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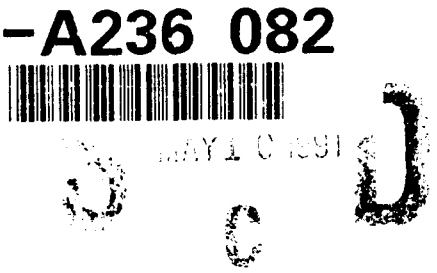

AAMRL-TR-90-050

GENOTOXICITY ASSESSMENT OF MIXED OLIGOMERS OF CHLOROTRIFLUOROETHYLENE USING A BATTERY OF *IN VITRO* AND *IN VIVO/IN VITRO* ASSAYS

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November 1990

Final Report for the Period 25 January 1989 through 1 November 1989

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TECHNICAL REVIEW AND APPROVAL

AAMRL-TR-90-50

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

JAMES N. McDOUGAL, Maj, USAF, BSC
Deputy Director, Toxic Hazards Division
Harry G. Armstrong Aerospace Medical Research Laboratory

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13. ABSTRACT (Maximum 200 words)

Halocarbon 3.1 oil, a potential hydraulic fluid consisting of mixed CTFE oligomers, was evaluated using *in vitro* bioassays to assess its potential genotoxic activity. The assays conducted were the *Salmonella*/Reverse Mutation Assay, the Chinese Hamster Ovary/Forward Mutation Assay, Sister Chromatid Exchange and Chromosome Aberration Assay, the BALB/c-3T3 Cell Transformation Assay an in *In Vivo/In Vitro* Unscheduled DNA Synthesis Assay and S-Phase Synthesis Assay. CTFE oligomers caused a weak evaluation in the mutant frequency in the forward mutation assay without metabolic activation and an increase in S-phase DNA synthesis in rat liver. The evaluation in the mutant frequency was considered too tenuous to warrant any consideration in risk assessment. Because CTFE was negative in all other assays, the results of this test battery would predict no genetic risk from CTFE however, the results from the S-phase synthesis assay indicate that the test article is hepatotoxic.

14. SUBJECT TERMS

Ames Assay, Chlorotrifluoroethylene, Chromosomal Aberration, Halocarbon 3.1 Oil, Hepatoxicity, Mutagenicity, Sister Chromatid Exchange, S-Phase DNA Synthesis, Transformation, Unscheduled DNA Synthesis

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PREFACE

The research reported in this document was conducted by Hazleton Laboratories America, Inc. under a subcontract to NSI Technology Services Corporation in support of the Toxic Hazards Research Unit (THRU). The THRU is the contractor-operated effort of the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory located at Wright-Patterson Air Force Base, OH. During the initiation and conduct of these studies Melvin E. Andersen, Ph.D., Lt Col Harvey J. Clewell, III, and Lt Col Michael B. Ballinger served consecutively as the contract technical monitor.

The experimental work reported here was begun on 25 January 1989 and completed 1 November 1989. The genotoxicity assays were conducted at the Hazleton Laboratories America facilities in Kensington, MD. The results of their work were reported to NSI in separate reports on each assay. These reports were edited by NSI and organized such that the results of each assay are provided in a separate paper. Each paper is authored by the investigator that conducted the study and a final discussion paper is provided to collectively consider the results from the individual studies. The final reports received from Hazleton Laboratories, copies of the raw data, Quality Assurance Statements, and Good Laboratory Practice Compliance and Certification Statements for each of the studies will be archived in the Quality Assurance Archive of the THRU.

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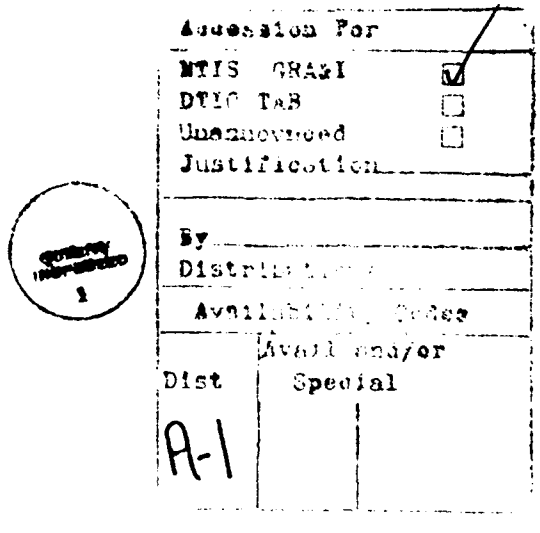

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SECTION 1

INTRODUCTION

Raymond S. Kutzman

Fluids of chlorotrifluoroethylene oligomers of various chain lengths are candidate materials for nonflammable hydraulic fluids and lubricating oils. A mixture of chlorotrifluoroethylene oligomers of six and eight carbons (CTFE) investigated in this study has been found to have low acute toxicity in rodents (Kinkead et al., 1987). However, inhalation exposure to 0.25, 0.50, and 1.00 mg CTFE/L resulted in dose-related effects in both male and female Fischer 344 rats with more severe effects in males (Kinkead et al.,1989). Effects included hepatomegaly and increased liver-to-body weight ratios. The enlarged livers were histologically characterized by multifocal to diffuse hepatocytomegaly due to increased cytoplasm. The abundant granular eosinophilic cytoplasm had abundant peroxisomes and smooth endoplasmic reticulum. This liver lesion was investigated at several timepoints for up to one-year postexposure. At 105 days postexposure more severe hepatocytic damage was observed that included markedly enlarged hepatocytes with pronounced cytoplasmic vascular degeneration. However, at 265 days and one-year postexposure, the hepatocytes of CTFE-exposed animals more closely resembled those of the control rats in that the cytoplasmic volume was reduced.

The induction of hepatocytic peroxisome proliferation by phthalate esters and hypolipidemic agents has been associated with the occurrence of liver neoplasia in laboratory rodents (Reddy and Lalwani, 1983). Additionally, some chlorinated hydrocarbons have been shown to cause hepatocytic peroxisome proliferation (Elcombe et al., 1985; Goldsworthy and Popp, 1987; Odum et al., 1988). Therefore, the carcinogenicity of CTFE, associated with its peroxisomal proliferation properties became of concern. In addition to studies to assess the initiation/promotion potential of CTFE and its metabolites, a battery of *in vitro* and *in vivo/in vitro* tests was conducted to assess the potential of CTFE to induce genetic damage. The tests conducted included bacterial and mammalian mutagenicity tests, mammalian cell transformation tests, sister chromatid exchange and chromosomal aberration assays, and an *in vivo/in vitro* unscheduled DNA synthesis test.

This report is arranged such that the results of each assay are provided in a separate paper. Each paper is authored by the investigator who conducted the work. A final discussion paper is provided to collectively consider the results from the individual studies.

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SECTION 2

MUTAGENICITY TEST ON CHLOROTRIFLUOROETHYLENE OLIGOMERS IN THE *SALMONELLA*/REVERSE MUTATION ASSAY (AMES TEST) PREINCUBATION METHOD

Lawlor, T. E.[a]

INTRODUCTION

The mutagenic activity of chlorotrifluoroethylene oligomer (CTFE) was examined in the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method. This assay evaluates the test article and/or its metabolites for their ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella* typhimurium tester strains both in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor-induced rat liver.

The *Salmonella*/Mammalian-microsome reverse mutation assay (Ames Test) detects point mutations, both frameshifts and/or base pair substitutions, in bacteria. The strains of *Salmonella* typhimurium used in this assay are histidine auxotrophs by virtue of conditionally lethal mutations in their histidine operon. When these histidine-dependent cells (his-) are exposed to the test article and grown under selective conditions (minimal media with a trace amount of histidine) only those cells which revert to histidine independence (his+) are able to form colonies. The trace amount of histidine in the media allows all the plated bacteria to undergo a few cell divisions. This growth is essential for mutagenesis to be fully expressed. The his+ revertants are readily discernable as colonies against the limited background growth of the his- cells. By utilizing several different tester strains, both base pair substitution mutations and frameshift mutations can be detected. The Ames Test has been shown to be a sensitive, rapid, and accurate indicator of the mutagenic activity of many materials including a wide range of chemical classes.

In addition to a mutation in the histidine operon, the tester strains contain two additional mutations which enhance their sensitivity to some mutagenic compounds. The rfa wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall. The resulting cell wall deficiency increases permeability to certain classes of chemicals such as those containing large ring systems (i.e. benzo[a]pyrene) that would otherwise be excluded by a normal intact cell wall. The second mutation, a deletion of the uvrB gene, results in a deficient DNA excision repair system which greatly

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enhances the sensitivity of these strains to some mutagens. Since the uvrB deletion extends through the bio gene, all of the tester strains containing this deletion also require the vitamin biotin for growth.

Strains TA98 and TA100 also contain the R-factor plasmid, pKM101, which further increases the sensitivity of these strains to some mutagens. The mechanism by which this plasmid increases sensitivity to mutagens has been suggested to be by modifying an existing bacterial DNA repair polymerase complex involved with the mismatch-repair process.

Tester strains TA98, TA1537, and TA1538 are reverted from histidine dependence (auxotrophy) to histidine independence (prototrophy) by frameshift mutagens. TA1535 is reverted by base substitution mutagens and TA100 is reverted by mutagens which cause both frameshifts and base substitutions.

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strain TA100 and 10 doses of test article ranging from 10,000 to 10.0 µg per plate, one plate per dose, both in the presence and absence of microsomal enzymes.

MATERIALS AND METHODS

The experimental materials, methods, and procedures are based on those described by Ames et al., (1975) and Yahagi et al. (1975).

The test article, chlorotrifluoroethylene oligomers (CTFE) (3.1 Oil®, Batch #N.87-23; Air Force #MLO 87-347), a clear, colorless liquid, was stored at room temperature.

A 10% F-68 Pluronic® solution (w/v in deionized water, sterilized with a 0.45 µm filter) was used as the vehicle and the test article formed a suspension at 200 mg/mL, which was the most concentrated stock dilution of test article prepared. This stock suspension was mixed with a Tissuemizer® for approximately 1 min to enhance the homogeneity of the suspension.

Media and Reagents

Top Agar for Selection of Histidine Revertants: Minimal top agar was prepared with 0.8% agar (w/v) and 0.5% NaCl (w/v). After sterilization by autoclaving, the molten top agar was distributed into sterile bottles and stored at room temperature. Immediately before its use in the mutagenicity assay, the top agar was melted and supplemented with 10 mL/100 mL agar of a sterile solution that contained 0.5 mM L-histidine and 0.5 mM D-biotin.

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

Nutrient Broth: Nutrient Broth used for growing overnight cultures of the tester strains was Vogel-Bonner salt solution supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Nutrient Bottom Agar: Nutrient bottom agar (for tester strain culture density determination) was Vogel-Bonner minimal medium E supplemented with 2.5% (w/v) Oxoid Nutrient Broth No. 2 (dry powder).

Exogenous Metabolic Activation

Liver Microsomal Enzymes – S9 Homogenate: S9 Liver homogenate for use in the mutagenicity assay, prepared as described below, was purchased commercially from Molecular Toxicology, Inc., College Park, MD, 20742, Lot #0264, 39.8 mg of protein/mL.

Species, Strain, Sex, Inducer: Liver microsomal enzymes were prepared from male Sprague-Dawley rats that had been injected with Aroclor 1254 (200 mg/mL in corn oil) at 500 mg/kg. Five days after intraperitoneal injection with the Aroclor, the rats were sacrificed by decapitation, and their livers were excised.

Homogenate Preparation: The preparation of the microsomal enzyme fraction was carried out with sterile glassware and solutions at 0 to 4°C. The livers were excised and placed in a beaker containing three volumes of 0.15M KCl (3 mL/g of wet liver) and homogenized. The homogenate was centrifuged at 9000 x g for 10 min. Small aliquots of the supernatant (referred to by Ames as the S9 fraction) were distributed into freezing ampules which were stored at ≤ -70°C.

S9 Characterization: The S9 homogenate was characterized (using the Ames Assay) for its ability to metabolize selected promutagens to their mutagenic forms, as described by deSerres and Shelby (1979).

S9 Mix: The S9 mix was prepared immediately before its use in the mutagenicity assay. One milliliter of the microsomal enzyme reaction mixture (S9 mix) contained the following components.

H_2O	0.70 mL
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
0.25M Glucose-6-phosphate	0.02 mL
0.10M NADP	0.04 mL
0.2M $MgCl_2$/0.825M KCl	0.04 mL
S9 Homogenate	0.10 mL
	1.00 mL

When S9 was required, 0.5 mL of the S9 mix was added to the preincubation mixture.

Sham S9 Mix: The Sham S9 mix was prepared immediately before its use in the mutagenicity assay One millileter of the Sham S9 mix contained the following components

H_2O	0.90 mL
1.00M NaH_2PO_4/Na_2HPO_4, pH 7.4	0.10 mL
	1.00 mL

When S9 was not required, 0.5 mL of the Sham S9 mix was added to the preincubation mixture, in place of the S9 mix.

Test System

Tester Strains: The tester strains used were the *Salmonella* typhimurium histidine auxotrophs TA98, TA100, TA1535, TA1537, and TA1538. The description of the tester strains that follows is a summarization of the description provided by Ames et al. (1975).

TESTER STRAIN GENOTYPES

Histidine Mutation			Additional Mutations		
hisG46	hisC3076	hisD3052	LPS	Repair	R Factor
TA1535	TA1537	TA1538	rfa	uvrB	–
TA100		TA98	rfa	uvrB	+ R

Source of Tester Strains: The tester strains in use at HLA were received directly from Dr. Bruce Ames, Department of Biochemistry, University of California, Berkeley.

Frozen Permanent Stocks: Frozen permanent stocks were prepared by growing fresh overnight cultures, adding DMSO (0.09 mL/mL of culture), and freezing small aliquots (approximately 1.5 mL) at ≤ -70° C.

Master Plates: Master plates were prepared by streaking each tester strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine (260 µM), biotin (3 µM), and for strains containing the R-factor, ampicillin (25 µg/mL). Tester strain master plates were stored at approximately 4° C.

Preparation of Overnight Cultures: Overnight cultures were prepared by transferring a colony from the appropriate master plate to a flask containing culture medium. In order to assure that cultures were harvested in late log phase, the length of incubation was determined by spectrophotometric monitoring. Inoculated flasks were placed in a shaker/incubator which was programmed to begin operation (shaking, 125 ± 12.5 rpm; incubation, 37 ± 2° C) so that the overnight cultures were in log phase or late log phase when turbidity monitoring began. Cultures were harvested once a predetermined turbidity was reached as determined by a percent transmittance

(%T) reading on a spectrophotometer. Overgrowth of cultures can result in their loss of sensitivity to some mutagens. Cultures were removed from incubation when the target %T was reached.

Confirmation of Tester Strain Genotypes: Tester strain cultures were checked for the following genetic markers on the day of their use in the mutagenicity assay.

rfa Wall Mutation: The presence of the rfa wall mutation was confirmed by demonstration of sensitivity to crystal violet. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of crystal violet was added. Sensitivity was demonstrated by inhibition of bacterial growth in a zone immediately surrounding the disk.

pKM101 Plasmid R-factor: The presence of the pKM101 plasmid was confirmed for tester strains TA98 and TA100 by demonstration of resistance to ampicillin. An aliquot of an overnight culture of each strain was overlaid onto plates containing selective media and an antibiotic sensitivity disk containing 10 µg of ampicillin was added. Resistance was demonstrated by bacterial growth in the zone immediately surrounding the disk.

Characteristic Number of Spontaneous Revertants: The mean number of spontaneous revertants per plate in the vehicle controls that are characteristic of the respective strains were demonstrated by plating 100 µL aliquots of the culture along with the appropriate vehicle on selective media.

Experimental Design

The tester strains used in this study were TA98, TA100, TA1535, TA1537, and TA1538. The assay was conducted using three plates per dose in the presence and absence of microsomal enzymes. Six doses of the test article were tested, from 10,000 to 333 µg/plate in both the presence and absence of S9. (It should be noted that in this report, the doses have been expressed as µg of test article/plate. This reflects the fact that the exposure of the test system to the test article does not cease at the end of the 20 min preincubation period. A dose of 10,000 µg/plate indicates that the bacteria are exposed to a concentration of 15,400 µg of test article/mL of preincubation mixture for 20 min prior to being combined with 2 mL of overlay agar and being overlaid onto 25 mL of bottom agar.)

Mutagenicity Assay: The mutagenicity assay was performed using tester strains TA98, TA100, TA1535, TA1537, and TA1538, both in the presence and absence of microsomal enzymes (S9 mix). Six doses of the test article were tested along with the appropriate vehicle and positive controls. The doses tested were selected based on the results of the dose rangefinding study.

Frequency and Route of Administration: The test system was exposed to the test article via the preincubation modification of the Ames Test originally described by Yahagi et al. (1975). This

methodology has been shown to detect mutagenicity with certain classes of chemicals, such as nitrosamines or volatiles, which may not be detected in the standard plate incorporation method. All doses of test article, vehicle controls, and positive controls were preincubated and plated in triplicate.

Dose Rangefinding Study: The dose rangefinding study was performed using tester strain TA100 both in the presence and absence of microsomal enzymes. Ten doses of test article were tested (one plate per dose). The dose rangefinding study was performed using the same methodology as was used for the mutagenicity assay. Cytotoxicity in this study is detectable as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Routinely, the maximum dose selected to be tested in the mutagenicity assay should demonstrate cytotoxicity if possible.

The growth inhibitory effect (cytotoxicity) of the test article on tester strain TA100 is generally representative of that observed on the other tester strains and because of TA100's comparatively high number of spontaneous revertants per plate, gradations of cytotoxicity can be readily discerned from routine experimental variation. Also, the cytotoxicity induced by a test article in the presence of microsomal enzymes may vary greatly from that observed in the absence of microsomal enzymes. Therefore, this would require that different test article dose ranges be tested in the mutagenicity assay based on the presence or absence of the microsomal enzymes.

Controls

Positive Controls: All combinations of positive controls and tester strains plated concurrently with the assay are listed below.

POSITIVE CONTROL AND TESTER STRAIN COMBINATIONS

Tester Strain	S9 Mix	Positive Control	Conc. per Plate
TA98	+	2-aminoanthracene	2.5 µg
TA98	–	2-nitrofluorene	1.0 µg
TA100	+	2-aminoanthracene	2.5 µg
TA100	–	sodium azide	2.0 µg
TA1535	+	2-aminoanthracene	2.5 µg
TA1535	–	sodium azide	2.0 µg
TA1537	+	2-aminoanthracene	2.5 µg
TA1537	–	ICR-191	2.0 µg
TA1538	+	2-aminoanthracene	2.5 µg
TA1538	–	2-nitrofluorene	1.0 µg

Source and Grade of Positive Control Articles:

2-aminoanthracene (CAS #613-13-8), Sigma Chemical Co., practical grade

2-nitrofluorene (CAS #607-57-8), Aldrich Chemical Co., 98%

sodium azide (CAS #26628-22-8), Sigma Chemical Co., practical grade

ICR-191 (CAS #1707-45-0), Polysciences Inc., >95% pure.

Vehicle Controls: Appropriate vehicle controls were plated for all tester strains both in the presence and absence of S9. The vehicle control was plated, using an aliquot of vehicle equal to the aliquot of test article dilution plated, along with an aliquot of the appropriate tester strain, on selective agar.

Sterility Determination: In order to determine the sterility of the test article, the highest test article dose used in the mutagenicity assay was checked for sterility by plating an aliquot volume equal to that used in the assay on selective agar. In order to determine the sterility of the S9 mix, a 0.5 mL aliquot was plated on selective agar.

Plating Procedures

The plating procedures employed are similar to those described by Ames et al. (1975) and Yahagi et al. (1975). These procedures were employed for both the Dose Rangefinding Study and the Mutagenicity Assay.

Test System Identification: Each plate was labeled with a code system which identified the test article, test phase, dose, and activation condition.

Test Article Plating Procedure: The test article was diluted and the S9 mix was prepared immediately before their use in any experimental procedure.

When S9 mix was required, 0.5 mL of S9 mix was added to 13- x 100-mm glass, screwcap, culture tubes, preheated to 37 ± 2° C. To these tubes were added 100 µL of appropriate tester strain and 50 µL of vehicle or test article dilution. When S9 mix was not required, 0.5 mL of Sham S9 (0.1M phosphate buffer) was substituted for the S9 mix. Once all components had been added to a tube, it was tightly capped, and after vortexing, the mixture was allowed to incubate for 20 ± 2 min at 37 ± 2° C. The caps then were removed and 2.0 mL of molten selective top agar was added to each tube and, after vortexing, the mixture was overlaid onto the surface of 25 mL of minimal bottom agar contained in a 15- x 100-mm petri dish. After the overlay had solidified, the plates were inverted and incubated for approximately 48 h at 37 ± 2° C.

Scoring Plates

Plates that were not scored immediately after the 48 ± 4.8 h incubation period were held at 4 ± 2° C until such time that scoring could occur.

Evaluation of the Bacterial Background Lawn: The condition of the background bacterial lawn was evaluated for evidence of cytotoxicity due to the test article by using a dissecting microscope. The cytotoxicity was scored relative to the vehicle control plate.

Colony Counting: Revertant colonies for a given tester strain and activation condition were counted either entirely by automated colony counter or entirely by hand. If the plates contained sufficient test article precipitate to interfere with automated colony counting, then they were counted manually.

Analysis of the Data: For all replicate platings, the mean number of revertants per plate was calculated and the standard deviation around the mean was also calculated.

Criteria for Determination of a Valid Test

The following criteria must be met for the assay to be considered valid.

rfa Wall Mutation: In order to demonstrate the presence of the deep rough mutation, all tester strain cultures must exhibit sensitivity to crystal violet.

pKM101 Plasmid R-Factor: In order to demonstrate the presence of the pKM101 Plasmid R-factor, all tester strains must exhibit resistance to ampicillin.

Characteristic Number of Spontaneous Revertants: All tester strain cultures must exhibit a characteristic number of spontaneous revertants per plate in the vehicle controls. The acceptable ranges are as follows.

TA98	10 – 60
TA100	80 – 240
TA1535	5 – 45
TA1537	3 – 21
TA1538	5 – 35

Tester Strain Culture Density: In order to ensure that appropriate numbers of bacteria are plated, tester strain culture density must be greater than or equal to 5.0×10^8 bacteria/mL.

Positive Control Values: All positive controls must exhibit at least a three-fold increase in the number of revertants per plate over the mean value for the vehicle control for the respective strain.

Cytotoxicity: A minimum of three nontoxic doses are required to evaluate assay data.

Evaluation of Test Results

Tester Strains TA98 and TA100: For a test article to be considered positive, it must cause at least a two-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

Tester Strains TA1535, TA1537 and TA1538: For a test article to be considered positive, it must cause at least a three-fold increase in the mean revertants per plate of at least one tester strain over the mean vehicle control value for that tester strain. This increase in the mean number of revertants per plate must be accompanied by a dose response to increasing concentrations of the test article.

RESULTS AND DISCUSSION

Dose Rangefinding Study

Doses of CTFE to be tested in the mutagenicity assay were selected based on the results of the dose rangefinding study conducted using tester strain TA100 in both the presence and absence of S9 (one plate per dose). Ten doses of test article, from 10,000 to 10.0 µg were tested and the results are presented in Table 2-1. No cytotoxicity was observed in either the presence or absence of S9 as evidenced by a normal background lawn and no observed decrease in the number of revertants per plate.

TABLE 2-1. DOSE RANGEFINDING STUDY FOR CTFE

	TA100 Revertants Per Plate			
	With S9		Without S9	
µg/Plate	Number of Colonies/Plate	Appearance of Background Lawn*	Number of Colonies/Plate	Appearance of Background Lawn*
0.00 (Vehicle) (50.0 µL)	93	1	86	1
CTFE				
10	112	1	110	1
33.3	86	1	104	1
66.7	97	1	81	1
100	111	1	100	1
333	91	1sp	80	1sp
667	86	1sp	121	1sp
1000	87	1sp	91	1sp
3330	93	1sp	67	1sp
6670	95	1sp	70	1sp
10000	83	1sp	80	1sp

* Background Lawn Evaluation Codes:

1 = normal
2 = slightly reduced
3 = moderately reduced
4 = extremely reduced
5 = absent
6 = obscured by precipitate
hp = heavy precipitate (requires hand count)
mp = moderate precipitate (requires hand count)
sp = slight precipitate

Mutation Assay

The results of the dose rangefinding study were used to select six doses to be tested in the mutagenicity assay. The doses selected for the mutation assay ranged from 10,000 to 333 µg per plate in the presence of S9 and from 10,000 to 333 µg per plate in the absence of S9 (Appendix 2-A).

In the mutagenicity assays for CTFE all data were acceptable, all criteria for a valid study were met, and no positive increases in the mean number of revertants per plate were observed with any of the tester strains either in the presence or absence of S9 (Appendix 2-B).

CONCLUSION

The results of the *Salmonella*/Reverse Mutation Assay (Ames Test), Preincubation Method, indicate that under the conditions of this study, CTFE did not cause a positive increase in the numbers of histidine revertants per plate of any of the tester strains either in the presence or absence of microsomal enzymes prepared from Aroclor-induced rat liver.

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APPENDIX 2-A
SUMMARY OF TEST RESULTS
TEST ARTICLE ID: Chlorotrifluoroethylene Oligomers
VEHICLE: 10% F-68 Pluronic (w/v in H_2O)
PLATING ALIQUOT: 50.0 µL

		Mean Revertants Per Plate With Standard Deviations										
		TA1535		TA1537[a]		TA1538		TA98		TA100		Background
	Dose/Plate	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Lawn*
MICROSOMES: None												
VEHICLE CONTROL		13	2	8	4	8	1	23	3	144	20	1
TEST ARTICLE	333 µg	10	1	8	1	7	4	20	4	122	11	1
	667 µg	13	1	7	3	6	2	22	3	129	14	1
	1000 µg	9	2	7	2	10	1	19	6	127	5	1sp
	3330 µg	12	4	7	3	5	2	17	6	134	5	1sp
	6670 µg	10	3	6	2	7	3	23	2	119	17	1sp
	10000 µg	10	0	6	2	9	4	19	3	136	11	1sp
POSITIVE CONTROL **		493	61	1135	12	286	38	185	15	616	43	1
MICROSOMES: Rat Liver												
VEHICLE CONTROL		15	6	10	4	11	3	28	3	162	14	1
TEST ARTICLE	333 µg	12	3	13	4	15	5	37	8	165	7	1
	667 µg	15	3	10	6	11	3	32	7	178	16	1
	1000 µg	16	2	9	2	12	3	39	5	167	4	1sp
	3330 µg	18	6	11	2	12	5	38	6	172	6	1sp
	6670 µg	13	2	10	4	14	3	30	9	165	8	1sp
	10000 µg	19	2	11	2	17	5	36	3	164	22	1sp
POSITIVE CONTROL ***		139	12	115	7	777	135	965	32	969	112	1

[a] The density of the culture of tester strain TA1537 (0.4×10^9) was below the acceptable range ($\geq 0.5 \times 10^9$). However, since all other tester strain characterization indicators were acceptable (culture turbidity, ampicillin, and crystal violet sensitivities, and positive and vehicle control values), the data generated with this culture of TA1537 has been accepted.

* Background Lawn Evaluation Codes:

1 = normal; 2 = slightly reduced; 3 = moderately reduced; 4 = extremely reduced; 5 = absent; 6 = obscured by precipitate; sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

** TA1535 sodium azide @10 µg/plate; TA1537 quinacrine mustard @ 5 µg/plate; TA1538 2-nitrofluorene @10 µg/plate; TA98 2-nitrofluorene @10 µg/plate; TA100 sodium azide @10 µg/plate

*** TA1535 2-aminoanthracene @ 2.5 µg/plate; TA1537 2-aminoanthracene @ 2.5 µg/plate; TA1538 2-aminoanthracene @ 2.5 µg/plate; TA98 2-aminoanthracene @ 2.5 µg/plate; TA100 2-aminoanthracene @ 2.5 µg/plate

APPENDIX 2-B
INDIVIDUAL PLATE COUNTS
TEST ARTICLE ID: Chlorotrifluoroethylene Oligomers
VEHICLE: 10% F-68 Pluronic (w/v in H_2O)
PLATING ALIQUOT: 50.0 µL

		Revertants Per Plate															
		TA1535			TA1537[a]			TA1538			TA98			TA100			Background
	Dose/Plate	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	Lawn*
MICROSOMES: None																	
VEHICLE CONTROL		12	15	11	6	12	5	7	8	8	20	23	26	138	166	128	1
TEST ARTICLE	333 µg	10	10	11	9	8	7	12	6	4	23	20	16	109	130	126	1
	667 µg	12	14	12	4	9	7	6	8	5	19	23	25	113	140	133	1
	1000 µg	8	12	8	8	8	5	11	11	9	16	26	16	123	126	133	1sp
	3330 µg	8	15	13	7	4	10	3	7	6	16	24	12	130	132	139	1sp
	6670 µg	8	13	8	7	7	4	9	3	9	22	22	26	110	138	108	1sp
	10000 µg	10	10	10	4	7	7	11	12	4	23	17	18	124	138	145	1sp
POSITIVE CONTROL **		548	502	428	1134	1123	1147	243	302	313	191	168	196	635	647	567	1
MICROSOMES: Rat Liver																	
VEHICLE CONTROL		22	11	11	7	14	9	12	8	13	25	31	29	157	178	152	1
TEST ARTICLE	333 µg	15	12	10	10	12	18	18	10	18	29	44	38	172	158	165	1
	667 µg	11	17	17	11	15	3	11	8	14	28	28	40	165	173	196	1
	1000 µg	17	17	14	10	7	10	13	9	14	35	39	44	166	171	164	1sp
	3330 µg	22	21	12	13	11	9	11	18	8	31	40	43	178	169	168	1sp
	6670 µg	13	14	11	6	9	14	18	12	13	22	30	39	174	160	161	1sp
	10000 µg	17	21	18	9	12	11	13	15	22	34	34	39	138	178	175	1sp
POSITIVE CONTROL ***		145	146	125	123	109	114	642	778	912	956	938	1000	1087	957	863	1

[a] The density of the culture of tester strain TA1537 (0.4×10^9) was below the acceptable range ($\geq 0.5 \times 10^9$). However, since all other tester strain characterization indicators were acceptable (culture turbidity, ampicillin, and crystal violet sensitivities, and positive and vehicle control values), the data generated with this culture of TA1537 has been accepted.

* Background Lawn Evaluation Codes:

1 = normal; 2 = slightly reduced; 3 = moderately reduced; 4 = extremely reduced; 5 = absent; 6 = obscured by precipitate; sp = slight precipitate; mp = moderate precipitate (requires hand count); hp = heavy precipitate (requires hand count)

** TA1535 sodium azide @ 10 µg/plate; TA1537 quinacrine mustard @ 5 µg/plate; TA1538 2-nitrofluorene @ 10 µg/plate; TA98 2-nitrofluorene @ 10 µg/plate; TA100 sodium azide @ 10 µg/plate

*** TA1535 2-aminoanthracene @ 2.5 µg/plate; TA1537 2-aminoanthracene @ 2.5 µg/plate; TA1538 2-aminoanthracene @ 2.5 µg/plate; TA98 2-aminoanthracene @ 2.5 µg/plate; TA100 2-aminoanthracene @ 2.5 µg/plate

APPENDIX 2-A

BACTERIAL BACKGROUND LAWN EVALUATION CODE

The condition of the background bacterial lawn is evaluated both macroscopically and microscopically (using a dissecting microscope) for indications of cytotoxicity and test article precipitate as follows.

	CODE DEFINITION	CHARACTERISTICS
1	Normal	A healthy microcolony lawn.
2	Slightly Reduced	A noticeable thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately Reduced	A marked thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely Reduced	An extreme thinning of the microcolony lawn and an increase in the size of the microcolonies compared to the vehicle control plate.
5	Absent	A complete lack of any microcolony lawn.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic and/or macroscopic test article precipitate.

Evidence of macroscopic test article precipitate on the plates is recorded by addition of the following precipitate code to the code number used to evaluate the condition of the background bacterial lawn.

	CODE DEFINITION	CHARACTERISTICS
SP	Slight Precipitate	Noticeable macroscopic precipitate on the plate, however, the precipitate does not influence automated counting of the plate.
MP	Moderate Precipitate	The amount of macroscopic precipitate on the plate would interfere with automated counting, thus, requiring the plate to be hand counted.
HP	Heavy Precipitate	The large amount of macroscopic precipitate on the plate makes the required hand counting difficult.

Example: 4-MP would indicate a plate observed to have an extremely reduced background lawn which had to be counted manually due to the marked amount of macroscopic test article precipitate.

SECTION 3

MUTAGENICITY TEST ON CHLOROTRIFLUOROETHYLENE OLIGOMERS IN THE CHO/HGPRT FORWARD MUTATION ASSAY

Young, R.R.[a]

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of chlorotrifluoroethylene (CTFE) to induce forward mutations at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in Chinese hamster ovary (CHO) cells under conditions with and without metabolic activation.

The test material was emulsified in the biologically compatible surfactant Pluronic® F-68 to permit preparation of homogeneous treatment media. The cells were exposed to the test material for four hours in the presence and absence of rat liver S9 metabolic activation. The test material remained in an emulsion during the 4-h treatment period. Dose-related toxicity was observed both without metabolic activation and with metabolic activation.

Without S9 metabolic activation, mutant frequencies of treated cultures varied randomly with dose at background levels with the exception of two doses, one of which was the highest surviving dose level. The two cultures had mutant frequencies that were significantly elevated over the background mutant frequency of the concurrent vehicle controls. A second nonactivation mutation assay was performed that used a modified dose range to investigate the response observed in the first nonactivation trial. The significance seen at the highest dose level in the first trial was confirmed in the second nonactivation mutation assay. The test material therefore was evaluated as weakly positive for inducing forward mutations at the HGPRT locus in CHO cells under conditions without metabolic activation. In the S9 metabolic activation trial, mutant frequencies of cultures treated with test material varied randomly with dose within a range comparable to the mutant frequencies of the concurrent vehicle controls. The test material was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells under conditions with S9 metabolic activation.

INTRODUCTION

Hypoxanthine-guanine phosphoribosyl transferase (HGPRT) is a cellular enzyme that allows cells to salvage hypoxanthine and guanine from the surrounding medium for use in DNA synthesis. The HGPRT enzyme utilizes the substrates 5-phosphoribosyl-1-pyrophosphate and hypoxanthine or

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guanine to catalyze the formation of inosine or guanosine monophosphate. If a purine analog such as 6-thioguanine (TG) is included in the growth medium, the analog will be phosphorylated via the HGPRT pathway and incorporated into nucleic acids, eventually resulting in cellular death. The HGPRT locus is located on the X chromosome. Since only one of the two X chromosomes is functional in the female Chinese hamster ovary (CHO) cells, a single-step forward mutation from HGPRT+ to HGPRT- in the functional X chromosome will render the cell unable to utilize hypoxanthine, guanine, or TG supplied in the culture medium. Such mutants are as viable as wild-type cells in normal medium because DNA synthesis may still proceed by *de novo* synthetic pathways that do not involve hypoxanthine or guanine as intermediates. The basis for the selection of HGPRT- mutants is the loss of their ability to utilize toxic purine analogs (e.g., TG), which enables only the HGPRT- mutants to grow in the presence of TG. Cells which grow to form colonies in the presence of TG are assumed to have mutated, either spontaneously or by the action of the test article, to the HGPRT- genotype.

The objective of this *in vitro* study was to evaluate the ability of CTFE to induce forward mutations at the HGPRT locus in the CHO-K1-BH_4 CHO cell line as assessed by colony growth in the presence of TG. Testing was performed both in the presence and absence of S9 metabolic activation.

MATERIALS

Test Material

The test material, chlorotrifluoroethylene (CTFE), was further identified as batch number N.87-23 (Air Force number MLO 87-347). The test material was stored at room temperature in the dark.

Indicator Cells

The indicator cells used for this study were CHO cells. The hypodiploid CHO-K1 cell line was originally derived from the ovary of a female Chinese hamster (Cricetulus griseus) (Kao and Puck, 1968). Characteristics of the cell line were high clonability (approximately 85%) and rapid doubling time (11 to 14 h). The particular clone used in this assay was CHO-K1-BH_4. The BH_4 subclone of CHO-K1 cells, isolated by Dr. A.W. Hsie (Oak Ridge National Laboratory, Oak Ridge, TN), has been demonstrated to be sensitive to many chemical mutagens.

The CHO-K1-BH_4 cells used in this study were obtained in October 1982 from Dr. Hsie. Master stocks of the cells were maintained frozen in liquid nitrogen. Laboratory cultures were maintained as monolayers at 37° C ± 1° C in a humidified atmosphere containing 5% ± 1.5% CO_2. Laboratory cultures were checked periodically for karyotype stability and for the absence of mycoplasma contamination. To reduce the negative control frequency (spontaneous frequency) of HGPRT-mutants to as low a level as possible, the cell cultures were exposed to conditions which selected against the HGPRT- phenotype. Cells were maintained in cleansing medium for two to three days,

placed in recovery medium for one day, and then returned to culture medium. Cleansed cultures were used to initiate mutation assays from three to seven days after having been removed from cleansing medium.

Media

The cells used during experimental studies were maintained in Ham's Nutrient Mixture F12 supplemented with L-glutamine, antibiotics, and fetal bovine serum (8% by volume), hereafter referred to as culture medium. Cleansing medium used for reducing the spontaneous frequency of HGPRT- mutants prior to experimental studies consisted of culture medium (5% serum) supplemented with 5.0×10^{-6} M thymidine, 1.0×10^{-5} M hypoxanthine, 1.0×10^{-4} M glycine, and 3.2×10^{-6} M of either aminopterin or methotrexate. Recovery medium was similar to cleansing medium except that the aminopterin or methotrexate component was removed and the fetal bovine serum was increased to 8% by volume. Selection medium for mutants was hypoxanthine-free F12 medium containing 4 µg/mL (24 mM) of TG and the fetal bovine serum component reduced to 5% by volume.

Control Articles

Negative (media) controls were performed for the cytotoxicity assay by carrying cells unexposed to the test article through all of the assay operations. In the activation portion of the assay, the negative control cultures were exposed to the S9 metabolic activation mix. Triplicate cultures were used in the cytotoxicity assays.

The test material was a liquid with poor solubility directly in water or culture medium. In order to effectively prepare primary 10X stocks, the test material was emulsified in sterile deionized water that contained 10% (w/v) Pluronic® F-68 (BASF-Wyandotte, lot number WPHB 500B and WPDJ 546B). The primary test material stocks then were diluted 1:10 into culture medium resulting in varying test material concentrations emulsified in 1% Pluronic® F-68 in the treatment medium. Therefore, concurrent vehicle controls were performed for each portion of the study by exposing cells to 1% Pluronic® F-68 in culture medium. In the activation portions of the study, the vehicle controls were also exposed to the rat liver S9 metabolic activation mix. Triplicate cultures were used in the cytotoxicity assays and duplicate cultures were used in the mutation assays. 5-Bromo-2`-deoxyuridine (BrdU) is a chemical that is reproducibly and highly mutagenic to CHO-K1 cells without S9 metabolic activation. BrdU (Sigma Chemical Co., lot number 81F-0082) was used at a concentration of 50 µg/mL as a concurrent positive control article for nonactivation mutation studies.

3-Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic to CHO-K1-BH_4 cells. 3-Methylcholanthrene (Sigma Chemical Co., lot number 70F-0306)

was used at 5 µg/mL as a concurrent positive control article for mutation assays performed with S9 activation.

S9 Metabolic Activation System

The *in vitro* metabolic activation system was comprised of rat liver enzymes (S9 fraction) and an energy producing system, CORE (nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, and an ion mix) prepared in a phosphate buffer. The enzymes were contained in a 9000 x g supernatant from liver homogenate prepared from Sprague-Dawley rats treated with 500 mg/kg of Aroclor 1254 five days prior to sacrifice (Molecular Toxicology, Inc., lot number 0249). The treatment with Aroclor 1254 was used to induce mixed function oxidase enzymes capable of transforming chemicals to more active forms. The S9 and reaction mixture (CORE) were retained frozen at about -80° C until used. The S9 fraction and CORE were thawed immediately before use and combined to form the activation system described below.

Component	Final Concentration in Cultures
NADP (sodium salt)	1.0 mM
Glucose-6-phosphate	5.0 mM
Calcium chloride	2.0 mM
Potassium chloride	6.6 mM
Magnesium chloride	2.0 mM
Phosphate	2.0 mM
S9 homogenate	20.0 µL/mL

The amount of S9 homogenate per culture depends upon the lot of S9 in use at any time. Before use in the assay, each lot of S9 homogenate was tested when purchased. Because the enzymatic activity of S9 homogenate varies among lots, S9 at various concentrations was tested against reference chemicals such as benzo(a)pyrene or 3-MCA. The optimum S9 concentration was selected based on induction of HGPRT-mutants in CHO cells, and this amount of S9 was used in all subsequent assays with that particular lot of S9.

Dosing Procedure

In order to achieve as uniform an exposure as possible for cell monolayers treated with the test material in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material. Pluronic® F-68 is a polyalcohol that is used frequently in cell cultures due to its low toxicity and ability to lower surface tension. When a preparation of 50 mg/mL of CTFE in 10% w/v Pluronic® F-68 in deionized water was agitated vigorously for about 1 min with a Tissuemizer®, a stable, white emulsion was formed. The test material remained dispersed into tiny droplets after diluting 1:10 into culture medium and did not coalesce upon sitting, even though dense droplets settled and collected on the bottom of the container. A brief mixing with a vortex caused complete dispersion.

It was noted that CTFE had no effect on polystyrene tissue culture flasks, whether placed directly on the surface of the flask or as an emulsion in Pluronic® F-68. Thus, after consultation with the sponsor, standard polystyrene tissue culture flasks were used in this study for test material treatment.

A fresh emulsion of CTFE was prepared in 10% w/v Pluronic® F-68 for each dose rangefinding study and mutation assay. A Tissuemizer® was used at a setting of 40 to 50 for 45 sec to 1 min to prepare the emulsions. The initial concentration of test material was prepared at 50 mg/mL, and lower concentrations were prepared by diluting the emulsions into 10% w/v Pluronic® F-68. The emulsions were mixed well by vortex before preparing the treatment media. The treatment media were prepared as 1:10 dilutions of the emulsions into F12 culture medium so that the Pluronic® F-68 content was diluted to 1% w/v.

Rangefinding Cytotoxicity Testing

After the selection of 10% Pluronic® F-68 as a suitable vehicle, a wide range of test article concentrations was tested for cytotoxicity both with and without S9 metabolic activation. Ten concentrations that spanned a three-log concentration range were used. The applied doses ranged from 0.005 mg/mL to 5.0 mg/mL. In addition, three negative (media) controls and three vehicle controls containing 1% Pluronic® F-68 were used in each cytotoxicity assay.

The cells were seeded quantitatively at 200 cells/dish, allowed to attach overnight (16 to 18 h), and exposed to the test or control article for 4 h. The cells then were washed twice with Dulbecco's phosphate buffered saline and incubated in F12 culture medium for seven additional days to allow colony development. Colonies then were fixed in alcohol, stained with Giemsa, and counted by eye, excluding those with approximately 50 cells or less. Cytotoxicity was expressed as a percentage of colony counts in treated cultures versus control cultures. The preliminary cytotoxicity information was used to select doses for the mutation assay that covered a range from approximately 0% to 90% reduction in colony-forming ability. Treatment conditions chosen for the nonactivation portion of the mutation assay covered a 100-fold range from 0.05 mg/mL to 5.0 mg/mL and for the activation assay a 10-fold range from 0.1 mg/mL to 5.0 mg/mL.

Nonactivation Mutagenicity Assay

The assay procedure was based on that reported by Hsie et al., (1975), and reviewed by Hsie et al., (1981), with modifications suggested by Myhr and DiPaolo (1978). The cleansed cells were plated at about 3×10^6 cells per T-75 (75 cm^2) tissue culture flask on the day before dosing. The time between plating and treatment was about 18 h. Cell cultures were treated with test or control material for 4 h. Cell cultures normally contain at least 4×10^6 cells by the time of treatment termination. After treatment, the cell monolayers were washed twice with phosphate buffered

saline, trypsinized, and suspended in culture medium. The cell suspension from each dose was counted using a Coulter Counter and replated at 1.5×10^6 cells into each of two 150-mm dishes and at 200 cells into each of three 60-mm dishes. The small dishes were incubated for seven days to permit colony development and the determination of the cytotoxicity associated with each treatment. The large dishes were incubated for seven days to permit growth and expression of induced mutations. The mass cultures were subcultured every two or three days during the expression period to maintain logarithmic cell growth. At each subculture the cells from the two 150-mm dishes from each dose were combined and reseeded at about 1.5×10^6 cells into each of two 150-mm dishes.

At the end of the expression period (seven days), each culture was reseeded at 2×10^5 cells per 100-mm dish (12 dishes total) in mutant selection medium. Also, three 60-mm dishes were seeded at 200 cells each in culture medium to determine the cloning efficiency of each culture. After incubation for seven to ten days, at 37° C ± 1° C in a humidified atmosphere with about 5% CO_2, the colonies were fixed with alcohol, stained with Giemsa, and counted to determine the number of TG-resistant colonies in mutant selection dishes and the number of colonies in the cloning efficiency dishes. The colonies were counted by eye, excluding those with approximately 50 cells or less.

Activation Mutagenicity Assay

The activation assay was performed independently with its own set of vehicle and positive controls. The procedure was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors during the 4-h treatment period. The fetal bovine serum content of the medium used for dosing was reduced to 5% by volume. The cofactors consisted of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt), glucose-6-phosphate, calcium chloride, potassium chloride, and magnesium chloride, all of which were in a pH 7.8 sodium phosphate buffer.

Data Presentation

The collected data were used to calculate several assay parameters. The chosen combination of raw data and calculated data allows a complete description of events for each treatment condition. The significance of each calculated parameter and its method of calculation are listed below.

Relative Survival to Treatment: This parameter gives the clonal cytotoxicity of each treatment by showing what percentage of the cells were able to form colonies after the treatment period in both the rangefinding cytotoxicity assays and the mutation assays relative to the concurrent vehicle controls. The average number of colonies in three dishes (seeded at 200 cells each) was determined for each treatment condition.

$$Relative\ Survival\,(\%) = \frac{Average\ no.\ of\ colonies\ per\ treated\ culture}{Average\ no.\ of\ colonies\ per\ vehicle\ control\ dish} \times 100\%$$

Relative Population Growth: This parameter shows the cumulative growth of the treated cell population, relative to the vehicle control growth, over the entire expression period and prior to mutant selection. In general, highly toxic treatments will reduce the growth rate as well as the survival.

Values less than 100% indicate growth inhibition. For example, 50% and 25% relative growth values would indicate treated cell populations that were one and two population doublings behind the negative control culture. Treated populations that are more than two or three doublings behind the control might not achieve maximum expression of the TG-resistant phenotype. The relative population growth is calculated from cell count data not presented in this report and is intended to provide only an approximate indication of growth during the expression period, since cells are easily lost or not completely released by trypsin during the subculture procedures.

$$Relative\ Population\ Growth\ (\%) = \frac{Treated\ culture\ population\ increase\ over\ the\ expression\ period}{Vehicle\ control\ population\ increase\ over\ the\ expression\ period} \times 100\%$$

Absolute Cloning Efficiency: The ability of the cells to form colonies at the time of mutant selection is measured by the absolute cloning efficiency (CE). This parameter is used as the best estimate of the CE of the mutant cells in the selection dishes. Thus, the observed number of mutant colonies can be converted to the frequency of mutant cells in the treated population.

$$Absolute\ CE\ (\%) = \frac{Average\ no.\ of\ viable\ colonies\ per\ dish}{200} \times 100\%$$

Mutant Frequency: The mutant frequency is the endpoint of the assay. It is calculated as the ratio of colonies found in thioguanine-selection medium to the total number of cells seeded and adjusted by the absolute CE. The frequency is expressed in units of 10^{-6}, e.g., the number of mutants per one million cells.

$$Mutant\ Frequency = \frac{Total\ mutant\ clones}{no.\ of\ dishes\ x\ 2\ x\ 105\ x\ abs.\ CE} \times 100\%$$

Assay Acceptance Criteria

An assay normally is considered acceptable for evaluation of the results only if all of the following criteria are satisfied. The activation and nonactivation portions of the mutation assay may be performed concurrently, but each portion is, in fact, an independent assay with its own positive and vehicle controls. The activation or nonactivation assays will be repeated independently, as needed, to satisfy the acceptance and evaluation criteria.

- The average absolute cloning efficiency of the vehicle controls should be between 70% and 115%. A value greater than 100% is possible because of errors in cell counts (usually ± 10%) and dilutions during cloning. Cloning efficiencies below 70% do not necessarily indicate substandard culture conditions or unhealthy cells. Assay variables can lead to artificially low cloning efficiencies in the range of 50 to

70% and still yield internally consistent and valid results. Assays with cloning efficiencies in this range will be conditionally acceptable and dependent on the scientific judgment of the Study Director. All assays below 50% cloning efficiency will be unacceptable.

- The background mutant frequency (average of the vehicle controls) is calculated separately for the activation and nonactivation assays, even though the same population of cells may be used for concurrent assays. The activation vehicle controls contain the S9 activation mix and may have a slightly different mutant frequency than the nonactivation vehicle controls. For both conditions, background frequencies for assays performed with different cell stocks are generally 0 to 10×10^{-6}. Assays with backgrounds greater than 15×10^{-6} will not be used for evaluation of a test article.

- A positive control is included with each assay to provide confidence in the procedures used to detect mutagenic activity. An assay will be acceptable in the absence of a positive control (loss due to contamination or technical error) only if the test article clearly shows mutagenic activity as described in the evaluation criteria. If the test article appears to have no or only weak mutagenic activity, an acceptable assay must have a positive control mutant frequency that is significantly elevated over the concurrent vehicle controls ($p \leq 0.01$).

- For test articles with little or no mutagenic activity, an acceptable assay should include applied concentrations that reduce the clonal survival to approximately 10% to 15% of the average of the vehicle controls, reach the maximum applied concentrations given in the evaluation criteria, reach a concentration that is approximately twice the solubility limit of the test article in culture medium, or include a high concentration that is at least 75% of an excessively toxic concentration. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

- Mutant frequencies are normally derived from sets of 12 dishes for the mutant colony count and three dishes for the viable colony count. To allow for contamination losses, an acceptable mutant frequency for treated cultures can be calculated from a minimum of eight mutant selection dishes and two cloning efficiency dishes.

- The mutant frequencies for five treated cultures are normally determined in each assay. A required number of different concentrations cannot be explicitly stated, although a minimum of three analyzed cultures is considered necessary under the most favorable test conditions in order to accept a single assay for evaluation of the test article.

Assay Evaluation Criteria

Mutation assays are initiated by exposing cell cultures to about six to eight concentrations of test article that are expected, on the basis of preliminary toxicity studies, to span a range of cellular responses from no observed toxicity to about 10% survival. Five doses then are usually selected for completion of the mutation assay. These doses should cover a range of toxicities with emphasis placed on the most toxic doses. An assay may need to be repeated with different concentrations to properly evaluate a test article.

The statistical tables provided by Kastenbaum and Bowman (1970) are used to determine whether the results at each dose are significantly different from the negative controls at 95% or 99% confidence levels. This test compares variables distributed according to Poissonian expectations by summing up the probabilities in the tails of two binomial distributions. The 95% confidence level must be met as one criterion for considering the test article to be active at a particular dose. In addition, the mutant frequency must meet or exceed 15×10^{-6} in order to compensate for random fluctuations in the 0 to 10×10^{-6} background mutant frequencies that are typical for this assay.

Observation of a mutant frequency that meets the minimum criteria for a positive response in a single-treated culture within a range of assayed concentrations is not sufficient evidence to evaluate a test article as a mutagen. The following test results must be obtained to reach this conclusion for either activation or nonactivation conditions.

- A dose-related or toxicity-related increase in mutant frequency should be observed. It is desirable to obtain this relation for at least three doses. However, this depends on the concentration steps chosen for the assay and the toxicity at which mutagenic activity appears. If an increase in mutant frequency is observed for a single dose near the highest testable toxicity, as defined previously, and the number of mutant colonies is more than twice the value needed to indicate a significant response, the test article generally will be considered mutagenic. Smaller increases at a single dose near the highest testable toxicity will require confirmation by a repeat assay.

- For some test articles, the correlation between toxicity and applied concentration is poor. The proportion of the applied article that effectively interacts with the cells to cause genetic alterations is not always repeatable or under control. Conversely, measurable changes in the frequency of induced mutants may occur with concentration changes that cause only small changes in observable toxicity. Therefore, either parameter, applied concentration or toxicity (percent survival), can be used to establish whether the mutagenic activity is related to an increase in effective treatment.

A test article is evaluated as nonmutagenic in a single assay only if the minimum increase in mutant frequency is not observed for a range of applied concentrations that extends to concentrations causing about 10% to 15% survival or extends to a concentration at least 75% of that causing excessive toxicity. If the test article is relatively nontoxic, the maximum applied concentrations normally will be 5 mg/mL (or 5 µL/mL) for water-soluble materials or 1 mg/mL (or 1 µL/mL) for materials in organic solvents. If a repeat assay does not confirm an earlier, minimal response as discussed above, the test article is evaluated as nonmutagenic in this assay system.

This presentation may not encompass all test situations, and the Study Director may use other criteria to arrive at a conclusion, especially when data from several repeat assays are available. The interpretation of the results in the Results and Discussion section provides the reasoning involved when departures from the above descriptions occur.

RESULTS AND DISCUSSION

Test Material Handling

The test material was found to give a slightly cloudy emulsion in dimethylsulfoxide (DMSO) at about 100 mg/mL. The 100 mg/mL stock solutions were diluted 1:10 and 1:100 into culture medium. Immiscible globules of test material formed at 10 mg/mL and 1.0 mg/mL that settled to the bottom of the glass vial. Therefore, DMSO was not a suitable solvent for preparing treatment media.

In order to achieve as uniform an exposure as possible for cell monolayers treated with the test material in culture medium, Pluronic® F-68 was investigated as an emulsifying agent for the test material. The surfactant Pluronic® F-68, manufactured by BASF Wyandotte, was used to prepare emulsions of the sample for use as 10X primary stocks. The Pluronic® F-68 was prepared at 10% w/v in sterile deionized water followed by filtration through a 0.45 µm filter. The initial stock for each assay was prepared by adding together the desired weight of test material and volume of Pluronic® F-68 in sterile deionized water. Stable emulsions were produced after this preparation was homogenized using a Tissuemizer®.

Rangefinding Cytotoxicity Assay

The sample, CTFE, was tested in the preliminary rangefinding cytotoxicity assay with and without S9 metabolic activation. Ten doses were used in each case that ranged from 0.005 mg/mL to 5.0 mg/mL.

The rangefinding cytotoxicity assay showed that the test material was toxic to CHO cells in culture both with and without S9 metabolic activation (Tables 3-1 and 3-2). Without activation, the test material was nontoxic from 0.005 to 0.1 mg/mL, followed by increased toxicity from 0.2 to 2.0 mg/mL. Total cell killing was obtained at 5.0 mg/mL. With activation, no toxicity was observed from 0.005 to 0.5 mg/mL, followed by increased toxicity from 1.0 to the maximum applied dose of 5.0 mg/mL. At 5.0 mg/mL, 14.2% relative clonal survival was obtained. The results from the preliminary rangefinding cytotoxicity assays were used to select doses for the mutation assays. Treatment conditions chosen for the nonactivation portion of the mutation assay covered a 100-fold range from 0.05 to 5.0 mg/mL and for the activation portion of the mutation assay a 10-fold range from 0.1 to 5.0 mg/mL.

TABLE 3-1. CLONAL CYTOTOXICITY ASSAY WITHOUT METABOLIC ACTIVATION OF CTFE

Sample	Applied Concentration mg/mL	Average Colonies/ Dish[a]	Relative Survival[b] (Percent)	Cloning Efficiency (Percent)
NC[c]	--------	179.3	98.4	89.7
VC, 1%[d]	--------	182.3	100.0	91.2
CTFE	0.005	178.3	97.8	
CTFE	0.01	171.0	93.8	
CTFE	0.02	179.3	98.4	
CTFE	0.05	166.5[e]	91.3	
CTFE	0.1	171.0[e]	93.8	
CTFE	0.2	121.5[e]	66.6	
CTFE	0.5	14.7	8.1	
CTFE	1.0	8.3	4.6	
CTFE	2.0	3.0	1.6	
CTFE	5.0	0.0	0.0	

[a] n = 3
[b] Relative to 1% VC for all treatments.
[c] NC = Negative Control, F12 Medium.
[d] VC = Vehicle Control, percent given for Pluronic® F-68.
[e] n = 2

TABLE 3-2. CLONAL CYTOTOXICITY ASSAY WITH METABOLIC ACTIVATION OF CTFE

Sample	Applied Concentration mg/mL	Average Colonies/ Dish[a]	Relative Survival[b] (Percent)	Cloning Efficiency (Percent)
NC[c]	--------	155.0	94.7	77.5
VC, 1%[d]	--------	163.7	100.0	81.9
CTFE	0.005	168.0	102.6	
CTFE	0.01	153.0	93.5	
CTFE	0.02	155.3	94.9	
CTFE	0.05	170.3	104.0	
CTFE	0.1	185.3	113.2	
CTFE	0.2	170.0	103.8	
CTFE	0.5	151.3	92.4	
CTFE	1.0	117.7	71.9	
CTFE	2.0	64.7	39.5	
CTFE	5.0	23.3	14.2	

[a] n = 3
[b] Relative to 1% VC for all treatments.
[c] NC = Negative Control, F12 Medium.
[d] VC = Vehicle Control, percent given for Pluronic® F-68.

Mutation Assay Without Metabolic Activation

Two independent mutation assays were performed with the test material using nonactivation conditions (Tables 3-3 and 3-4). In each trial the test material produced dose-related toxicity. In the first trial, 10 doses were used that ranged from 0.05 to 5.0 mg/mL (Table 3-3). The culture treated with 5.0 mg/mL had <10% relative clonal survival and was terminated prior to plating for mutant selection. Two nontoxic doses, 0.05 mg/mL and 0.4 mg/mL, were not plated for mutant selection due to a sufficient number of nontoxic doses available for analysis. The remaining seven doses showed a

TABLE 3-3. MUTATION ASSAY WITHOUT METABOLIC ACTIVATION OF CTFE – TRIAL 1

Nonactivation Test Condition	Survival To Treatment (% Vehicle Control)	Relative Population Growth (% of Control)	Total Mutant Colonies	Absolute CE ± SD (%)	Mutant Freq in 10^{-6} Units[a]
Vehicle Control[b]	96.8	109.3	21	96.2 ± 14.8	9.1
Vehicle Control[b]	103.2	90.5	12	97.7 ± 6.7	5.1
Positive Control (50 µg/mL BrdU)[c]	69.1	40.5	242	88.9 ± 5.1	113.4[d]
CTFE (mg/mL)					
0.05	101.1	NS[e]	-	-	-
0.1	100.7	70.0	42	96.9 ± 9.4	18.1[d]
0.2	92.6	81.9	4	95.4 ± 1.3	1.7
0.3	89.8	87.6	9	104.2 ± 5.5	3.6
0.4	80.8	NS	-	-	-
0.5	70.6	81.5	12	109.5 ± 4.3	4.6
0.75	57.4	45.8	12	88.2 ± 3.1	5.7
1.0	63.1	47.0	12	99.5 ± 5.2	5.0
2.0	37.0	27.0	62	94.4 ± 8.6	27.4[d]
5.0	6.5	T[f]	-	-	-

[a] Mutant Frequency = Total mutant colonies/(No. of dishes x 2 x 10^5 x absolute CE).
[b] Vehicle Control = 1% Pluronic® F-68.
[c] BrdU = 5-Bromo-2'-deoxyuridine.
[d] Significant increase: Kastenbaum-Bowman test $p \leq 0.01$ and mutant frequency r 15 x 10-6.
[e] NS = Not plated for selection due to sufficient surviving higher dose levels.
[f] T = Terminated due to excessive toxicity.

TABLE 3-4. MUTATION ASSAY WITHOUT METABOLIC ACTIVATION OF CTFE – TRIAL 2

Nonactivation Test Condition	Survival To Treatment (% Vehicle Control)	Relative Population Growth (% of Control)	Total Mutant Colonies	Absolute CE ± SD (%)	Mutant Freq in 10^{-6} Units[a]
Vehicle Control[b]	101.3	108.6	9	80.5 ± 5.3	4.7
Vehicle Control[b]	98.8	91.4	20	78.0 ± 4.8	10.7
Positive Control (50 µg/mL BrdU)[c]	73.3	51.1	146	73.5 ± 1.3	82.8[d]
CTFE (mg/mL)					
0.1	87.5	160.1	17	79.4 ± 1.6	8.9
1.0	56.2	48.3	6	85.7 ± 2.5	2.9
2.0	27.0	29.2	6	81.0 ± 4.8	3.1
3.0	18.1	18.7	2	92.2 ± 8.3	0.9
4.0	5.2	8.2	5	82.0 ± 10.5	2.5
5.0	3.8	4.1	36	78.9 ± 5.0	19.0[d]

[a] Mutant Frequency = Total mutant colonies/(No. of dishes x 2 x 10^5 x absolute CE).
[b] Vehicle Control = 1% Pluronic® F-68.
[c] BrdU = 5-Bromo-2'-deoxyuridine.
[d] Significant increase: Kastenbaum-Bowman test $p \leq 0.01$ and mutant frequency $\geq$ 15 x 10-6.

clonal survival range relative to the concurrent vehicle controls of 100.7% to 37.0%. As shown in Table 3-3, mutant frequencies of treated cultures were within the acceptable range for background mutant frequency variation of between 0 and 15×10^{-6} with the exception of the cultures treated with 0.1 mg/mL and 2.0 mg/mL. Those cultures had mutant frequencies of 18.1×10^{-6} and 27.4×10^{-6}, respectively. The mutant frequencies of those two cultures were also statistically elevated over the mutant frequencies of the concurrent vehicle control cultures. The mutant frequencies of the other cultures varied randomly with dose and no other culture had a significantly elevated mutant frequency. Assay evaluation criteria required a second mutation assay to properly evaluate the elevated mutant frequency at the highest acceptable dose.

The second nonactivation mutation assay used a modified dose range to focus on the toxic range where significance occurred in the first trial. Six doses were used that ranged from 0.1 to 5.0 mg/mL (Table 3-4). The six doses analyzed had relative survivals that ranged from 87.5% at 0.1 mg/mL to 3.8% for the culture at 5.0 mg/mL. The two highest doses had relative clonal survivals of $<10\%$. Cultures with $<10\%$ relative clonal survival are not used as primary evidence in the evaluation of a compound but may be used as supporting evidence to confirm a response observed in another trial. The mutant frequencies of all the treated cultures varied randomly with dose and were within the range for acceptable background mutant frequencies with the exception of the culture treated with the highest concentration, 5.0 mg/mL. This culture had a mutant frequency of 19.0×10^{-6} that was significantly elevated over the mutant frequencies of the concurrent vehicle controls.

Two independent trials had toxic doses with significantly elevated mutant frequencies. While toxic doses did show a significant increase in mutant frequency in both trials, there was no agreement between the two trials as to the level of toxicity required for a response. Neither trial alone had a sufficient response for a positive evaluation as defined by the assay evaluation criteria. However, because two independent trials showed a significant increase in mutant frequency at toxic treatment doses, CTFE was evaluated as weakly positive for inducing forward mutations at the HGPRT locus in CHO cells under the nonactivation conditions used in the study.

The positive control treatment with 50 µg/mL 5-bromo-2'-deoxyuridine induced a large, significant ($p \leq 0.01$) increase in mutant frequency. The background mutant frequencies of the two vehicle controls were acceptable. The assay results achieved all assay acceptance criteria, which provided confidence in the assumption that the recorded data represented a typical response of the test material in the nonactivation assay system.

Mutation Assay With Metabolic Activation

Under S9 metabolic activation test conditions, the cultures treated with CTFE showed a dose-related decrease in both relative survival and relative population growth (Table 3-5). All eight

doses survived treatment. An intermediate dose, 1.5 mg/mL, was not plated for mutant selection due to a sufficient number of nontoxic doses available for analysis. Seven doses were plated for mutant selection and were available for analysis.

TABLE 3-5. MUTATION ASSAY WITH METABOLIC ACTIVATION OF CTFE – TRIAL 1

Activation Test Condition	Survival To Treatment (% Vehicle Control)	Relative Population Growth (% of Control)	Total Mutant Colonies	Absolute CE ± SD (%)	Mutant Freq in 10^{-6} Units[a]
Vehicle Control[b]	99.8	84.9	22	93.5 ± 8.3	9.8
Vehicle Control[b]	100.2	15.9	4	92.9 ± 3.3	1.8
Positive Control (5 µg/mL 3-MCA)[c]	97.4	43.7	760	94.0 ± 7.9	336.9[d]
CTFE (mg/mL)					
0.1	97.0	72.4	28	101.8 ± 2.5	11.5[e]
0.5	111.8	66.0	17	90.9 ± 6.4	7.8
1.0	89.9	59.9	15	94.9 ± 3.3	6.6
1.5	81.6	NS[f]	-	-	-
2.0	73.3	56.6	25	91.5 ± 4.5	11.4[g]
3.0	76.3	56.1	14	102.3 ± 3.9	5.7
4.0	51.2	30.2	26	105.5 ± 2.6	10.3[g]
5.0	48.6	26.4	1	107.0 ± 2.2	0.4

[a] Mutant Frequency = Total mutant colonies/(No. of dishes x 2 x 10^5 x absolute CE.)
[b] Vehicle Control = 1% Pluronic® F-68.
[c] 3-MCA = 3-Methylcholanthrene.
[d] Significant increase: Kastenbaum-Bowman test $p \leq 0.01$ and mutant frequency $\geq 15 \times 10^{-6}$.
[e] Kastenbaum-Bowman test $p \leq 0.01$ but mutant frequency is within acceptable background range ($< 15.0 \times 10^{-6}$).
[f] NS = Not plated for selection due to sufficient surviving higher dose levels.
[g] Kastenbaum-Bowman test $p \leq 0.05$ but mutant frequency is within acceptable background range ($< 15.0 \times 10^{-6}$).

With S9 metabolic activation, the mutant frequency of cultures treated with the test material varied within the acceptable range of vehicle control mutant frequency variation which is 0 to 15×10^{-6}. There was no positive correlation of mutant frequency with dose and no treated culture had a mutant frequency that was both significantly elevated over the average background mutant frequency of the concurrent vehicle controls and higher than the acceptable range of background mutant frequencies. Three random cultures with mutant frequencies less than 15×10^{-6} were significantly elevated over the mutant frequencies of the concurrent vehicle controls. The mutant frequency of these cultures appeared to represent normal assay variation. Therefore, CTFE was evaluated as negative for inducing forward mutations at the HGPRT locus in CHO cells in the presence of S9 metabolic activation.

The positive control treatment with 5 µg/mL 3-MCA induced a large, significant ($p \leq 0.01$) increase in mutant frequency which demonstrated the effectiveness of the S9 metabolic activation system and the ability of the test system to detect known mutagens. The background mutant frequencies of the 1% Pluronic® F-68 vehicle controls were within the acceptable background range and comparable to the historical activation control data (see Appendix 3-A). The assay results

achieved all assay acceptance criteria and provided confidence in the assumption that the recorded data represented typical responses of the test material in the assay system.

CONCLUSIONS

The test material, CTFE, is considered weakly positive for inducing forward mutations at the HGPRT locus in Chinese hamster ovary cells under nonactivation conditions of the assay. With S9 metabolic activation, the test material was evaluated as negative in the same assay.

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APPENDIX 3-A

HISTORICAL CHO/HGPRT ASSAY CONTROL MUTANT FREQUENCY DATA

A. Nonactivation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$3.9 \pm 2.9 \times 10^{-6}$
Range	$0 \text{ to } 16.8 \times 10^{-6}$
Number of experiments	50
Number of controls	88

2. Positive controls (50 µg/mL 5-bromo-2'-deoxyuridine)

Mean (± SD)	$121.6 \pm 27.9 \times 10^{-6}$
Range	$38.7 \text{ to } 165.6 \times 10^{-6}$
Number of experiments	50
Number of controls	59

B. Activation Studies

1. Pooled negative and solvent controls

Mean (± SD)	$2.9 \pm 2.1 \times 10^{-6}$
Range	$0 \text{ to } 10.0 \times 10^{-6}$
Number of experiments	50
Number of controls	86

2. Positive controls (5 µg/mL 3-methylcholanthrene)

Mean (± SD)	$370.0 \pm 173.3 \times 10^{-6}$
Range	$152.3 \text{ to } 941.6 \times 10^{-6}$
Number of experiments	50
Number of controls	61

The historical control data was compiled from the most recent 50 experiments. Because some experiments contained duplicate controls, the number of independent control cultures exceeded the number of experiments.

SECTION 4

MUTAGENICITY TEST ON CHLOROTRIFLUOROETHYLENE OLIGOMERS IN AN *IN VITRO* CYTOGENETIC ASSAY MEASURING SISTER CHROMATID EXCHANGE AND CHROMOSOMAL ABERRATION FREQUENCIES IN CHINESE HAMSTER OVARY CELLS

Murli, H.[a]

ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of chlorotrifluoroethylene (CTFE) oligomers to induce sister chromatid exchange (SCE) and chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation. Single cultures of CHO cells were incubated in the SCE assays with 0.167 to 5000 µg/mL of the test material in a half-log series. Complete cellular toxicity was observed at 500, 1670, and 5000 µg/mL in the nonactivation assay and at 5000 µg/mL in the activation assay. No significant increase in SCE was observed at the concentrations analyzed (except for a weak response at 500 µg/mL in the activation assay).

Based on the evaluation of cell-cycle kinetics from the SCE assay, 10-h harvests were selected for the aberrations assays. Duplicate cultures of CHO cells were incubated with 20.0 to 300 µg/mL of the test material in the nonactivation aberrations assay and with 200 to 2000 µg/mL in the aberrations assay with activation. No increase in chromosomally aberrant cells was observed at the concentrations analyzed under conditions of nonactivation and activation.

The test article, CTFE, is considered negative for inducing SCE and for inducing chromosomal aberrations in CHO cells under both nonactivation and activation conditions of this assay.

INTRODUCTION

Sister chromatid exchanges (SCE) are seen at metaphase as reciprocal interchanges of the two chromatid arms within a single chromosome. These exchanges presumably require enzymatic incision, translocation, and ligation of the two DNA strands. The frequency of SCE is thought to be a very sensitive indicator of damage to the genetic material, DNA. Increases in the frequency of SCE are caused by many chemical agents known to be mutagens/carcinogens. Thus SCE test is a relevant cytogenetic test for potentially genotoxic chemicals.

The SCE test involves treating cultured cells with a test compound, growing cells with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) for ~2 cell cycles, and making chromosome preparations that are stained for SCE. The chromosomes of dividing cells consist of two identical

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halves, or sister chromatids. By growing cells with BrdU for two cell cycles one chromatid contains half as much BrdU as the other and is stained more intensely by Giemsa, while its pair, or sister, is pale.

This assay also is designed to establish whether the test article or its metabolites can interact with cells to induce chromosomal breaks. Chemically induced lesions may result in breaks in chromatin that are either repaired by the cell in such a way as to be undetectable or result in visible damage. Aberrations are a sequence of failure or mistakes in repair processes such that breaks do not rejoin or rejoin in abnormal configurations (Evans, 1962).

The chromosomal aberrations assay is designed to examine cells in the first mitosis after chemical exposure. This design limits loss of aberrant cells during the division process or conversion into complex derivatives during subsequent cell cycles. In the case of Chinese hamster ovary (CHO) cells most dividing cells examined 8 to 12 h after treatment are in the first mitosis (M_1 cells). However, many test articles cause severe delay of progression through the cell cycle, and the assay has been designed to detect this delay and allow for slower growth of damaged cells by adjustments in the time between treatment and cell fixation.

The objective of this *in vitro* assay was to evaluate the ability of CTFE to induce sister chromatid exchange and chromosomal aberrations in CHO cells, with and without metabolic activation.

EXPERIMENTAL DESIGN

In the SCE assay, CHO cell cultures which were exposed to the test article for approximately two cell cycles were analyzed to determine cellular toxicity and effects of the test article on cell generation time. If necessary and possible, the assay was extended in cultures at affected doses to allow for the progression to second generation cells. The doses used in the assay ranged from 0.167 µg/mL of the test article solution through 5000 µg/mL in a half-log series. Single cultures were used for the negative control, solvent control, each of two doses of the positive control, and 10 doses of the test material. Sister chromatid exchange frequencies were analyzed from cultures treated with the four highest doses with second generation cells and from a negative, solvent, and positive control culture. Cell-cycle kinetics of the treated cultures also were evaluated.

Summary of SCE Assay Treatment Schedule in Hours

Test	Test Article	Wash	BrdUrd	Wash	Colcemid®	Fixation
-S9	-2.25		0	22.75	23	25.5
+S9	-2.25	-0.25	0		23	25.5

Cell-cycle kinetics from the SCE assay were used to determine the dose range to be used in the chromosomal aberrations assay, and to determine the optimal time of harvest of the dosed cells so

that primarily metaphase cells which were in the first metaphase since exposure to the test article would be analyzed for chromosomal aberrations. The aberrations assay was conducted at the 10-h harvest time for those chemicals which did not induce any cell-cycle delay and at the 20-h harvest time for those chemicals that induced cell-cycle delay.

In the chromosomal aberration assays duplicate cultures were used at each dose. Single cultures were used for the negative control, solvent control, and at each of two doses of the positive control. In the nonactivation and the activation assays 10-h harvests were conducted. Chromosomal aberrations were analyzed from the four highest doses from which results could be obtained and from only one of the positive control doses. A summary of the treatment schedule for the chromosomal aberrations assays is given below.

Summary of Chromosomal Aberrations Assay Treatment Schedule in Hours

Test	Test Article	Wash	Colcemid®	Fixation
-S9	0	7.25	7.5	10
+S9	0	2	7.5	10

MATERIALS AND METHODS

Test Material

The CTFE used in these studies was provided by the U.S. Air Force. The clear, colorless liquid was further identified as Batch # N.87-23, Air Force #ML087-347.

Indicator Cells

The Chinese hamster ovary cells (CHO-WBL) used in this assay were from a permanent cell line and were originally obtained from the laboratory of Dr. S. Wolff, University of California, San Francisco. The cells have since been recloned to maintain karyotypic stability. This cell line has an average cycle time of 12 to 14 h with a modal chromosome number of 21.

Cell Culture Medium

The CHO cells were grown in McCoy's 5a culture medium which was supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% penicillin and streptomycin, at about 37°C, in an atmosphere of about 5% CO_2 in air.

Negative and Solvent Controls

In the nonactivation assays, negative controls were cultures which contain only cells and culture medium. Solvent controls were cultures containing the solvent for the test article at the same

concentration used in test cultures. In the activation assays, the negative and solvent controls were as in the nonactivation assays but the S9 activation mix also was added.

Positive Control Agents

The positive control agents which were used in the assays were mitomycin C (MMC) for the nonactivation series and cyclophosphamide (CP) in the metabolic activation series. Mitomycin C is a clastogen that does not require metabolic activation. Cyclophosphamide does not act directly but must be converted to active intermediates by microsomal enzymes. In the SCE assay two doses of MMC (0.005 and 0.010 µg/mL) and CP (1.5 and 2.0 µg/mL) were used. In the chromosomal aberrations assays two concentrations of MMC (0.500 and 1.00 µg/mL) and CP (25.0 and 50.0 µg/mL) were used to induce chromosomal aberrations in the CHO cells. Only one dose of the positive control was actually analyzed in each of the SCE and aberration assays.

Sister Chromatid Exchange Assays

In these assays, the cells were cultured for approximately 24 h prior to treatment by seeding approximately 1.0×10^6 cells per 75 cm^2 flask into 10 mL of complete McCoy's 5a culture medium. The thymidine analog, BrdU, was added at a final concentration of 10 µM approximately two hours after the initial exposure of the cells to the test article.

Nonactivation Assay: The cultures were dosed with the test article for ~2.25 h when BrdU was added at a final concentration of 10 µM. The cultures then were reincubated for approximately 23 h. Approximately 2.75 h prior to the harvest of the cells, the test article was washed from the cells with phosphate buffered saline and fresh complete medium with BrdU (10 µM), and Colcemid® (final concentration 0.1 µg/mL) was added.

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures also were evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. Only flasks from the highest seven (nonactivation assay) and six (activation assay) surviving doses from which metaphase cells for analysis were expected were harvested (See Section on Harvest). Medium was collected and the cultures centrifuged. The mitotic cells were fixed but medium was replaced on the remaining cell monolayer in the flasks. A test slide was made from fixed cells treated with the highest doses of test compound, and stained with Hoechst 33258 stain (0.5 µg/mL in phosphate buffer, pH 6.8). The slides were examined under ultraviolet fluorescence microscopy. Since there was no marked cell-cycle delay, a second cell collection was not made. The harvested cells were differentially stained for the analysis of SCE using a modified

fluorescence-plus-Giemsa (FPG) technique (See Sections on Harvest and Slide Preparation and staining).

Assay with Metabolic Activation: In this assay, the CHO cells were exposed to the test article for two hours in the presence of a rat liver S9 reaction mixture (S9 15 µL/mL, NADP 1.5 mg/mL, and isocitric acid 2.7 mg/mL). The S9 fraction was derived from the liver of male Sprague-Dawley rats which had been treated previously with Aroclor 1254 to induce the mixed function oxidase enzymes which are capable of metabolizing chemicals to more active forms. The 2-h incubation time was used because prolonged exposure to the S9 mixture might be toxic to the cells and the enzyme activity of S9 is lost rapidly at 37°C. The medium did not have FCS during the exposure period to avoid possible inactivation of short-lived and highly reactive intermediates produced by the S9 enzymes by binding to serum proteins.

In this assay, the CHO cells were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in the growth medium without FCS. After the exposure period, the cells were washed twice with buffered saline. Complete McCoy's 5a medium with 10 µM BrdU was added to the cultures which then were incubated for approximately 23 h. Colcemid® (final concentration 0.1 µg/mL) then was added and the cultures then were reincubated for 2.5 h, harvested, and examined for any cell delay. Slides were prepared and stained as described for the nonactivation assay. Delayed fixation was not required for any of the cultures that survived exposure to the test article.

Nonactivation Aberrations Assays

Cultures were initiated by seeding approximately 1.5×10^6 cells per 75 cm^2 flask into 10 mL of complete McCoy's 5a medium. One day after culture initiation, the CHO cells to be used in the nonactivation trial were treated with the test article at predetermined doses for 7.25 h. The cultures then were washed with buffered saline and complete McCoy's 5a medium containing 0.1 µg/mL Colcemid® was placed back onto the cells. Two and one-half hours later the cells were harvested and air-dried slides were made. The slides then were stained in pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations.

Aberrations Assays with Metabolic Activation

Cultures were initiated by seeding approximately 1.5×10^6 cells per 75 cm^2 flask into 10 mL of complete McCoy's 5a medium. One day after culture initiation, the cultures that were treated under the conditions of metabolic activation were incubated at 37°C for two hours in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FCS. After the 2-h exposure period the cells were washed twice with buffered saline and refed with complete McCoy's 5a medium. The cells were incubated for an additional 7.75 h with 0.1 µg/mL Colcemid® present during

the last 2.5 hours of incubation. The metaphase cells then were harvested and prepared for cytogenetic analysis.

Harvest Procedure

Prior to the harvest of the cultures visual observations of toxicity were made. These observations included an assessment of the percent confluence of the cell monolayer within the culture flasks. The cultures also were evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. The metaphase cells were collected by mitotic shakeoff (Terasima and Tolmach, 1961) and treated with 0.075 M KCl hypotonic solution. This treatment helps to swell the cells and thus disperse the chromosomes. The cultures then were fixed with an absolute methanol: glacial acetic acid (3:1) fixative and were washed several times before air-dried slides were prepared.

Slide Preparation and Staining

Slides were prepared by dropping the harvested cultures on clean slides. The slides from the rangefinding assays were differentially stained using a modified FPG technique (after Perry and Wolff, 1974; Goto et al., 1978). The slides were stained in Hoechst 33258 stain, exposed to ultraviolet light, and then stained with Giemsa Azure B stain. The slides prepared from the aberrations assay were stained with pH 6.8 buffered 5% Giemsa solution for the analysis of chromosomal aberrations. All slides then were air-dried and coverslipped using Depex® mounting medium.

SCE Analysis and Assay Evaluation

Fifty cells per dose were analyzed from each of the top four doses from which sufficient M_2 metaphase cells were available. Fifty cells were read from each of the negative and solvent controls, and at least 20 cells were read from one of the positive control doses. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells were selected for scoring on the basis of good morphology and clear sister chromatid differentiation along the entire length of all chromosomes; only cells with the number of centromeres equal to the modal number 21 ± 2 (range of 19 to 23) were analyzed.

The slides were examined for the presence of delayed cells. One hundred metaphase cells were scanned and classified as M_1, M_{1+}, or M_2 from each dose and the positive, negative, and solvent controls to give an estimate of cell-cycle inhibition. In those doses where more than one harvest was carried out, cells were analyzed for cell-cycle kinetics and SCE from the earliest harvest time from which sufficient M_2 cells were available for analysis. Controls were analyzed only at the normal harvest time (25 to 26 hours).

Data were collected on standard forms. The data were summarized in tables showing the numbers of cells scored, total SCE, SCE per chromosome, and SCE per cell. The cell-cycle kinetics also were calculated.

If an increase in SCE was observed, one of the following criteria must normally be met to assess the compound as positive.

- Two-fold increase: Approximately a doubling in SCE frequency over the "background" (solvent and negative control) levels at one or more doses.
- Dose response: A positive assessment may be made in the absence of a doubling if there was a statistically significant increase at a minimum of three doses and evidence for a positive dose response.

In some cases, statistically significant increases were observed with neither a doubling nor a dose response. These results were assessed according to repeatability, the magnitude of the response, and the proportion of the doses affected.

Statistical analysis employed a Student t-test (Bancroft, 1957; Hollander and Wolfe, 1973) to compare SCE frequencies in the tested cultures with the negative and solvent controls. The final evaluation of the test article was based upon scientific judgment.

Aberrations Analysis and Assay Evaluation

Cells were selected for good morphology and only cells with the number of centromeres equal to the modal number 21 ± 2 (range 19 to 23) were analyzed.

One hundred cells, if possible, from each duplicate culture at four doses of the test article and from each of the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962). At least 25 cells were analyzed for chromosomal aberrations from one of the positive control cultures. For control of bias, all slides except for the positive controls were coded prior to analysis. Cells with aberrations were recorded on the data sheets by the microscope stage location.

The following factors were taken into account in the evaluation of the chromosomal aberrations data:

The overall chromosomal aberration frequencies.

The percentage of cells with any aberrations.

The percentage of cells with more than one aberration.

Any evidence for increasing amounts of damage with increasing dose, i.e., a positive dose response.

The estimated number of breaks involved in the production of the different types of aberrations which were observed, i.e., complex aberrations may have more significance than simple breaks.

Chromatid and isochromatid gaps, if observed, were noted in the raw data and were tabulated. They were not, however, considered in the evaluation of the ability of the test article to induce chromosomal aberrations since they may not represent true chromosomal breaks and may possibly be induced by toxicity.

A cell classified as "GT" is considered to contain 10 aberrations for statistical purposes but a ">" also is included in the tables for this classification to indicate that it is a minimum number.

Statistical analysis employed the Fisher's Exact Test with an adjustment for multiple comparisons (Sokal and Rohlf, 1981) to compare the percentage of cells with aberrations in each treatment group with the results from the pooled solvent and negative controls (the solvent and negative controls were evaluated statistically for similarity prior to the pooled evaluation). Test article significance was established where $p \leq 0.01$. All factors as stated previously were taken into account and the final evaluation of the test article was based upon scientific judgment.

RESULTS AND DISCUSSION

Solubility, Stability, and Dose Determination

Solubility was tested in 10% (w/v) solution of Pluronic® F-68 solution. Stable emulsions were produced after this solution was homogenized using a Tissuemizer®. A top stock of 50.0 mg/mL was selected for testing in these assays. Final concentrations were achieved by a 1:10 dilution of the 50.0 mg/mL stock solution or the serial dilutions prepared from this stock solution. A half-log series of concentrations from 0.167 µg/mL through 5010 µg/mL was tested in the SCE assay.

Sister Chromatid Exchange Assay Without Metabolic Activation

Prior to harvest, a precipitate was observed at 1670 and 5000 µg/mL. Dead cell monolayer with no visible mitotic cells was obtained at 500, 1670, and 5000 µg/mL. An extremely unhealthy cell monolayer, severe reduction in visible mitotic cells, floating debris, and ~50% reduction in cell monolayer confluence were observed at 167 µg/mL. Fluorescent examination of the prepared slides indicated no significant cell-cycle delay at 167 µg/mL. An unhealthy cell monolayer and reduction in visible mitotic cells were observed at 50.0 µg/mL. A slightly unhealthy cell monolayer and a slight reduction in visible mitotic cells were still observed at 16.7 µg/mL. Results were evaluated at 5.00, 16.7, 50.0, and 167 µg/mL (Table 4-1). No significant increase in SCE was observed at the concentrations analyzed. The sensitivity of the cell culture for induction of SCE is shown by the

increased frequency of SCE in the cells exposed to the positive control agent. The test article is considered negative for inducing SCE under conditions of nonactivation.

Sister Chromatid Exchange Assay With Metabolic Activation

A precipitate was observed before harvest at 1670 and 5000 µg/mL. A dead cell monolayer with no visible mitotic cells was observed at 5000 µg/mL. At 1670 µg/mL, there were floating dead cells and debris, severe reduction in visible mitotic cells and ~60% reduction in visible mitotic cells. Fluorescent examination of the prepared slides indicated no significant cell-cycle delay at 1670 µg/mL. No toxicity was discernible at the subsequent concentration of 500 µg/mL. Results were evaluated at 50.0, 167, 500, and 1670 µg/mL (Table 4-2). A weakly positive increase in SCE was observed only at 500 µg/mL and not at the other dose levels analyzed. This increase was not substantiated at the higher dose level of 1670 µg/mL, and the increase at 500 µg/mL is not significantly greater than the results in the negative control culture. The successful activation of the metabolic system is illustrated by the increased frequency of SCE in the cells induced with the positive control agent. The test article is considered negative for inducing SCE under conditions of metabolic activation.

Chromosomal Aberrations Assay Without Metabolic Activation

Cell-cycle kinetics from the SCE assay indicated only a slight cell-cycle delay at 167 and 50.0 µg/mL. A 10-h harvest was selected for the aberrations assay testing a dose range of 20.0, 50.0, 100, 150, 200, 250, and 300 µg/mL. Unhealthy cell monolayer, floating dead cells and debris, severe reduction in visible mitotic cells, and ~30% reduction in cell monolayer confluence were observed at 300 µg/mL. No toxicity was discerned at the subsequent concentration of 250 µg/mL. Results were evaluated at 150, 200, 250, and 300 µg/mL (results pooled from the replicate cultures are in Table 4-3, and results from individual cultures are in Table 4-4). No significant increase in chromosomally aberrant cells was observed at the concentrations analyzed. The sensitivity of the cell culture for induction of chromosomal aberrations is shown by the increased frequency of aberrations in the cells exposed to the positive control agent. The test article is considered negative for inducing chromosomal aberrations under nonactivation conditions.

TABLE 4-1. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CTFE

Treatment	Dose µg/mL	Total Cells Scored	# of Chromo-somes	# of SCE	SCE/ Chromo-some	SCE/Cell Mean ± SE	Time in BrdU (hr)	Cell Cycle Stages (%) M_1	M_1 +	M_2	M_2 +	% SCE Increase Over Solvent	Confluence % Solvent Control
Controls													
Negative: McCoys 5a		50	1047	461	0.44	9.22 ± 0.44	25.5		0.5	84.5	15		
Solvent: 10% Pluronic®		50	1042	417	0.40	8.34 ± 0.39	25.5	0.5	9	88.5	2		100
Positive: MMC	0.005	20	415	608	1.47	30.40 ± 1.48*	25.5		2.5	97.5		266	100
CTFE	5.00	50	1046	430	0.41	8.60 ± 0.42	25.5	0.5	7	90.5	2	3	100
	16.7	50	1046	431	0.41	8.62 ± 0.34	25.5	0.5	9.5	90.0		3	88
	50.0	50	1042	456	0.44	9.12 ± 0.44	25.5	3.5	21.0	75.5		9	88
	167	50	1045	425	0.41	8.50 ± 0.37	25.5	12	24.5	63.5		2	50
	500**												25

* Significantly greater than the solvent control, $p<0.01$.
** Toxic dose level.

TABLE 4-2. SISTER CHROMATID EXCHANGE IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CTFE

Treatment	Dose µg/mL	Total Cells Scored	# of Chromo-somes	# of SCE	SCE/ Chromo-some	SCE/Cell Mean ± SE	Time *in* BrdU (hr)	Cell Cycle Stages (%) M_1	M_1 +	M_2	M_2 +	% SCE Increase Over Solvent	Confluence % Solvent Control
Controls													
Negative:													
McCoys 5a		50	1045	467	0.45	9.34 ± 0.45	25.5		3.0	92.0	5		
Solvent:													
10% Pluronic®		50	1042	401	0.38	8.02 ± 0.35	25.5		2.0	94.0	4		100
Positive:													
MMC	1.5	20	420	969	2.31	48.45 ± 1.73*	25.5		16.5	83.0	0.5	501	100
CTFE	50.0	50	1038	425	0.41	8.50 ± 0.41	25.5		7	86.5	6.5	7	100
	167	50	1047	420	0.40	8.40 ± 0.32	25.5	0.5	4.0	94.5	1.0	4	100
	500	50	1044	486	0.47	9.72 ± 0.41*	25.5	1.5	9.5	82.0	7.0	21	100
	1670	50	1047	470	0.45	9.40 ± 0.46	25.5	9.5	36.5	54.0		17	63
	5000**												25

* Significantly greater than the solvent control, $p<0.01$.
** Toxic dose level.

TABLE 4-3. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CTFE (RESULTS FROM DUPLICATE CULTURES)

			Number and Type of Aberration													No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberrations
			Not Computed			Simple			Complex									
		Cells Scored	TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
Controls																		
Negative and Solvent		200	12	1									1			0.01	0.5	0.0
Positive: MMC	1.00 µg/mL	25	7	5		1	3		1	2	5					0.48	24.0*	8.0*
Test Article:	150 µg/mL	200	16	2									1			0.01	0.5	0.0
	200 µg/mL	200	6	1			2									0.01	0.5	0.5
	250 µg/mL	200	17	1	1								2			0.01	1.0	0.0
	300 µg/mL	200	13										1			0.01	0.5	0.0

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

TABLE 4-4. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITHOUT METABOLIC ACTIVATION OF CTFE (RESULTS FROM INDIVIDUAL CULTURES)

			Number and Type of Aberration													No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberrations
			Not Computed			Simple			Complex									
		Cells Scored	TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
Controls																		
Negative:	McCoy's 5a	100	8	1									1			0.01	1.0	0.0
Solvent:	10% Pluronic® 100 ul/mL	100	4													0.00	0.0	0.0
Positive:	MMC 1.00 µg/mL	25	7	5		1	3		1	2	5*					0.48	24.0	8.0
Test Article:	150 µg/mL A	100	9	1												0.00	0.0	0.0
	B	100	7	1									1			0.01	1.0	0.0
	200 µg/mL A	100	1				2									0.02	1.0	1.0
	B	100	5	1												0.00	0.0	0.0
	250 µg/mL A	100	10										2			0.02	2.0	0.0
	B	100	7	1	1											0.00	0.0	0.0
	300 µg/mL A	100	9										1			0.01	1.0	0.0
	B	100	4													0.00	0.0	0.0

Chromosomal Aberrations Assay With Metabolic Activation

Cell-cycle kinetics from the SCE assay indicated only a slight cell-cycle delay at 1670 µg/mL and a 10-h harvest was selected for the aberrations assay testing concentrations of 200, 500, 1000, 1500, 2000, and 2500 µg/mL. An unhealthy cell monolayer, floating dead cells and debris, ~30% reduction in cell monolayer confluence, and a severe reduction in visible mitotic cells were observed at 2500 µg/mL. No toxicity was observed at the subsequent concentration of 2000 µg/mL. Results were evaluated at 1000, 1500, 2000, and 2500 µg/mL (results pooled from the replicate cultures are in Table 4-5, and results from individual cultures are in Table 4-6). No significant increase in chromosomally aberrant cells was observed at the doses analyzed. The successful activation of the metabolic system is illustrated by the increased incidence of chromosomally aberrant cells in the cultures induced with cyclophosphamide, the positive control agent. The test article is considered negative for inducing chromosomal aberrations under conditions of metabolic activation.

CONCLUSION

The test article, CTFE, is considered negative for inducing sister chromatid exchanges in CHO cells under both the metabolic activation and nonactivation conditions of this assay.

The test article, CTFE, is considered negative for inducing chromosomal aberrations in CHO cells under both the metabolic activation and nonactivation conditions of this assay.

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TABLE 4-5. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CTFE (RESULTS POOLED FROM DUPLICATE CULTURES)

			Number and Type of Aberration															
			Not Computed			Simple				Complex						No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberrations
		Cells Scored	TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
Controls																		
Negative and Solvent		200	7	1		1	1						1			0.02	1.5	0.0
Positive: CP	25.0 µg/mL	25	9	5			4				4					0.32	20.0*	4.0
Test Article:	1000 µg/mL	200	5	1						1						0.01	0.5	0.0
	1500 µg/mL	200	9	5									1			0.01	0.5	0.0
	2000 µg/mL	200	10	4	1		1			1			3			0.03	2.5	0.0
	2500 µg/mL	200	12	4			1									0.01	0.5	0.0

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

TABLE 4-6. CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS WITH METABOLIC ACTIVATION OF CTFE (RESULTS FROM INDIVIDUAL CULTURES)

			Number and Type of Aberration													No. of Aberrations Per Cell	% Cells with Aberrations	% Cells with >1 Aberrations
			Not Computed			Simple				Complex								
		Cells Scored	TG	SG	UC	TB	SB	DM	ID	TR	QR	CR	D	R	CI			
Controls																		
Negative:	McCoy's 5a	100	2			1										0.01	1.0	0.0
Solvent:	10% Pluronic® 100 ul/mL	100	5	1			1						1			0.02	2.0	0.0
Positive:	CP 25.0 µg/mL	25	9	5			4				4					0.32	20.0	4.0
Test Article:	1000 µg/mL A	100	2	1												0.00	0.0	0.0
	B	100	3							1						0.01	1.0	0.0
	1500 µg/mL A	100	3	1												0.00	0.0	0.0
	B	100	6	4									1			0.01	1.0	0.0
	2000 µg/mL A	100	5	3			1						2			0.03	3.0	0.0
	B	100	5	1	1					1			1			0.02	2.0	0.0
	2500 µg/mL A	100	7	1			1									0.01	1.0	0.0
	B	100	5	3												0.00	0.0	0.0

APPENDIX 4-A

DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA-STAINED CELLS NOT COMPUTED

NOT COMPUTED

TG	Chromatid Gap:	"tid gap" - An achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks.
SG	Chromosome Gap:	"isochromatid gap, IG" - Same as chromatid gap but at the same locus in both sister chromatids.
UC	Uncoiled Chromosome:	Failure of chromatin packing. Probably not a true aberration.
PP	Polyploid cell:	A cell containing multiple copies of the haploid number (n) of chromosomes. Only indexed if very common. Not counted in the cells scored for aberrations.
E	Endoreduplication:	4n cell in which separation of chromosome pairs has failed. Only indexed if very common. Not counted in the cells scored for aberrations.

SIMPLE

TB	Chromatid Break:	An achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced.
SB	Chromosome Break:	Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated. This classification now includes the acentric fragment (AF). The AF was different from the SB only in that it was not apparently related to any specific chromosome.
DM	"Double Minute" fragment:	These are small double dots, some of which are terminal deletions and some interstitial deletions and probably small rings. Their origins are not distinguishable.

COMPLEX

ID	Interstitial Deletion:	Length of chromatid "cut out" from midregion of a chromatid resulting in a small fragment or ring lying beside a shortened chromatid or a gap in the chromatid.
TR	Triradial:	An exchange between two chromosomes, or one chromosome and an acentric fragment, which results in a three-armed configuration.

QR	Quadriradial:	As triradial, but resulting in a four-armed configuration.
CR	Complex Rearrangement:	An exchange among more than two chromosomes or fragments which is the result of several breaks.
D	Dicentric:	An exchange between two chromosomes which results in a chromosome with two centromeres. This is often associated with an acentric fragment in which case it is classified as DF.
DF		Dicentric with fragment.
TC	Tricentric:	An exchange involving three chromosomes and resulting in a chromosome with three centromeres. Often associated with two to three AF. Such exchanges can involve many chromosomes and are named as follows.
QC	Quadricentric:	four centromeres, up to four AF
PC	Pentacentric:	five centromeres, up to five AF
HC	Hexacentric:	six centromeres, up to six AF
R	Ring:	A chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as RF.
RC	Ring Chromatid:	Single chromatid ring (acentric).
RF		Ring with associated acentric fragment.
CI	Chromosome Intrachange:	Exchange within a chromosome; e.g., a ring that does not include the entire chromosome.
T	Translocation:	Obvious transfer of material between two chromosomes resulting in two abnormal chromosomes. When identifiable, scored as "T" not "2Ab."
AB		Abnormal monocentric chromosome. This is a chromosome whose morphology is abnormal for the karyotype, and often the result of a translocation, pericentric inversion, etc. Classification used if abnormality cannot be ascribed to; e.g., a reciprocal translocation.
OTHER		
GT/>		A cell which contains more than 10 aberrations. A heavily damaged cell should be analyzed to identify the types of aberrations and may not actually have >10; e.g., multiple fragments such as those found associated with a tricentric.

SECTION 5

GENETIC TOXICITY TESTS ON CHLOROTRIFLUOROETHYLENE OLIGOMERS IN THE *IN VITRO* TRANSFORMATION OF BALB/c-3T3 CELLS ASSAY WITH AND WITHOUT METABOLIC ACTIVATION

Myhr, B.[a]

ABSTRACT

Chlorotrifluoroethylene (CTFE) oligomers was assayed for its ability to induce morphological cell transformation in BALB/c-3T3 cell cultures in the absence and presence of a rat liver S9 metabolic activation system. The material was applied as emulsions in Eagle's minimum essential medium culture medium containing 1% w/v Pluronic® F-68. The toxicity, determined from the clonal survival of ouabain-resistant cells in the presence of a wildtype monolayer, ranged from 95% to 4% survival for a dose range of 40 to 400 µg/mL without S9. The effect of the S9 activation system was to reduce the toxicity of CTFE. In two experiments with S9, treatments covering a dose range of 75 to 1000 µg/mL caused a survival range of 98% to 8%. The number of transformed foci in the treated cultures in the absence of S9 did not change from the corresponding negative (solvent) controls. With S9, a small, unexplainable, inverted dose response was observed in one trial that was not repeatable in the independent study. The results were evaluated as showing the test material to be negative for the induction of morphological transformation in BALB/c-3T3 cell cultures.

INTRODUCTION

BALB/c-3T3 mouse cells multiply in culture until a uniform monolayer is achieved and then cease further division (Kakunaga, 1973; Rundell[b], 1984). These nontransformed cells, if injected into immunosuppressed mice (1×10^7 cells/animal), do not produce neoplastic tumors (Kakunaga, 1973; Rundell, 1983; Rundell[a], 1984). However, BALB/c-3T3 cells treated *in vitro* with some chemical carcinogens give rise to foci of morphologically altered cells superimposed on the contact-inhibited cell monolayer. If foci picked from cell cultures are grown to larger cell numbers and are injected into immunosuppressed mice, a malignant tumor will be obtained in most cases. Thus, the appearance of foci of altered cells is correlated with malignant transformation.

The ability of BALB/c-3T3 to metabolize test articles from various chemical classes can be enhanced by the addition of an exogenous S9 metabolic activation system to the cultures during the treatment period. However, the standard treatment period of 72 hours is reduced to only four hours

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because of S9 toxicity and the degradation of the NADPH-dependent P450 enzyme system, so this assay modification may not always detect procarcinogens. The procarcinogen dimethylnitrosamine (DMN) does not transform BALB/c-3T3 cells in the absence of S9 (Rundell, 1983), but DMN treatments with the S9 activation system usually induce statistically significant increases in the frequency of transformed foci (Matthews et al.). Similarly, S9-dependent induction of transformed foci by DMN has been reported for another mouse line (Tu et al., 1984).

An appropriate dose range for toxic test articles is selected by a novel method for determining clonal survival under the mass culture conditions of the transformation assay. Normal BALB/c-3T3 cells are sensitive to the cell membrane poison, ouabain, and are quickly killed. However, a mutant BALB/c-3T3 cell line has been established in this laboratory that is ouabain-resistant but otherwise as sensitive to test articles as the parent (wildtype), ouabain-sensitive cells. When a few (for example, 250) ouabain-resistant cells are mixed with a large number of wildtype cells (for example, 3×10^4 cells), the clonal survival of the ouabain-resistant cells can be determined by the addition of 4 mM ouabain to the culture medium after the treatment period. In this manner, the test article toxicity is determined under the same cellular exposure conditions that will occur in the transformation assay mass cultures.

The objective of this assay was to evaluate a mixture of six and eight carbon oligomers of CTFE for its ability to induce the malignant transformation of cultured BALB/c-3T3 mouse cells in the absence and presence of a rat liver S9 metabolic activation system. Transformation is recognized as a dense, piling up of morphologically altered cells, called a focus, superimposed on a monolayer of contact-inhibited cells (Heidelberger et al., 1983; Rundell[a], 1984; Rundell[b], 1984).

MATERIALS AND METHODS

Indicator Cells

Clone 1-13 of BALB/c-3T3 mouse cells was obtained from Dr. T. Kakunaga (Kakunaga, 1973). A subclone of these cells with a low spontaneous frequency of transformants was used for the transformation assays in this study. Stocks of cells were maintained in liquid nitrogen and have been checked to ensure the absence of mycoplasma contamination. Laboratory cell cultures were grown in Eagle's minimum essential medium (EMEM) supplemented with fetal bovine serum (FBS), L-glutamine, and antibiotics.

Controls

Negative (Solvent) Control: The negative control (also referred to as the solvent control) was EMEM culture medium containing the emulsifier, Pluronic® F-68, at a concentration of 1% w/v. In addition, for the S9 activation assay, the S9 activation system was added to the medium.

Medium Control: A medium control consisting only of EMEM culture medium or medium containing the S9 activation system also was included with each assay. This control was used to detect any significant effects that might be caused by the 1% Pluronic® F-68 component.

Positive Controls: A known carcinogen, 3-methylcholanthrene (MCA), was used at 2.5 µg/mL as a positive control for the transformation of BALB/c-3T3 cells in the absence of S9. A second known carcinogen, DMN, was used as the positive control for the transformation of BALB/c-3T3 cells in the presence of the S9 activation system. Dimethylnitrosamine requires activation by S9 microsomal enzymes to become transforming, and two or more concentrations of DMN within the 0.5 to 4 µL/mL range were chosen to demonstrate a significant response for at least one concentration of DMN.

Test Material: The test material, CTFE, was a clear, colorless liquid. It was stored at room temperature in its original glass container in a chemical cabinet.

S9 Metabolic Activation System

S9: A 9000 x g supernatant fraction (Ames et al., 1984) prepared from the livers of Aroclor 1254-induced Sprague-Dawley male rats was purchased commercially and used for this study. The S9 was prescreened and selected for relatively low toxicity to BALB/c-3T3 cells and for the conversion of DMN to toxic substances. The concentration of S9 selected for the assay was 40 µL/mL, which corresponded to 1.5 mg S9 protein/mL in the treatment medium.

Core: The S9 activation system included a NADPH regenerating system (CORE) composed of NADP and isocitric acid in the presence of S9. The final concentrations of the CORE components in the treatment medium were approximately 236 µg/mL of NADP (sodium salt) and approximately 1552 µg/mL of isocitrate. The CORE solution was prepared fresh as a 10X stock solution in culture medium and then was combined with S9 to give a 5X S9 mix. The mix was held on ice until used in the assay.

EXPERIMENTAL DESIGN

Test Material Preparation

Initial studies with 3.1 Oil® showed that this material was immiscible with dimethylsulfoxide (DMSO) at approximately 100 mg/mL. After mixing to obtain a slightly cloudy emulsion, the suspension was diluted 1:10 and 1:100 into culture medium containing 10% serum. White insoluble material formed in both cases and settled upon standing. Dimethylsulfoxide therefore was considered to be ineffectual as a solvent for this study.

In order to achieve as uniform an exposure as possible for cell monolayers bathed in culture medium, Pluronic® F-68 was investigated as an emulsifying agent. This polyalcohol frequently is used in cell cultures due to its low toxicity and ability to lower surface tension. When a preparation of

50 mg/mL of 3.1 Oil® in 10% w/v Pluronic® F-68 in deionized water was agitated vigorously with a Tissuemizer®, a stable, milky-white emulsion was formed. The material remained dispersed into tiny droplets after diluting 1:10 into culture medium and did not coalesce, even though the dense droplets settled and collected on the bottom of the container. A brief mixing by swirling easily resuspended the droplets throughout the medium.

3.1 Oil® also was tested for reaction with polystyrene and polyester. A small amount of a 5 mg/mL emulsion in culture medium was applied to the surface of both types of plastic. After washing off the material after 30 minutes, no visible changes to the surfaces were observed. Thus, standard polystyrene cultureware was considered to be appropriate for the cell cultures in this study, but glass containers were used for the preparation of the test material emulsions and dilutions prior to treating the cells.

For both the dose rangefinding study and the transformation assays, 3.1 Oil® was prepared fresh as 50 mg/mL emulsions in either 10% w/v Pluronic® F-68 (rangefinding) or 5% w/v Pluronic® F-68 (assays). The Tissuemizer® was operated for 30 to 60 sec at a midrange setting (30 to 50 power setting or 13,500 rpm). Lower concentrations were prepared by dilutions into 5% w/v Pluronic® F-68. The emulsions were mixed well by vortex before preparing the treatment media. The treatment media were prepared by diluting the Pluronic® F-68 emulsions 1:5 into EMEM culture medium so that the Pluronic® F-68 was diluted to 1% w/v.

Preliminary Dose Rangefinding

Plastic culture flasks with approximately 25 cm^2 of surface area were seeded concurrently with approximately 250 ouabain-resistant 3T3 cells and 3×10^4 wildtype cells. The following day, three cultures were exposed to each of 15 dose levels with and without S9, starting at 5000 µg/mL and diluting in two-fold steps. Negative (solvent) and medium control cultures were prepared in triplicate for both (±S9) test conditions. After an exposure period of ~four hours in the presence of the S9 activation system or ~72 hours without S9, the cells were washed with a physiological solution and refed with EMEM culture medium containing 4 mM ouabain. The cultures were refed with 4 mM ouabain in EMEM culture medium 3 to 6 days later. The cultures were terminated 6 to 9 days after treatment, and the surviving colonies were fixed with methanol, stained with Giemsa, and counted manually.

A relative survival for each treatment condition was obtained by comparing the number of surviving colonies to the average colony count for the solvent control cultures. This survival information was used to select appropriate doses for the transformation assay that would span an anticipated survival range of 10% to 100%.

Transformation Assay

The transformation assay procedure was adapted from that reported by Kakunaga (1973). Culture flasks having a monolayer growth area of approximately 25 cm^2 were used. Each culture was seeded with approximately 3×10^4 cells for the transformation assay and 3×10^4 cells plus approximately 250 ouabain-resistant cells for the concurrent clonal survival assay. On the day after seeding, 18 cultures were exposed to each selected treatment with the test material, the positive controls, and the medium control. Thirty-six cultures were used as solvent control for each test condition ($\pm$ S9). Three mixed cultures of wildtype and ouabain-resistant cells were exposed to each treatment and control condition.

The treatments were conducted at 37 $\pm$ 1°C for ~four hours with the S9 activation system and ~72 hours without S9. The treatments were initiated by adding 1.0 mL of a 5X suspension of the test material or solution of positive control to each culture containing 4 mL of culture medium (with or without the S9 activation system). After treatment, all cultures were washed with Hanks' balanced salt solution. The transformation assay cultures were refed with fresh EMEM culture medium, and the incubation was continued for approximately four weeks with refeedin ; twice a week. The clonal survival cultures were refed with medium containing 4 mM ouabain. Th. cultures without S9 were refed with 4 mM ouabain four days later and were terminated nine days after treatment. The S9 activation cultures were refed with 4 mM ouabain three days after treatment and terminated 10 days after treatment.

The cultures were terminated by fixing the cells with methanol and staining with 10% Giemsa in tap water. Stained cultures were examined by eye and by microscope to determine the number of foci of transformed cells and the colony survival. The transformation assay cultures were coded with random numbers prior to evaluation for foci.

Evaluation of Transformed Foci

At the end of the incubation period, cultures of cells with a normal phenotype yield a uniformly stained monolayer of round, contact-inhibited cells. Transformed cells form a multilayered mass of cells, or focus, that stains deeply and is superimposed on the surrounding monolayer of cells (Kakunaga, 1973; Rundell[b], 1984). The foci are variable in size and exhibit several variations in morphological features. Many scored foci consist of a dense piling up of cells with a random, crisscross orientation of fibroblastic cells at the periphery of the focus and extensive invasiveness into the contiguous monolayer. Other scored foci are composed of more rounded cells with little crisscrossing at the periphery but with necrosis at the center caused by the dense piling up of a large number of cells. A third variation is a focus without the necrotic center and large number of cells but which exhibits the crisscross pattern of overlapping cells throughout most of the colony.

Some densely stained areas are not scored as transformed foci because the random orientation of fibroblastic cells is not observed. Microscopic examination is employed routinely for scoring and for the final judgment of the transformed character of each focus.

All foci that exhibited the transformed characteristics were scored. In the raw data, a record of focus size was maintained by scoring foci greater than 4 mm in diameter as + + + and those of 2- to 4-mm in diameter as + +. No significance currently is attached to this categorization. The sum of all scored foci (+ + + and + +) was reported for each culture and was used for the assay analysis.

Assay Acceptance Criteria

The clonal survival assay conducted simultaneously with the transformation assay was considered acceptable for evaluation of the test results by meeting the following three criteria.

1. The negative (solvent) control cultures must have macroscopically visible BALB/c-3T3 cell colonies representing a cloning efficiency of 15% or greater.

2. At least one of the test material treatments resulted in $\geq$ 50% cell survival.

3. A cytotoxic dose response was obtained for the test material treatments, unless the test material was nontoxic at 10 mg/mL or its solubility limit in culture medium was exceeded.

The transformation assay was considered acceptable for evaluation of test results by meeting the following five criteria.

1. Negative (solvent) control, positive control, and test material treatments resulted in contiguous monolayers of cells to be evaluated.

2. Negative control spontaneous frequencies of transformation did not exceed an average of approximately two foci per culture.

3. At least one of the positive control treatments resulted in an average number of foci per culture vessel that was significantly different from the negative control at the 99% confidence level ($p \leq 0.01$).

4. A minimum number of 12 culture vessels per test condition was available for analysis.

5. A minimum number of three treatment levels of the test material was available for analysis.

In addition, the cytotoxicity dose-related data from the preliminary and simultaneous clonal survival assays should be qualitatively similar over a comparable range of test chemical treatments.

Assay Evaluation Criteria

The appearance of transformed foci usually occurs as a general increase in foci for all cultures exposed to a transforming dose. However, large numbers of foci may appear at random in one or

more culture vessels in a treatment set, resulting in skewing of the mean number of foci in that set. This skewing could be caused by factors such as mechanical disruption and respreading of transformed foci cells or a culture-conditioning effect caused by the early appearance of a focus. The appearance of occasional dishes with numerous foci is a random process and occurs in both treated and control cell cultures. In our laboratory, we have utilized a $\log_{10}$ mathematical transformation to handle this non-normal distribution of BALB/c-3T3 cell transformed foci data (Rundell, 1983); however, other mathematical models also have been proposed (Whorton et al., 1982). After performing a $\log_{10}$ transformation of the data, Bailey's modification of the Student's t-test (Bailey, 1959) was used to evaluate positive control and test chemical treatment transforming activity for significant differences from the negative control. The possible spectrum of responses was routinely subdivided into three levels for the evaluation of each treatment.

Evaluation of Individual Treatments

Strong positive response	=	$p \leq 0.01$
Weak positive response	=	$p \leq 0.05$
Negative response	=	$p > 0.05$

The results of each treatment condition were evaluated in relation to the observed activities of model compounds, and scientific judgment was exercised in the evaluation of each test material. In general, a response at only one dose level attaining a 95% confidence level is not considered sufficient evidence for activity in this assay. However, responses at two or more treatment levels attaining the 95% confidence level and exhibiting evidence of a dose relationship are considered as evidence for transformation. Responses achieving the 99% confidence level for one or more test material treatments usually are considered sufficient for a positive evaluation.

RESULTS AND DISCUSSION

Clonal Survival

As shown in Table 5-1, CTFE was highly toxic or lethal to BALB/c-3T3 cells at 313 µg/mL and higher concentrations. Little or no toxicity was obtained at 39.1 µg/mL and lower concentrations. Thus, a dose range of 40 to 400 µg/mL covered the entire survival range appropriate for the transformation assay without S9. Six doses were chosen at appropriate increments over this range to help define the response curve.

TABLE 5-1. CYTOTOXIC ACTIVITY OF CTFE IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITHOUT S9 ACTIVATION

Treatment Condition	Colonies/Culture 1	2	3	Average Colonies/ Culture	Relative Cell Survival %
Solvent Control[a]	126	139	137	134.0	100.0
Medium Control[b]	122	130	132	128.0	95.5
CTFE (µg/mL)					
0.305	131	131	135	132.3	98.7
0.610	120	143	114	125.7	93.8
1.22	131	130	134	131.7	98.3
2.44	119	121	101	113.7	84.9
4.88	117	132	101	116.7	87.1
9.77	134	120	121	125.0	93.3
19.5	110	97	103	103.3	77.1
39.1	115	118	110	114.3	85.3
78.1	80	112	90	94.0	70.1
156	42	46	45	44.3	33.1
313	9	8	5	7.3	5.4
625	0	0	0	0	0
1250	0	0	0	0	0
2500	0	0	0	0	0
5000	0	0	0	0	0

[a] EMEM culture medium containing 1% Pluronic® F-68.
[b] EMEM culture medium without Pluronic® F-68.

The effect of adding the rat liver S9 metabolic activation system was to reduce the toxicity of CTFE (Table 5-2). The survival was 65.0% at 1250 µg/mL but then dropped sharply to no surviving colonies when the dose was doubled to 2500 µg/mL. These results were used to initiate a transformation assay with 10 doses ranging from 100 to 2000 µg/mL in an attempt to fully cover the toxicity curve. This assay was uninterpretable due to a high spontaneous transformation frequency, but the associated clonal survival results (data not shown) allowed a selection of six doses ranging from 125 to 1500 µg/mL for the repeat assays.

Transformation Assay Without S9

Table 5-3 summarizes the results obtained for CTFE in the transformation assay without S9. For treatments with 40 to 400 µg/mL (approximately 95% survival to 4% survival), the frequency of transformed foci remained essentially equivalent to the solvent control. The highest frequency was observed for the lowest and nontoxic dose, but the small increase was not significant. In contrast, the MCA positive control induced a significant increase in focus formation. Thus, no evidence was obtained for transforming activity by the test material without S9 in BALB/c-3T3 cell cultures.

TABLE 5-2. CYTOTOXIC ACTIVITY OF CTFE IN THE PRELIMINARY CLONAL SURVIVAL ASSAY WITH S9 ACTIVATION

Treatment Condition	Colonies/Culture 1	2	3	Average Colonies/ Culture	Relative Cell Survival %
Solvent Control[a]	116	112	101	109.7	100.0
Medium Control[b]	102	111	89	100.7	91.8
CTFE (µg/mL)					
0.305	97	85	137	106.3	96.9
0.610	115	122	80	105.7	96.4
1.22	110	133	94	112.3	102.4
2.44	99	87	99	95.0	86.6
4.88	97	94	93	94.7	86.3
9.77	75	94	50	73.0	66.5
19.5	119	106	97	107.3	97.8
39.1	C	104	101	102.5	93.4
78.1	116	94	96	102.0	93.0
156	93	71	70	78.0	71.1
313	91	80	80	83.7	76.3
625	49	38	71	52.7	48.0
1250	59	86	69	71.3	65.0
2500	0	0	0	0	0
5000	0	0	0	0	0

[a] EMEM culture medium containing 1% Pluronic® F-68.
[b] EMEM culture medium without Pluronic® F-68.
C = Contaminated.

TABLE 5-3. TRANSFORMING ACTIVITY OF CTFE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITHOUT S9 ACTIVATION

Treatment Condition	Average Colony Count[a]	Relative Cell Survival[b] (%)	Focus Data Total Cultures	Total Foci	Average Foci/ Culture	Transforming[c] Activity Mean Foci/ Culture
Solvent Control[d]	105.7	100.0	32	16	.50	0.33
Positive Control[e]	12.0	13.2	18	64	3.56	3.40*
Medium Control[f]	91.0	86.1	18	2	0.11	0.08
CTFE (µg/mL)						
40	100.0[g]	94.6	18	14	0.78	0.54
80	86.0	81.4	18	3	0.17	0.12
100	71.3	67.5	18	10	0.56	0.42
200	15.3	14.5	18	10	0.56	0.39
300	10.7	10.1	14	3	0.21	0.14
400	4.3	4.1	18	3	0.17	0.10

[a] Clonal survival assay performed concurrently with the transformation assay; average of three cultures.
[b] Colony survival relative to the solvent control, except for the positive control, which is relative to the medium control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68.
[e] Positive Control: The positive control treatment was 3-methylcholanthrene at 2.5 µg/mL.
[f] Medium Control: EMEM culture medium without Pluronic® F-68.
[g] Average of two cultures; one culture lost to contamination.
* $p \leq 0.01$

Transformation Assay with S9

Results were obtained for CTFE in two independent trials of the transformation assay with S9. These results are summarized in Tables 5-4 and 5-5. These two trials actually represented the third and fourth trials performed in this experimental study. The first two trials were not acceptable for evaluation and were not scored completely because the spontaneous transformation frequencies were excessively high at 3.5 and 7.9 foci/culture, respectively.

TABLE 5-4. TRANSFORMING ACTIVITY OF CTFE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITH S9 ACTIVATION – TRIAL 1

Treatment Condition	Average Colony Count[a]	Relative Cell Survival[b] (%)	Focus Data			Transforming[c] Activity
			Total Cultures	Total Foci	Average Foci/ Culture	Mean Foci/ Culture
Solvent Control[d]	367.0	100.0	36	40	1.11	0.81
Positive Control[e]						
DMN, 0.5 µg/mL	257.7	71.1	17	34	2.00	1.65*
DMN, 1.0 µg/mL	217.3	59.9	17	154	9.06	8.11**
DMN, 2.0 µg/mL	158.7	43.8	16	253	15.81	14.68**
Medium Control[f]	362.7	98.8	18	21	1.17	0.79
CTFE (µg/mL)						
125	337.3	91.9	18	57	3.17	2.35**
250	189.0	51.5	18	57	3.17	2.11*
500	115.7	31.5	18	40	2.22	1.48
750	86.7	23.6	18	36	2.00	1.62*
1000	29.0	7.9	18	28	1.56	1.24
1500	0.7	0.2	D	-	-	-

[a] Clonal survival assay performed concurrently with the transformation assay; average of three cultures.
[b] Colony survival relative to the solvent control, except for the positive controls, which are relative to the medium control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68.
[e] Positive Control: The positive control treatment was dimethylnitrosamine (DMN) at the concentrations shown.
[f] Medium Control: EMEM culture medium without Pluronic® F-68.
D = Discontinued due to excessive toxicity.
* $p \leq 0.05$
** $p \leq 0.01$

In the first trial, small but statistically significant increases in the transformation frequency were observed in the treated cultures (Table 5-4). However, an inverted response pattern for transformed foci with respect to the dose and clonal survival did not indicate the presence of transforming activity. The highest response occurred for the low dose of 125 µg/mL, which was essentially nontoxic at 91.9% survival. Transforming activity under such high survival conditions would be very unusual. As the dose was increased, the arithmetic transformation frequency declined, despite the increased interaction with the cells as shown by the declining survival. Treatment with 1500 µg/mL was excessively toxic and the transformation cultures were terminated. In the second trial, no significant increases in foci were obtained for seven doses in the 75 to 1250 µg/mL

concentration range (Table 5-5). The survivals to the treatments followed the same toxicity curve as established in the first trial. The treatment with 1250 µg/mL was highly toxic, however, and only nine of the 18 cultures recovered sufficiently to allow the scoring of foci on a background monolayer of normal cells. Because fewer than 12 cultures survived, this dose was not included in the formal evaluation. Nevertheless, no evidence was obtained for transforming activity over a wide toxicity range, and the unexplained, inverse response obtained in Trial 1 was not repeatable.

In contrast to the test material, the DMN positive control treatments clearly demonstrated dose-related increases in transformation frequency in both trials. Thus, the S9 metabolic activation system was active under the conditions of the assays, and the cells were responsive to transforming treatments in the presence of S9.

TABLE 5-5. TRANSFORMING ACTIVITY OF CTFE ASSESSED IN THE TRANSFORMATION ASSAY USING BALB/c-3T3 CELLS WITH S9 ACTIVATION – TRIAL 2

Treatment Condition	Average Colony Count[a]	Relative Cell Survival[b] (%)	Focus Data			Transforming[c] Activity
			Total Cultures	Total Foci	Average Foci/ Culture	Mean Foci/ Culture
Solvent Control[d]	127.7	100.0	36	3	0.08	0.06
Positive Control[e]						
DMN, 1.0 µL/mL	62.0	48.3	18	12	0.67	0.56*
DMN, 2.0 µL/mL	40.7	31.7	18	38	2.11	1.39*
Medium Control[f]	128.3	100.5	18	3	0.17	0.12
CTFE (µg/mL)						
75	122.0	95.5	18	3	0.17	0.10
125	125.3	98.1	18	3	0.17	0.12
250	120.0	94.0	18	0	0	0
500	70.7	55.4	18	1	0.06	0.04
750	34.3	26.9	18	4	0.22	0.17
1000	10.3	8.1	18	0	0	0
1250	8.0	6.3	9	3	0.33	0.26

[a] Clonal survival assay performed concurrently with the transformation assay; average of three cultures.
[b] Colony survival relative to the solvent control, except for the positive controls, which are relative to the medium control.
[c] The mean transforming activity is expressed as the anti-log of the $\log_{10}$ mean minus one.
[d] Solvent Control: EMEM culture medium containing 1% Pluronic® F-68.
[e] Positive Control: The positive control treatment was dimethylnitrosamine (DMN) at the concentrations shown.
[f] Medium control: EMEM culture medium without Pluronic® F-68.
* $p \leq 0.01$

CONCLUSION

The test material, CTFE, was evaluated using doses ranging from 40 to 400 µg/mL without S9 and from 75 to 1000 µg/mL inclusive for two trials with S9 activation. Both dose ranges covered wide survival ranges. No increases in transformed foci were obtained without S9. Small increases obtained in an inverted dose-response pattern with S9 in the first trial were not observed in the second trial

with S9. These results were evaluated as showing the test material to be negative for transforming BALB/c-3T3 cells in culture.

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SECTION 6

MUTAGENICITY TEST ON CHLOROTRIFLUOROETHYLENE OLIGOMER IN THE *IN VIVO/IN VITRO* RAT PRIMARY HEPATOCYTE UNSCHEDULED DNA SYNTHESIS AND S-PHASE INDUCTION ASSAY

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ABSTRACT

In the *In Vivo/In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis Assay, the test material, a mixture of six and eight carbon oligomers of chlorotrifluoroethylene (CTFE) did not induce significant changes in the nuclear labeling of rat hepatocytes over a dose range of approximately 625 to 5000 mg/kg. Three male Fischer 344 (F-344) rats were treated by oral gavage at each of four doses with the test material suspended in corn oil. About 16 h after treatment with the test material, primary hepatocyte cultures were prepared. Viabilities of the hepatocytes obtained ranged from 83.8% to 96.9%. After attachment, the cultures were incubated with 10 µCi/mL ^{3}H-thymidine (^{3}HTdr) for about 4 h. The cultures were prepared for analysis of nuclear labeling 16.4 to 17.0 h after removal of the radioactivity and addition of 0.25 mM thymidine. None of the criteria used to indicate unscheduled DNA synthesis was approached by the treatments and no dose-related response was observed. The test material, CTFE, therefore was evaluated as inactive in the *In Vivo/In Vitro* Rat Hepatocyte Unscheduled DNA Synthesis Assay.

In the *In Vivo/In Vitro* Rat Primary Hepatocyte DNA Synthesis (S-Phase) Induction Assay, the test material, CTFE, induced significant increases in the number of S-phase cells over a dose range of approximately 625 to 5000 mg/kg. In the assay described in this report, at least three male F-344 rats were treated by oral gavage at each of four doses with the test material dissolved in corn oil. About 48 h later, primary hepatocyte cultures were prepared for analysis of S-phase. Viabilities of the hepatocytes prepared for S-phase analysis ranged from 76.0% to 96.3%. After attachment, the cultures were incubated with 10 µCi/mL ^{3}HTdr for about 4 h. The cultures were prepared for analysis of labeling 16.9 to 17.5 h after removal of the radioactivity and addition of 0.25 mM thymidine. All of the treatments with the test article caused significant elevations in the level of S-phase cells when compared to the levels observed in hepatocyte cultures obtained from the control animals. The test material, CTFE, therefore was evaluated as active in the *In Vivo/In Vitro* Rat Hepatocyte DNA Synthesis (S-Phase) Induction Assay.

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INTRODUCTION

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Only a small percentage of the cells enter S-phase (replicative DNA synthesis). Therefore, if ^{3}H-thymidine (^{3}HTdr) is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. Pretreatment *in vivo* of rats with a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by nondividing cells is known as unscheduled DNA synthesis (UDS) and can be measured by determining the amount of ^{3}HTdr incorporated into DNA. In this assay, an autoradiographic technique is used to determine the number of grains per nucleus caused by ^{3}HTdr incorporation. Autoradiographic measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977). Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system. The use of autoradiographic techniques allows simultaneous measurements of UDS and S-phase in hepatocyte cultures because S-phase cells are very heavily labeled and easily distinguished from lightly labeled cells exhibiting UDS. Hepatotoxicants such as carbontetrachloride and dinitrotoluene induce an increase in cell proliferation to replace necrotic tissue. Other compounds may induce S-phase synthesis in the absence of hepatotoxicity.

The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS or S-phase induced in rat primary hepatocytes *in vivo*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain counts in hepatocytes obtained from treated animals when compared to those from untreated animals. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including ^{3}HTdr) into DNA during a short *in vitro* culture period. Quantification of cells in S-phase (DNA replication) has been shown to be useful for the evaluation of chemicals that may cause increased cell proliferation in the liver (Butterworth et al., 1987).

MATERIALS AND METHODS

Indicator Cells

The indicator cells for this assay were hepatocytes obtained from adult male Fisher 344 rats, (CDF) weighing from 212.0 to 244.8 g, purchased from Charles River Breeding Laboratories, Incorporated. The animals scheduled for this assay were fed Purina-Certified® Rodent Chow (Formula 5002) and water *ad libitum*. Animals were identified by ear tag after random assignment to the study. Animals were quarantined a minimum of five calendar days prior to use.

Animals were anesthetized prior to surgery using about 60 mg/kg sodium pentobarbitol (V-Pento) and were exsanguinated during the liver perfusion. The cells were obtained by perfusion of the livers *in situ* with a collagenase solution. Monolayer cultures were established in culture dishes and were used the same day for analysis of the UDS activity. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

Media

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamicin (WME+). WME+ without serum is referred to as WMEI. After the establishment period, cultures were refed with WMEI containing 10 µCi/mL ^{3}HTdr (40-60 Ci/mM)(WME-treat).

Control Compounds

A vehicle negative control consisting of a minimum of three rats treated with Mazola 100% pure corn oil (Lot No. 021489) by oral gavage was performed in all cases and at all timepoints. The dosing volumes did not exceed 5.2 mL/kg (a target of 5.0 mL/kg ± 10%).

The positive control articles used are known to induce UDS *in vivo* in rat hepatocytes. Dimethylnitrosamine (DMN, CAS 62-75-9), delivered by oral gavage at approximately 15 mg/kg was used for the UDS timepoint. For the S-phase timepoint, DMN was administered by oral gavage at 20 mg/kg.

TEST MATERIAL

The test material, predominately six and eight carbon oligomers of CTFE was a clear, colorless liquid.

Dosing Procedure

For both the UDS and S-phase assays, rats were treated by oral gavage with the test article dissolved in corn oil. The total volume of the test article solution administered did not exceed 5.2 mL/kg (target of 5.0 mL/kg ± 10%). Dimethylnitrosamine was solubilized in sterile deionized water and dosed as described previously. Fresh preparations of test article in vehicle were used for any testing purpose.

Dose Selection and Perfusion Time

For the UDS assay, the highest dose selected was 5000 mg/kg. Three additional doses of test material were prepared using approximate two-fold dilution steps and a minimum of three animals per dose. The timepoint for UDS was 16.6 to 17.2 h after the administration of a single dose of the test article by oral gavage.

The dose of 5000 mg/kg was selected as the maximum dose for the S-phase induction assay. Three additional doses of test material were prepared using approximate two-fold dilution steps and a minimum of three animals per dose. S-phase analysis was performed at 48.2 to 48.9 h after the administration of a single dose of the test article by oral gavage.

UDS and S-Phase Assays

These assays were based on the procedures in rats described by Williams (1980), Mirslis, Tyson, and Butterworth (1982), and Butterworth et al., (1987). The hepatocytes for both assays were obtained by perfusion of livers *in situ* for about four minutes with Hanks' balanced salts (Ca^{++}-Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis (a-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50 to 100 units/mL of collagenase was perfused through the liver for about 10 to 11 min. Depending upon the condition of the liver, this time may be altered by 2 min. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. The suspended tissue and cells were allowed to settle to remove cell clumps and debris. The cell suspension was centrifuged and the cell pellet resuspended in WME+. After obtaining a viable cell count, a series of 35-mm culture dishes (at least six per animal containing a 25-mm round, plastic coverslip and at least two per animal to assess attachment efficiency) was inoculated for each animal with approximately 0.5×10^6 viable cells in 3 mL of WME+ per dish.

An attachment period of 1.5 to 2 h at 37°C in a humidified atmosphere containing 5% CO_2 was used to establish the cell cultures. Unattached cells then were removed and the cultures were refed with 2.5 mL WME-treat. Any remaining cultures were kept for analysis in the event of technical problems with autoradiography. Attachment efficiency was determined for two cultures from each animal using trypan blue dye exclusion and *in situ* analysis.

After a labeling period of about 4 h, labeled cultures were refed with WMEI containing 0.25 mM thymidine. The cells were returned to the incubator for 16 to 20 h. After the incubation period, the hepatocyte nuclei were swollen by addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 10 to 11 min. The cells were next fixed in acetic acid:ethanol (1:3) and dried for at least 24 h. The fixed coverslips were mounted on glass slides, dipped in Kodak NTB2 emulsion, and dried. The emulsion-coated slides were stored for seven days at 4°C in light-tight boxes containing Drierite. The emulsions then were developed in D19, fixed, and stained with Williams' modified hematoxylin and eosin procedure.

For the UDS assay, the cells were examined microscopically at approximately 1500x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. The 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 analyzed for UDS. Only normally appearing nuclei were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. The mean net nuclear grain count was determined from triplicate coverslips (150 total nuclei) for each animal and each treatment condition. Occasionally, a coverslip is recounted at a later date by a different technician. Since a different cell population is generally scored, the average count for 50 cells is used in the calculation of the mean for the triplicate treatment.

Cells undergoing DNA replication are easily distinguished from nonreplicating cells in autoradiographic preparations. For the S-phase timepoint (48 h), the percentage of cells in S-phase (%S) was calculated manually as those cells exhibiting nuclei blackened by grains too numerous to count. Approximately 2000 cells were counted from randomly selected areas of each slide. Replicating and nonreplicating cells were counted separately using a two-position hand counter. For each dose, 6000 cells (three slides) were scored for each of three animals (18,000 total cells per dose), unless indicated otherwise.

Assay Acceptance Criteria

An assay normally will be considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied. This listing may not encompass all test situations, so the study director must exercise scientific judgment in modifying the criteria or considering other causes that might affect assay reliability and acceptance.

1. The viability of the hepatocytes collected from the perfusion process normally exceeds 70%. A variety of factors can affect cell yield and viability, so values below 70% are not uncommon nor necessarily detrimental. Toxicity of treatment with test article may be reflected in perfusion viability, therefore no lower limit will be set.

2. The viability of the monolayer cell cultures used for the assay must be 70% or greater. Normally, the viability of attached cells is about 85%.

3. The positive control is used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS. For test materials causing weak or no UDS activity, the average response to the positive control treatments must exceed both criteria used to indicate UDS. The positive control for S-phase must have greater than 1% of the cells in scheduled DNA synthesis. For test materials clearly causing a dose-related UDS activity or S-phase activity, an assay will be acceptable in the absence of a positive control lost for

technical reasons. Historical control values for the positive control are NNG = 26.65 ± 7.69 (range, 15.47 to 37.25) and % r5 grains per nucleus = 93.1% ± 6.2% (range, 83.3% to 100.0%). Historical control values for the negative control are NNG = -0.81 ± 1.18 (range, -3.27 to 0.47) and % r5 grains per nucleus = 0.23% ± 0.35% (range, 0% to 0.7%).

4. Grain count data obtained per animal is acceptable as part of the evaluation if obtained from two replicate cultures and at least 50 nuclei per culture. Grain count data should be available from two of the three animals treated.

5. A minimum of three doses will be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed doses in the first trial to achieve a total of three concentrations but must include one dose previously assayed as acceptable.

ASSAY EVALUATION CRITERIA

UDS Assay

Several criteria have been established which, if met, provide a basis for evaluation of a test material as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of the historical control data as described by Casciano and Gaylor (1983).

The test material is considered active in the UDS assay at doses that cause the following.

1. An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent negative control value leading to a positive number, and/or

2. An increase in the number of nuclei having five or more net grains such that the percentage of these nuclei in test cultures is 10% above the negative control cultures.

Generally, if the first condition is satisfied, the second also will be met. However, satisfaction of only the second condition also can indicate UDS activity. Different DNA-damaging agents can give a variety of nuclear labeling patterns, and weak agents may strongly affect only a minority of the cells. Therefore, both of the above conditions are considered in an evaluation. In cases where increases are not observed in all three animals, the test material will be considered active for that condition if cells from two of the three animals show increases. If the negative control shows an average < -5 or > 1 grain per nucleus, the assay normally will be considered invalid.

The test material is considered inactive in this assay if the following is true.

1. The mean net nuclear grain counts for all dosed groups is < 1.0 net nuclear grain count above the concurrent negative control value and/or

2. The percent of nuclei with five or more net grains does not increase more than 2% above the concurrent negative control.

When results are neither clearly positive nor clearly negative, the presence of a dose response, the frequency distribution of cellular responses, and the reproducibility of data among slides is considered; the test article then is classified as "negative", "weak positive" or "equivocal". Groups in which one out of three animals show increases will be decided on a case-by-case basis depending on the level of activity in cells from the active animal, the level of activity in cells from the inactive animals, and the presence or absence of activity in surrounding groups.

The positive control nuclear labeling is not used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test material. Unscheduled DNA Synthesis elicited by test agents in this assay is probably more dependent on the type of DNA damage inflicted and the available repair mechanisms than on the potency of the test agent as a mutagen or carcinogen. Some forms of DNA damage are repaired without the incorporation of new nucleic acids. Thus, the positive controls are used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

S-Phase Induction

The test article will be considered positive if the % S in cells from an animal is significantly greater than the vehicle control and exceeds 1% of the population. Significance will be determined using Student's t-test modified for unpaired observations with unequal variances where necessary.

The test article will be considered negative if the % S is $<1.0\%$ of the population. (Negative controls usually have $< 1\%$ of the cells undergoing DNA replication.) The data base for S-phase is not extensive and some judgment may be needed in the evaluation of a response.

RESULTS AND DISCUSSION

UDS Assay

The test article, CTFE, was dissolved in corn oil at concentrations ranging from 125.1 to 1007 mg/mL. All dosing stocks were prepared just prior to use. Each dose was prepared by two-fold serial dilutions with the vehicle after preparation of an initial stock. The test material appeared to form a clear, pale yellow solution in the vehicle. Three rats per dose were treated with 5000, 2500, 1250, and 625 mg/kg in volumes which did not exceed 5.2 mL/kg.

For the UDS assay, perfusions were initiated 16.6 to 17.2 h after administration of a single dose of the test article by oral gavage. The hepatocytes collected for the UDS assay ranged in viability (determined by trypan blue exclusion) from 83.8% to 96.9% of the total cells collected in the perfusate (Table 6-1). At 1250 mg/kg, one culture was unacceptable because of low-cell attachment (16.5%). Another culture at 1250 mg/kg also had low attachment (30.9%), but the cells had good morphologies and were acceptable for analysis. The attachment efficiencies of the remaining

cultures ranged from 51.9% to 82.4%. The viabilities of the attached cells were very good, ranging from 85.7% to 97.8%.

TABLE 6-1. CULTURE DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH OLIGOMERS OF CHLOROTRIFLUOROETHYLENE (16-HOUR TIMEPOINT)

Target Dose (mg/kg)	Animal Number	Perfusion[a] Viability (%)	Attachment[b] Efficiency (%)	Viability[b]
VC	3187	90.5	72.6	92.7
VC	3206	85.1	73.3	88.8
VC	3212	90.7	72.2	91.0
625	3195	95.8	70.0	86.3
625	3198	87.2	82.4	93.0
625	3216	86.1	73.8	92.0
1250	3191	93.8	16.5	90.7
1250	3192	94.7	68.8	85.7
1250	3203	94.7	30.9	96.3
2500	3199	96.9	51.9	94.2
2500	3213	94.2	61.8	96.6
2500	3215	93.2	68.9	95.2
5000	3183	88.2	75.6	95.6
5000	3188	85.5	75.4	95.4
5000	3193	83.8	61.9	95.1
DMN	3189	89.3	61.7	97.0
DMN	3204	87.7	81.0	96.8
DMN	3220	84.4	74.8	96.7
DMN	3222*	95.0	62.7	97.8
DMN	3197*	93.0	62.7	96.5

[a] At time of hepatocyte collection (determined by trypan blue exclusion).
[b] Two culture dishes per animal were counted *in situ* after the attachment period using dilute trypan blue to determine viability.
DMN = Positive control, dimethylnitrosamine, 15 mg/kg.
VC = Vehicle control, 5 mL/kg of corn oil.
* Cells from these animals not chosen for the analysis of UDS.

The minimum criteria for a UDS response were determined by comparison to the averages of the concurrent vehicle control treatments. A positive response consisted of mean net nuclear grain counts exceeding 4.53 or at least 10.7% of the nuclei containing five or more grains. None of the treatments with the test material samples caused nuclear labeling significantly different from the vehicle control (see Table 6-2). Furthermore, no dose-related trend was evident. In contrast, the DMN treatments induced large increases in nuclear labeling that greatly exceeded both criteria used to indicate UDS. Since the positive control animals were responsive to DMN, the test results were considered to provide conclusive evidence for the lack of UDS induction by the test material samples.

Heavily labeled nuclei (blackened with numerous grains) represent cells undergoing DNA replication as opposed to DNA repair. The number present in the UDS study was low and did not

interfere with the assay although a slight elevation in S-phase cells was observed with some of the chemical treatments (see Table 6-2). Only 29 cells (0.64%) among the 4500 control cells screened were heavily labeled.

TABLE 6- 2. UDS DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH OLIGOMERS OF CHLOROTRIFLUOROETHYLENE (16-HOUR TIMEPOINT)

Target Dose (mg/kg)	Animal Number	Mean (± SD) Net Nuclear Grains[a]	Mean Cyto Grains[b]	% Nuclei with >5 Net Grains	% S[c]
VC	3187	-0.21 ± (0.47)	7.94	2.0	0.53
VC	3206	-0.59 ± (0.38)	7.75	0.0	0.73
VC	3212	-0.62 ± (0.52)	7.20	0.0	0.67
625	3195	-1.77 ± (0.31)	6.39	0.7	1.07
625	3198	-1.22 ± (0.50)	8.66	0.7	2.33
625	3216	0.41 ± (1.05)	6.81	8.0	1.73
1250[d]	3191				
1250	3192	-1.67 ± (0.44)	8.20	2.0	3.53
1250	3203	-0.32 ± (0.68)	5.75	4.0	1.00
2500	3199	-0.16 ± (0.59)	6.39	1.3	0.60
2500	3213	0.95 ± (1.20)	6.39	8.0	0.13
2500	3215	-1.01 ± (0.54)	7.27	2.0	0.87
5000	3183	-0.72 ± (0.92)	6.61	2.7	1.07
5000	3188	-1.36 ± (0.34)	6.56	0.7	1.26
5000	3193	-0.55 ± (0.33)	6.01	2.0	0.93
DMN	3189	10.15 ± (2.13)	7.15	68.7	0.27
DMN	3204	11.02 ± (1.17)	6.09	71.3	0.67
DMN	3220	15.40 ± (1.07)	5.61	84.7	0.73

[a] UDS = Mean net nuclear grain count from triplicate coverslips (150 total cells).
[b] Triplicate coverslips (150 total cells).
[c] Percent of S-phase nuclei on triplicate coverslips (1500 total cells scored)
[d] not readable due to poor cell attachment.
VC = Vehicle Control; 5 mL/kg of corn oil.
DMN = Positive control dimethylnitrosamine, 15 mg/kg.

S-Phase Assay

In order to determine the amount of cell proliferation induced by the test material, three rats per dose were sacrificed at about 48 h after a single dose of the chemical was administered. The test material was dosed at about 5000, 2500, 1250, and 625 mg/kg.

The hepatocytes collected for S-phase analysis ranged in viability from 76.0% to 96.3% of the total cells collected in the perfusate (Table 6-3). The attachment efficiencies of the analyzed cultures ranged from 28.3% to 98.4%. Cells from two animals with attachment efficiencies of 23.3% and 62.1% were not analyzed because of poor cell morphologies. Most of the low attachment efficiencies probably reflect toxicity since the lowest viabilities were seen in cells from the high dose animals and

the positive control animals. The viabilities of the attached cells were very good, ranging from 74.4% to 94.9%.

TABLE 6- 3. CULTURE DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH OLIGOMERS OF CHLOROTRIFLUOROETHYLENE (48-HOUR TIMEPOINT)

Target Dose (mg/kg)	Animal Number	Perfusion[a] Viability (%)	Attachment[b] Efficiency (%)	Viability[b]
VC	3185	92.5	86.2	90.3
VC	3186	90.7	41.8	89.3
VC	3217	92.6	98.4	91.4
625	3196	76.0	62.1	74.4
625	3207	96.3	87.7	90.3
625	3208	84.8	71.3	93.1
1250	3190	77.3	75.4	94.9
1250	3205	81.4	85.8	92.7
1250	3210	93.1	71.7	92.1
2500	3200	86.1	69.1	91.9
2500	3201	90.1	70.6	88.5
2500	3214	94.4	97.0	89.8
5000	3184	95.7	28.3	78.1
5000	3194	87.1	23.3	76.4
5000	3218	94.1	70.8	89.6
DMN	3202	88.7	73.3	84.4
DMN	3209*	91.8	35.7	77.1
DMN	3211	87.2	71.2	88.6
DMN	3219*	92.3	32.4	88.8
DMN	3221	79.5	41.8	86.8

[a] At time of hepatocyte collection (determined by trypan blue exclusion).
[b] Two culture dishes per animal were counted *in situ* after the attachment period using dilute trypan blue to determine viability.
DMN = Positive control, dimethylnitrosamine, 20 mg/kg.
VC = Vehicle control, 5 mL/kg of corn oil.
* Cells from these animals not chosen for the analysis of S-phase.

A significant increase in DNA synthesis (S-phase) was determined by comparison to the averages of the concurrent vehicle control treatments. Significance was determined using a Student's t-test, modified for unpaired observations with unequal variances when an F-test performed on the means determined that the variances were unequal. A standard t-test was used when the variances were equal. All of the treatments with the test material induced significant elevations in replicative DNA synthesis in each of the rats treated (Table 6-4). The levels of S-phase varied from a low of 3.3% to a high of 6.0% in the treated groups. Increases in S-phase were not directly correlated with increases in dose. In fact, there may have been a trend toward a decrease in S-phase at the higher doses. This lack of a clear dose response may have been related to toxic effects in the animal.

Significant elevation of S-phase also was observed in all three of the positive control animals. These results provided conclusive evidence of the induction of S-phase by the test article treatments.

TABLE 6- 4. S-PHASE DATA SUMMARY FOR HEPATOCYTES FROM RATS TREATED WITH OLIGOMERS OF CHLOROTRIFLUOROETHYLENE

Target Dose (mg/kg)	Animal Number	Total[a] S-Phase	Total[a] Cells Analyzed	% S-Phase (± SD)
VC	3185	116	6000	1.9 (± 1.78)
VC	3186	53	6000	0.9 (± 0.31)
VC	3217	103	6000	1.7 (± 1.40)
625[b]	3196			
625	3207	306	6000	5.1* (± 0.80)
625	3208	355	6000	5.9* (± 0.96)
1250	3190	228	6000	3.8* (± 0.35)
1250	3205	361	6000	6.0* (± 0.21)
1250	3210	283	6000	4.7* (± 0.21)
2500	3200	266	6000	4.4* (± 0.67)
2500	3201	261	6000	4.4* (± 1.30)
2500	3214	225	6000	3.8* (± 0.50)
5000	3184	194	6000	3.3* (± 1.10)
5000[b]	3194			
5000	3218	340	6000	5.7* (± 0.44)
DMN	3202	351	6000	5.9* (± 1.63)
DMN	3211	176	6000	2.9* (± 0.54)
DMN	3221	653	6000	10.9* (± 1.85)

[a] Triplicate coverslips, 2000 cells/coverslips.
[b] Not analyzable - poor cell morphology.
VC = Vehicle Control; 5 mL/kg of corn oil.
DMN = Positive control, dimethylnitrosamine, 20 mg/kg.
* Significantly different ($p<0.05$) from pooled control values with sample size of nine coverslips, a mean of 1.51% and a standard deviation of ± 1.24 using an unpaired t-test modified for unequal variances, if necessary.

CONCLUSIONS

The test material, CTFE, did not induce significant changes in the nuclear labeling of rat primary hepatocytes for a dose range of about 625 to 5000 mg/kg. Chlorotrifluoroethylene was therefore evaluated as inactive in the *In Vivo/In Vitro* Rat Primary Hepatocyte UDS Assay.

A significant S-phase response was observed for the test material over a dosing range of about 625 to 5000 mg/kg. The observed increases ranged from 2.2 to 4.0-fold above the average vehicle control S-phase synthesis. Therefore, CTFE was evaluated as active in the S-Phase Induction Assay.

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SECTION 7

CHLOROTRIFLUOROETHYLENE OLIGOMERS GENOTOXICITY SUMMARY EVALUATION

Myhr, B.[a]

3.1 Oil®, which is composed of chlorotrifluoroethylene (CTFE) oligomers, was tested for potential genotoxicity by application to several *in vitro* assays as described below, and an *in vivo* assay for DNA damage and induction of DNA replication. Because the substance has little or no solubility in aqueous medium, the polyalcohol Pluronic® F68 was used as an emulsifying agent for the *in vitro* assays. After homogenization in 10% w/v Pluronic® F68, a stable suspension of tiny droplets was obtained that did not coalesce when diluted into culture media at Pluronic® F68 concentrations of 0.8 to 1% w/v. The CTFE emulsion showed no visible reaction with a polystyrene surface, so standard polystyrene cultureware was used for the treatment phase of each *in vitro* assay; primary emulsions and dilutions of CTFE were handled with glass containers and pipets.

The Ames *Salmonella* reverse mutation assay was performed with the preincubation methodology in order to maximize contact between the bacteria and CTFE emulsions. Strains TA98, TA100, TA1535, TA1537, and TA1538 were used in the presence and absence of a rat liver S9 metabolic activation system (Aroclor 1254-induced). Doses up to 10,000 µg/plate were tested with and without S9. A slight precipitate was observed at 1000 µg/plate, but CTFE caused no toxicity and no increases in revertants and thus showed no mutagenic activity.

In mammalian cell culture, CTFE was tested for mutagenic activity at the hypoxanthine guanine phosphoribosyltransferase (HGPRT) locus in CHO cells. A 4-h exposure period was used in the presence and absence of the rat liver S9 activation system. The S9 system resulted in some protection against CTFE toxicity and allowed concentrations as high as 5000 µg/mL to be used with only moderate toxicity. In contrast, CTFE at 5000 µg/mL was highly toxic or lethal in the absence of S9. Two independent trials of the assay were performed without S9 in order to assess isolated, but apparently significant, increases in mutant frequency. An elevated mutant frequency of 27.4×10^{-6} in Trial 1 at 2000 µg/mL (37% survival) was not repeated in Trial 2, where a slightly higher toxicity (27% survival) was achieved at 2000 µg/mL. However, Trial 2 yielded an elevated mutant frequency (19.0×10^{-6}) at 5000 µg/mL at extremely high toxicity (4% survival). These increases just exceeded the typical background mutant frequency ($\leq 15 \times 10^{-6}$) and were cited as evidence for weak mutagenic activity. However, the mutagenic activity is clearly erratic and at the limit of resolution for this assay

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and would be very difficult to demonstrate convincingly among different laboratories. With S9 activation, no mutagenic activity was detected over a dose range of 100 µg/mL to 5000 µg/mL and toxicity range of 112% to 49% survival.

Chinese hamster ovary (CHO) cells also were examined for cytogenetic damage in assays for CTFE-induced chromosomal aberrations and sister chromatid exchanges (SCEs). Chlorotrifluoroethylene was highly toxic at 167 µg/mL without S9 and at 1670 µg/mL with S9, causing cellular debris and 50 to 60% reduction in mitotic cells but no significant delay in cell-cycle time. Accordingly, a normal harvest time of 10 h was used to analyze a dose range of 150 µg/mL to 300 µg/mL without S9 and 1000 µg/mL to 2500 µg/mL with S9. No increases in chromosomal aberrations were observed. The assay for SCE showed no increases for doses up to 167 µg/mL without S9 and 1670 µg/mL with S9. The next highest doses of 500 µg/mL without S9 and 5000 µg/mL with S9 were lethal. Therefore, no evidence was obtained for any cytogenetic damage by CTFE *in vitro*.

Chlorotrifluoroethylene was assayed for its ability to induce morphological transformation *in vitro* in cultures of mouse BALB/c-3T3 cells, both in the presence and absence of a rat liver S9 metabolic activation system. The treatment periods were two hours with S9 and 72 hours without S9. The treatments in the presence of S9 resulted in less toxicity, as measured by the clonal survivals of ouabain-resistant cells in the presence of the wildtype monolayer cultures. Without S9, no response was obtained for a dose range of 40 µg/mL to 400 µg/mL, which yielded a survival range of 95% to 0.2%. With S9, two independent trials were required to interpret an unusual, inverted response pattern obtained in the first trial. The second trial showed no responses for a dose range of 75 µg/mL to 1250 µg/mL, which caused a survival range of 98% to 6%. Therefore, no evidence was obtained for transformation activity *in vitro* by CTFE.

An *in vivo* assessment of genetic activity was performed by dosing male Fischer 344 rats with four dose levels of CTFE ranging from 625 mg/kg to the maximum amount of 5000 mg/kg. After single oral administrations, primary hepatocyte cultures were established to determine the degree of DNA repair (unscheduled DNA synthesis, UDS) and DNA synthesis (S-phase) by labeling with ^{3}H-thymidine, followed by autoradiographic analysis. The cultures were prepared approximately 16 h after treatment of the animals for the UDS analysis and approximately 48 hours after treatment of additional animals for S-phase induction. None of the treatments caused any increases in nuclear labeling or the percent of cells in DNA repair relative to control rats. Therefore, CTFE did not induce any detectable UDS. However, CTFE damage to the liver was indicated by a nearly constant increase in S-phase cells at all four dose levels. In control animals, approximately 1.5% of the hepatocytes were in S-phase, whereas the treatments with CTFE yielded approximately 5% hepatocytes in

S-phase. This increase in hepatocyte replication indicated a significant and saturable degree of hepatotoxicity caused by CTFE.

The results of the above genetic tests indicate that CTFE has no significant interaction with genetic material. For five of the six genetic endpoints, no responses were obtained. An erratic response observed for mutagenesis (without S9 only) at the HGPRT locus in CHO cells was too tenuous to warrant any consideration in risk assessment. In rat liver, no damage to DNA was detected, but the CTFE treatments did cause sufficient cytotoxicity to detect an increase in dividing hepatocytes. Thus, the results of the genetic test battery would predict no genetic risk from CTFE, but physiological stress to liver upon CTFE ingestion is indicated by the S-phase results.

SECTION 8

APPENDICES

APPENDIX A

SUMMARY OF TESTING ON CHLOROTRIFLUOROETHYLENE OLIGOMERS WITH RAT PRIMARY HEPATOCYTES

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ABSTRACT

An attempt was made to evaluate six and eight carbon oligomers of chlorotrifluoroethylene (CTFE) in the *In Vitro* Rat Primary Hepatocyte Unscheduled DNA Synthesis (UDS) Assay. The test material required that testing be performed in closed containers. Several containers and conditions were tried and none resulted in acceptable levels of survival. While conditions for the UDS assay were being developed, an attempt was made to obtain toxicity information. Five cytotoxicity assays were initiated but the data from the first four studies was unacceptable or difficult to interpret. The fifth study demonstrated that the test material was excessively toxic to rat primary hepatocytes at concentrations above 1.0 µg/mL. An attempt was made to perform the UDS assay. Hepatocytes from two rats were treated with CTFE. Erratic toxicity curves were observed and the cells had morphologies that were unacceptable for analysis. The studies were terminated because acceptable conditions for performing the UDS assay would require further developmental studies.

INTRODUCTION

Fresh hepatocytes obtained from rat liver will attach to a surface in culture and continue to metabolize for several days without undergoing cell division. Only a small percentage of the cells enter S-phase (replicative DNA synthesis). Therefore, if 3H-thymidine (3HTdr) is introduced in the culture medium, little or no label will be incorporated into nuclear DNA. The addition of a test material that interacts with the DNA often stimulates a repair response in which the altered portion of DNA is excised and the missing region replaced by DNA synthesis. This synthesis of DNA by nondividing cells is known as unscheduled DNA synthesis (UDS) and can be measured by determining the amount of 3HTdr incorporation using an autoradiographic method. Cells involved in DNA replication are recognized by heavy labeling of the nuclei and are excluded from the evaluation of UDS activity. Autoradiographic measurement of DNA repair is highly sensitive and appears to correlate very well with the known mutagenic or carcinogenic activities of chemicals (Williams, 1977). Furthermore, the use of primary hepatocytes has the advantage that these cells have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system.

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The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring UDS in rat primary hepatocytes *in vitro*. The existence and degree of DNA damage was inferred from an increase in net nuclear grain counts in treated hepatocytes when compared to untreated hepatocytes. The types of DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of new bases (including 3HTdr) into DNA.

MATERIALS AND METHODS

Indicator Cells

The indicator cells for these assays were hepatocytes obtained from adult male Fischer 344 rats (150 to 300g), purchased from Charles River Breeding Laboratories, Incorporated. The animals scheduled for this assay were fed Purina-Certified® Rodent Chow (Formula 5002) and water *ad libitum*. Two animals, identified by cage card, were used for each trial of the UDS assay after a minimum quarantine period of five days. One rat was used for each cytotoxicity assay.

The cells were obtained by perfusion of the liver *in situ* with a collagenase solution. Monolayer cultures were established on plastic coverslips in culture dishes and were used the same day for initiation of the UDS assay. All cultures were maintained as monolayers at about 37°C in a humidified atmosphere containing approximately 5% CO_2.

Medium

The cell cultures were established in Williams' Medium E supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 µg/mL streptomycin sulfate, and 150 µg/mL gentamicin (WME+). In some cases 25 mm Hepes buffer (pH 7.2) was included in the medium. After the establishment period, the serum was removed. This latter culture medium is referred to simply as WMEI. This also contained Hepes buffer. If Hepes buffer was not added, the cultures were blanketed with 5% CO_2 to maintain the correct pH range.

Controls

A negative control consisting of assay procedures performed on cells exposed only to the test material solvent was performed. The solvent for the UDS assay was deionized water containing 1.0% F68 Pluronic®. The final concentration of solvent in the medium was 10%. An untreated control consisting of assay procedures performed on mock-exposed cells also was included.

The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetylaminofluorene (2-AAF) at 4.48×10^{-7}M (0.10 µg/mL) was used as the positive control.

EXPERIMENTAL DESIGN

Dosing Procedure

The test material, a mixture of predominately six and eight carbon oligomers of CTFE, was dissolved at the highest desired concentration in deionized water containing 10% F68 Pluronic®. Chlorotrifluoroethylene was immiscible with the solvent and was dispersed with a Tissuemizer® to obtain a milky-white suspension. Lower concentrations then were prepared by dilution with 10% F68 (initial studies) or 1% F68 (later studies including UDS assay). The dosing treatments were prepared by performing 1:10 dilutions of the stocks into WMEI containing ^{3}HTdr (final concentration, 5 µCi/mL).

Fresh preparations of test material in the vehicle were used for the biological testing. Treatments were initiated by replacing the medium on the cell cultures with WMEI containing the test material at the desired concentrations and 5 µCi/mL ^{3}HTdr (20 Ci/mM). The stability of the test material in the solvent is the responsibility of the Sponsor.

Dose Selection

Preliminary cytotoxicity assays were performed to determine the doses to be initiated in the UDS assay. The initial cytotoxicity assay was initiated with treatments from about 5000 µg/mL to about 50.0 µg/mL. The concentrations tested were lowered when it was observed that the test material was highly toxic. Five cytotoxicity assays were performed.

UDS and Cytotoxicity Assays

This assay was based on the procedures described by Williams (1977, 1980). The hepatocytes were obtained by perfusion of livers *in situ* for about four minutes with Hanks' balanced salts (Ca^{++}-Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis (a-aminoethyl ether)-N, N-tetraacetic acid (EGTA), and HEPES buffer at pH 7.2. Then WMEI containing 50 to 100 units/mL of collagenase was perfused through the liver for about 10 min. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing the WMEI culture medium and collagenase. Clumps of cellular tissue and debris were removed by allowing the clumps to settle to the bottom of the plate. The supernatant was centrifuged and the cell pellet resuspended in WME+. After obtaining a viable cell count, a series of 8-dram Shell vials (some containing a 15-mm round, plastic coverslip) was inoculated with viable cells in WME+.

An attachment period of 1.5 to 2 h at approximately 37°C in a humidified atmosphere containing about 5% CO_2 was used to establish the cell cultures. Unattached cells then were removed and the cultures were refed with WMEI. The assays were initiated within three hours by replacing the media in the culture dishes with 2.5 mL WMEI containing 5 µCi/mL ^{3}HTdr, (20 Ci/mM) and the test material at

the desired concentration. After treatment for 18 to 20 h, the assays were terminated by washing the cell monolayers twice with WMEI. Cytotoxicity assays were initiated without the added label. After treatment for 18 to 20 h, the assays were terminated by washing the cell monolayers twice with WMEI. Cytotoxicity assays were initiated without the added label. For the cytotoxicity assays, two cultures were used to monitor toxicity. For the UDS assay, three of the cultures from each treatment were washed with WMEI containing 1 mM thymidine and were further processed as described below. Another two cultures used to monitor the toxicity of each treatment were refed with WMEI and returned to the incubator. At 20 to 24 h after the initiation of the treatments, viable cell counts (trypan blue exclusion) were determined to estimate cell survival relative to the negative control.

The nuclei in the labeled cells were swollen by addition of 1% sodium citrate to the coverslips for eight to 10 min, and then the cells were fixed in acetic acid:ethanol (1:3) and dried for at least 24 h. The coverslips were mounted on glass slides, dipped in an emulsion of Kodak NTB2, and dried. The coated slides were stored for seven to 10 days at 4°C in light-tight boxes containing packets of Drierite®. The emulsions then were developed in D19, fixed, and stained using Williams' modified hematoxylin and eosin procedure.

The cells were examined microscopically at approximately 1500 x magnification under oil immersion and the field was displayed on the video screen of an automatic counter. Unscheduled DNA Synthesis could not be measured because of the morphological appearance of the cells.

RESULTS AND DISCUSSION

The test material, CTFE, was insoluble in medium and formed large globules that settled to the bottom of the vessel. In order to disperse the test material in the solvent and allow even exposure of the target hepatocyte cells, 10% F68 (w/v in water) was used to suspend the test material. Chlorotrifluoroethylene was mixed with 10% F68 Pluronic® and the test material was dispersed using a Tissuemizer®. A milky-white suspension formed that appeared to be stable. The highest concentration prepared was 50 mg/mL and, in most cases, this suspension was diluted with 10% F68 Pluronic® to obtain a series of stock solutions. In some of the later experiments, the 10% F68 Pluronic® was reduced to 1% (for diluting the stocks) in order to reduce any possible effects of the Pluronic® on the hepatocytes. The final dosing concentrations were prepared by performing 1:10 dilutions of the F68 Pluronic® stocks with WME medium containing ^{3}HTdr (final concentration, 5 µCi/mL). No labeled thymidine was added to the treatments used for cytotoxicity tests. The test material formed a good suspension in the medium. The cytotoxicity and UDS assays were initiated by replacing the medium on the cells with medium containing the appropriate concentration of test material (and ^{3}HTdr, if appropriate).

Because of the volatile nature of CTFE compounds, and in order to maintain a stable and representative concentration of the test material during the dosing period, it was necessary to perform the assays in closed containers. The cells from two rats were seeded into 8-dram glass shell vials containing 15-mm polyester coverslips instead of the usual 36-mm plastic culture dishes containing 25-mm round polyester coverslips. In the early experiments, 25-mm Hepes buffer was included in the medium to maintain the appropriate pH. It was later found to be slightly toxic and the cultures were blanketed with 5% CO_2, which also maintained the appropriate pH. The use of closed containers proved to be a continual problem throughout the studies. A brief description of studies performed to alleviate this problem is shown in the Appendix B.

In order to choose the appropriate doses for the UDS assay, it was decided that a preliminary cytotoxicity assay would be performed. This is not usually necessary for this assay but was instituted because of the special conditions and the request for a two-rat study. Trial 1 was initiated with treatments from about 50.0 to about 5000 µg/mL. This assay was terminated because all the cells were dead, even the solvent control cells. The controls in Trial 2 were better but all doses were dead. Trial 3 was initiated at lower concentrations (0.05 to 50.1 µg/mL) but all the cells had abnormal morphologies. In Trial 4, the test material appeared toxic above 1.0 µg/mL although the cells still did not have a normal flat morphology. Six treatments from 0.005 to 1.00 µg/mL were initiated in Trial 5 (Table 8-A-1) and the control cells had acceptable morphologies. The test material was highly toxic at 1.00 µg/mL (43.1% survival). Lower concentrations induced survivals that ranged from 75.9% to 105.5% of the solvent control survival.

The UDS assay was initiated with cells from two rats. The cytotoxicity curve was erratic in both studies and the cells were unacceptable for analysis. The assay was terminated.

TABLE 8-A-1. SUMMARY OF DATA FROM TRIAL 5 OF THE RAT HEPATOCYTE CYTOTOXICITY ASSAY TESTING CHLOROTRIFLUOROETHYLENE

Test Condition	Concentration	Survival (at 20.7 %)*
Negative Control Media	----------	88.2
Solvent Control 1% F68	10%	100.0
CTFE (µg/mL)	1.00	43.1
	0.500	92.5
	0.100	75.9
	0.050	104.8
	0.010	81.5
	0.005	105.5

* Survival = Number of viable cells per unit area relative to the solvent control.

REFERENCES

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APPENDIX B

SUMMARY OF ATTEMPTS TO PERFORM UDS ASSAY IN CLOSED CONTAINERS

Three unscheduled DNA synthesis (UDS) studies were requested that required the assays to be performed in closed glass containers. This is difficult in the UDS assay because hepatocytes do not attach well to glass and do not survive well in closed containers. The requirement for glass coverslips was waived when the polyester coverslips routinely used were shown to be resistant to the chemicals. However, it was still a requirement that closed containers be used. A preliminary experiment was performed to determine the technical problems associated with this requirement.

In the preliminary experiment, several glass containers containing coverslips were seeded with cells; Hepes buffer was added to some of the cultures to control the pH. The cells attached very well after 1.5 to 2 h and the toxicity tests were initiated using Shell vials. When the toxicity tests were performed, no cells were alive 18 to 20 h later. It appeared that cell attachment was normal but long-term survival was a problem. Several experiments then were performed to determine the parameters that affected survival. The following changes were made.

- Hepes buffer was not used and the cultures were gassed with 5% CO_2 to maintain an acceptable pH.
- The culture vessels were pretreated with medium containing 20% serum.
- The number of cells seeded was reduced.
- The volume of medium used was increased.
- Other culture vessels were tried again but rejected because they currently were not available or because of technical reasons.
- The dosing and feeding procedures were changed to make the cell handling more gentle.

Cytotoxicity studies were initiated again but were only partially successful. Additional studies were performed and the following changes were made.

- 1% serum was added to the dosing medium.
- At least twice the number of cultures was set up.
- The requirement for 50% survival of the cells in the control cultures was waived.

The cytotoxicity tests were again initiated and were partially successful and doses could be chosen. The UDS assays were initiated.

In the UDS assays initiated, the results were variable. In some cases, cells appeared to survive but the toxicities were erratic. Slides were prepared and autoradiography was performed. When the slides were developed, UDS could not be determined because most of the cells were rounded and cell labeling was abnormal. In addition, all of the positive control cultures were excessively toxic. None of the changes that were instituted avoided the problem of random loss of cultures or whole dose groups. It appeared that the cells were more fragile under conditions where closed containers were used and the parameters involved were not readily controlled. Reliable results could not be obtained from the cells under the conditions requested.

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