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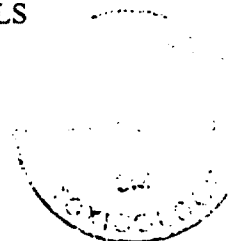
T6358

MEASURING CHROMOSOMAL ABERRATIONS IN
CHINESE HAMSTER OVARY (CHO) CELLS

FINAL REPORT

AUTHOR

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LABORATORY PROJECT IDENTIFICATION

CHV Study No.: 17388-0-437

SUBMITTED TO

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QUALITY ASSURANCE STATEMENT

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Quality Assurance inspections of the study and review of the final report of the above referenced project were conducted according to the Standard Operating Procedures of the Quality Assurance Unit and according to the general requirements of the appropriate Good Laboratory Practice regulations. Findings from the inspections and final report review were reported to management and to the study director on the following dates:

<u>Inspection/Date</u>	<u>Findings Reported</u>	<u>Auditor</u>
Harvest/02/14/1996	02/14/1995	S. Ballenger
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Quality Assurance Unit 4/25/96
Date Released

STUDY COMPLIANCE AND CERTIFICATION

The study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Food and Drug Administration (FDA) Title 21 of the U.S. Code of Federal Regulations Part 58, issued December 22, 1978, (effective June 20, 1979) 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. All raw data, documentation, records, protocol and a copy of the final report generated as a result of this study will be archived in the storage facilities of Corning Hazleton Inc. 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 Corning Hazleton Inc. for an additional period of time, or sent to a storage facility designated by the Sponsor.

Submitted by:

Study Director:

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Mammalian Cytogenetics
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4/25/96
Study Completion
Date

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ABSTRACT

The objective of this in vitro assay was to evaluate the ability of T6358 to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without metabolic activation.

For the dose rangefinding assays with and without metabolic activation, the test article was dissolved in sterile deionized water at a concentration of 501 mg/ml. Concentrations of 0.167, 0.501, 1.67, 5.01, 16.7, 50.1, 167, 501, 1670, and 5010 µg/ml were tested in the dose rangefinding assays. All dosing was achieved using a dosing volume of 1% (10.0 µl/ml). Complete cytotoxicity was observed in the cultures dosed with 501, 1670, and 5010 µg/ml in the nonactivation assay and with 1670 and 5010 µg/ml in the assay with metabolic activation. Very few metaphases were available in the culture treated with 167 µg/ml and a severe cell cycle delay was observed. No cell cycle delay was observed in the cultures analyzed in the metabolic activation assay.

Based on the data from the dose rangefinding assay, replicate cultures of CHO cells were incubated with 12.5, 25.0, 50.0, 75.0, 100, 150, and 200 µg/ml in a 20.0 hour aberrations assay without metabolic activation. Replicate cultures were treated with 125, 250, 500, 750, 1000, and 1500 µg/ml with a 20.0 hour aberrations assay with metabolic activation. Cultures treated with 75.0, 100, 150, and 200 µg/ml from the assay without metabolic activation and cultures treated with 125, 250, 500, and 750 µg/ml from the assay with metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations was observed at the concentrations analyzed.

The test article, T6358, was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation.

Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells
With T6358

- 1.0 SPONSOR: 3M
- 2.0 MATERIAL (TEST ARTICLE):
 - 2.1 Client's Identification: T6358
 - 2.2 Date Received: January 16, 1996
 - 2.3 Physical Description: White powder
 - 2.4 Genetics Assay No.: 17388
- 3.0 TYPE OF ASSAY: Chromosomal Aberrations in Chinese Hamster Ovary (CHO) Cells
- 4.0 PROTOCOL NO.: 437, Edition 15
- 5.0 STUDY DATES:
 - 5.1 Initiation Date: January 18, 1996
 - 5.2 Experimental Start Date: January 30, 1996
 - 5.3 Experimental Termination Date: March 20, 1996
- 6.0 SUPERVISORY PERSONNEL:
 - 6.1 Study Director: Hemalatha Murli, Ph.D.
 - 6.2 Laboratory Supervisor: Carol S. Spicer, B.S.
- 7.0 OBJECTIVE:

The objective of this in vitro assay was to evaluate the ability of the test article, T6358, to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells, with and without metabolic activation.

8.0 RATIONALE:

The assay is designed to establish whether the test article or its metabolites can interact with cells to induce chromosome 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 consequence of failure or mistakes in repair processes such that breaks do not rejoin or rejoin in abnormal configurations (Evans, 1962).

The assay is designed to examine cells in the first mitosis after initiation of treatment with a chemical. This design limits loss of aberrant cells during the division process or conversion into complex derivatives during subsequent cell cycles. In the case of CHO cells, most dividing cells examined 8-12 hours after treatment are in the first mitosis (M_1 cells). 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.

9.0 EXPERIMENTAL DESIGN:

Results from the rangefinding 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. In the rangefinding assay, the cultures were incubated for 25-26 hours with 5-bromo-2'-deoxyuridine (BrdUrd). This enabled the majority of cells to progress through about two cell divisions, thus providing a full assessment of cell cycle kinetics. A summary of the treatment schedule for the rangefinding assay is given below.

Summary of Rangefinding Assay Treatment Schedule in Hours

Test	Test Article	Wash	BrdUrd	Wash	Colcemid®	Fixation
- S9	0	-	2.2	25.4	25.7	27.7
+ S9	0	2	2.2	-	25.7	27.7

In the chromosomal aberrations assays, replicate cultures were used at each dose level. Single cultures were used for the negative control, solvent control, and at each of two doses of the positive control. In the aberrations assay with and without metabolic activation, 20 hour harvests were conducted. Chromosomal aberrations were analyzed from the cultures treated at the four highest dose levels and from 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	17.6	18	20
+ S9	0	2	18	20

10.0 MATERIALS AND METHODS:

10.1 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 hours with a modal chromosome number of 21.

10.2 Cell Culture Medium:

The CHO cells were grown in McCoy's 5a culture medium which was supplemented with 10 % fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin and streptomycin, at approximately 37°C, in an atmosphere of about 5% CO₂ in air.

10.3 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, sterile deionized water, at the highest concentration used in test cultures (1%, i.e., 10 µl/ml). In the activation assays, the negative and solvent controls were the same as described in the nonactivation assays but with the S9 activation mix included.

10.4 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 (CAS# 50-07-7, Sigma, Lot # 25H0619) is a clastogen that does not require metabolic activation. Cyclophosphamide (CAS # 6055-19-2, Sigma, Lot # 67F0155) does not act directly but must be converted to

active intermediates by microsomal enzymes. In the rangefinding assay, two concentrations of MMC (0.25 and 0.50 µg/ml) and CP (15.0 and 20.0 µg/ml). In the chromosomal aberrations assays, two concentrations of MMC (0.08 and 0.10 µg/ml) and CP (10.0 and 15.0 µg/ml) were used to induce chromosomal aberrations in the CHO cells. One of the dose levels was analyzed in each of the aberration assays. Both MMC and CP were dissolved in water.

10.5 Rangefinding Assays:

In these assays, the cells were cultured for approximately 24 hours prior to treatment by seeding approximately 0.3×10^6 per 25 cm² flask into 5 ml of complete McCoy's 5a culture medium.

10.5.1 Assay Without Metabolic Activation:

The cultures were dosed with the test article for 2.2 hours when 5-bromo-2'-deoxyuridine (BrdUrd) was added at a final concentration of 10 µM. The cultures were washed 23.3 hours later with phosphate buffered saline and fresh complete medium containing BrdUrd at a final concentration of 10 µM, and Colcemid® (final concentration 0.1 µg/ml) was added. The cultures were then harvested two hours later and differentially stained for the analysis of cell cycle delay using a modified fluorescence-plus-Giemsa (FPG) technique (See Sections on Harvest and Slide Preparation and Staining).

10.5.2 Assay With Metabolic Activation:

In this assay, the CHO cells were exposed to the test article for two hours at ≈37°C 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 (Molecular Toxicology, Inc., Lot #0583) was derived from the liver of male Sprague-Dawley rats which had been previously treated with Aroclor 1254 to induce the mixed function oxidase enzymes which are capable of metabolizing chemicals to more active forms. The two hour 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 FBS 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.

After the exposure period the cells were washed twice with buffered saline. Complete McCoy's 5a medium containing BrdUrd (final concentration 10 μ M) was added to the cultures which were then incubated for 25.5 hours with Colcemid® (final concentration 0.1 μ g/ml) added for the last 2.0 hours. The cultures were then harvested, fixed, and slides were prepared and stained as was described for the nonactivation rangefinding assay.

10.5.3 Assay Evaluation:

One hundred consecutive metaphases from three dose levels with metaphases from the assay without and with metabolic activation (103 from the solvent control from the activation assay) were assessed for the number of cell cycles through which the cells had progressed while in the presence of BrdUrd.

10.6 Aberrations Assay Without Metabolic Activation:

Cultures were initiated by seeding approximately 1.2×10^6 cells per 75 cm² flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cells were incubated at $\approx 37^\circ\text{C}$ with the test article at predetermined doses for 17.8 hours. The cultures were then washed with buffered saline and complete McCoy's 5a medium containing 0.1 μ g/ml Colcemid® was placed back onto the cells. Two hours later, the cells were harvested and air dried slides were made. The slides were then stained in 5 % Giemsa solution for the analysis of chromosomal aberrations.

10.7 Aberrations Assay With Metabolic Activation:

Cultures were initiated by seeding approximately 1.2×10^6 cells per 75 cm² flask into 10 ml of complete McCoy's 5a medium. One day after culture initiation, the cultures were incubated at $\approx 37^\circ\text{C}$ for 2 hours in the presence of the test article and the S9 reaction mixture in McCoy's 5a medium without FBS. After the 2 hour exposure period, the cells were washed twice with buffered saline and the cells were refed with complete McCoy's 5a medium. The cells were incubated for the rest of the culture period up to the time of harvest with 0.1 μ g/ml Colcemid® present during the last 2.0 hours of incubation. The metaphase cells were then harvested and prepared for cytogenetic analysis.

10.8 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 were also evaluated for the presence of mitotic (large rounded cells) or dead cells floating in the medium. The metaphase cells were collected by a mitotic shake-off (Terasima and Tolmach, 1961) and were treated with 0.075 M KCl hypotonic solution. This treatment helps to swell the cells and thus disperse the chromosomes. The cultures were then fixed with an absolute methanol: glacial acetic acid (3:1, v:v) fixative and were washed several times before air-dried slides were prepared.

10.9 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 fluorescence-plus-Giemsa (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 5% Giemsa solution for the analysis of mitotic chromosomal aberrations. All slides were then air-dried and cover slipped using Depex[®] mounting medium.

10.10 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 19-23) were analyzed.

One hundred cells from each replicate culture at four dose levels of the test article, and from the negative and solvent control cultures were analyzed for the different types of chromosomal aberrations (Evans, 1962; See Section 15.0). 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:

1. The overall chromosomal aberration frequencies.

11.2 Rangefinding Assay Without Metabolic Activation

Floating debris, no cell monolayers, and no visible mitotic cells were observed in the cultures dosed with 1670 and 5010 $\mu\text{g/ml}$. Unhealthy cell monolayer, floating debris, $\approx 15\%$ reduction in the cell monolayer confluence, and no visible mitotic cells were observed in the culture treated with 501 $\mu\text{g/ml}$. Unhealthy cell monolayer, floating debris, $\approx 15\%$ reduction in the cell monolayer confluence, and severe reductions in the number of visible mitotic cells were observed in the culture treated with 167 $\mu\text{g/ml}$. Toxicity was manifested on the slides prepared from this culture by the availability of only 89 metaphases. Cell cycle kinetics were evaluated in the cultures treated with 16.7, 50.1, and 167 $\mu\text{g/ml}$ (Table 1). Severe cell cycle delay was observed in the culture dosed with 167 $\mu\text{g/ml}$. Based on these results, a 20 hour harvest was selected for testing concentrations of 12.5, 25.0, 50.0, 75.0, 100, 150, and 200 $\mu\text{g/ml}$ in the nonactivation aberrations assay.

11.3 Rangefinding Assay With Metabolic Activation

Floating debris, no cell monolayers, and no visible mitotic cells were observed in the cultures dosed with 1670 and 5010 $\mu\text{g/ml}$. No visual signs of toxicity were observed in any of the other cultures. Cell cycle kinetics were evaluated in the cultures treated with 50.1, 167, and 501 $\mu\text{g/ml}$ (Table 1). No cell cycle delay was observed in the cultures analyzed. Based on these results, a 20 hour harvest was selected for testing concentrations of 125, 250, 500, 750, 1000, and 1500 $\mu\text{g/ml}$ in the aberrations assay with metabolic activation.

11.4 Chromosomal Aberrations Assay Without Metabolic Activation

Unhealthy cell monolayers, $\approx 30\%$ reduction in the cell monolayer confluence, and severe reductions in the number of visible mitotic cells were observed in the culture treated with 200 $\mu\text{g/ml}$. Toxicity was manifested on the slides prepared from these cultures by the presence of many interphase cells and sparse numbers of metaphases. Unhealthy cell monolayers, $\approx 15\%$ reduction in the cell monolayer confluence, and reductions in the number of visible mitotic cells were observed in the culture treated with 150 $\mu\text{g/ml}$. Slightly unhealthy cell monolayers, slight reductions in the numbers of visible mitotic cells, and $\approx 15\%$ reduction in the cell monolayer confluence were observed in the culture treated with 100 $\mu\text{g/ml}$. Chromosomal aberrations were evaluated from the cultures treated with 75.0, 100, 150, and 200 $\mu\text{g/ml}$ (Table 2). No significant increase in cells with chromosomal aberrations 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 mitomycin C, the positive control agent. The test article is considered negative for inducing chromosomal aberrations under nonactivation conditions.

11.5 Chromosomal Aberrations Assay With Metabolic Activation

No cell monolayers and no visible mitotic cells were observed in the cultures dosed with 1500 µg/ml. Dead cell monolayers, no visible mitotic cells, and <5% cell monolayer confluence were observed in the cultures dosed with 1000 µg/ml. Slightly unhealthy cell monolayers, floating dead cells, and ≈ 15% reduction in the cell monolayer confluence were observed in the culture treated with 750 µg/ml. Toxicity was manifested on the slides prepared from these cultures by the availability of sparse numbers of metaphases. Chromosomal aberrations were evaluated from the cultures treated with 125, 250, 500, and 750 µg/ml (Table 3). No significant increase in cells with chromosomal aberrations 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 cyclophosphamide, the positive control agent. The test article is considered negative for inducing chromosomal aberrations under conditions of metabolic activation.

12.0 CONCLUSION:

The test article, T6358, was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation.

13.0 REFERENCES:

Armitage, P. Statistical Methods in Medical Research, John Wiley & Sons, Inc., New York, NY, 1971.

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Goto, K., Maeda, S., Kano, Y., and Sugiyama, T.: Factors involved in differential Giemsa- staining of sister chromatids. *Chromosoma*, 66:351-359, 1978.

Perry, P. and Wolff, S.: New Giemsa method for the differential staining of sister chromatids. Nature, 251:156-158, 1974.

Sokal, R.R., and Rohlf, F.J.: Biometry, Ed. 2, W.h. Freeman and Company, New York, 1981.

Terasima, T. And Tolmach, L.J.: changes in X-ray sensitivity of HeLa cells during the division cycle. Nature, 190:1210-1211, 1961.

14.0 EXPERIMENTAL DATA TABLES

CHV Study No.: 17388-0-437

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000881

TABLE 1

RANGEFINDING ASSAY FOR ASSESSING TOXICITY

Assay No.: 17388 Trial No.: 1 Date: 01/30/96 Lab No.: CY1296

Compound: T-6358

Metabolic Activation: -S9

Treatment			M1	M1+	≥M2	Confluence * % Solvent Control
POSITIVE CONTROL	MMC	0.250 µg/ml	51	43	6	100
NEGATIVE CONTROL	McCoy's 5a		0	3	97	100
SOLVENT CONTROL	Water	10.0 µl/ml	1	0	99	100
TEST ARTICLE		16.7 µg/ml	0	3	97	100
		50.1 µg/ml	0	10	90	100
		167 µg/ml**	72	15	13	86
		501 µg/ml*	-	-	-	86
		1670 µg/ml*	-	-	-	0

Metabolic Activation: +S9

Treatment			M1	M1+	≥M2	Confluence * % Solvent Control
POSITIVE CONTROL	CP	15.0 µg/ml	49	49	2	100
NEGATIVE CONTROL	McCoy's 5a		0	0	100	100
SOLVENT CONTROL	Water	10.0 µl/ml***	1	2	97	100
TEST ARTICLE		50.1 µg/ml	0	5	95	100
		167 µg/ml	1	2	97	100
		501 µg/ml	0	1	99	100
		1670 µg/ml*	-	-	-	0

* This endpoint is based upon visual observations which are made prior to the harvest of the metaphase cells. Actual cell counts are not taken and any hypertrophy of the attached cells cannot be evaluated. At the time of the confluence observation the flasks are also evaluated for the appearance of floating mitotic cells and dead cells.

* Toxic dose level.

** Toxic dose level, only 89 cells available for analysis.

*** 103 metaphases analyzed.

TABLE 2
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
 Cells Fixed 20.0 Hours After Treatment

Assay No.: 17388

Trial #: 1

Date: 02/13/96

Lab #: CY2126

Metabolic Activation: -S9

Compound: T-6358

NUMBER AND TYPE OF ABERRATION																		# OF ABERRA- TIONS PER CELL	% CELLS WITH ABERRA- TIONS	% CELLS WITH >1 ABERRA- TIONS	
CELLS SCORED	NOT COMPUTED			SIMPLE		COMPLEX								OTHER GI							
	TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF								
CONTROLS																					
NEGATIVE	McCoy's 5a	A 100	1	2															0.00	0.0	0.0
SOLVENT:	Water	10.0 µl/ml	B 100	1					I										0.01	1.0	0.0
	Negative + Solvent	200	2	2					I										0.01	0.5	0.0
POSITIVE:	MMC	0.080 µg/ml	25			2		2			I			3					0.32	24.0*	8.0*
TEST ARTICLE:		75.0 µg/ml	A 100	2									I						0.01	1.0	0.0
			B 100	2	I														0.00	0.0	0.0
			A+B 200	4	I								I						0.01	0.5	0.0
		100 µg/ml	A 100		2		I												0.01	1.0	0.0
			B 100	1															0.00	0.0	0.0
			A+B 200	1	2		I												0.01	0.5	0.0
		150 µg/ml	A 100	3			I												0.01	1.0	0.0
			B 100	2															0.00	0.0	0.0
			A+B 200	5			I												0.01	0.5	0.0
		200 µg/ml	A 100	3	2														0.00	0.0	0.0
			B 100										I						0.01	1.0	0.0
			A+B 200	3	2								I						0.01	0.5	0.0

* Significantly greater than the solvent controls, p<0.01.

McCoy's 5a = culture medium MMC = Mitomycin C

000883

TABLE 3
CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
 Cells Fixed 20.0 Hours After Treatment

Assay No.: 17388

Trial #: 1

Date: 02/13/96

Lab #: CY2126

Metabolic Activation: +S9

Compound: T-6358

NUMBER AND TYPE OF ABERRATION																				
			CELLS SCORED	NOT COMPUTED			SIMPLE		COMPLEX								OTHER	# OF ABERRATIONS PER CELL	% CELLS WITH ABERRATIONS	% CELLS WITH >1 ABERRATIONS
				TG	SG	UC	TB	SB	ID	TR	QR	CR	D	R	CI	DF				
CONTROLS																				
NEGATIVE:																				
SOLVENT:																				
			McCoy's 5a	A 100	2	2							1				0.01	1.0	0.0	
			Water	10.0 µl/ml	B 100	1			1				1		1		0.03	2.0	1.0	
			Negative + Solvent	200	3	2			1				1	1	1		0.02	1.5	0.5	
POSITIVE:			CP	10.0 µg/ml	25				3		6	6				1	>1.00	48.0*	20.0*	
TEST ARTICLE																				
			125 µg/ml	A 100	4									2			0.02	2.0	0.0	
				B 100	4												0.00	0.0	0.0	
				A+B 200	8									2			0.01	1.0	0.0	
			250 µg/ml	A 100	2												0.00	0.0	0.0	
				B 100	1			1						1			0.02	2.0	0.0	
				A+B 200	3			1						1			0.01	1.0	0.0	
			500 µg/ml	A 100	2									1			0.01	1.0	0.0	
				B 100	3	1		2	1				1				0.04	2.0	1.0	
				A+B 200	5	1		2	1				1				0.03	1.5	0.5	
			750 µg/ml	A 100	1	1		1			1	1	1				0.04	2.0	1.0	
				B 100	6	2		1				1	1			1	0.04	3.0	1.0	
				A+B 200	7	3		2			1	2	2			1	0.04	2.5	1.0	

* Significantly greater than the solvent controls, $p < 0.01$.
 McCoy's 5a = culture medium CP = Cyclophosphamide

TABLE 4

CONTROL DATA OF CHROMOSOME ABERRATIONS IN CHINESE HAMSTER OVARY CELLS
8/95 THROUGH 12/95

	Activation		# Of Aberrations Per Cell	% Of Cells With Aberrations	% Of Cells With >1 Aberrations
Negative Control	Without	MIN	0.00	0.0	0.0
		MAX	0.04	4.0	0.5
		AVG	0.012	1.04	0.01
		N	36	36	36
Solvent Control	Without	MIN	0.00	0.0	0.0
		MAX	0.05	5.0	0.5
		AVG	0.012	0.93	0.01
		N	36	36	36
Positive Control Mitomycin C	Without	MIN	0.24	20.0	0.0
		MAX	2.96	84.0	60.0
		AVG	0.770	39.70	16.52
		N	27	27	27
Negative Control	With	MIN	0.00	0.0	0.0
		MAX	0.03	2.5	1.0
		AVG	0.017	1.40	0.10
		N	31	31	31
Solvent Control	With	MIN	0.00	0.0	0.0
		MAX	0.12	7.0	4.0
		AVG	0.023	1.78	0.26
		N	29	29	29
Positive Control Cyclophosphamide	With	MIN	0.40	32.0	8.0
		MAX	8.88	100.0	100.0
		AVG	1.728	57.17	32.83
		N	24	24	24

15.0 DEFINITIONS OF CHROMOSOME ABERRATIONS FOR GIEMSA STAINED CELLS

NOT COMPUTED

TG	Chromatid gap:	("tid gap"). An achromatic (unstained) region in one chromatic, the size of which is equal to or smaller than the width of a chromatic. 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. Not counted in the cells scored for aberrations.
E	Endoreduplication:	4n cell in which separation of chromosome pairs has failed. Not counted in the cells scored for aberrations.

SIMPLE

TB	Chromatic 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.
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 of ring lying beside a shortened chromatid of 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	Quadri radial:	As triradial, but resulting in a four-armed configuration.
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 between two chromosomes which results in a chromosome with three centromeres. Often associated with two to three acentric fragment (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.

CHV STUDY NO. _____
PROTOCOL NO. 437, EDITION 15

CORNING Hazleton

**CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER
OVARY (CHO) CELLS**

Corning Hazleton Inc. (CHV) will conduct this study in compliance with Good Laboratory Practice (GLP) Regulations. This protocol, critical phase(s) of the work in progress and the final report will be subject to audit by Quality Assurance in accordance with SOPs at Corning Hazleton Inc. The study will be conducted by CHV at 9200 Leesburg Pike, Vienna, Virginia 22182.

PART 1. SPONSOR INFORMATION AND APPROVALS

I. SPONSOR IDENTIFICATION

Company Name: 3M

Address: St. Paul, MN

II. TEST ARTICLE IDENTIFICATION: T-6358

III. TEST ARTICLE ANALYSIS

Determination of the test article stability and the test article characteristics as defined in the GLP regulations is the responsibility of the Sponsor.

IV. NOTIFICATION OF REGULATORY SUBMISSION

In order to comply with the GLP regulations, consulting laboratories must be notified if all or part of a study is intended for regulatory submission. CHV 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	☐	OTHER _____

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V. STUDY DATES

Proposed Experimental Start Date: _____

Proposed Experimental Termination Date: _____

VI. APPROVAL OF STUDY PROTOCOL

Study Director: _____

H. Murli, Ph.D.

Date: _____

Sponsor: _____

Steven C. Gordon

Date: _____

7/12/95

Protocol No. 437, Edition 15

PART 2 - STUDY PROTOCOL

CHROMOSOMAL ABERRATIONS IN CHINESE HAMSTER
OVARY (CHO) CELLS

I. OBJECTIVE

The objective of this *in vitro* assay is to evaluate the ability of a test article to induce chromosomal aberrations in Chinese hamster ovary (CHO) cells with and without an exogenous metabolic activation system.

II. DEFINITIONS

Structural changes and rearrangements result from damage to the genetic material and can be analyzed in cultured cells. Chromosomal breaks are mutations; many carcinogens cause chromosomal damage and chromosomal changes have been associated with cancer. Chromosomal aberrations are therefore a relevant test for potential mutagens and carcinogens.

Numerical aberrations or variations from the normal chromosomal number are not scored in a continuous cell line such as CHO cells, and are not detected in an assay such as this in which cells pass through only one division cycle before analysis.

- Chromosomal aberration: damage expressed in both sister chromatids at the same locus, or the exchange between both chromatids of more than one chromosome.
- Chromatid aberration: damage seen in a single chromatid, or exchange between chromatids.

III. RATIONALE

The assay aims 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 consequence of failure or mistakes in repair processes such that breaks do not rejoin or rejoin in abnormal configurations (reviewed by Evans, 1962).

Aberrations are examined when cells enter mitosis for the first time after chemical exposure, before they can be lost during the division process or be converted into complex derivatives during subsequent cell cycles. In the case of the CHO cells used here, most dividing

cells examined 8 to 12 hours after treatment are in their first mitosis (M_1 cells). However, many test articles cause severe delay of progression through the cell cycle, and this 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 (see Section V.B.).

IV. MATERIALS

A. Test Cells

The CHO cell line was derived from an ovarian biopsy of a Chinese hamster. Cells to be used in this assay (CHO-WBL) are a subclone originally obtained from Dr. S. Wolff, University of California, San Francisco. The cells were subsequently recloned in this laboratory to maintain karyotypic stability. The CHO-WBL subclone is a permanent cell line with an average cycle time of 12 to 14 hours with a modal chromosome number of 21.

B. Cell Culture Conditions

CHO cells will be grown in McCoy's 5a culture medium supplemented with approximately 10% fetal calf serum, L-glutamine (2mM), penicillin (100 units/ml) and streptomycin (100 µg/ml). Cultures will be incubated with loose caps in a humidified incubator at $37^\circ\text{C} \pm 2^\circ\text{C}$ in an atmosphere of $5\% \pm 1.5\% \text{CO}_2$ in air.

C. Test For Mycoplasma Contamination

Mycoplasma testing is performed routinely by examination of slides stained with Hoechst 33258 and observed under UV microscopy. Mycoplasma are detected as small, intensely fluorescent specks over the cytoplasmic region of the cells. Any contamination would be rapidly recognized both from the fluorescent-stained appearance and from a decreased uptake of BrdUrd in the cell cycle delay test (Section V.B.2.e.).

D. Karyotype Stability

Karyotype stability is under constant scrutiny because the end-point of the assay is cytogenetic analysis. To ensure maximum stability, cells will be used that have been cloned (CHO-WBL) and subsequently stored in liquid nitrogen. These stocks have undergone 5-8 transfers in culture before cryopreservation. Samples will be thawed periodically, recultured twice a week, and discarded after about eight weeks of continuous culture.

E. Test Article

Solid or liquid test articles are suitable for this assay. The test article is dissolved in an appropriate solvent such as serum-free culture medium, water, dimethyl sulfoxide (DMSO), ethanol, or acetone. Serial dilutions will be carried out so as to achieve desired final concentrations by addition of 0.05 ml per 5 ml culture medium, unless insolubility requires a larger volume. The maximum final concentrations of solvent in the culture will be, unless otherwise specified by the Sponsor, up to 80% culture medium, up to 10% water, 1% DMSO or ethanol, or 0.5% acetone. The test compound solution will be prepared immediately before use.

F. Control Articles1. Negative and Solvent Controls

Without activation: Negative controls will be cultures which contain only cells and culture medium. Solvent controls will be cultures containing the solvent for the test article at the same concentration used in test cultures.

With activation: Negative and solvent controls, as defined above, will be treated with S9 activation mix in the same way as the test cultures.

2. Positive Controls

Known mutagenic and chromosome breaking agents will be used at two concentrations to ensure adequate results, but only one concentration will be chosen for aberration analysis.

Without activation: Mitomycin C (MMC) is a clastogen that does not require metabolic activation. MMC will be dissolved in water and used at final concentrations of 0.2 to 0.5 µg/ml in the rangefinding assay, 0.5 to 1.0 µg/ml for fixation at 8-12 hours, and 0.040 to 0.100 µg/ml for later fixation.

With activation: Many mutagens do not act directly but must be converted to active intermediates by enzymes found in microsomes. An example is cyclophosphamide (CP), used here as the positive control to demonstrate the activity of the S9 metabolic activation system. CP will be dissolved in water and used at final concentrations of 10 to 30 µg/ml in the rangefinding assay, 10 to 50 µg/ml for fixation at 8 to 12 hours, and 10 to 25 µg/ml for later fixation.

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G. The Metabolic Activation System

The *in vitro* metabolic activation system consists of rat liver enzymes and an energy producing system necessary for their function (NADP and isocitric acid). Enzymes are contained in a preparation of liver microsomes (S9 fraction) from rats treated with Aroclor 1254 to induce enzymes capable of transforming chemicals to more active forms. The S9 fraction, prepared in sucrose or in potassium chloride from male Sprague Dawley rats, is purchased from commercial suppliers and is retained frozen at approximately -80°C until use. Aliquots of this S9 fraction will be thawed immediately before use and added to a "core" reaction mixture to form the activation system described below:

<u>Component</u>	<u>Concentration in Cultures</u>
NADP (sodium salt)	1.5 mg/ml (1.8 mM)
Isocitric acid	2.7 mg/ml (10.5 mM)
Homogenate (S9 fraction)	15.0 µl/ml*

V.

EXPERIMENTAL DESIGNA. Solubility and Dose Determination

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. A range of doses covering five orders of magnitude in a half-log series will be tested in the preliminary rangefinding test unless a dose range is specified by the Sponsor. If no solubility information has been provided by the Sponsor, a preliminary solubility test will be carried out with sterile deionized water, serum-free culture medium, DMSO, ethanol, or acetone. Stock solutions in DMSO or ethanol will be diluted one-hundred-fold in culture medium in a precipitate test. Stock solutions in acetone will be diluted 1:200 in culture medium in a precipitate test. The solvent selected will be the one which gives the best solubility after dilution in medium.

* The amount of S9 homogenate per culture will depend on the lot of S9 in use at any time. Each lot of S9 homogenate is tested when purchased before use in the assay. Because the protein content of S9 homogenate varies among lots, S9 at various concentrations is tested against reference chemicals, such as cyclophosphamide, benzo(α)pyrene or dimethylnitrosamine. The optimum S9 concentration is selected, based on induction of sister chromatid exchanges in CHO cells, and this amount of S9 is used in subsequent assays with that particular lot of S9.

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In some cases, test articles are apparently insoluble in solvents 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.

The top dose tested for liquid test articles in the rangefinding assay will be 5 mg/ml, which may also be calculated from the specific gravity. Solubility testing will be the same as described above.

B. Rangefinding and Determination of Fixation Time

1. Rationale

Most known chemical clastogens (chromosome breaking agents) require a period of DNA synthesis to convert initial DNA damage into chromosome alterations visible at mitosis. Many cells bearing aberrations may not complete the division process successfully and may die. The time of fixation for chromosome preparation is thus selected to allow cells to go through DNA synthesis (S-phase) and reach the first mitosis after treatment. Since under normal circumstances the cell cycle of CHO-WBL cells is about 13 hours, fixation 8 to 11 hours after addition of test article will collect cells in the first mitosis after the beginning of treatment. However, many clastogens inhibit progression of cells through the cycle, and damaged cells which recover enough to progress to mitosis will not be collected at an 8-11 hour fixation time. As an alternative to carrying out several tests in which cells are fixed at a series of times in order to avoid missing a "peak" of aberration yield, a preliminary assessment of cell cycle delay will be made by differential staining. Cells will be grown in the presence of the base analogue 5-bromo-2'-deoxyuridine (BrdUrd); after two cell cycles, one-half of a chromosome (sister chromatid) will be more heavily substituted with BrdUrd than the other. This chemical difference causes the chromatids to differ in the reaction with the stain. The characteristic staining pattern of a cell that has undergone two complete cell cycles with BrdUrd (M_2 cells) is a clear differentiation between chromatids along the complete length of every chromosome. In the CHO-WBL cell system, approximately 85%-100% of cells fixed after 26 hours exposure to BrdUrd show this M_2 staining pattern. After only one cell cycle, chromatids are evenly stained (M_1 cells). After more than one cycle but less than two complete cycles, intermediate patterns are found in which only parts of the chromosomes are differentially stained (M_1+). Thus, if the

cell cycle is slowed down by the test article, both M_1 and M_1+ cells will appear in the preparations made after 26 hours in BrdUrd. An estimate of the degree of cell cycle delay may be made from the frequencies of M_1 cells. The fixation time for the subsequent chromosome aberration test will then be selected to ensure that most cells are in their first metaphase after treatment, allowing the progression of damaged cells to the division stage.

2. Preliminary Test for Toxicity and Cell Cycle Delay

Cultures are seeded at about 0.3×10^6 cells per 25 cm² plastic flasks in 5 ml of fresh culture medium. One flask will be initiated for each treatment (10 dose levels or the Sponsor requested doses, solvent, negative and positive controls).

a. Test without metabolic activation

The day after culture initiation, solvent or test article will be added to the cultures. Incubation at about 37°C will be continued for about 2.0 to 2.5 hours when BrdUrd (final concentration 10 μ M) will be added and the cultures will be returned to the incubator for a total of about 25.5 to 26.5 hours before fixation. Approximately 2.0 to 2.5 hours before fixation, the cell monolayers will be washed with phosphate buffered saline to remove the test article, and fresh medium will be added which contains Colcemid® at 0.1 μ g/ml and BrdUrd at 10 μ M. Incubation with Colcemid® will continue for about 1.0 to 2.0 hours.

b. Test with the metabolic activation system

One day after culture initiation, culture medium will be removed and the cells will be washed once with phosphate buffered saline to remove serum. Cells will then be incubated at about 37°C for about 2 hours in the presence of the test article, the S9 reaction mixture, and growth medium without fetal calf serum (FCS). The short incubation time is used because prolonged exposure to the S9 mixture is toxic to cells; also enzyme activity is lost rapidly at 37°C. Serum is omitted to avoid possible inactivation, by binding to serum proteins, of short-lived, highly reactive intermediates produced by S9 enzymes. After the 2 hour exposure period, the cells will be washed at least twice with buffered saline, and

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normal growth medium containing 10% FCS and 10 μ M BrdUrd will be added. Incubation will be continued for about 25.5 to 26.5 hours, with Colcemid® (0.1 μ g/ml) added for the last 1.0 to 2.0 hours of incubation.

c. Summary of Treatment Schedule*

Test	SCHEDULE (HOURS)					
	Chemical	Wash	BrdUrd	Wash	Colcemid	Fixation
-S9	-2.25	-	0	24.25	24.5	25.5-26.5
+S9	-2.25	-0.25	0	-	24.5	25.5-26.5

*Times given are approximate and may vary according to the size of the assay.

d. Modification for test articles that are volatile and/or alter pH

For test articles that are known to be markedly volatile, the culture flasks will be incubated with tightly closed caps until the article is washed off. To compensate for lack of CO₂ circulation, HEPES buffer (25 mM final concentration) will be included in the medium.

If marked pH changes are noticed (i.e., color changes in medium) during the solubility test, HEPES buffer will be included in the medium to attempt to counteract the pH change. Further neutralization (e.g., with HCl or NaOH) will be performed to maintain normal culture pH range (approximately 6.8 to 7.4) only after consultation with the Sponsor.

e. Cell fixation, staining, and scoring for cell cycle delay

Before fixation, flasks will be examined under an inverted microscope to estimate cytotoxicity expressed as growth inhibition and mitotic suppression. Observations of the degree of confluency and apparent availability of mitotic cells (large, rounded, healthy cells on the surface of the monolayer or floating in the medium) will be made. Cells will be harvested from the highest dose level expected to yield any metaphases and from at least 2 lower dose levels. Metaphase cells will be collected by mitotic shake-off (Terasima and Tolmach, 1961), swollen with 75 mM KCl, fixed in methanol: glacial acetic acid (3:1, v/v), dropped onto glass slides, and air dried.

Differential staining is accomplished by a modified fluorescence-plus-Giemsa (FPG) technique (after Perry and Wolff, 1974; Goto, *et al.*, 1978). Slides will be stained for at least 10 minutes with Hoechst 33258 (5 µg/ml) in phosphate buffer (pH 6.8), mounted in the same buffer, and exposed at 55°-65°C to "black-light" from 15 watt tubes for the amount of time required for differentiation between chromatids (3-20 minutes). Finally, slides will be stained with 5% Giemsa for 3 to 20 minutes and air dried.

The slides from the highest dose levels "harvested" will be examined for presence of delayed cells. An attempt will be made to scan and classify one hundred metaphase cells as M_1 , $M_1 +$ or $\geq M_2$.

3. Determination of Dose and Fixation Time for the Aberration Test

The doses selected for the assay will be based on the observations of toxicity (confluence reduction and cell appearance) and cell cycle delay from the preliminary test, and the maximum dose selected will be the highest dose at which analyzable metaphase cells are expected to be obtained. In the absence of observed cell cycle delay, a fixation time 8 to 11 hours after addition of chemical will be used. If a cell cycle delay is observed, a later fixation time such as 18 hours or 24 hours will be selected based on the estimated time to give a minimum of 50% cells in M_1 at the lowest dose. Two different fixation times will be used if necessary (e.g., if the top dose causes delay but the lower doses do not).

C. Chromosome Aberration Test

Cultures will be seeded at about 1.0 to 1.5×10^6 cells per 75 cm² plastic flask in 10 ml of fresh culture medium. A minimum of five doses will be used, depending on the results of the range-finding test (Section V.B). The doses may be more closely spaced than half-log dilutions depending on the pattern of toxicity observations.

Duplicate flasks will be used for each dose level. Single flasks will be used for solvent and negative controls and for each of two doses of positive control compounds.

1. Assay without the metabolic activation system

One day after culture initiation, the culture medium will be replaced with fresh medium and the cells will be treated with the test article. The test article will be left in cultures for the entire period, the length of which will be determined from the preliminary test (usually 8 to 30 hours). About 2.0 hours before fixation, cultures will be washed with phosphate buffered saline, and fresh medium will be added which contains Colcemid® (0.1 µg/ml final concentration). Presence of Colcemid® helps to accumulate the dividing cells.

2. Assay with the metabolic activation system

One day after culture initiation, the culture medium will be removed and the cells will be washed once with saline. The cells will then be incubated at about 37°C for about 2 hours in the presence of the test article, S9 reaction mixture, and growth medium without fetal calf serum (FCS). After the 2 hour exposure period, cells will be washed at least twice with phosphate buffered saline, and normal growth medium containing 10% FCS will be added. Incubation will be continued for a further period of approximately 6 to 28 hours with Colcemid® (0.1 µg/ml final concentration) present during the last 1.0 to 2.0 hours to accumulate the dividing cells.

3. Cell staining and scoring

Metaphase cells will be subsequently collected and fixed as in section V.B.2.e. Those flasks with excessive toxicity will not be harvested. The slides will be air dried and stained with Giemsa. After drying, the slides will be rinsed in xylene and mounted permanently. For control of bias, the slides will be coded for analysis except for the positive controls, which will be checked first to make sure the aberration frequency is adequate.

Normally, two hundred cells per dose level (100 from each of the duplicate flasks), if available, will be read from each of the top four doses. If two hundred cells were not available from each of these doses, lower doses may be evaluated. One hundred cells will be read from each of the negative and solvent controls. At least twenty-five cells will be read from one positive control and from those cultures that have greater than 50% of cells with one or more aberrations. The complete number of 100 cells per culture may not be available due to toxicity or quality of preparation. Cells will be

selected for scoring on the basis of good morphology, and only cells with the number of centromeres equal to the modal number 21 ± 2 (range of 19 to 23) will be analyzed.

Standard forms will be used to record analyses. For each cell bearing an aberration, the microscope stage location will be noted so the cell may be relocated if necessary. The complete list of aberrations scored and their definitions is attached to this protocol (pages 16-18).

VI. DATA

A. Data Presentation and Evaluation

Data will be summarized in tables showing the numbers of cells analyzed, types of aberrations found, frequencies of aberrations per cell, and percentages of cells bearing aberrations. Chromatid and isochromatid gaps will be noted but will not be added into the totals for aberration assessment since they may not be true breaks.

B. Assay Acceptance Criteria

An assay will be considered accepted for evaluation of test results only if all of the following criteria are satisfied. Activation and nonactivation sections of the aberrations assay form independent units and will be repeated independently, as needed, to satisfy the acceptance criteria.

● Unsatisfactory Controls

The assay will be repeated if:

1. The negative or the solvent control is more than twice the upper limit of the range of historical control values (upper limit 5% or more of cells with aberrations).
2. The positive control result is not significantly higher ($p < 0.01$) than the pooled negative and solvent controls. If the positive control result in the test with S9 is adequate in an assay where activation and nonactivation assays are run concurrently but the positive control in the nonactivation assay fails, the test need not be repeated as the activation positive control demonstrates the sensitivity of the cells.

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- Lack of Toxicity

If the aberration results are negative and there is no significant reduction in confluence or delay in cell cycle progression, the test may be repeated at higher doses if higher doses can be achieved (i.e. not limited by solubility or not greater than 5 mg/ml).

- Excess Toxicity

The assay will be repeated if results are not available for at least three dose levels.

- Sporadic Increase

If a significant increase is seen at one or more dose levels but not in consecutive doses and if there is no clear evidence for a positive dose response, the assay will be repeated to verify the significance.

If the assay has to be repeated because of equivocal results, only three dose levels will be analyzed, instead of four, in the second assay.

C. Assay Evaluation

The following factors are taken into account in evaluation of the test article data:

- Overall aberration frequencies.
- Percentage of cells with aberrations.
- Percentage of cells with more than one aberration.
- Evidence for increasing amounts of damage with increasing dose, i.e., a dose related increase in aberrations. Cells with complex aberrations may be considered to have more significance than simple aberrations.

Statistical analysis employs the Fisher's Exact Test to compare the percentage of cells with aberrations in treated cells with pooled results from solvent and negative controls (Sokal and Rohlf, 1981). A linear trend test of increasing number of cells with aberrations with increasing dose (Armitage, 1971) will also be performed. The difference is considered significant when $p < 0.01$. The final evaluation of the test article will be based upon scientific judgment.

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VII. REFERENCES

1. Armitage, P. Statistical Methods in Medical Research. John Wiley & Sons, Inc., New York, NY, 1971.
2. Evans, H.J.: Chromosomal aberrations produced by ionizing radiation. International Review of Cytology, 13:221-321, 1962.
3. Goto, K., Maeda, S., Kano, Y., and Sugiyama, T.: Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma, 66:351-359, 1978.
4. Perry, P. and Wolff, S.: New Giemsa method for the differential staining of sister chromatids. Nature, 251:156-158, 1974.
5. Sokal, R.R. and Rohlf, F.J.: Biometry. Freeman, 1981.
6. Terasima, T. and Tolmach L.J.: Changes in X-ray sensitivity of HeLa cells during the division cycle. Nature, 190:1210-1211, 1961.

VIII. REPORT FORMAT

CHV 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 CHV 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.
- Study director and senior technician.
- Methods.
- Evaluation criteria.
- Interpretation of results.
- Conclusions.
- References.
- Test results presented in tabular form.

IX. 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.

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X. RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocols, and final reports generated as a result of this study will be archived in the storage facilities of Corning Hazleton Inc. 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 Corning Hazleton Inc. for an additional period of time or sent to a storage facility designated by the sponsor.

DEFINITIONS OF CHROMOSOMAL ABERRATIONS FOR GIEMSA STAINED CELLS

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. |
| E | Endoreduplication: | 4n cell in which separation of chromosome pairs has failed. Only indexed if very common. |

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. |

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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".

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COMPLEX (Continued)

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

Greater than 10 aberrations: 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.