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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: Methylene bis (thiocyanate)

Project No: 9-1025
Tox. Chem. No.: 565
BARCODE No.: 241264
EPA ID No: 61756

TO: John Lee, PM # 31
Registration Division (H7505C)

THRU: Roger Gardner, Section Head
Review Section 1
Toxicology Branch
Health Effects Division (H7509C)

FROM: Nguyen Bich Thoa, Ph.D
Review Section I
Toxicology Branch I
Health Effects Division (H7509C)

Registrant: ICI Americas Inc., Farmington, Connecticut
(Methylene bis (thiocyanate) Task Force)

ACTIONS REQUESTED:

Review of the 3 following mutagenicity studies:

1. Salmonella/Mammalian-Microsome Plate Incorporation Mutagenicity Assay (Ames Test) with a Confirmatory Assay (84-2a) (MRID 410037-01).
2. Micronucleus Cytogenetic Assay in Mice (84-2b) (MRID 410037-02).
3. Unscheduled DNA synthesis in Rat Primary Hepatocytes (84-2c) (MRID 410037-03).

CONCLUSIONS:

1. The mutagenic potential of methylene bis (thiocyanate) was studied in 5 histidine auxotrophs bacterial strains derived from Salmonella Typhimurium (TA- 98, 100, 1535, 1537, and 1538), according to the reverse mutation test method of Ames. According to the results of the study, methylene bis (thiocyanate), at concentrations of 0.1-20 ug/plate in assays without metabolic

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activation or 0.3-50 ug/plate in assays with metabolic activation, was negative in the Ames Test. The dosage range used were based on a range finding study with TA 100 strain, in which concentrations ≥ 33 ug/plate (without metabolic activation) or ≥67 ug/plate (with metabolic activation) were 100% cytotoxic. The study is acceptable and satisfies the toxicological data requirements of guidelines 82-2a (Mutagenicity).

2. The mutagenic potential of methylene bis (thiocyanate) was studied in an in vivo test system, the Mouse micronucleus cytogenetic assay. According to the results of the study, doses of 0.3, 1.3, or 2.6 mg/kg injected IP to ICR mice of both sexes, were negative in this test. The LD_{50} was about 3.2 mg/kg. The study is acceptable and satisfies the toxicological data requirements of guidelines 82-2b (Mutagenicity).

3. The mutagenic potential of methylene bis (thiocyanate) was studied using the Unscheduled DNA Synthesis in Rat Hepatocytes Test. Adult male Fisher 344 rats were used to prepare the primary hepatocytes ($5x10^5$ cells/culture). ^3H-Thymidine (10 uCi/ml medium) was incorporated into the hepatocytes DNA and the number of net nuclear grain per cell nucleus as well as the number of cells in repair (≥5 net nuclear grain/cell nucleus) were recorded. Methylene bis (thiocyanate), 0.03-1.5 ug/ml, was negative in the Unscheduled DNA Synthesis in Rat Hepatocytes Test.Concentrations from 3 to 10 ug/ml were cytotoxic (survival rate decrease ≥50%. The study is acceptable and satisfies the toxicological data requirements of guidelines 82-2c. (Mutagenicity).

DERs for the above referenced studies are attached.

Reviewed by: Nguyen B. Thoa, Ph.D.
Section 1, Toxicology Branch I
Secondary Reviewer: Roger Gardner
Section 1, Toxicology Branch I

02-13-91

DATA EVALUATION RECORD

STUDY TYPE: Reverse mutation (Ames) test (84-2a)

TOX CHEM NUMBER: 565

MRID NUMBER: 410037-01

EPA REC.NUMBER: 241264

TEST MATERIAL: Methylene Bis (thiocyanate) (99% purity), Lot No 70846-M
SYNONYMNS: None

STUDY NUMBER(S): T8013.501014

SPONSOR: Methylene Bis (thiocyanate) Task Force, ICI Americas Inc., Farmington, Connecticut

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

TITLE OF REPORT: SALMONELLA/MAMMALIAN-MICROSOME PLATE INCORPORATION MUTAGENICITY ASSAY (AMES TEST) WITH A CONFIRMATORY ASSAY

AUTHOR(S): Timothy E. Lawlor, M.A.

DATE REPORT ISSUED: 08-12-88

CONCLUSIONS: The mutagenic potential of Methylene Bis (thiocyanate) was studied in 5 histidine auxotrophs bacterial strains derived from Salmonella typhimurium (TA 98, TA 100, TA 1535, TA 1537, and TA 1538), according to the reverse mutation test method of Ames.

Positive controls were 2-amino anthracene (2 ug/plate) for mutagenic testing with metabolic activation in all 5 strains, and Sodium azide (1 ug/plate), 9-amino acridine (75 ug/plate), or 2-nitro fluorene (3 ug/plate), for mutagenic testing without metabolic activation, sodium azide in the TA 100 and TA 1535 strains, 9-amino acridine in the TA 1537 strain, and 2-nitro fluorene in the TA 1538 and TA 98 strains.

Methylene Bis (thiocyanate) at concentrations of 0.1-20 ug/plate in assays without metabolic activation or 0.3-50 ug/plate in assays with metabolic activation was negative in the Ames reverse Mutation test with all 5 strains studied. The dosage ranges used

were based on a range finding study with TA 100 strain, in which concentrations of Methylene Bis (thiocyanate) ≥ 33 ug/plate (without metabolic activation) or ≥ 67 ug/plate (with metabolic activation) were 100% cytotoxic.

The positive controls all induced positive reverse mutation in the bacterial strains studied.

CORE CLASSIFICATION:ACCEPTABLE

MATERIALS AND METHODS:

Sterile procedures were used throughout the preparation of materials and the execution of the reverse mutation tests.

Bacterial Tester Strains: Five bacterial tester strains, TA 98, TA 100, TA 1535, TA 1537, and TA 1538, all histidine auxotrophs derived from Salmonella tiphymurium, were obtained from Dr. B. Ames, from UC Berkeley, California. TA 1535 strain was used to detect mutations involving base-pair substitutions, TA 98, TA 1537 and TA 1538 strains to detect frameshift mutations, and TA 100 strain to detect both types of mutation. Samples (1.5 ml) of fresh bacteria cultures in nutrient broth containing dimethylsulfoxide (DMSO, 0.14 ml) were stored at $\leq$-80 Degrees C (frozen permanent stock). Aliquots of thawed stock were streaked onto culture media, and stored at 4° C (master plates). Overnight cultures were obtained by transferring colonies of tester strains from their respective master plates into culture flasks, and incubating them for 12 hours, at 37° C, with slight shaking. Acceptable working bacterial tester strain cultures have a density of 1-2 X 10^9 cells/ml medium. The following ranges of spontaneous revertants per culture plate were considered characteristic and acceptable: 10-50 for TA 98 strain, 80-240 for TA 100, 5-45 for TA 1535, 3-21 for TA 1537, and 5-35 for TA 1538.

Test Chemical: Methylene Bis (thiocyanate) was dissolved in DMSO (certified A.C.S., Fisher Sci. Co.). The concentrations used in tests without metabolic activation were 0.1, 0.3, 1.0, 3.3, 10, and 20 ug/culture plate. Those used in tests with metabolic activation were 0.3, 1.0, 3.3, 10, 33, and 50 ug/culture plate. These dosage choices were based on the results of a preliminary range finding test using the TA 100 strain, showing that concentrations of Methylene Bis (thiocyanate) $\geq$ 33 ug/culture plate (without metabolic activation) or $\geq$ 67 ug/culture plate (with metabolic activation) were 100% cytotoxic (absence of any revertant colonies and complete clearing of background lawn).

Reference Chemicals: The following chemicals mutagens were used as positive controls: 2-amino anthracene (2 ug/plate) for mutagenic testing with metabolic activation in all 5 strains, and Sodium azide (1 ug/plate), 9-amino acridine (75 ug/plate), or 2-nitro fluorene (3 ug/plate), for mutagenic testing without metabolic activation, sodium azide in the TA 100 and TA 1535 strains, 9-amino acridine in the TA 1537 strain, and 2-nitro fluorene in the TA 1538 and TA 98 strains. Sodium azide and 2-amino anthracene were of practical grade (Sigma Chem. Co.). The 2 remaining chemicals were 98% pure (Aldrich Chem. Co.).

Preparation of the metabolic activating system (S-9 mix): A S-9 fraction was prepared from livers of Sprague-Dawley male rats induced with a single dose of Aroclor 1254 (500 mg/kg, IP, 5 days prior to sacrifice). The S-9 mix was prepared immediately before

each test by combining the following: S-9 fraction (1 ml), 0.2M MgCl2/0.835M KCL (0.4 ml), 0.05M glucose-6-phosphate (1 ml), 1.0M NaH_2PO_4/K_2HPO_4, PH 7.4 (1 ml), 0.04M NADP (1 ml), and H_2O (5.6 ml).

Preparation of Top Agar: Top agar is molten agar (0.8 % agar + 0.5 % NaCl) to which was added 0.5 mM L-Histidine and 0.5 mM D-Biotin in proportions of 10:1, v/v.

Minimal Bottom Agar: Minimal bottom agar is Vogel- Bonner minimal medium E (Vogel and Bonner, 1956).

Nutrient Broth: Nutrient broth is Vogel-Bonner minimal medium E + 2.5% Nutrient broth No. 2 (w/v).

EXPERIMENTAL PROCEDURES:

For each of the 5 bacterial tester strain, six concentrations of the test material (0.1, 0.3, 1.0, 3.3, 10, and 20 ug/culture plate without metabolic activation, and 0.3, 1.0, 3.3, 10, 33, and 50 ug/culture plate with metabolic activation) were used. Vehicle controls (0.05 ml DMSO) and appropriate positive controls (chemical names and concentrations mentioned under reference chemicals above) were also included. The mutation test was started by the quick addition to molten Top agar (2 ml in tests with metabolic activation and 2.5 ml in tests without metabolic activation) of the following:
- 0.1 ml of fresh bacterial culture
- 0.05 ml DMSO alone (vehicle controls), or containing either the test material or an appropriate reference chemical.
- 0.5 ml of S-9 mix (only in tests with metabolic activation).

The samples were mixed, overlaid on bottom ager plates , incubated at 37 degrees C for 48 hours, and the number of colonies reverting to histidine prototrophy were counted, using a automatic colony counter. Positive reverse mutation is said to have occured when the number of revertant colonies is at least greater than 2 folds that of the solvent controls and there is a dose related response. Positive controls should produce at least a three fold increase in the number of revertant colonies compared to the solvent controls. All samples were done in triplicates, and the mutation test repeated once, for confirmation.

The results were expressed as mean + S.D. of the number of revertant colonies per experimental point.

REPORTED RESULTS:

The attached table 1 shows the results of the range finding study using TA 100 as the representative bacterial tester strain. It could be observed that all background lawn had disapeared at concentrations $\geq$ 33 ug/plate without metabolic activation, and at

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concentrations ≥ 67 ug/plate with metabolic activation. Consequently, the respective concentrations of 20 and 50 ug/plate were chosen as the highest concentrations for the main study with and without metabolic activation.

The attached tables 22 and 23 show the results of the main and confirmatory studies. It could be shown that: 1. The observed number of spontaneous revertants in the vehicle control plates were all within acceptable ranges for each of the bacterial tester strain used,

2. Methylene Bis (thiocyanate) did not induce any appreciable increase in the number of revertant colonies of any strain, at any dose level, with or without the presence of a metabolic activation system. In contrast, all the positive control chemicals induced mutagenicity,

3. The negative mutagenicity potential of Methylene Bis (thiocyanate) was again demonstrated in the confirmatory experiment, and

4. Methylene Bis (thiocyanate) was 100% cytotoxic to all 5 tester strains at 20 ug/plate in tests without metabolic activation, and at 50 ug/plate in tests with metabolic activation.

DISCUSSION:

Author's discussion:

Under the conditions of this study, Methylene Bis (thiocyanate) is negative in the Ames reverse mutation tests, with or without metabolic activation.

Reviewer's comments:

Adequate data were presented to support the author's conclusions. The range of Methylene Bis (thiocyanate) concentrations used in both the main and confirmatory studies was adequate, since the two highest levels, 20 ug/plate without metabolic activation, and 50 ug/plate with metabolic activation, were shown to be highly cytotoxic to all 5 bacterial tester strains.

CORE Classification: Acceptable

REFERENCES:

Ames, B. N., J. McCann, and E. Yamasaki: Methods for Detecting Carcinogens and Mutagens with the Salmonella/Mammalian - Microsome Mutagenicity test, Mutation Res., 31, 347-363, 1975.

Maron, D. M., and B. Ames: Revised Methods for the Salmonella Mutagenicity Test, Mutation Res., 113, 173-215, 1983.

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Vogel, H. J., and D. M. Bonner: Acetylornithinase of E. coli: Partial Purification and some Properties, J. Biol. Chem., 218: 97-106, 1956.

PAGES 9 THROUGH 11 HAVE BEEN REMOVED. THOSE PAGES CONSIST OF REGISTRANT-SUBMITTED DATA.

00827

Reviewed by: Nguyen B. Thoa, Ph.D.
Section 1, Toxicology Branch I
Secondary Reviewer: Roger Gardner
Section 1, Toxicology Branch I

03-13-91

DATA EVALUATION RECORD

STUDY TYPE: Micronucleus Cytogenetic Assay in Mice (84-2b)

TOX CHEM NUMBER: 565

MRID NUMBER: 410037-02

EPA REC.NUMBER: 241264

TEST MATERIAL: Methylene Bis (thiocyanate) (99% purity), Lot No 70846-M
SYNONYMNS: None

STUDY NUMBER(S): T8013.122

SPONSOR: Methylene Bis (thiocyanate) Task Force, ICI Americas Inc., Farmington, Connecticut

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

TITLE OF REPORT: Micronucleus Cytogenetic Assay in Mice

AUTHOR(S): Donald L. Putman

DATE REPORT ISSUED: 10-18-88

CONCLUSIONS: The mutagenic potential of Methylene Bis (thiocyanate) was studied in an In vivo test system, the mouse micronucleus cytogenetic assay.

ICR mice of both sexes were injected IP with single doses of Methylene Bis (thiocyanate) (0.3, 1.3, or 2.6 mg/kg). The selection of this HDT was based on the results of a preliminary acute toxicity study showing that the LD_{50} was approximatively 3.2 mg/kg. Vehicle controls were injected with 1% carboxy methyl cellulose and positive controls with Triethylenemelamine (TEM, 0.25 mg/kg). Five males and 5 females treated with TEM were sacrificed 24 hr after dosing. Five mice/sex in all the other groups were sacrificed each time at 24, 48, or 72 hrs after dosing. Bone marrows from the animals' femurs were prepared for microscopy under oil immersion and 1000 polychromatic erythrocites (PE) per mouse were scored for incidences of micronucleated polychromatic erythrocytes (MPE). Positive mutagenic effect through a clastogenic action is identified if there is a statistically significant increase in the MPE/1000 PE ratio above vehicle controls values, accompanied by a either a

positive dose response relationship or by 2 time- adjacent positive responses.

Under the experimental conditions of the study, Methylene Bis (thiocyanate) did not exhibit any mutagenic potential in the mouse micronucleus assay, at any dose, and at any of the 3 observation times. In contrast, the reference chemical TEM exhibited a positive mutagenic effect at 24 hr.

CORE CLASSIFICATION: ACCEPTABLE

MATERIALS AND METHODS:

Test Animals: Test animals were pathogen free ICR mice, 6-8 weeks old, obtained from Harlan Sprague Dawley Inc., Frederick, Md. Maryland. The mice were quarantined for at least 7 days prior to the studies. They were housed 6/cage, in a controlled environment (74 ± 6° F, 50 ± 20% relative humidity, and 12:12 light/dark cycle). They received tap water and certified lab. rodent chow ad libitum. In the preliminary acute toxicity study, conducted to determine the dose range for the mutagenicity test (micronucleus assay), the males weighed 30-37 g and the females 23-31 g. In the mutagenicity study, the males weighed 22-31 g and the females 20-28 g.

Materials: The test material was Methylene Bis (thiocyanate), lot No 70846-M. The purity of the test material was not stated. However, lot No. 70846-M purity is 99% according to study MRID 411719-02. It was stored at room temperature, protected from light. Working solutions were in 1% carboxy methyl cellulose (CMC)(Sigma Chem. Co). The reference chemical (positive control) was Triethylenemelamine (TEM) (lot No 45272, Polysciences Inc., St. Louis). It was dissolved in sterile distilled water.

EXPERIMENTAL PROCEDURES:

Preliminary Study: Mice were randomly assigned into 6 groups (5 mice/sex/group). The vehicle controls were injected IP with 1% CMC (1 ml/100g B.W.) and the 5 test groups with Methylene Bis (thiocyanate) 1, 2, 4, 6, or 10 mg/kg (1 ml/100 g B.W.). They were observed for clinical signs and mortality in the period immediately after dosing, then daily afterward for 7 days. Body weights were recorded immediately before dosing, and on days 2 and 4. The results showed that all animals dosed with 6 and 10 mg/kg died within 24 hrs, five males and 3 females dosed with 4 mg/kg at unspecified times, and one male dosed with 1 mg/kg shortly after dosing. Clinical signs in mice dosed with 4 mg/kg included lethargy, diarrhea, ataxia, piloerection, and difficulty in breathing. The approximate LD_{50} was 3.2 mg/kg, calculated by the method of probit analysis. Consequently, 2.6 mg/kg (80% of the LD_{50} dose) was selected as the HDT for the micronucleus assay in mice.

Micronucleus Assay: Mice were weighed and randomly assigned into 13 groups (5 mice/sex/group). Three vehicle control groups received IP injections of 1% CMC as described above. Three LDT, MDT, and HDT groups received Methylene Bis (thiocyanate) at the respective doses of 0.3, 1.3, or 2.6 mg/kg (route and dosage volume as described above). The positive control group received TEM (0.25 mg/kg, IP route). Positive controls (5/sex/group) were sacrificed 24 hr later and all other mice groups (5/sex/group) were sacrificed 24, 48, or 72 hrs after their respective dosings, by CO_2 asphyxiation. The femurs were immediately removed, and the

bone marrow aspirated into fetal bovine serum (FbS). The resulting suspension was centrifuged at 100 X g for 5 minutes, and the bone marrow cells were resuspended in FBS. Drops of the resuspended bone marrow cells preparations were spread onto glass slides (2-4 slides/mouse). The slides were fixed in methanol, stained with May-Gruenwald-Giemsa, and permanently mounted. The slides were coded blindly, and 1000 polychromatic erythrocytes (PE) were scored for the presence of micronucleated polychromatic erythrocytes (MPE) for each mouse. The ratio of PE/total erythrocytes were also counted for detection of toxicity to the bone marrow proliferation process. Mutagenicity response was expressed as the individual groups mean ± S.D. of incidences MPE/1000 PE at 24, 48, and 72 hrs. Toxicity to the bone marrow was expressed as the individual groups mean ± S.D. of PE/total erythrocytes at 24, 48, and 72 hrs.

Evaluation of Tests Results: The statistical evaluation of the data was done using the Kastenbaum and Bowman "tables for determining the statistical significance of mutation frequencies" (Kastenbaum and Bowman, 1970). Positive mutagenic effect through a clastogenic action is identified if there is a statistically significant increase ($p \leq 0.05$) in the MPE/1000 PE ratio over vehicle controls levels, accompanied by a either a positive dose response relationship or positive responses at 2 time adjacent sacrifice times. A test is considered valid only if the mean incidence of MPE in the vehicle controls is ≤ 5/1000 PE, and if the positive controls exhibited a positive clastogenic effect as defined above.

REPORTED RESULTS:

The following results could be observed in the attached table 2:
1. In both male and female vehicle control groups, at all 3 observation times (24, 48, and 72 hr), the incidences of MPE/1000 PE were below the acceptable ratio of 5/1000,
2. In both males and female groups treated with Methylene Bis (thiocyanate) 0.3, 1.3, and 2.6 mg/kg, IP, and at all 3 observation times, the incidences of MPE/1000 PE were not significantly different from those observed in the vehicle control groups,
3. In both male and female positive control groups, positive mutagenicity by clastogenic action was exhibited at 24 hr after dosing, and
4. Toxicity to the bone marrow was not observed in any group, at any of the observation times.

DISCUSSION:

Author's discussion:

Under the conditions described in the report, Methylene Bis (thiocyanate) does not induce MPE in male or female ICR mice.

Reviewer's comments: 00827

Adequate data were presented to support the author's conclusions. The dosage range of Methylene Bis (thiocyanate) used in the micronucleus assay in mice was adequate, since the HDT, 2.6 mg/kg, was 80% of the approximate LD_{50} of 3.2 mg/kg, which was obtained in a preliminary acute IP toxicity test.

CORE CLASSIFICATION: ACCEPTABLE

REFERENCES:

Heddle, J. A.: A rapid in vivo test for chromosomal damage. Mutation Res. 18:187-190, 1973.

Heddle, J. A., M. Hite, B. Kirkhart, K. Mavournin, J. T. Mcgregor, G. W. Newell, and M. Salamone: The induction of micronuclei as a measure of genotoxicity. A report of the U.S. Environment Protection Agency Gene-Tox Program. Mutation Res., 123:61-118, 1983.

Kastenbaum, M. A., and K. O. Bowman: Tables for determining the statistical significance of mutation frequencies. Mutation Res., 9:527-549, 1970.

Mackey, B.E., and J. T. Mcgregor: The micronucleus test; Statistical design and analysis. Mutation Res, 64:195-204. 1979.

Matter B. E., and J. Grauwiler: Micronuclei in mouse bone marrow cells. A simplr in vivo model for the evaluation of drugs-induced chromosomal aberrations. Mutation Res., 23:239-249. 1974.

PAGE 17 HAS BEEN REMOVED. THE MATERIAL ON THAT PAGE CONSISTED OF REGISTRANT-SUBMITTED DATA.

00827

Reviewed by: Nguyen B. Thoa, Ph.D.
Section 1, Toxicology Branch I
Secondary Reviewer: Roger Gardner
Section 1, Toxicology Branch I

7-13-91

DATA EVALUATION RECORD

STUDY TYPE: Unscheduled DNA Synthesis in Rat Primary Hepatocytes (84-2c)

TOX CHEM NUMBER: 565

MRID NUMBER: 410037-03

EPA REC.NUMBER: 241264

TEST MATERIAL: Methylene Bis (thiocyanate) (99% purity), Lot No 70846-M

SYNONYMNS: None

STUDY NUMBER(S): T8013.380

SPONSOR: Methylene Bis (thiocyanate) Task Force, ICI Americas Inc., Farmington, Connecticut

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

TITLE OF REPORT: Unschduled DNA Synthesis in Rat Primary Hepatocytes

AUTHOR(S): Roger D. Curren, Ph.D.

DATE REPORT ISSUED: 08-12-88

CONCLUSIONS: The mutagenic potential of Methylene Bis (thiocyanate)(MBT)was studied using the test of Unscheduled DNA Synthesis (UDS) in primary hepatocytes from the rat.

Male adult Fisher 344 rats were used. The primary hypatocytes (5×10^5 viable cells/culture dish) were prepared according to the methods of Williams (1,2) and Bradlaw (3).The following experimental groups (3 cultures/group) were incubated ($37\pm1^{\circ}$ C, humid atmosphere containing $5\pm1\%$ CO_2) with ^{3}H-thymidine (10 uCi/ml medium) for 18-20 hrs: medium control, vehicle control (with DMSO 10 ul/ml medium), test (with 10 levels of MBT from 0.03-10 ug/ml medium), and positive control (DMBA 3 and 5 ug/ml medium). The MBT dose range selection was based on a preliminary test in which MBT 5 ug/ml medium was shown to cause a significant increase in the level of the cytotoxic enzyme marker LDH in the incubation medium. Following the incubation period, the hepatocytes were treated for autoradiography, fixed, and stained.

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The incorporation of ^{3}H-thymidine into the hepatocytes DNA (a measure of DNA repair) was measured by the number of net nuclear grains/cell nucleus. The number of cell in repair (≥5 net nuclear grains/nucleus) were also reported.

Under the experimental conditions of the study, Methylene Bis (thiocyanate) did not exhibit any mutagenic potential in the UDS test. MBT levels of 0.03-1.5 ug/ml induced neither any change in the number of net nuclear grains/nucleus, nor any increase in the number of cells in repair. Levels of 3-10 ug/ml induced a decrease in relative survival rate greater than 50%. In contrast, both doses of the reference chemical DMBA were devoid of cytotoxic action but significantly increased both the number of net nuclear grains/nucleus, and the number of cells in repair.

CORE CLASSIFICATION: ACCEPTABLE

MATERIALS AND METHODS:

Test Media: 1. EGTA Solution: EGTA solution for liver perfusion contained 0.5 mM EGTA in Ca^{++} and Mg^{++} free Hanks' balanced salts solution.

2. Collagenase Solution: Collagenase solution for liver perfusion contained Williams medium E, supplemented wiyh L-glutamine (2 mM), gentamycine (50 ug/ml), and collagenase type 1, (80-100 U/ml).

3. Hepatocytes Seeding Medium: Seeding medium is Williams medium E , supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), and gentamicin (50 ug/ml).

4. Experimental Medium: Experimental medium is FBS-free seeding medium.

All solutions and media were adjusted to pH 7.3 with 0.01 M HEPES buffer.

Materials: The test material was Methylene Bis (thiccyanate) (MBT), lot Nc 70846-M, an orange granular solid of 99% purity. It was stored at room temperature, protected from light. Stocks solutions were made in DMSO (Aldrich, Lot No.00730MP). The reference chemical was 7,12-dimethylbenz(a)anthracene (DMBA), (Kodak, Lot No. 15A). It was dissolved in DMSO. The final concentration of DMSO in the experimental culture media was $\leq$ 1%, v:v.

Preparation of Primary Hepatocytes: Adult male Fisher 344 rats, previously quarantined for $\geq$ 7 days, were anesthetized by metofane inhalation, and their livers were perfused in situ with EGTA solution then with collagenase solution before they were removed. The hepatocytes were dissociated and seeded (5×10^5 viable cells/35 mm culture dish). The culture dishes were placed in an incubator ($37 \pm 1^{\circ}$ C, 5±1% CO_2 and unspecified relative humidity), for 90-150 minutes to permit a good attachment of the hepatocytes to the coverslips. Cellular and other debris were then washed from the cultures, fresh medium was added, and the cultures were ready for experimental use (Williams, 1977 and 1979, and Bradlaw).

EXPERIMENTAL PROCEDURES:

Preliminary Cytotoxicity Study to establish a Dose Range for the Main Study: Duplicates hepatocytes cultures, seeded 90-180 minutes earlier, were treated with 10 concentrations of MBT (0.15 to 5000 ug/ml medium). Controls included both medium controls and vehicle controls (DMSO 10 ug/ml). The cultures were incubated for 18-20 hours as described above, then aliquots of each medium were analyzed for lactic acid dehydrogenase (LDH), the enzyme marker for cellular toxicity. The results (shown in the attached table 1), showed that MBT, at 5 ug/ml medium, induced the greatest leakage of LDH observed. Less LDH was recovered in media of cells

cultures treated with higher MBT concentrations. According to the author this unexpected effect was probably due to an interference of high concentrations of MBT with the enzyme assay. Abnormal morphology changes, evidenced by microscopy, were observed with MBT concentrations ≥ 0.5 ug/ml. Concentrations higher than 500 ug/ml were precipitated into the media. Consequently, the HDT for the main study was established at 10 ug/ml medium. Triton X- 100, a cell membrane lyser, is normally used in this type or toxicity study to elicit 100% loss of LDH from the cells (100% toxicity, 0% survival). In the present study, Trito X-100 was omitted by mistake. Therefore,the cytotoxic effect of the various doses of MBT were expressed as % of the effect observed with 5 ug/ml medium.

Unscheduled DNA Synthesis (UDS) Test (Main Study): Hepatocytes cultures seeded 90-150 minutes earlier were used. The experimental groups included: Ten test groups (0.003-10 ug MBT/ml medium), 2 positive control groups (DMBA 3 and 5 ug/ml medium), 1 vehicle control group (DMSO 10 ug/ml medium), and 1 medium control group. Triplicate cultures were used in each experimental group. After ^{3}H-thymidine was added to all experimental cultures (final concentration of 10 uCi/ml medium), they were incubated for 18-20 hrs as described above, then were washed. The cells were swelled with 1% Na citrate, fixed in ethanol-acetic acid, air dryed, and kept in cold darkness for 10 days. The coverslips were then developed and fixed for autoradiography (Kodak developer and fixer), and were stained with hematoxylin-Na acetate-eosin for microscopic examination. The incorporation of ^{3}H-thymidine into the hepatocytes DNA was measured and expressed as the number of net nuclear grains/cell nucleus (number of nuclear grains per nucleus minus average number of cytoplasmic grains from 3 adjacent nuclear-sized cytoplasmic areas). Randomly selected nuclei, 150/MBT concentration level, were scored. A parallel cytotoxicity study was conducted with the same MBT dose range, with 100% relative cytotoxicity provided by the use of 1% Triton X-100. The results of the UDS test are expressed as group mean± S.D. of the net nuclear grain count per 150 nuclei/concentration of MBT. The % of hepatocytes in repair were also computed. The results of the parallel cytotoxicity test are expressed as mean % toxicity relative to Triton X-100.

Evaluation of the Results: 1. The test material is considered a positive inducer of UDS if it causes an increase of 5 or more net nuclear counts/nucleus (Williams, 1977, 1979), with either a dose response relationship or with positive UDS effects with 2 consecutive dose levels.

2. Cells with ≥ 5 net nuclear grains/nucleus are defined as cells "in repair".

3. Means, S.D., and percent survival are computed using a Lotus 1-2-3 program.

REPORTED RESULTS:

The following results from the parallel cytotoxicity test could be observed in the attached table 2: 1. The 100% cytotoxicity obtained with Triton X-100 translated into 620 LDH U/L in the incubation medium (100% toxicity).

2. MBT was relatively non cytotoxic at levels ≤ 0.3 ug/ml medium, was relatively toxic at 0.3, 1, and 1.5 ug/ml medium (relative cytotoxicities were 13, 38, and 42% respectively), and was maximally toxic at levels ≥3 ug/ml medium (relative toxicities > 50%),

3. Both concentrations of MDBA used were relatively non cytotoxic, and

4. The combination of 1% Triton X-100 and MBT 10 ug/ml produced less LDH recovery in the incubation medium than Triton X alone. It would seem that MBT 10 ug/ml had interfered with the enzyme assay.

The following results from the UDS test were reported in the attached table 3: 1. MBT levels of 0.03-1.5 ug/ml induced neither any change in the number of net nuclear grains/nucleus, nor any increase in the number of cells in repair. The mean ± S.D. number of net nuclear grains/nucleus was -1 ± 1.6 for MBT 1.5 ug/ml.

2. In contrast, both doses of the reference chemical DMBA significantly increased both the number of net nuclear grains/nucleus (mean ± S.D. was 17.6 ± 5.9 for 3 ug/ml and 19.2 ± 7.1 for 5 ug/ml), and the number of cells in repair (98% of cells were affected).

Excessive cytotoxic effects were observed with MBT ≥ 3 ug/ml, as evidenced by microscopic observations of abnormal cell morphology, and of abnormal small and darkly stained nuclei. Consequently, the data observed with these concentrations in the UDS test were not considered.

DISCUSSION:

Author's discussion: Under the conditions described in the report, Methylene Bis (thiocyanate) does not induce UDS in rats' primary hepatocytes. None of the MBT doses used caused a significant increase in the mean nuclear silver grains count/nucleus. In contrast, a significant increase in these counts was observed with both doses of the positive control.

Reviewer's comments: Adequate data were presented to support the author's conclusions. The dosage range of Methylene Bis (thiocyanate) used in UDS test was adequate. The cytotoxic effects of MBT 3-10 ug/ml may be underreported since these doses may be high enough to have caused interferences with the LDH assay. Several defects were noted in this study : 1. The use of rats from 2 different sources was not explained,

2. There was no mention of the relative humidity in the incubator used in both toxicity and UDS studies,

3. Hepatocytes used in the preliminary toxicity study were used 90-180 hrs after their seeding instead of 90-150 hrs as stated in the protocol.

4. Triton X-100 was not used in the preliminary toxicity study to provide 100% toxicity, and all toxicity results were therefore graded relative to that produced by MBT 5ug/ml.

5. The purity of the test material was not stated in this study. However, it is supposed to be 99% purity (lot number 7-0846 M stated purity was 99% in the teratology study MRID 411719-02).

None of these defects were however serious enough to affect the outcome of the study.

CORE CLASSIFICATION: Core ACCEPTABLE

REFERENCES:

1. Williams,G.M.: The detection of chemical mutagens/carcinogens by DNA repair and mutagenesis in liver cultures. In: Chemical Mutagens. Vol VI, F.J.DeSerres and A. Hollaender, eds. Plenum Press, New York, pp 71-79, 1979.

2. Williams,G.M.: Carcinogen-induced DNA repair in primary rat liver cell cultures, a possible screen for chemical carcinogens. Canc. Lett., 1: 231-237, 1977.

Bradlaw, J.: FDA, personal communication.

PAGES 24 THROUGH 26 HAVE BEEN REMOVED. THOSE PAGES CONSIST OF REGISTRANT-SUBMITTED DATA.