Final Report

Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures With PFOS

PREPARED FOR:
3M Corporate Toxicology

COVANCE STUDY NUMBER:
20784-0-447

COVANCE
THE DEVELOPMENT SERVICES COMPANY

FINAL REPORT

UNSCHEDULED DNA SYNTHESIS IN RAT LIVER PRIMARY CELL CULTURES WITH PFOS

AUTHOR

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PERFORMING LABORATORY

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LABORATORY PROJECT IDENTIFICATION

Covance Study No.: 20784-0-447
Sponsor Study No.: T6295.19

SUBMITTED TO

3M Corporate Toxicology
3M Center
Building 220-2E-02
St. Paul, MN 55144-1000

STUDY COMPLETION DATE

November 9, 1999

QUALITY ASSURANCE STATEMENT

Unscheduled DNA Synthesis In Rat Liver Primary Cell Cultures
With PFOS

The report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc., in accordance with the Good Laboratory Practice regulations as set forth in the Environmental Protection Agency (EPA-TSCA), Title 40 of the U.S. Code of Federal Regulations Part 792, and with any applicable amendments. The following inspections were conducted and the findings reported to the Study Director and study director management. Written status reports of inspections and findings are issued to Covance management according to standard operating procedures.

Inspection Dates	Phase	Dates Reported to Study Director and Study Director Management	Auditor
08/31/99	Weighing of Test Article	08/31/99	P. Cáceres
11/1,2/99	Protocol and Draft Report Review	11/2/99	P. Cáceres
11/09/99	Final Report Review	11/09/99	C. Smith

Representative, Quality Assurance Unit

11/9/99
Date

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STUDY COMPLIANCE AND CERTIFICATION

The described study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Environmental Protection Agency (EPA-TSCA), Title 40 of the U.S. Code of Federal Regulations Part 792, and with any applicable amendments. There were no significant deviations from the aforementioned regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria. All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director.

Study Director:

Maria A. Cifone

Maria A. Cifone, PhD
Genetic and Cellular Toxicology

11-9-99
Study Completion Date

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ABSTRACT

The test article, PFOS, was tested in the *in vitro* assay for unscheduled DNA synthesis in rat liver primary cell cultures. PFOS was insoluble in water and Williams' Medium E. In dimethylsulfoxide (DMSO), the test article formed a suspension with precipitate that settled to the bottom of the tube at 501 mg/mL. The precipitate was absent at 401 mg/mL. DMSO was chosen as the vehicle. In the assay described in this report, rat hepatocytes were exposed to the test article prepared in DMSO and dosed into media. In medium, a suspension with fine precipitate was observed at 4010 µg/mL

Based on solubility in DMSO and medium, a concentration of 4000 µg/ml was selected as the top dose for the *in vitro* UDS assay. Fifteen concentrations of PFOS were applied, ranging from 0.250 µg/mL to 4000 µg/mL. Cytotoxicity was determined by comparing the cell density of the treated plates to the cell density of the control plates approximately 23 hours after initiation of treatment. The test article was excessively cytotoxic at and above 50.0 µg/ml, weakly cytotoxic at 25.0 µg/ml, and noncytotoxic at and below 10.0 µg/ml. The cellular morphology was suitable for analysis at and below 25.0 µg/ml. No evidence for an increase in UDS was observed. None of the dose levels showed an elevation in mean net nuclear grains (NNG) or in the number of nuclei containing 5 or more net grains. The test article, PFOS, was therefore evaluated as negative in the *in vitro* assay for unscheduled DNA synthesis in rat liver primary cell cultures.

STUDY INFORMATION

Sponsor

3M Corporate Toxicology

Test Article

Sponsor's Identification: PFOS (FC-95), Lot 217
Date Received: August 19, 1999
Physical Description: White crystalline powder
Storage Conditions: Ambient temperature

Assay Information

Type of Assay: Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures
Protocol No.: 447 Edition 17
Covance Study No.: 20784
3M Study No.: T6295.19

Study Dates

Initiation Date: August 20, 1999
Experimental Start Date: September 1, 1999
Experimental Termination Date: September 22, 1999

Supervisory Personnel

Study Director: Maria A. Cifone, PhD
Laboratory Supervisor: Agnes Nourbakhsh, BS

OBJECTIVE

The objective of this *in vitro* assay was to evaluate the ability of the test article, PFOS, or a metabolite, to induce DNA damage in rat primary hepatocytes by measuring unscheduled DNA synthesis (UDS).

TEST SYSTEM RATIONALE

This assay aims to establish whether the test article or its metabolites interacts with rat liver primary cell (hepatocyte) cultures to induce DNA damage followed by DNA repair measured as UDS. Hepatocytes were isolated from liver and exposed *in vitro* to the test article and tritiated thymidine (^{3}H-TdR). Primary hepatocytes have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system. An autoradiographic technique described by Williams (1977) was used to measure incorporation of ^{3}H-TdR into DNA. Because only a small percentage of the cells enter S-phase (replicative DNA synthesis) during the exposure period, the incorporation of ^{3}H-TdR into DNA is used as a measure of the repair of DNA damage caused by treatment with the test article. The existence and degree of DNA

damage is inferred from an increase in nuclear grain counts in treated hepatocytes compared to nuclear grain counts from untreated hepatocytes. The types of detectable DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of nucleic acid bases including ^{3}H-TdR into the DNA.

MATERIALS AND METHODS

Test System

The indicator cells for this assay were hepatocytes obtained from a single adult male, Fischer 344 rat, weighing 214.0 grams. The animal was purchased from Charles River Laboratories, Raleigh, NC, and was housed in a shipping crate and received food supplied for shipping by the vendor and water *ad libitum*. The rat was used for the surgical procedure within 2 days of receipt, and was anesthetized before surgery with about 60 mg/kg sodium pentobarbital by intraperitoneal injection and exsanguinated during the procedure.

The cells were obtained by perfusion of the liver *in situ* with a collagenase solution, WMEC (see Cell Collection and Culture in the section on the UDS assay). Monolayer cultures were established on plastic coverslips in culture dishes and were used the same day for initiation of the UDS assay.

Media and Cell Culture Conditions. Williams' Medium E supplemented with 2 mM L-glutamine, 100 µg/ml streptomycin sulfate, and 150 µg/ml gentamicin (WMEI) was the base culture medium, and was modified for each specific requirement. The hepatocytes were obtained by perfusion of livers *in situ* with HBSS/EGTA followed by WMEC:Hanks' balanced salts (Ca^{++}-and Mg^{++}-free) containing 0.5 mM ethyleneglycol-bis(β-aminoethyl ether)-N, N-tetra-acetic acid and 50 mM HEPES buffer at pH 7.2 (HBSS/EGTA), and WMEI containing 50-100 units/ml of collagenase and 50 mM HEPES buffer at pH 7.2 (WMEC). The cultures were established in WMEI supplemented with 10% fetal bovine serum (WME+). All cell cultures were maintained as monolayers in a humidified incubator at 35 to 37.5° C in an atmosphere of 4 to 6% CO_2 in air. After the established period, the culture labeling was initiated using WMEI containing 10 µCi/ml ^{3}H-TdR at 40 to 60 Ci/mMole (WME-treat).

Control Articles

Vehicle Control Article. The vehicle control cultures received a concentration of the vehicle equivalent to the vehicle in the test article-treated cultures and were subjected to all of the experimental manipulation that the test article-treated cultures received. Dimethylsulfoxide (DMSO; CAS 67-68-5, Lot #128H3460, Sigma Chemical Co.) at a concentration of 1% was used for all trials.

Positive Control Article. The positive control compound is known to induce UDS in rat hepatocyte primary cell cultures. 2-Acetylaminofluorene (2-AAF, CAS# 53-96-3, Lot#57H0293, Sigma Chemical Co.) was dissolved in DMSO and diluted 1:100 into WME-treat for a final concentration of 0.10 µg/ml (4.48×10^{-7} M).

EXPERIMENTAL DESIGN

Dosing Procedure

A preliminary solubility test was performed with the test article, PFOS, in water, Williams' Media E and DMSO. The test article was insoluble in all vehicles but formed an acceptable suspension in DMSO at 401 mg/mL. DMSO was therefore chosen as the vehicle. When dosed into media for mammalian cells, the test article formed a suspension/translucent liquid with very fine particles of precipitate at 4010 µg/mL. Based on this information, a concentration of 4000 µg/ml was selected as the top dose.

A fresh preparation of test article in the vehicle was used for the assay. A suspension/solution of test article in DMSO was serially diluted with DMSO to obtain the final desired concentrations of test article. DMSO stocks were dosed 1:100 into media to create the desired dosing concentrations.

The sponsor is responsible for the determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

UDS Assay

Dose Determination. A concurrent cytotoxicity assessment was used to select appropriate doses for the particular, fresh primary culture of hepatocytes used in this study. A range of fifteen concentrations from 0.25 µg/ml to 4000 µg/ml was applied to the cells. A viable cell count, based on trypan blue dye exclusion, was then obtained approximately 23 hours after initiation of the treatments. This information was used to choose six concentrations for analysis of nuclear labeling starting with the highest dose that resulted in a sufficient number of surviving cells with intact morphologies and proceeding to successively lower doses.

Cell Collection and Culture. This assay was based on the procedures described by Williams (1980), and Butterworth *et al.* (1987). The hepatocytes were obtained by perfusion of liver *in situ* for four minutes with HBSS/EGTA then for 11 minutes with WMEC. The hepatocytes were obtained by mechanical dispersion of excised liver tissue in a culture dish containing WMEC. The suspended tissue and cells were then allowed to settle to remove cell clumps and debris prior to collection. The collected 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 5 per dose level) were inoculated with approximately 0.5×10^6 viable cells in 3 ml of WME+ per dish. Culture dishes used for the UDS analysis contained round plastic coverslips, while dishes used to assess attachment efficiency and toxicity had no coverslips.

To establish the cell cultures, cells were allowed an attachment period of 2 hours at 35 to 37.5°C in a humidified atmosphere containing 4 to 6% CO_2. Unattached cells were then removed and the cultures were refed with WMEI. The UDS assay was initiated 1.4 hours later by replacing the media in the culture dishes with 2.5 ml WME-treat containing the test article at the desired

concentration. Each treatment, including the positive and vehicle controls, was performed on at least five cultures, two of which were used for cytotoxicity measurements.

Termination and Cytotoxicity Measurement. After an exposure period of 19.6 to 20 hours, the UDS assay was terminated by washing the cell monolayers twice with WMEI. The cultures used for the cytotoxicity measurements were refed with WMEI and returned to the incubator. At approximately 23 hours after the initiation of the treatments, cytotoxicity was assessed as cellular morphology and cell survival relative to the vehicle control. Viable cell counts were obtained using trypan blue dye exclusion.

The triplicate labeled cell cultures for UDS analysis were refed with WMEI containing 1 mM thymidine and incubated for 30 minutes. The nuclei in the cells were swollen by addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 12 minutes, and then the cells were fixed with cold acetic acid:ethanol (1:3) and dried overnight. The coverslips were mounted on glass slides, dipped in a solution of Kodak NTB2 emulsion and deionized water, and dried. The coated slides were stored for 6 days at 2 to 8°C in light-tight boxes containing packets of Drierite®. The emulsions were then developed in Kodak D19, fixed, and stained using Williams' modified hematoxylin and eosin procedure.

Slide Analysis. 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. UDS was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized cytoplasmic areas adjacent to each nucleus (cytoplasmic 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 fifty randomly selected cells on each coverslip. Only nuclei with normal morphologies were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis (S-phase) occurred rather than repair synthesis. Normally, 150 cells per dose level were read (50 from each of the triplicate coverslips). The complete number of 150 cells per dose may not be available due to toxicity or quality of preparation. Grain count data obtained for a given treatment is acceptable as part of the evaluation if obtained from at least two replicate cultures and at least 100 cells per dose. The mean net nuclear grain count is determined from the triplicate coverslips for each treatment condition.

Assay Acceptance Criteria

An assay normally is 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, thus the study director exercises scientific judgment in modifying the criteria or considering events that might affect assay reliability and acceptance.

Cell Culture Conditions. 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

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70% are not uncommon nor necessarily detrimental. A lower limit for acceptability is set at 50% in order to avoid the possible use of a damaged, unrepresentative sample of cells.

The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 90%.

The number of viable cells in the vehicle control cultures should remain reasonably stable over the experimental time period because rapidly declining (dying) cultures may not respond in a representative manner to the test article treatments. Therefore, the average number of viable cells in the vehicle control cultures must not be less than 50% of the cell number at the beginning of the treatment period.

Acceptable Controls. The average net nuclear labeling in the vehicle control cultures must be in the range of -5.00 to 1.00. In addition, no more than 10% of the cells should contain five or more net nuclear grains. Failure to meet either of these criteria will invalidate the assay.

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 articles causing weak or no UDS activity, the average response to the positive control treatments must exceed both criteria used to indicate UDS (see Assay Evaluation Criteria section of this report). For a test article clearly causing dose-related UDS activity, an assay is acceptable in the absence of a positive control lost for technical reasons.

Acceptable High Dose. It is not necessary to include toxic doses (less than 95% survival) in the UDS analysis but it is necessary to show that an increase in concentration results in excessive toxicity as measured by either survivals below 50% or observation of poor cellular morphology. Highly toxic doses can result in reduced incorporation of ^{3}H-TdR resulting in decreased grain counts or poor cellular morphology and may lead to artifacts during grain analysis. The highest analyzed dose must approach excessive toxicity, or result in test article insolubility, or reach the highest applicable dose of 5 mg/ml.

Acceptable Number of Doses. A minimum of six dose levels must be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed dose levels in the first trial to achieve a total of six different concentrations.

Grain count data obtained for a given treatment is acceptable as part of the evaluation if obtained from at least two replicate cultures and at least 100 cells per dose.

Assay Evaluation Criteria

Several criteria have been established which, if met, will provide a basis for evaluation of a test article as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of Covance historical data and calculation of the required minimum increase in nuclear labeling using an approach described by Casciano and Gaylor (1983).

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The test article is considered active in the UDS assay at applied concentrations that cause:

- An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent vehicle control value, and/or;

- An increase in the number of nuclei with five or more net grains such that the percentage of these nuclei in test cultures is 10% above the percentage observed in vehicle control cultures.

Generally, if the first condition is satisfied, the second often will be met. However, satisfaction of only the second condition can also indicate UDS activity. When net nuclear labeling is elevated above the vehicle control labeling but does not reach the statistically significant minimum, the overall pattern of the response is considered in the evaluation. 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.

A dose-related increase in UDS for at least two consecutive applied concentrations is also desirable to evaluate a test article as active in this assay. In some cases, UDS can increase with dose and then decrease to near-zero with successively higher doses. If this behavior is associated with increased toxicity, the test article can be evaluated as active. If an isolated increase occurs for a treatment far removed from the toxic doses, the UDS is considered spurious.

The test article is considered negative if none of the above criteria are met.

When, in the judgment of the study director, 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 positive control nuclear labeling is not to be used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test article. UDS 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.

RESULTS

A preliminary solubility test was performed and the test article, PFOS, was insoluble in water and Williams' Media E. In DMSO, the test article formed a translucent light-yellow suspension with a few undissolved pieces of test article on the bottom of the vial at a concentration of 501 mg/mL. At 401 mg/mL, a translucent light-yellow suspension with a small amount of foam on top was observed. When dosed into media for mammalian cells, the test article formed a suspension/translucent liquid with very fine particles of precipitate at 4010 µg/mL. At concentrations from 253 mg/mL to 15.6 mg/mL in DMSO, PFOS formed a translucent off-white to white homogenous suspension with a small amount of foam on top. When dosed into media, 312 µg/mL formed a transparent media-colored solution. Based on this information, a concentration of 4000 µg/mL was selected as the top dose.

In the UDS assay, the test article formed a translucent light-yellow homogeneous suspension with a small amount of foam on top in DMSO at 400 mg/mL. The stock became a transparent colorless solution at 50.0 mg/mL. In media prior to treatment, a precipitate was present from 4000 µg/mL to 1000 µg/mL. Fifteen concentrations of PFOS were applied, ranging from 4000 µg/mL to 0.250 µg/mL. The test article was excessively cytotoxic at and above 50.0 µg/mL, weakly cytotoxic at 25.0 µg/mL, and noncytotoxic at and below 10.0 µg/mL. The cellular morphology was suitable for analysis at and below 25.0 µg/mL.

The hepatocytes were collected at a calculated viability of 92.3% based on trypan blue exclusion. The treatments were initiated within 3 hours with cell monolayers that were 96.7% viable, with an attachment efficiency of 94.5%. After an additional 22.7 hours in culture (which encompassed the 19.6-hour treatment period), the average viable cell count in the control cultures was 86.0% of the viable count at the beginning of the treatments. This stability in cell number and the normal morphological appearance of the cells indicated that the hepatocyte cultures were in good metabolic condition for the UDS assay.

The vehicle control values were a mean net nuclear grain count of -4.74 and a mean percent nuclei containing five or more net grains of 2.67%. These values were used to determine the minimum criteria for a positive UDS response. For a treatment to be considered positive, there must be an increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent vehicle control value, and/or an increase in the number of nuclei with five or more net grains such that the percentage of these nuclei in test cultures is 10% above the percentage observed in vehicle control cultures. The criteria for a positive response in this assay were a mean net nuclear grain count exceeding 0.26 and /or at least 12.67% of the nuclei containing five or more net grains. None of the treatments with the test article samples caused a positive response in either the mean nuclear grain count or in the nuclei containing five or more net grains.

The 2-AAF treatment induced large increases in nuclear labeling that exceeded both criteria used to indicate UDS. This positive response confirmed that the hepatocyte cultures were metabolically active during the UDS assay.

Heavily-labeled nuclei that were blackened with numerous grains represent cells undergoing DNA replication (S-phase) as opposed to DNA repair. The number present in this study was low and did not interfere with the assay.

CONCLUSION

The test article did not induce unscheduled DNA synthesis, as measured by mean net nuclear grain counts or the percentage of nuclei containing five or more net grains. Doses were chosen for each assay based on cell counts determined at 20-24 hours after dosing and the analyzed treatments covered a good range of cytotoxicity for evaluation of UDS. The test article, PFOS, was therefore evaluated as negative in the *in vitro* assay for unscheduled DNA synthesis in rat liver primary cell cultures.

RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocol and the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna for at least one year following submission of the final report to the Sponsor. After the 1-year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna for an additional period of time, or sent to a storage facility designated by the Sponsor.

REFERENCES

Butterworth, B.E., J. Asby, E. Bermudez, D. Casciano, J. Marsalis, G. Probst and G. Williams (1987) A protocol and guide for te in vitro rat hepatocyte DNA-repair assay, Mutation Research, 189, 113-121.

Casciano, D.A. and D.W. Gaylor (1983) Statistical criteria for evalutating chemicals as positive or negative in the hepatocytes DNA repair assay, Mutation Research, 122:81-86.

Williams, G.M. (1977) Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture, Cancer Research, 37:1845-1851.

Williams, G.M. (1980) The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures, in: F. de Serres and A. Holaender (Eds.), Chemical Mutagens, Plenum Press, N.Y, Vol. 6, pp. 61-79.

DATA TABLES

TABLE 1. SUMMARY OF DATA FROM THE RAT HEPATOCYTE UDS ASSAY

TEST ARTICLE: PFOS
COVANCE STUDY NO.: 20784-0-447
TRIAL INITIATION DATE: 1-September-99

Test Condition	Concentration		Mean Net Nuclear Grains (NNG) (1)	% Cells w/ ≥ 5 Mean NNG (2)	Mean Cyto. Grains (3)	% Survival At 23.3 hours (4)
Vehicle Control - DMSO						
	1.00	%	-4.74	2.67	19.03	100.0
Positive Control						
2-AAF	0.100	µg/mL	19.35	96.00	16.17	88.5
Test Material						
	25.0	µg/mL	-2.81	6.67	20.81	65.2
	10.0	µg/mL	-3.71	6.00	22.61	103.3
	5.00	µg/mL	-4.51	3.33	18.77	96.8
	2.50	µg/mL	-3.17	5.33	15.84	109.3
	1.00	µg/mL	-4.04	0.67	17.39	96.3
	0.500	µg/mL	-4.87	1.33	19.29	102.2

(1) Average of net nuclear grain counts on triplicate coverslips (150 total cells). Net nuclear grains = Nuclear grain count - Average cytoplasmic grain count.
(2) Average percentage of cells with greater than or equal to 5 net nuclear grains on triplicate coverslips (150 total cells).
(3) Average of cytoplasmic grain counts on triplicate coverslips (150 total cells).
(4) Survival = Number of viable cells per unit area relative to the solvent control.

2-AAF = 2-acetylaminofluorene
DMSO = Dimethyl sulfoxide

TABLE 2. INDIVIDUAL SLIDE DATA

ASSAY NO. 20784-0-447
TRIAL INITIATION DATE: 09/01/99

Slide Code	Dose (µg/mL)	Mean Net Nuclear Grains	Standard Deviation	Mean Cytoplasmic Grains	% Cells w/ ≥ 5 Net Nuclear Grains
8	VC	-4.38		16.86	2.00
6	VC	-4.16		17.44	4.00
4	VC	-5.68		22.80	2.00
Dose Average		**-4.74**	**0.82**	**19.03**	**2.67**
Cells in S-Phase 0/1500 screened for S-Phase = **0.00%**					
9	PC	23.60		18.06	98.00
7	PC	20.66		14.58	100.00
2	PC	13.78		15.86	90.00
Dose Average		**19.35**	**5.04**	**16.17**	**96.00**
Cells in S-Phase 7/1500 screened for S-Phase =**0.47%**					
5	25.0	-1.42		15.88	8.00
3	25.0	-3.32		21.64	8.00
1	25.0	-3.68		24.90	4.00
Dose Average		**-2.81**	**1.21**	**20.81**	**6.67**
Cell in S-Phase 4/1500 screened for S-Phase = **0.27%**					
18	10.0	-3.30		21.38	4.00
17	10.0	-2.72		22.72	6.00
16	10.0	-5.12		23.74	8.00
Dose Average		**-3.71**	**1.25**	**22.61**	**6.00**
Cells in S-Phase 5/1500 screened for S-Phase = **0.33%**					
15	5.00	-5.94		20.06	4.00
14	5.00	-4.94		22.94	4.00
13	5.00	-2.66		13.32	2.00
Dose Average		**-4.51**	**1.68**	**18.77**	**3.33**
Cells in S-Phase 2/1500 screened for S-Phase = **0.13%**					

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TABLE 2. INDIVIDUAL SLIDE DATA (Continued)

ASSAY NO. 20784-0-447
TRIAL INITIATION DATE: 09/01/99

Slide Code	Dose (µg/mL)	Mean Net Nuclear Grains	Standard Deviation	Mean Cytoplasmic Grains	% Cells w/ ≥ 5 Net Nuclear Grains
21	2.50	-3.66		13.92	4.00
20	2.50	-4.30		17.48	2.00
19	2.50	-1.54		16.12	10.00
Dose Average		**-3.17**	**1.44**	**15.84**	**5.33**
Cells in S-Phase 4/1500 screened for S-Phase = **0.27%**					
24	1.00	-4.38		16.08	0.00
23	1.00	-3.96		16.56	0.00
22	1.00	-3.78		19.52	2.00
Dose Average		**-4.04**	**0.31**	**17.39**	**0.67**
Cells in S-Phase 0/1500 screened for S-Phase = **0.00%**					
12	0.500	-4.14		19.98	2.00
11	0.500	-3.46		15.02	2.00
10	0.500	-7.02		22.86	0.00
Dose Average		**-4.87**	**1.89**	**19.29**	**1.33**
Cell in S-Phase 1/1500 screened for S-Phase = **0.07%**					
Total Cells in S-Phase 23/12000 screened for S-Phase = **0.19%**					

VC = Vehicle Control
PC = Positive Control

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HISTORICAL CONTROL DATA

HISTORICAL VEHICLE CONTROLS
IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

Study Number	UDS Grains/ Nucleus ± SD [a]	% of Nuclei with ≥ 5 Net Nuclear Grains [b]	Average Cyto Grains [b]
1	-3.53	0.0	15.44
2	-2.68	2.7	16.17
3	-0.37	6.7	8.78
4	0.74	8.0	10.59
5	-0.74	6.0	13.28
6	-1.24	7.3	17.47
7	-1.41	1.3	16.87
8	-0.86	8.0	14.54
9	-0.47	5.3	10.30
10	-0.87	5.3	21.57
11	-0.99	9.3	14.69
12	0.16	9.3	10.73
13	-1.45	7.3	14.62
14	-0.80	6.7	12.95
15	-0.73	6.0	18.63
16	-1.36	3.3	11.29
17	-2.78	0.7	11.50
18	-1.83	0.7	10.49
19	-2.67	4.0	16.49
20	-4.63	3.3	20.05
Average	-1.43 ± 1.28	5.06 ± 2.91	14.32 ± 3.54
Range:			
Low	-3.53	0.0	8.29
High	0.74	9.3	21.57

Number of data points is 20. Data included from 5/90 to 1/99
[a]Average of net nuclear grain counts triplicate or duplicate coverslips (150 cells analyzed)
[b] Average values for triplicate or duplicate coverslips (150 cells) analyzed
SD = Standard deviation

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HISTORICAL CONTROL DATA (continued)

HISTORICAL POSITIVE CONTROLS
IN VITRO UNSCHEDULED DNA SYNTHESIS ASSAY

Study Number	UDS Grains/ Nucleus ± SD [a]	% of Nuclei with ≥ 5 Net Nuclear Grains [b]	Average Cyto Grains [b]
1	29.45	100.0	25.71
2	15.53	92.0	11.52
3	7.35	63.3	12.53
4	12.35	88.7	11.31
5	14.18	97.3	16.25
6	14.45	92.7	16.05
7	16.09	100.0	15.73
8	9.51	78.7	15.98
9	13.17	90.7	14.27
10	16.92	97.3	10.51
11	17.38	86.7	15.68
12	4.46	46.0	10.89
13	15.59	99.3	10.73
14	10.69	84.8	11.68
15	13.79	92.7	14.09
16	14.66	89.3	17.85
17	14.33	91.3	12.22
18	14.63	96.7	11.50
19	16.81	98.7	10.49
20	17.51	97.3	16.49
Average	14.44 ± 4.89	89.19 ± 13.36	14.07 ± 3.65
Range:			
Low	4.46	46.00	10.49
High	29.45	100.00	25.71

Number of data points is 20. Data included from 5/90 to 1/99
The positive control used is 10 µg/ml 2-AAF
[a]Average of netnuclear grain counts triplicate or duplicate coverslips (150 cells) analyzed
[b] Average values for triplicate or duplicate coverslips (150 cells) analyzed
SD = Standard deviation

C01907

COVANCE
THE DEVELOPMENT SERVICES COMPANY

AMENDMENT TO THE STUDY PROTOCOL

STUDY TITLE: Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures

PROTOCOL NO.: 447 Edition 17

COVANCE STUDY NO.: 20784-0-447

Amendment #1.

1. Section 4.2.3 Termination and Cytotoxicity Measurement

   The third sentence in paragraph 3 is changed from "The cells will be fixed with cold acetic acid:ethanol (1:3) and dried for at least 24 hours." to "The cells will be fixed with cold acetic acid:ethanol (1:3) and dried at least overnight."

   Reason: Overnight allows for sufficient drying.

2. Section 4.2.3 Termination and Cytotoxicity Measurement

   The third sentence in paragraph 4 is changed from "The emulsion-coated slides will be stored for 7 to 10 days at 2 to 8°C in light-tight boxes containing a desiccant." to "The emulsion-coated slides will be stored for 6 to 9 days at 2 to 8°C in light-tight boxes containing a desiccant."

   Reason: Previous studies indicated that a shorter exposure period was needed.

3. The reference to Mirsalis, Tyson and Butterworth, 1982 is deleted from Section 4.2.2 of the protocol:

   Reason: The reference refers to procedures for an *in vivo/in vitro* UDS assay.

Study Director:

Maria A. Cifone — 9-2-99

Maria A. Cifone, Ph.D. — Date
Genetic and Cellular Toxicology

COVANCE
THE DEVELOPMENT SERVICES COMPANY

AMENDMENT TO THE STUDY PROTOCOL

STUDY TITLE: Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures

PROTOCOL NO.: 447 Edition 17

COVANCE STUDY NO.: 20784-0-447

Amendment #2.

1. Section 1.0 Sponsor Identification

The name of the sponsor is changed from 3M Corporation to 3M Corporate Toxicology.

Reason: Sponsor request.

Study Director:

Maria A. Cifone

Maria A. Cifone, Ph.D.
Genetic and Cellular Toxicology

11-8-99
Date

COVANCE
THE DEVELOPMENT SERVICES COMPANY

COVANCE STUDY NO. 2C784-C-447
PROTOCOL NO. 447 EDITION 17

UNSCHEDULED DNA SYNTHESIS IN RAT LIVER PRIMARY CELL CULTURES

Covance Laboratories Inc. (Covance) will conduct this study in compliance with Good Laboratory Practice (GLP) regulations and standards. This protocol, at least one critical phase of the work in progress, and the final report will be subject to audit by Quality Assurance in accordance with Standard Operating Procedures maintained at Covance. The study will be conducted by Covance at 9200 Leesburg Pike, Vienna, Virginia 22182 (Covance-Vienna).

PART 1. SPONSOR INFORMATION AND APPROVALS

1.0 SPONSOR IDENTIFICATION

Company Name: 3M Corporation
Address: 3M Center
Building 220-2E-02
St. Paul, MN 55144-1000

2.0 TEST ARTICLE IDENTIFICATION

PFOS

3M Study No. T6295.19

3.0 TEST ARTICLE ANALYSIS

The Sponsor will be responsible for the determination and documentation of the analytical purity and composition of the test article and the stability and strength of the dosing solutions.

4.0 NOTIFICATION OF REGULATORY SUBMISSION

In order to comply with GLP regulations and standards, consulting laboratories must be notified if all or part of a study is intended for regulatory submission. Covance maintains a master schedule of studies which fall under regulatory review. Please indicate which agency, if any, might receive the results of this study:

☐ Undetermined ☐ FDA ☒ EPA-TSCA ☐ EPA-FIFRA
☐ MAFF ☐ MOHW ☐ OECD ☐ None ☐ Other_____

5.0 STUDY DATES

Proposed Experimental Start Date: September 1999

Proposed Experimental Termination Date: October 1999

6.0 APPROVAL OF STUDY PROTOCOL

Study Director:

Maria A. Cifone Date: 8/20/99

Maria A. Cifone, Ph.D.

Testing Facility Management:

Brian C. Myhr Date: 8/20/99

Brian C. Myhr, Ph.D.
Associate Director

Sponsor's Authorized Representative:

Marvin Case Date: 16 August 1999

Marvin T. Case, D.V.M., Ph.D.

PART 2. STUDY PROTOCOL

UNSCHEDULED DNA SYNTHESIS IN RAT LIVER PRIMARY CELL CULTURES

1.0 OBJECTIVE

The objective of this *in vitro* assay is to evaluate the ability of a test article, or a metabolite, to induce DNA damage in rat primary hepatocytes by measuring unscheduled DNA synthesis (UDS).

2.0 TEST SYSTEM RATIONALE

This assay aims to establish whether the test article or its metabolites can interact with rat liver primary cell (hepatocyte) cultures to induce DNA damage followed by DNA repair measured as UDS. Hepatocytes will be isolated from liver and exposed *in vitro* to the test article and tritiated thymidine (^{3}H-TdR). Primary hepatocytes have sufficient metabolic activity to eliminate the need for the addition of a microsomal activation system. An autoradiographic technique described by Williams (1977) is used to measure incorporation of ^{3}H-TdR into DNA. Because only a small percentage of the cells will enter S-phase (replicative DNA synthesis) during the exposure period, the incorporation of ^{3}H-TdR into DNA can be used as a measure of the repair of DNA damage caused by treatment with the test article. The existence and degree of DNA damage will be inferred from an increase in nuclear grain counts in treated hepatocytes compared to nuclear grain counts from untreated hepatocytes. The types of detectable DNA damage are unspecified but must be recognizable by the cellular repair system and result in the incorporation of nucleic acid bases including ^{3}H-TdR into the DNA.

3.0 MATERIALS

3.1 Test System

The indicator cells for this assay will be hepatocytes obtained from a single adult, male, Fischer 344 rat (weighing 150 to 300 g), which will be purchased from the Charles River Breeding Laboratories, Incorporated or another qualified dealer. Animals will be received by animal care personnel, examined, and housed according to applicable Covance SOPs. Food and water will be supplied *ad libitum*. The animals will be used for the surgical procedure within two days of receipt. Animals will be anesthetized prior to surgery to obtain the hepatocytes (sodium pentobarbital at about 60mg/kg by intraperitoneal injection) and exsanguinated during the procedure.

The cells will be obtained by perfusion of the liver *in situ* with a collagenase solution, WMEC, (see Section 4.2, UDS ASSAY). Monolayer cultures will be established on plastic coverslips in culture dishes and used the same day for initiation of the UDS assay.

3.1.1 Media and Cell Culture Conditions

Williams' Medium E supplemented with 2 mM L-glutamine, 100 μg/ml streptomycin sulfate, and 150 μg/ml gentamicin (WMEI) is the base culture medium, and is modified for each specific requirement. The hepatocytes will be obtained by perfusion of livers *in situ* with HBSS/EGTA followed by WMEC: Hanks' balanced salts (Ca++-and Mg++-free) containing 0.5 mM ethyleneglycol-bis(β-aminoethyl ether)-N, N-tetra-acetic acid and 50 mM HEPES buffer at pH 7.2 (HBSS/EGTA), and WMEI containing 50 to 100 units/ml of collagenase (WMEC). The cultures are established in WMEI supplemented with 10% fetal bovine serum (WME+). All cell cultures will be maintained as monolayers in a humidified incubator at 35 to 37.5°C in an atmosphere of 4 to 6% CO_2 in air. After the establishment period the culture labeling is initiated using WMEI containing 10 μCi/ml ^{3}H-TdR at 40 to 60 Ci/mMole (WME-treat). When water is the vehicle of choice, the concentration of ^{3}H-TdR will be adjusted so that the final concentration of label in the medium (after a 1:10 dilution of the water stocks) will be about 10 μCi/ml.

3.2 Test Article

Solid or liquid test articles are suitable for this assay. The test article is identified in Part 1 of this protocol. Storage conditions will be as specified by the Sponsor.

3.3 Control Articles

3.3.1 Vehicle Control Article

The vehicle control article will be the vehicle selected for the test article. The vehicle may be specified by the Sponsor or selected by the process described in Section 4.1, Test Article Handling. The vehicle will be used in the vehicle control cultures at a concentration equivalent to the vehicle in the test article-treated cultures. The vehicle control cultures will be handled identically to the test article treated cultures. An untreated (negative) control, consisting of assay procedures performed on cells exposed to WME-treat only, will be included when acetone is used as the vehicle.

3.3.2 Positive Control Article

The positive control article, 2-acetylaminofluorene (2-AAF; CAS # 53-96-3), is known to induce UDS in rat hepatocyte primary cell cultures. The 2-AAF will be dissolved in DMSO and diluted 1:100 into WME-treat for a final concentration of 0.10 μg/ml (4.48×10^{-7}M).

4.0 EXPERIMENTAL DESIGN

4.1 Test Article Handling

If solubility information is not provided by the Sponsor, a preliminary solubility test will be carried out with serum-free culture medium or sterile deionized water, dimethyl sulfoxide (DMSO; CAS #67-68-5), ethanol (CAS #64-17-5), or acetone (CAS #67-64-1). If the vehicle of choice is culture medium, the test article will be dissolved at the highest desired concentration in WME-treat. Stock solutions in water will be diluted 1:10, in DMSO or ethanol will be diluted 1:100, and in acetone will be diluted 1:200 in WME-treat. The vehicle selected will be the one which gives the best solubility and dispersion characteristics after dilution in culture medium. In some cases, test articles are apparently insoluble in vehicles that are compatible with tissue culture. In such cases, the vehicle chosen and the highest dose tested will be that in which an evenly dispersed suspension can be prepared.

4.2 UDS Assay

4.2.1 Dose Determination and Culture Conditions

The maximum dose will be determined on a case by case basis taking into account both solubility and any relevant cytotoxicity information available on the test article. The highest dose tested will be 5 mg/ml, unless higher doses are specified by the Sponsor. Relatively insoluble test articles will be tested up to approximately 2 times the solubility limit, if insoluble article does not interfere with the enumeration of nuclear grains.

The top dose tested for liquid test articles will be 5 mg/ml, which may be determined by weight or calculated from the specific gravity. Solubility testing will be the same as described above.

If marked pH changes are noticed during the solubility test, neutralization, with HCl or NaOH, will be performed to maintain a normal culture pH range (6.8 to 7.4) only after consultation with the Sponsor.

A concurrent cytotoxicity assessment is an integral part of the UDS assay used to select appropriate doses for a particular, fresh primary culture of hepatocytes. A range of 15 concentrations will usually be applied to the cells. Fewer concentrations may be initiated when toxicity information is available. A viable cell count (trypan blue dye exclusion) will then be obtained 20 to 24 hours after initiation of the treatments. At least six concentrations will be chosen for analysis of nuclear labeling, starting with the highest dose that results in a sufficient number of surviving cells with intact morphologies and proceeding to successively lower doses. Repeat trials may contain as few as 5 dose levels at the discretion of the study director. The test article solution will be prepared immediately before use. The maximum final concentrations of vehicle in WME-treat will be up to 10% water, 1% DMSO or ethanol, or 0.5% acetone.

4.2.2 Cell Collection and Culture

This assay is based on the procedures described by Williams, (1980), Mirsalis, Tyson and Butterworth, (1982) and Butterworth *et al*, (1987). The hepatocytes will be obtained by perfusion of livers *in situ* with HBSS/EGTA for about 4 minutes followed by WMEC for about 11 minutes. Depending on the condition of the liver, this time may be altered by ± 2 minutes. The hepatocytes will be obtained by mechanical dispersion of excised liver tissue in a culture dish containing WMEC. The suspended tissue and cells will then be allowed to settle to remove cell clumps and debris prior to collection. The collected cell suspension will be centrifuged and the cell pellet resuspended in WME+. After obtaining a viable cell count, a series of culture dishes (at least 5 per dose level) will be inoculated with approximately 0.5 x 10^6 viable cells in 3 ml of WME+. Culture dishes that will be used for UDS analysis will contain round plastic coverslips while dishes used to assess attachment efficiency and toxicity will have no coverslips.

An attachment period of 1.5 to 2 hours at 35 to 37.5°C in a humidified atmosphere containing 4 to 6% CO_2 will be used to establish the cell cultures. Unattached cells will then be removed and the cultures will be refed with WMEI.

The UDS assay will be initiated within three hours by replacing the media in the culture dishes with 2.5 ml WME-treat containing the test article at the desired concentration. Each control and test article treatment will be performed with at least five cultures. Three of these replicate cultures will be used for UDS analysis and two replicates will be used for the cytotoxicity measurements. Dishes will be labeled per Covance SOP.

4.2.3 Termination and Cytotoxicity Measurement

After an exposure period of 18 to 20 hours, the treatments and labeling will be terminated by washing the cell monolayers with medium. The cultures used for the cytotoxicity measurements will be refed with WMEI and returned to the incubator.

At 20 to 24 hours after the initiation of the treatments, cytotoxicity will be assessed as cellular morphology and cell survival relative to the vehicle control. Viable cell counts will be obtained using trypan blue dye exclusion.

The triplicate labeled cell cultures for the UDS assay will be refed with WMEI containing 1 mM thymidine. The nuclei will be swollen by addition of 1% sodium citrate to the coverslips (containing the cell monolayers) for 8 to 12 minutes. The cells will be fixed with cold acetic acid:ethanol (1:3) and dried for at least 24 hours. The coverslips will be mounted on glass slides.

The cell survival and morphology obtained from the cytotoxicity cultures will be use to chose doses levels for autoradiography. Slides will be dipped in an emulsion of Kodak NTB2 and water, and dried. The emulsion-coated slides will be stored for 7 to 10 days at 2 to 8°C in light-tight boxes containing a desiccant. The emulsions will be developed with Kodak D19 developer and Kodak Rapid Fixer, then stained with a modified hematoxylin and eosin procedure.

4.2.4 Slide Analysis

The cells will be examined microscopically at approximately 1500x magnification under oil immersion and the field displayed on the video screen of an automatic counter. UDS will be measured by counting nuclear grains and subtracting the average number of grains in three

nuclear-sized cytoplasmic areas adjacent to each nucleus (cytoplasmic count). This value is referred to as the net nuclear grain count. The coverslips will be coded to prevent bias in grain counting.
The net nuclear grain count will be routinely determined for 50 randomly selected cells on each coverslip. Only nuclei with normal morphologies will be scored, and any occasional nuclei blackened by grains too numerous to count will be excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis. Normally, 150 cells per dose level will be read (50 from each of the triplicate coverslips). The complete number of 150 cells per dose may not be available due to toxicity or quality of preparation. Grain count data obtained for a given treatment is acceptable as part of the evaluation if obtained from at least two replicate cultures and at least 100 cells per dose. The mean net nuclear grain count will be determined from the triplicate coverslips for each treatment condition. Occasionally, a coverslip may be recounted at a later date or by a different technician. Since a different cell population will generally be scored, the average count for 50 cells will be used in the calculation of the mean for the triplicate treatment.

5.0 DATA

5.1 Data Presentation

The final report will include the following information in tabular form for the vehicle control, positive control, and each analyzed treatment:

- The mean net nuclear grain count for triplicate cultures with standard deviation (usually a total of 150 cells).
- The mean percentage of cells having five or more net nuclear grains for triplicate cultures.
- The mean cytoplasmic grain count for triplicate cultures (usually three areas per cell in a total of 150 cells).
- The mean survival for duplicate cultures, which is the number of viable cells per unit area in the culture relative to the vehicle control value x 100%.

Information regarding test article solubility in the culture medium, viability of the hepatocyte cultures, and the percent of cells in DNA replicative synthesis (S-phase) will also be provided.

5.2 Assay Acceptance Criteria.

An assay 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, thus the study director exercises scientific judgment in modifying the criteria or considering events that might affect assay reliability and acceptance.

5.2.1 Cell Culture Conditions

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. A lower limit for acceptability is set at 50% in order to avoid the possible use of a damaged, unrepresentative sample of cells.

The viability of the monolayer cell cultures used for the assay treatments must be 70% or greater. Normally, the viability of attached cells is about 90%.

The number of viable cells in the vehicle control cultures should remain reasonably stable over the experimental time period because rapidly declining (dying) cultures may not respond in a representative manner to the test article treatments. Therefore, the average number of viable cells in the vehicle control cultures must not be less than 50% of the cell number at the beginning of the treatment period.

5.2.2 Acceptable Controls

The average net nuclear labeling in the vehicle control cultures must be in the range of -5.00 to 1.00. In addition, no more than 10% of the cells should contain five or more net nuclear grains. Failure to meet either of these criteria will invalidate the assay.

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 articles causing weak or no UDS activity, the

average response to the positive control treatments must exceed both criteria used to indicate UDS (see Section 5.3). For a test article clearly causing a dose-related UDS activity, an assay will be acceptable in the absence of a positive control lost for technical reasons.

5.2.3 Acceptable High Dose

It is not necessary to include toxic doses (less than 95% survival) in the UDS analysis but it is necessary to show that an increase in concentration results in excessive toxicity as measured by either survivals below 50% or observation of poor cellular morphology. Highly toxic doses can result in reduced incorporation of ^{3}H-TdR resulting in decreased grain counts or poor cellular morphology and may lead to artifacts during grain analysis. The highest analyzed dose must approach excessive toxicity, or result in test article insolubility, or reach the highest applicable dose of 5 mg/ml.

5.2.4 Acceptable Number of Doses

A minimum of six dose levels will be analyzed for nuclear grain counts. Repeat trials need only augment the number of analyzed dose levels in the first trial to achieve a total of six different concentrations.

Grain count data obtained for a given treatment is acceptable as part of the evaluation if obtained from at least two replicate cultures and at least 100 cells per dose.

5.3 ASSAY EVALUATION CRITERIA

Several criteria have been established which, if met, will provide a basis for evaluation of a test article as active in the UDS assay. The criteria for a positive response are based on a statistical analysis of Covance historical data and calculation of the required minimum increase in nuclear labeling using an approach described by Casciano and Gaylor (1983).

The test article will be considered active in the UDS assay at applied concentrations that cause:

- An increase in the mean net nuclear grain count to at least five grains per nucleus above the concurrent vehicle control value, and/or;

- An increase in the number of nuclei with five or more net grains such that the percentage of these nuclei in test cultures is 10% above the percentage observed in vehicle control cultures.

Generally, if the first condition is satisfied, the second often will be met. However, satisfaction of only the second condition can also indicate UDS activity. When net nuclear labeling is elevated above the vehicle control labeling but does not reach the statistically significant minimum, the overall pattern of the response is considered in the evaluation. 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 will be considered in an evaluation.

A dose-related increase in UDS for at least two consecutive applied concentrations is also desirable to evaluate a test article as active in this assay. In some cases, UDS can increase with dose and then decrease to near-zero with successively higher doses. If this behavior is associated with increased toxicity, the test article can be evaluated as active. If an isolated increase occurs for a treatment far removed from the toxic doses, the UDS will be considered spurious.

The test article is considered negative if none of the above criteria are met.

When, in the judgement of the study director, 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 positive control nuclear labeling will not be used as a reference point to estimate mutagenic or carcinogenic risk associated with the UDS activity of the test article. UDS 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 will be used to demonstrate that the cell population employed was responsive and the methodology was adequate for the detection of UDS.

6.0 REFERENCES

Butterworth, B.E., J. Ashby, E. Bermudez, D. Casciano, J. Mirsalis, G. Probst and G. Williams (1987) A protocol and guide for the in vitro rat hepatocyte DNA-repair assay, Mutation Research, 189, 113-121.

Casciano, D.A. and D.W. Gaylor (1983) Statistical criteria for evaluating chemicals as positive or negative in the hepatocytes DNA repair assay, Mutation Research, 122:81-86.

Williams, G.M. (1977) Detection of chemical carcinogens by unscheduled DNA synthesis in rat liver primary cell culture, Cancer Research, 37:1845-1851.

Williams, G.M. (1980) The detection of chemical mutagens-carcinogens by DNA repair and mutagenesis in liver cultures, in: F. de Serres and A. Hollaender (Eds.), Chemical Mutagens, Plenum Press, NY, Vol. 6, pp. 61-79.

7.0 REPORT FORMAT

Covance employs a standard report format for each assay design. The final report will provide the following information.

- Sponsor identification.
- Quality Assurance statement.
- Statement of GLP compliance.
- Signature of study director.
- Test article identification and Covance Study Number. A physical description of the test article and date of receipt will be included in this section.
- Type of assay and protocol number.
- Dates of study initiation and completion.
- Identification of study director and senior technician.
- Methods.
- Evaluation criteria.
- Interpretation of results.
- Conclusions.
- References.
- Test results presented in tabular form.
- Historical control data.

8.0 CHANGES OR REVISIONS

Any changes or revisions of this approved protocol will be documented, signed by the study director, dated, and maintained with this protocol. The Sponsor will be notified of any changes or revisions.

9.0 ANIMAL CARE AND USE STATEMENT

In the opinion of the Study Director, no alternative testing methods are appropriate, the study does not duplicate any previous work with this test article, and the number and species selected are appropriate. This protocol will be reviewed by the Covance-IACUC for compliance with regulatory guidelines concerning the care and use of animals. If not in compliance, a modification will be required. Any changes or revisions of this approved protocol will be sent to the Covance-IACUC for their review.

10.0 RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocol, and the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna for at least one year following submission of the final report to the Sponsor. After the one year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna for an additional period of time or sent to a storage facility designated by the Sponsor.