Study No. 17037-0-401; Dow Study No. M-003993-036; Study Completion Date: November 26, 1996 (Study Report Reformatted January 22, 1997). (Unpublished) MRID NUMBER: 44460501

SPONSOR: Dow Chemical Co., Midland, MI

EXECUTIVE SUMMARY: In a series of microbial preincubation mutagenicity studies (MRID No. 44460501), Salmonella typhimurium strain TA100 was exposed to 1,3-dichloropropene (unspecified purity). The test substance was purified by passage through a silicic acid column, did not contain an epoxidized soybean oil stabilizer and was stored at 5±3°C under nitrogen. Trials were conducted in the absence of exogenous metabolic activation (100-450 μg/plate); with mouse lung S9 derived from control mice in both the presence and absence of glutathione (GSH) at doses of 150-1000 μg/plate (-GSH) or 75-1000 μg/plate (+GSH); and in the presence of mouse lung S9 derived from mice pretreated by inhalation with 63 ±7 ppm 1,3-D, 5 days/week for 2.5 weeks both with and without GSH at doses of 75-1000 μg/plate. The mouse lung S9 fractions were characterized prior to use and found to convert the known promutagen, 2-aminoanthracene to an active form. The test material was delivered to the test system in ethanol.

Cytotoxicity was observed at ≥450 μg/plate -S9. Under all conditions of mouse lung activation (+/-GSH), 1,3-D was lethal at ≥600 μg/plate and caused a marginal cytotoxic effect at 300 μg/plate. The positive controls induced a clear response in S. typhimurium TA100 under all assay conditions. **There was, however, no indication that 1,3-dichloropropene induced a mutagenic effect at any dose in either the presence or absence of control or pretreated mouse lung S9 fractions with or without GSH. It was concluded that the physical state of the test material (i.e., without the epoxidized soybean stabilizer), method of purification (passage through silicic acid column) and/or storage conditions (at 5±3°C under N_2) probably accounts for the lack of a mutagenic response in the nonactivated phase of testing. It was further concluded that the absence of mutagenicity in the S9-activated phase of the study was likely associated with**

the low level of microsomal/oxidative proteins in the lung preparations since there is independent confirmation from Watson et al., (1987)[1] and Schneider et al., (1998)[2] that 1,3-D is mutagenic in *S. typhimurium* TA100 (See Section D. Reviewers' Discussion/Conclusions).

The study is classified as Unacceptable (Nonguideline) for a negative response and does not contribute to the mode of action (MOE) understanding for carcinogenesis.

COMPLIANCE: Signed and dated GLP, Quality Assurance and Data Confidentiality statements were present.

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[1]Watson, W.P., Brooks, T.M., Huckle, K.R., Hutson, D.H., Lang, K.L., Smith, R.J. and Wright, A.S. (1987). Microbial mutagenicity studies with (Z)-1,3-dichloropropene. Chem. Biol. Interactions 61:17-30.

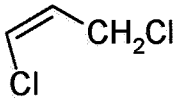
[2] Schneider, M., Quistad, G.B., and Casida, J.E. (1998). 1,3-Dichloropropene epoxides: Intermediates in Bioactivation of the Promutagen 1,3-Dichloropropene. Chem. Res. Toxicol. 11, 1137-1144.

I. MATERIALS AND METHODS

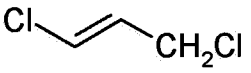
A. MATERIALS:

1. Test Material: 1,3-Dichloropropene (1,3-D)

Description: Clear colorless liquid
Lot/batch number: Not listed
Purity: Unspecified purity. The test material was purified by passage through a silicic acid column and did not contain an epoxidized soybean stabilizer.
Receipt date: July 19, 1995
Stability: Owing to the volatile nature of the test material, it was stored at 5±3°C under nitrogen.
CAS number: 542-75-6
Structure:



Z-1,3-D



E-1,3-D

Solvent used: Ethanol (ETOH)
Other provided information: Dilutions of the test material were prepared immediately prior to use and dosing solutions, preincubation tubes and test plates containing the test material were sealed to prevent compound loss.

2. Control Materials:

Negative: None
Solvent/final concentration: 50-155 µL ETOH/plate

Positive:

Nonactivation:
Sodium azide 2.0 µg/plate TA100

Activation:
2-Aminoanthracene (2-AA) 2.5 µg/plate TA100

3. Activation:

LIVER:
S9 was derived from male Sprague-Dawley

x Aroclor 1254	x induced	x rat	x liver
_____ phenobarbital	_____ noninduced	_____ mouse	_____ lung
_____ none		_____ hamster	_____ other
_____ other		_____ other	

The liver S9 homogenate (Batch 0614) was purchased from Molecular Toxicology, Inc., Annapolis, MD and contained 36.4 mg protein/mL. The rat liver S9 was only used in the mouse lung S9 characterization experiments.

Liver S9 mix composition:

Component:	Amount/mL of S9 mix
1 M NaH_2PO_4/Na_2HPO_4 (pH 7.4)	0.10 mL
0.25 M Glucose-6-phosphate	0.02 mL
0.10 M NADP	0.04 mL
0.825 M KCl/0.2 M $MgCl_2$	0.04 mL
H_2O	0.70 mL
S9	0.10 mL for 10% S9
	0.20 mL for 20% S9

Note: When 20% S9 was used the water content was reduce to yield a total volume of 1.00 mL S9 mix.

LUNG:±
S9 was derived from male B6C3F1 mice

____ Aroclor 1254	____ induced	____ rat	____ liver
_____ phenobarbital	_____ noninduced	__x__ mouse	__x__ lung
_____ none		_____ hamster	_____ other
_____ other		_____ other	

The lung S9 homogenates were prepared from the lungs of untreated control mice (MLu-C S9) or from the lungs of mice exposed via inhalation to 63±7 ppm 1,3-D, 5 days/week for 2.5 weeks (MLu-T S9). The protein content was 17.6 mg/mL or 22.0 mg/mL for the MLu-C and MLu-T S9 homogenates, respectively. The ability of the mouse lung fractions to convert 2-AA and benzo(a)pyrene to mutagens was characterized prior to initiation of the dose-range finding and mutagenicity assays. Results of the characterization experiments indicated that neither the MLu-T nor the MLu-C converted B(a)P to a mutagenic form and that the 20% MLu-C was slightly more efficient in converting 2-AA to a mutagen than 10% MLu-C. The data further indicate that 10% MLu-C S9 was ≈50% less effective in conversion of 2-AA to an active form than 10% Aroclor-induced rat liver. Accordingly, 2-AA was selected as the positive control at 2.5 μg/plate; a concentration of 10% mouse lung S9 was chosen to prepare the S9 mixes for the S9-activated assays.

Lung S9 mix composition:

Component:	Amount/mL of S9 mix
1 M NaH_2PO_4/Na_2HPO_4 (pH 7.4)	0.10 mL
0.25 M Glucose-6-phosphate	0.02 mL
0.10 M NADP	0.04 mL
0.825 M KCl/0.2 M $MgCl_2$	0.04 mL
0.2 M Glutathione (GSH)	0.00 or 0.01 mL
H_2O	0.70 or 0.69 mL
S9	0.10 mL for 10% S9
	0.20 mL for 20% S9

Note: When 20% S9 was used the water content was reduce to yield a total volume of 1.00 mL S9 mix.

4. Test Organism Used: S. typhimurium strains
_____ TA97 ___ TA98 __x__ TA100 _____ TA102 _____ TA104
____ TA1535 ____ TA1537 _____ TA1538; list any others:
Test organisms were properly maintained: Yes .
Checked for appropriate genetic markers (rfa mutation, R factor): Yes.

5. Test Compound Concentrations Used:

(a) Dose range finding assays:

Trial 1: Four doses (3.24, 10.8, 32.4 and 108 µg/plate) were evaluated with strain TA100 in the presence and absence of MLu-C S9 activation. Single cultures were plated in triplicate per dose, per condition; positive controls were included in the study.

Trial 2: Four doses (667, 1000, 3330 and 5000 µg/plate) were evaluated with strain TA100 in the absence of S9 activation. Single cultures were plated in triplicate per dose, per condition; the nonactivated positive control was included in the study.

Trial 3: Four doses (150, 300, 450 and 600 µg/plate) were evaluated as described for Trial 2.

(b) Mutation assay: Doses and experimental conditions were as follows:

Nonactivated conditions: 100, 150, 225, 300 and 450 µg/plate

S9-activated conditions:
75.0, 150, 300, 600 and 1000 µg/plate + MLu-C S9 + GSH
150, 300, 600 and 1000 µg/plate + MLu-C S9 - GSH

75.0, 150, 300, 600 and 1000 µg/plate + MLu-T S9 + GSH
75.0, 150, 300, 600 and 1000 µg/plate + MLu-T S9 - GSH

Note: All mutation trials were performed using single cultures plated in triplicate per dose, per condition. Positive controls were included.

B. TEST PERFORMANCE:

1. Type of Salmonella Assay:
_____ Standard plate test
__x__ Pre-incubation (20 + 2) minutes
_____ "Prival" modification
_____ Spot test
_____ Other (describe)

2. Protocol:

(a) Preliminary cytotoxicity/mutation assays: Similar procedures were used for the preliminary cytotoxicity and the mutation assays.

Approximately 1.5×10^8 cells (0.31 mL of a $\geq 0.5 \times 10^9$ cells/mL late log phase culture) of S. typhimurium TA100 and 155 µL of the appropriate test material dose or solvent were added to single tubes containing 1.55 mL 0.1 M phosphate buffer (nonactivated series). For the S9-activated series, 1.55 mL of the appropriate S9-cofactor mix replaced the phosphate buffer. Tubes were sealed with a screw cap and the

contents of each tube were mixed and incubated for 20±2 minutes at 37±2°C with shaking. Following preincubation, reaction mixtures (in 650 μL-volumes) were added in triplicate to tubes containing 2 mL of molten top agar supplemented with 0.05 mM histidine/biotin, vortexed and poured over Vogel-Bonner minimal medium E plates. Plates were sealed in bags and incubated at 37±2°C for 48±8 hours. Mutant colony counts were either determined immediately after incubation or plates were refrigerated until counting. Mean revertant colonies and standard deviations of the means were calculated from the counts of triplicate plates per strain, per dose, per condition. Positive controls were included in both the preliminary dose-range finding and mutation assays.

(b) Sterility controls: The sterility of the highest test dose and the S9 mix were determined by plating on selective agar.

3. Evaluation Criteria:

(a) Assay validity: The assay was considered acceptable if: (1) the rfa mutation and the R-factor plasmid were present in the tester strain; (2) the number of spontaneous revertants fell within specified range; (3) the density of the tester strain cultures was $\geq 0.5 \times 10^9$ bacteria/mL; and (4) the nonactivated and S9-activated positive controls induced at least a 3-fold increase in the number of revertants over the vehicle control.

(b) Positive response: The test material was considered positive if it caused a reproducible dose-related and 3-fold increase in the mean number of revertants per plate of S. typhimurium TA100.

C. REPORTED RESULTS:

1. Test Material Solubility The test material was soluble in ETOH at the highest concentration prepared (100 mg/mL).

2. Cytotoxicity Assays: Three trials of the dose-range finding study were performed. In Trial 1, cytotoxicity was not seen at any assayed dose (3.24-108 μg/plate) without or with MLu-C S9. For the second trial, nonactivated levels of 667-5000 μg/plate were tested and found to be lethal. Accordingly, a third trial was conducted with nonactivated doses of 150-600 μg/plate. Data from this trial showed that concentrations of 1,3-D ≥450 μg/plate were also lethal. At 300 μg/plate, revertant colonies were unaffected by treatment but a slight reduction in the background lawn of growth was scored. For the remaining dose (150 μg/plate), an ≈2-fold increase in revertants of TA100 was observed. Based on these data, the mutation assays were performed with dose ranges of 100-450 μg/plate -S9; 75-1000 μg/plate in the presence of 1,3-D pretreated lung (MLu-T) S9 homogenate with or without GSH; 75-1000 μg/plate in the presence of untreated lung (MLu-C) S9 homogenate with GSH; and 150-1000 μg/plate in the presence MLu-C S9 homogenate without GSH.

3. Mutation Assays:

S9-activated Conditions: Summarized findings from the mouse lung S9-activated series of mutation assays with the test material are presented in

Study Report Table 7, pp. 36 (see Attachment I). As shown, under all conditions of mouse lung activation, 1,3-D was lethal at concentrations ≥600 μg/plate. A slight cytotoxic effect, indicated by a reduction in the revertant colony count and/or the background lawn of growth, was recorded at 300 μg/plate (all conditions). The remaining doses (75.0 or 150 μg/plate) were neither cytotoxic nor mutagenic.

Nonactivated Conditions: In agreement with the preliminary findings, the test substance at 450 μg/plate was cytotoxic to strain TA100, causing a marked reduction in the background lawn of growth and an ≈63% reduction in revertant colonies. Although slight increases in histidine revertants (1.5- to 1.6-fold) were calculated at 150-300 μg/plate, the effect was not dose related and the values fell within the performing laboratories historical spontaneous revertant range for TA100. These increases are, therefore, not indicative of a mutagenic response (see Attachment II, Study Report Table 8, pp. 37).

By contrast, the corresponding positive control induced marked increases in revertant colonies of S.typhimurium TA100 both in the presence and in the absence of 10% mouse lung S9 fractions.

Based on the overall results, the study author concluded that 1,3-D was negative under the conditions of this preincubation microbial test system.

D. REVIEWER'S DISCUSSION/CONCLUSIONS: The study design (use of single cultures for each data point, plated in triplicate) is a departure from the standard assay. However, our reviewers assume that this approach was adopted because of the volatility of the test material and that the use of this modification to the standard protocol did not affect the outcome of the study. We agree with the study author's conclusion that 1,3-D was evaluated up to severely cytotoxic doses (450 μg/plate -S9; ≥600 μg/plate +MLu-T or MLu-C S9) but failed to induce a mutagenic effect in S. typhimurium TA100. The absence of direct mutagenic activity of silicic acid purified 1,3-D, stored under N2 to prevent autoxidation to mutagenic impurities has been previously demonstrated by Watson et al. (1987)[c]. Similarly, the ability of mono-oxygenases in rat liver microsomes to convert purified 1,3-D to a mutagen for *S. typhimurium* TA100 has been shown by Watson et al. (1987). Our reviewers further noted that in the series of experiments by Watson et al. (1987), the mutagenic response was enhanced in the presence of increasing levels of washed microsomes with the maximum effect attained in the presence of 0.9 mg microsomal protein/plate. These data support the investigators' conclusion that an oxidative metabolite was responsible for the mutagenic activity seen with TA100. Independent confirmation that 1,3-D is oxidized to mutagenic epoxides via a minor metabolic pathway *in vivo* in the livers of mice treated with 1,3-D and also *in vitro* by the mouse liver cytochrome P450-dependent microsome-NADPH system has been presented by Schneider, et al. (1997)[d]. In this study, equal caution was taken to prevent

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[c]Watson, W.P., Brooks, T.M., Huckle, K.R., Hutson, D.H., Lang, K.L., Smith, R.J. and Wright, A.S. (1987). Microbial mutagenicity studies with (Z)-1,3-dichloropropene. Chem. Biol. Interactions 61:17-30.

[d] Schneider, M., Quistad, G.B., and Casida, J.E. (1998). 1,3-Dichloropropene epoxides: Intermediates in Bioactivation of the Promutagen 1,3-Dichloropropene. Chem. Res. Toxicol. 11, 1137-1144.

and/or identify the formation of reactive impurities and/or autoxidation products. Findings indicate the *in vivo* and *in vitro* generation of *both cis* and *trans* 1,3-D-epoxides with the *cis* form generated at a higher yield than the *trans* epoxide both *in vitro* and *in vivo* and that the *cis* isomer reacts slower than the *trans* epoxide with GSH alone or catalyzed by GSH S-transferase. Similarly, the *cis* epoxide was more potent in *S. typhimurium* TA100 than the *trans* isomer (≈1850 revertants at 50 nmol *cis* isomer versus ≈850 revertants at 50 nmol for the *trans* isomer). Overall the results of Watson et al. (1987)and Schneider, et al. (1997) dispute the data presented in the current submission.

Based on these considerations, we conclude that the physical state of the test material (i.e., without the epoxidized soybean stabilizer), method of purification (passage through silicic acid column) and/or storage conditions (at 5±3°C under N_2) probably accounts for the lack of a mutagenic response in the nonactivated phase of testing. We further conclude that the absence of mutagenicity in the S9-activated phase of the study was likely associated with the low level of microsomal/oxidative proteins in the lung preparations and likely represents a "false negative" in light of the positive results achieved by independent investigators using mouse and rat liver enzyme activation.

We assess, therefore, that the study does not provided acceptable evidence of a negative response in this preincubation bacterial gene mutation assay under the specified assay and storage conditions of the purified test substance and can not be used for regulatory purposes.

E. STUDY DEFICIENCIES: NONE