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BAKELITE® Epoxy Resin ERL-2774

In Vitro Mutagenesis Studies: 3-Test Battery

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Sponsor: Union Carbide Corporation

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SUMMARY

Epoxy Resin ERL-2774 was evaluated for potential mutagenic activity with a battery of three in vitro tests, which were: the Chinese Hamster Ovary (CHO) Mutation test, the Sister Chromatid Exchange (SCE) test and an assay for induction of Unscheduled DNA Synthesis (UDS) in rat liver cells. The results indicated that Epoxy Resin ERL-2774 produced a statistically significant and dose-related effect in the SCE test with CHO cells and additional (but less definitive) positive effects in the CHO mutation test and the UDS test with hepatocytes. Epoxy Resin ERL-2774 was considered a probable positive mutagenic agent based on the suggestive pattern of activity in the 3 tests. Because definitive positive mutagenic activity was observed in only the SCE test, additional studies would be necessary to confirm this result.

RESULTS AND INTERPRETATION

Selection of Test Concentrations - Preliminary experiments were performed to select an appropriate range of test concentrations in which the maximum concentration would allow survival of approximately 10% of the treated cells. A maximum concentration of 30×10^{-4}% (by weight) was chosen for the highest dose-level in the first experiment and a total of five concentrations of Epoxy Resin ERL-2774 was tested. In a second repeat test with higher concentrations, 100×10^{-4}% was used as the highest dose level.

CHO Mutation Test - Epoxy Resin ERL-2774 produced a statistically significant effect on the frequency of mutations of CHO cells at only one concentration in tests with the incorporation of a liver S9 metabolic activation system. The lack of a dose-related effect on the mutation frequency complicated the definitive classification of the test chemical as mutagenic or non-mutagenic. However, the highly statistically significant increase in mutants obtained in the test with metabolic activation was clearly outside our historical control range of variation for this test. Because the data were equivocal, and further testing would be necessary to establish the existence of a dose-response relationship, the results from this test could not be used to classify the test chemical as either active or inactive. However, literature references concerning similar studies suggest that this single positive value may be a biologically significant effect.

SCE Test - Epoxy Resin ERL-2774 produced a dose-related and highly statistically significant effect on the frequency of SCE in CHO cells in tests without the incorporation of an S9 metabolic activation system. An overall range of concentrations between 120 x 10^{-4}% to 7.5 x 10^{-4}% (by weight) was used. All five of the concentrations tested produced a highly statistically significant effect on the SCE frequency. This result prompted an unequivocal consideration of Epoxy Resin ERL-2774 as a highly active mutagenic chemical in this test.

UDS Test - Epoxy Resin ERL-2774 produced statistically significant effects on UDS activity at two of eight concentrations between 1000 x 10^{-4}% to 3 x 10^{-4}% (by weight). Epoxy Resin ERL-2774 was considered to be active in the present test with the hepatocyte test system because of the high degree of statistical significance of the increases in UDS produced by apparently non-cytotoxic doses of the test chemical.

Comparative Mutagenicity - The pattern of responses produced by Epoxy Resin ERL-2774 in the 3-test battery to determine potential mutagenicity indicated that Epoxy Resin ERL-2774 was an active mutagenic agent. In the SCE test, Epoxy Resin ERL-2774 was comparable in activity to the historical data obtained with the positive control (EMS), a known mutagen and carcinogen.

Contradictory reports exist in the literature on the potential carcinogenicity of Epoxy Resin ERL-2774 (references 2, 3 and 4) but there is consistent agreement that this chemical possesses mutagenic potential in in vitro tests (references 2, 4 and this report). A comparison of data for the CHO mutation test from this study and a previous study performed at the Oak Ridge National Laboratory (reference 4) indicated that the weak positive result obtained in the CHO test with S9 metabolic activation was a reproducible effect in two laboratories. Thus, all the test results in this study would be consistent with the identification of Epoxy Resin ERL-2774 as an active mutagen.

SAMPLE

Quantity: 8 ounces

CHF Sample No.: 42-138

Submitted by: W. C. Kuryla, for Toxicology Advisory Group

Date Received: March 15, 1979

Division: Specialty Chemicals and Plastics
Union Carbide Corporation
South Charleston, WV 25303

Identification: Yellow, viscous liquid

CAS#: 1675-54-3

BAKELITE® Epoxy Resin ERL-2774

In Vitro Mutagenesis Studies: 3-Test Battery

Sponsor: Union Carbide Corporation

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OBJECTIVE

The purpose of this study was to evaluate the potential of Epoxy Resin ERL-2774 to induce genetic damage in mammalian cells at the gene, chromosome and/or DNA (deoxyribonucleic acid) level of molecular organization. A battery of three in vitro, short-term tests which detect each of these genetic endpoints was employed to evaluate Epoxy Resin ERL-2774 for potential mutagenic activity. A general description of the theoretical basis of these three tests is presented in Appendices I, II and III attached to the complete report.

SAMPLE CHARACTERISTICS

A commercial sample of Epoxy Resin ERL-2774 was received for testing on March 15, 1979 and it was assigned sample number 42-138. The physical and chemical information available from the Toxicology Data Bank or from "Material Safety Data Sheets" for this product are attached to this report as Appendix IV.

METHODS

A description of the technical procedures used in the CHO test, the SCE test and the UDS assay are presented in greater detail in Appendices I, II and III, respectively (attached to the complete report). Testing was performed as part of the development and validation of the in-house battery of mutagenicity assays at the Bushy Run Research Center and deviations from current standard operating procedures are noted in the individual test results.

1. CHO Test (Detailed procedures in Appendix I):

A. Dose Selection - Appropriate concentrations of Epoxy Resin ERL-2774 for testing were determined by measurements of cytotoxicity to CHO cells of six concentrations tested both in the presence and absence of a liver S9 metabolic activation system. Selection of a maximum concentration for testing depended upon an estimate of a dose level which would permit survival of at least 10% of the treated cells. Glass-distilled dimethylsulfoxide (DMSO) was used as the solvent and solvent control.

To simplify tables and to allow comparisons between different tests, concentrations of Epoxy Resin ERL-2774 in the following sections of the report are given in terms of weight/volume percentages x 10^{-4}% to eliminate zeros in the lower concentration values (eg. 0.00075 % = 7.5 x 10^{-4}%).

B. Mutation - CHO cells were exposed for 5 hours to a minimum of five concentrations of Epoxy Resin ERL-2774 both with and without the addition of an S9 metabolic activation system. Two separate experiments were performed to insure that an adequate range of doses of the test agent were tested. Dilutions of Epoxy Resin ERL-2774 for testing were prepared by weighing the test material in a sterile tube, preparing a stock solution for the highest concentration using DMSO as a solvent and then by making sequential one-half dilutions from the stock solution using DMSO as the diluent. The surviving fraction was determined at 20 to 24 hours after treatment and the mutant fraction was determined after a 7-day period to allow "expression" of the mutant phenotype. Only the data from the top five concentrations which allowed sufficient cell survival for assessment of survival and induction of mutants are usually presented in the tables. The percentage of cells surviving the treatment, the frequencies of mutant colonies and the number of mutants/10^6 viable cells are presented in tabular form.

2. SCE Test (Detailed procedures in Appendix II):

Production of SCE's following exposure to various concentrations of Epoxy Resin ERL-2774 was studied in CHO cells without the incorporation of an S9 metabolic activation system. Selection of a maximum dose level which would permit survival of at least 50% of the treated cells was based on the prescreening test for cytotoxicity performed as part of the CHO Mutation test. Dilutions of Epoxy Resin ERL-2774 for testing were prepared by weighing the test chemical in a sterile tube, making a stock solution for the highest concentration using DMSO as a solvent and then making sequential one-half dilutions of the stock solution using DMSO as the diluent. For determination of direct mutagenic action, CHO cells were exposed to Epoxy Resin ERL-2774 and appropriate controls for 5 hours without S9 activation. Indirect mutagenic action, requiring metabolic activation by liver S9 homogenate, was not studied because the highly significant positive response obtained in the test without metabolic activation indicated that the test chemical was highly active without metabolic conversion. Bromodeoxyuridine (BrdU) required to differentiate between the individual "sister" chromatids by SCE staining, was present at a concentration of 3 ug/ml in the growth medium during treatment and during the culture period following exposure. A total of 15 cells/dose level and 5 dose levels, tested without metabolic activation, were examined. The number of SCE/cell, mean number of SCE/chromosome and the level of statistical significance of the increases above the concurrent solvent control values are presented in tabular form.

3. UDS Test (Detailed procedures in Appendix III):

Induction of primary DNA damage in rat liver cells (hepatocytes) was studied with eight dose levels which spanned a 333-fold range of concentrations. Cells were treated with Epoxy Resin ERL-2774 for 2 hours in culture medium containing ^{3}H-thymidine, hydroxyurea and appropriate dilutions of Epoxy Resin ERL-2774 prepared in DMSO. Determination of UDS activity was

performed by analyses of incorporation of ^{3}H-thymidine into isolated hepatocyte nuclei or in DNA (precipitated from aliquots of the isolated nuclei) using a Searle Analytic Model 81 or Packard Model 2650 scintillation spectrometer. Data are presented in tabular form with an indication of the level of statistical significance above the concurrent solvent control values.

4. Controls - Positive, negative and solvent controls were tested concurrently with the test sample to assure the sensitivity of the test system and the concurrence of the results to previous test performance. For the CHO and SCE assays, dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) were used as positive control agents to assure the sensitivity of the test system for detecting indirect and direct-acting mutagens, respectively. Deionized water, sterilized by membrane filtration, and glass-distilled dimethylsulfoxide (DMSO) were used as the negative and solvent controls, respectively.

   In the UDS assay, DMN and ethylmethanesulfonate (EMS) were used as positive controls representing indirect- and direct-acting mutagens, respectively. DMSO was used as the solvent and the solvent control.

5. Metabolic Activation - S9 liver homogenate, prepared from Arochlor 1254-induced, Sprague-Dawley male rats, was purchased from Litton Bionetics. The S9 preparation used for the CHO test in the first test contained 38.5 mg/ml protein and had a benzo(a)pyrene hydroxylase (BPH) activity of 21.6 nmol of hydroxybenzpyrene/20 min/mg protein, (assayed by Litton). A final concentration of 480 ug/ml of S9 protein was used in the culture media. In experiment #2, the same lot of S9 homogenate was used and 360 ug/ml of 9 protein was used as the final concentration in the culture media.

   For the SCE test, the same lot of S9 homogenate, was used at a final concentration of 360 ug/ml of S9 protein in the culture media.

6. Statistical Analyses - Data from the SCE and UDS tests were analyzed by appropriate parametric tests following Standard Operating Procedures for statistical analyses at the Bushy Run Research Center. Data from the CHO test do not follow a normal distribution according to experience with historical controls. Thus, the Student's t-test was used after transformation of the mutation frequencies (MF) according to the method of Irr and Snee $(MF + 1)^{0.15}$ (Irr, J. D. and R. Snee, Proceedings of the Cold Spring Harbor-Banbury Conference, II (1979), 263-274).

   Rounding of data to either two decimal places or to the appropriate number of significant figures was performed for presentation on tables. Although statistically significant decreases in mutation indices can occur because of cytotoxic responses, only statistically significant increases in responses above control values are indicated on Tables for simplicity. The degree of statistical significance is denoted by: a: $0.05 > p > 0.01$, b: $0.01 > p > 0.001$, or c: $p < 0.001$. No superscript (or NS) indicates $p > 0.05$.

7. Raw Data Storage - Copies of the final report, statistical analyses, analytical data and data used to prepare the final report are stored in the BRRC Archives. Slides are stored in the Genetic Toxicology slide storage area.

RESULTS

SECTION I - CHO MUTATION TEST - BAKELITE® Epoxy Resin ERL-2774

A. Test Dates - Initiated: June 14, 1979
Completed: September 11, 1980

B. Selection of Test Concentration (Data not shown in tables)

CHO cells were exposed for five hours to concentrations of Epoxy Resin ERL-2774 which spanned a concentration range from 1000 x 10^{-4}% to 3 x 10^{-4}% by weight. The percentage of cells which survived the exposure, both in the presence and absence of an S9 metabolic activation system, was determined by counting the number of colonies produced by the survivors after a 5- to 7-day incubation period. A concentration of 30 x 10^{-4}% was selected as the maximum concentration for testing with and without S9 activation. A total of five concentrations were tested in the later mutation tests and the prescreening results indicated that moderately higher concentrations (100 x 10^{-4}%) would produce excessive cytotoxicity.

C. Determination of Mutation Induction

1. Survival (Cytotoxicity)

Table 1 presents the cytotoxicity data for CHO cells treated with Epoxy Resin ERL-2774 in the presence and absence of a liver S9 metabolic activation system. Only a moderate cytotoxic effect with the test agent was observed following exposure in tests with or without S9 activation. A second test, at higher concentrations, was performed to assure that adequate dose levels of the test chemical were tested and these results are shown in Table 3. In this repeat experiment, dose-related cytotoxic effects were obtained for tests both with and without S9 metabolic activation.

2. Mutation

Table 2 presents the data for induction of mutants by Epoxy Resin ERL-2774 and control agents. Epoxy Resin ERL-2774 did not produce a dose-related effect on the frequency of mutants/10^6 viable cells over the 16-fold range of Epoxy Resin ERL-2774 concentrations tested both with and without the presence of an S9 metabolic activation system. No concentration of Epoxy Resin ERL-2774 produced an increase in the mutation frequency which was statistically significant from the concurrent solvent control. Small numerical increases in the mutant frequency obtained at some dose levels were not considered to be biologically significant because occasional increases of similar magnitude have been obtained in previous tests with solvent or negative controls. However, relatively low mutation values were

obtained with both positive controls and the absence of cytotoxicity at the highest dose level suggested that higher concentrations should be tested to assure the reproducibility of the data. In the repeat experiment, summarized on Table 4, a single, highly statistically significant increase in the mutation frequency was obtained in the test which included addition of a metabolic activation system. This result was definitely outside the range of the historical control values for this test but no dose-related effect was observed. Also, the induction of mutants at this same concentration in experiment #1 (Table 2) did not demonstrate this mutagenic effect. No definitive classification of the test agent was possible following these equivocal responses and the results of the other mutagenicity tests were used to discern the existence of a possible pattern of mutagenic potential.

In experiment #1, mutation frequencies for the solvent controls for tests both with and without S9 activation were in an acceptable and low range based upon experience with historical control values. In the repeat experiment (Table 4), the value for the DMSO solvent control was abnormally high in the test without S9 activation. The lack of significant numbers of mutants induced by the test chemical indicated that this high result for the solvent control probably did not influence the statistical evaluation of the test chemical data. Statistically significant increases in the mutation frequencies were obtained for the DMN and EMS positive controls for both experiments #1 and #2 and these values were within the expected range of values observed in historical control data.

D. Deviations from Standard Procedures

Experiment #1. A dilution error for the DMSO-treated cells was made during the survival determination and comparisons to the negative (H_2O) control were used to calculate relative differences. Only 0.9×10^6 cells (total) were plated for the determination of mutation induction by DMN because cells treated with DMN grew poorly and not enough cells were available to assess the usual number of 1.0×10^6

Experiment #2. Aliquots of the diluted test sample were reduced in this second experiment in comparison to experiment #1 because a different dilution series was used. Thus, the volume of the solvent control was reduced from 20 ul/ml to 10 ul/ml. An extended 13-day expression period was used for this experiment to attempt to improve the sensitivity of the assay when we noted that a high degree of cytotoxicity was retarding the growth rate of the treated cells.

E. Conclusions

Epoxy Resin ERL-2774 was apparently inactive as a mutagenic agent for CHO cells when tested with and without the incorporation of an S9 metab lic activation system over a wide range of concentrations. Only one statistically significant increase above the concurrent solvent control was produced at the lowest dose level (30×10^{-4}%) tested with metabolic activation in the repeat test. The high degree of statistical significance of this single result, in comparison to the concurrent control, was also outside the range of historical control values for this test, and prevented a definitive classification of positive or negative mutagenic potential.

SECTION II - SCE TEST - BAKELITE® Epoxy Resin ERL-2774

A. Test Dates - Initiated: December 5, 1979
Completed: March 24, 1980

B. Selection of Test Concentrations

A maximum concentration of 120 x 10^{-4}% of Epoxy Resin ERL-2774 was chosen as the top dose level for testing with and without S9 activation respectively, based on cytotoxicity data from the CHO mutation test. Higher concentrations were expected to produce delays in the mitotic cycle and to decrease the number of cells with SCE staining. A 16-fold range of Epoxy Resin ERL-2774 concentrations from 120 x 10^{-4}% to 7.5 x 10^{-4}% was examined in the SCE experiment without S9 activation. Testing with the addition of a metabolic activation was not performed because the results of this first study clearly indicated that the test chemical was highly active without metabolic conversion.

C. Determinations of SCE Induction

The data for SCE induction in CHO cells treated with various dose levels of Epoxy Resin ERL-2774 or with positive, negative or solvent control agents without an S9 metabolic activation system are summarized in Table 5. Highly statistically significant increases in the SCE frequency were produced by all five of five dose levels of Epoxy Resin ERL-2774 tested for direct action in the absence of a metabolic activation system. Also, the dose-related effect of the test chemical on the frequency of SCE was considered a definitive indication of a biologically significant effect and an indication of the direct action of Epoxy Resin ERL-2774.

The number of SCE produced by the concurrent EMS positive control was highly statistically significant from the concurrent solvent control and these data indicated an appropriate sensitivity of the test system comparable to our historical positive control data. The numbers of SCE obtained with the solvent and controls were also in an acceptable range of values included in the variability encountered in our historical control values for this test.

D. Deviations from Standard Procedures - Two prior experiments were performed but are not reported because cytotoxicity of the test chemical reduced the mitotic index and the chromosomes could not be scored for SCE.

E. Conclusions

Epoxy Resin ERL-2774 produced highly statistically significant increases in the frequency of SCE over the 16-fold range of concentrations tested without addition of an S9 metabolic activation system. Definite and dose-related effects of Epoxy Resin ERL-2774 exposure on the SCE frequency were evident and the test agent was considered to be highly active in the present in vitro assay.

SECTION III - UDS TEST - Epoxy Resin ERL-2774

A. Test Dates - Initiated: August 23, 1979
Completed: March 31, 1980

B. Selection of Test Concentrations

Epoxy Resin ERL-2774 was tested over a wide range of concentrations from 1000 x 10^{-4}% to 3 x 10^{-4}% by weight. The maximum dose-level was selected with consideration of the cytotoxicity data obtained in the CHO Mutation test which indicated that higher values would result in excessive cell killing.

C. Determination of UDS Induction

Values for "unscheduled" incorporation of ^{3}H-thymidine into the DNA of hepatocytes exposed to Epoxy Resin ERL-2774 or to appropriate positive and negative controls are presented in Tables 6 and 7. In hepatocytes treated with Epoxy Resin ERL-2774, two concentrations tested for potential activity induced a statistically significant increase in the amount of ^{3}H-thymidine incorporation when UDS was assessed by measuring incorporation in whole cell lysates (Table 6). The stimulation of the UDS values was also observed when the amounts of DNA in the lysates were determined and values were expressed as radioactivity per microgram of DNA; but the increases were not statistically significant by this second method of measurement. The degree of statistical significance of the UDS induced by the test chemical and the similarity of the increases to values observed with the concurrent positive controls indicated that Epoxy Resin ERL-2774 produced a probable, biologically significant effect in the hepatocyte UDS test. Decreased values for ^{3}H-thymidine incorporation at dose levels of 30 x 10^{-4}% or above are a probable indication of the cytotoxic effects of the test chemical.

Both of the positive control agents, NQO and DMN, induced numerically elevated and statistically significant increases in UDS over values obtained with the solvent control. Recent improvements in this test system have increased the sensitivity of measurements but this study was not repeated because the results of this test were considered unequivocal for evaluating the significance of the data.

D. Deviations from Standard Procedures - One previous test was performed but was not reported because the positive controls did not produce a significant response for an acceptable test. Testing of Epoxy Resin ERL-2774 was performed during the development of this procedure and an older, less-sensitive procedure was employed which differed from the current method described in Appendix III. The new procedures (not used for the test reported) involve incubating hepatocytes in suspension *vs.* in petri plates and isolating nuclei prior to DNA precipitation. A repeat test with this newer method would presumably detect even higher levels of activity which would not affect the positive conclusions from these test results.

E. Conclusion

Epoxy Resin ERL-2774 stimulated a highly significant increase in the incorporation of radioactive thymidine in hepatocytes treated with 2 of 8 test concentrations which did not produce overt cytotoxicity. Epoxy Resin ERL-2774 was considered to be active in the present test with the hepatocyte test system.

Reviewed and Approved by:

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Unscheduled DNA Synthesis Assay	W. Christopher Hengler, M.S. Assistant Scientist

WPC/1125-4

REFERENCES

A search of the major computer data files concerning potential mutagenicity/carcinogenicity of Epoxy Resin ERL-2774 produced only one pertinent reference.

Andersen, et al., (see reference #1 below) reported that Epikote resin 848 (Shell Chemicals), the diglycidylether of bisphenol A, was the "most active compound of the resins tested, and . . . more mutagenic than the positive control, ECH (epichlorohydrin)" in tests with the Salmonella Ames Test. In those studies, Epikote resin 848 was mutagenic both with and without S9 metabolic activation, but mutagenic effects were more definitive and greater when S9 metabolic activation was present. This report quoted other studies demonstrating positive carcinogenic potential for additional epoxy resins (2) and a negative dermal carcinogenicity study (3) reported by our laboratory on Epoxy Resin ERL-2774. Positive dermal carcinogenicity and mutagenicity were observed in a study on this chemical performed at Oak Ridge National Laboratory (4).

1. Andersen, M., Kiel, P, Larsen, H. and Maxild, J. (1978). Mutagenic action of epoxy resins. Nature 276, 391-392.

2. Kotin, P. and Falk, H. L. (cited in reference above) (1963). Radiat. Res. Suppl. 3, 193-211.

3. Weil, C. S., Condra, N., Haun, C. and Striegel, J. A. (cited in #1) (1963). Am. Ind. Hyg. Assoc. J., 24, 305-325 (from data in CHF Report #23-99 and #27-152).

4. Holland, J. M., Gosslee, D. G., Gipson, L. C. and Whitaker, M. J. Epidermal carcinogenicity of bis[2,3-epoxycyclopentyl]ether,2,2-bis (p-glycidyloxyphenyl)propane, and m-phenylenediamine in C3H and C57BL/6 male and female mice. Oak Ridge National Laboratory Report, March 1978, Contract #W-7405-eng-26.

Table 1
Chinese Hamster Ovary (CHO) Mutation Assay:
Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period

Chinese Hamster Ovary (CHO) Mutation Assay:
Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
Experiment #1

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
		Without S9 Activation		
[Epoxy Resin ERL-2774] (%, w/v)				
30.0×10^{-4}	429	800	53.6	48.3
15.0×10^{-4}	500	800	62.5	56.2
7.5×10^{-4}	8	800	1.0	0.9
3.75×10^{-4}	390	800	48.8	43.9
1.875×10^{-4}	520	800	65.0	58.5
Controls				
DMSO (20 ul/ml) - Solvent	889	800	111.1	100.0
H_2O (20 ul/ml)	543	800	67.9	61.1
EMS (200 ug/ml)	445	800	55.6	50.1
		With S9 Activation		See Footnote [1]
[Epoxy Resin ERL-2774] (%, w/v)				
30.0×10^{-4}	393	800	49.1	85.2
15.0×10^{-4}	534	800	66.8	115.8
7.5×10^{-4}	425	800	53.1	92.2
3.75×10^{-4}	470	800	58.8	102.0
1.875×10^{-4}	502	800	62.8	108.9
Controls				
DMSO (20 ul/ml) - Solvent[1]	-	-	-	-
H_2O (20 ul/ml)	461	800	57.6	100.0
DMN (3700 ug/ml)	368	800	46.0	79.8

[1] A dilution error was made at this step and values in last column were calculated as a percentage of the negative control.

Abbreviations: H_2O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

Table 3
Chinese Hamster Ovary (CHO) Mutation Assay:
Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
Experiment #2

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
Without S9 Activation				
[Epoxy Resin ERL-2774] (%, w/v)				
100.0×10^{-4}	2	800	0.2	0.3
50.0×10^{-4}	0	400	0	0
25.0×10^{-4}	131	400	32.8	32.9
12.5×10^{-4}	328	400	82.0	82.4
6.25×10^{-4}	457	400	114.2	114.8
Controls				
DMSO (20 ul/ml) - Solvent	398	400	99.5	100.0
H_2O (20 ul/ml)	465	400	116.2	116.8
EMS (200 ug/ml)	424	400	106.0	106.5
With S9 Activation				
[Epoxy Resin ERL-2774] (%, w/v)				
100.0×10^{-4}	138	800	17.2	31.2
60.0×10^{-4}	238	800	29.8	53.8
30.0×10^{-4}	295	800	36.9	66.7
Controls				
DMSO (10 ul/ml) - Solvent	221	400	55.2	100.0
H_2O (10 ul/ml)	222	400	55.5	100.5
DMN (3700 ug/ml)	105	400	26.2	47.5

Abbreviations: H_2O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

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Table 2
Chinese Hamster Ovary (CHO) Mutation Assay:
Results on Evaluation of Mutant Induction by Epoxy Resin ERL-2774
Experiment # 1

Test Chemicals	Plating Efficiency			Mutation Induction		
	Total # Colonies	Total # Cells Plated	Viable Fraction	Total # Mutant Colonies	Total # Cells Plated	Mutants[1] 10^6 Viable Cells
Without S9 Activation						
[Epoxy Resin ERL-2774] (%, w/v)						
30.0×10^{-4}	449	400	1.122	3	1×10^6	2.7
15.0×10^{-4}	264	400	0.660	0	1×10^6	0
7.5×10^{-4}	270	400	0.675	1	1×10^6	1.5
3.75×10^{-4}	310	400	0.775	0	1×10^6	0
1.875×10^{-4}	249	400	0.622	0	1×10^6	0
Controls:						
DMSO (20 ul/ml) - Solvent	363	400	0.908	0	1×10^6	0
H_2O (20 ul/ml)	379	400	0.948	3	1×10^6	3.2
EMS (200 ug/ml)	289	400	0.722	27	1×10^6	37.4[b]
With S9 Activation						
[Epoxy Resin ERL-2774] (%, w/v)						
30.0×10^{-4}	314	400	0.785	6	1×10^6	7.6
15.0×10^{-4}	325	400	0.812	2	1×10^6	2.5
7.5×10^{-4}	395	400	0.988	1	1×10^6	1.0
3.75×10^{-4}	277	400	0.692	0	1×10^6	0
1.875×10^{-4}	259	400	0.648	2	1×10^6	3.1
Controls:						
DMSO (20 ul/ml) - Solvent	410	400	1.025	0	1×10^6	0
H_2O (20 ul/ml)	336	400	0.840	2	1×10^6	2.4
DMN (3700 ug/ml)	227	400	0.568	9	0.9×10^6	17.6[a]

[1]Total # mutant colonies per 10^6 cells plated divided by viable fraction.
Statistical significance above solvent control: a: $0.05 > p > 0.01$; b: $0.01 > p > 0.001$;
No superscript indicates $p > 0.05$.

Abbreviations: H_2O - water; S-9 - liver homogenate; DMSO - dim thylsulfoxide; EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine.

WPC/1125-1

Table 3

Chinese Hamster Ovary (CHO) Mutation Assay:
Determination of Toxic Effects of Chemical Treatment During 5 Hr Mutation Induction Period
Experiment #2

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
Without S9 Activation				
[Epoxy Resin ERL-2774] (%, w/v)				
100.0×10^{-4}	2	800	0.2	0.3
50.0×10^{-4}	0	400	0	0
25.0×10^{-4}	131	400	32.8	32.9
12.5×10^{-4}	328	400	82.0	82.4
6.25×10^{-4}	457	400	114.2	114.8
Controls				
DMSO (20 ul/ml) - Solvent	398	400	99.5	100.0
H_2O (20 ul/ml)	465	400	116.2	116.8
EMS (200 ug/ml)	424	400	106.0	106.5
With S9 Activation				
[Epoxy Resin ERL-2774] (%, w/v)				
100.0×10^{-4}	138	800	17.2	31.2
60.0×10^{-4}	238	800	29.8	53.8
30.0×10^{-4}	295	800	36.9	66.7
Controls				
DMSO (10 ul/ml) - Solvent	221	400	55.2	100.0
H_2O (10 ul/ml)	222	400	55.5	100.5
DMN (3700 ug/ml)	105	400	26.2	47.5

Abbreviations: H_2O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide
EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine

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Table 4
Chinese Hamster Ovary (CHO) Mutation Assay:
Results on Evaluation of Mutant Induction by Epoxy Resin ERL-2774
Experiment # 2

Test Chemicals	Plating Efficiency: Total # Colonies	Plating Efficiency: Total # Cells Plated	Plating Efficiency: Viable Fraction	Mutation Induction: Total # Mutant Colonies	Mutation Induction: Total # Cells Plated	Mutants[1] / 10^6 Viable Cells
Without S9 Activation						
[Epoxy Resin ERL-2774] (%, w/v)						
100.0×10^{-4}	406	400	1.015	0	1×10^6	0
50.0×10^{-4}	-	-	SEE FOOTNOTE #2	-	-	-
25.0×10^{-4}	443	400	1.108	11	1×10^6	9.9
12.5×10^{-4}	149	400	0.372	0	1×10^6	0
6.25×10^{-4}	234	400	0.585	0	1×10^6	0
Controls:						
DMSO (20 ul/ml) - Solvent	170	400	0.425	11	1×10^6	25.9
H_2O (20 ul/ml)	-	-	SEE FOOTNOTE #2	-	-	-
EMS (200 ug/ml)	440	400	1.100	216	1×10^6	196.4[c]
With S9 Activation						
[Epoxy Resin ERL-2774] (%, w/v)						
100.0×10^{-4}	185	400	0.462	1	1×10^6	2.2
60.0×10^{-4}	299	400	0.748	9	1×10^6	12.0
30.0×10^{-4}	373	400	0.932	77	1×10^6	82.6[c]
Controls:						
DMSO (10 ul/ml) - Solvent	189	400	0.472	3	1×10^6	6.3
H_2O (10 ul/ml)	232	400	0.580	10	1×10^6	17.2
DMN (3700 ug/ml)	129	400	0.322	78	1×10^6	241.9[c]

[1]Total # mutant colonies per 10^6 cells plated divided by viable fraction.
Statistical significance above solvent control: c: $p < 0.001$
No superscript indicates $p > 0.05$. Data analyzed by Student's t-test.

[2]Lost during expression period following incubator malfunction during routine trypsinization step.

Abbreviations: H_2O - water; S-9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine.

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Table 5

Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Epoxy Resin (ERL-2774) Without S9 Metabolic Activation
5 Hour Treatment

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell[1]	Mean Number SCE/Chromosome[2] ± S.D.	Significance Above Solvent Control[3]
[Epoxy Resin ERL-2774] (%, w/v)					
120×10^{-4}	309	869	57.93	2.793 ± 1.010	c
60×10^{-4}	299	613	40.87	2.049 ± 0.256	c
30×10^{-4}	302	448	29.87	1.490 ± 0.344	c
15×10^{-4}	299	357	23.80	1.193 ± 0.249	c
7.5×10^{-4}	300	306	20.40	1.022 ± 0.272	c
Controls					
DMSO (5 ul/ml) -Solvent	291	182	12.13	0.628 ± 0.214	-
H_2O (5 ul/ml)	294	200	13.33	0.680 ± 0.169	NS
EMS (100 ug/ml)	298	423	28.20	1.417 ± 0.242	c

[1]Fifteen cells examined per dose level.

[2]Mean value of SCE/chromosome determined from the values of the individual cells examined.

[3]Statistical significance above solvent control: c: $p < 0.001$; NS: $p > 0.05$.

Abbreviations: H_2O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate; S.D. - standard deviation

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Table 6
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

DPM/10^6 cells: all DPM values are calculated from DNA precipitated per 10^6 viable hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in DNA Avg. DPM ± S.D.	% of Solvent Control ± S.D.	Significance Above Solvent Control[1]
Solvent - DMSO	3.3%	22848 ± 926	100.0% ± 4.1%	-
Positive Controls:				
EMS	400 ug/ml	28618 ± 4161	125.3% ± 18.2%	b
	200 ug/ml	28573 ± 3735	125.1% ± 16.3%	b
	100 ug/ml	32107 ± 1083	140.5% ± 4.7%	c
	50 ug/ml	26715 ± 549	116.9% ± 2.4%	a
DMN	80 ug/ml	28753 ± 3744	125.8% ± 16.4%	b
	40 ug/ml	27290 ± 1686	119.4% ± 7.4%	a
	20 ug/ml	30930 ± 859	135.4% ± 3.8%	c
	10 ug/ml	24270 ± 1468	106.2% ± 6.4%	NS
Test Chemical: [Epoxy Resin ERL-2774] (%, w/v)	1000×10^{-4}%	4100 ± 247	17.9% ± 1.1%	NS
	600×10^{-4}%	3290 ± 27	14.4% ± 0.1%	NS
	300×10^{-4}%	5328 ± 126	23.3% ± 0.6%	NS
	100×10^{-4}%	11825 ± 1876	51.8% ± 8.2%	NS
	60×10^{-4}%	8956 ± 1956	39.2% ± 8.6%	NS
	30×10^{-4}%	15371 ± 2438	67.3% ± 10.7%	NS
	10×10^{-4}%	38488 ± 240	168.5% ± 1.1%	c
	3×10^{-4}%	35198 ± 2793	154.1% ± 12.2%	c

[1]Statistical significance above solvent control: a: $0.05 > p > 0.01$; b: $0.01 > p > 0.001$; c: $p < 0.001$; NS: $p > 0.05$. Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; EMS - ethylmethane sulfonate; DMN - dimethylnitrosamine; DPM - disintegrations per minute; S.D. - standard deviation

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Table 7
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

DPM/ug DNA: all DPM values are calculated per DNA precipitated from rat hepatocytes. Each average is calculated from duplicate samples, except for DMSO which was done in quadruplicate.

Test Chemical	Concentration	Radioactivity in DNA Avg. DPM ± S.D.	% of Solvent Control ± S.D.	Significance Above Solvent Control[1]
Solvent - DMSO	3.3%	847 ± 201	100.0% ± 23.7%	-
Positive Controls:				
EMS	400 ug/ml	1474 ± 330	174.0% ± 38.9%	a
	200 ug/ml	1491 ± 124	175.9% ± 14.6%	a
	100 ug/ml	1210 ± 2	142.8% ± 0.2%	NS
	50 ug/ml	1157 ± 134	136.5% ± 15.9%	NS
DMN	80 ug/ml	1252 ± 103	147.8% ± 12.2%	NS
	40 ug/ml	1394 ± 20	164.5% ± 2.3%	a
	20 ug/ml	1673 ± 351	197.4% ± 41.4%	c
	10 ug/ml	1639 ± 292	193.4% ± 34.4%	c
Test Chemical:				
[Epoxy Resin ERL-2774] (%, w/v)	1000 x 10^{-4}%	157 ± 27	18.6% ± 3.2%	NS
	600 x 10^{-4}%	146 ± 20	17.2% ± 2.3%	NS
	300 x 10^{-4}%	206 ± 18	24.3% ± 2.1%	NS
	100 x 10^{-4}%	570 ± 135	67.2% ± 15.9%	NS
	60 x 10^{-4}%	476 ± 3	56.1% ± 0.3%	NS
	30 x 10^{-4}%	809 ± 52	95.5% ± 6.1%	NS
	10 x 10^{-4}%	1282 ± 434	151.2% ± 51.2%	NS
	3 x 10^{-4}%	1220 ± 597	143.9% ± 70.4%	NS

[1]Statistical significance above solvent control: a: $0.05 > p > 0.01$; c: $p < 0.001$; NS: $p > 0.05$. Data analyzed by Duncan's Multiple Range Analysis.

Abbreviations: DMSO - dimethylsulfoxide; EMS - ethylmethane sulfonate; DMN - dimethylnitrosamine; DPM - disintegrations per minute; S.D. - standard deviation

APPENDIX I

Chinese Hamster Ovary (CHO) Mutation Assay

Theoretical Basis

Mutation is a heritable alteration in a cell in which a gene specifying the genetic code for a specific protein is modified in structure and/or function. Mutations, induced by chemical or physical agents, of the HGPRT (hypoxanthine-guanine phosphoribosyltransferase) gene may be detected by the growth of colonies of "mutant" cells which are resistant to the purine analogs 6-thioguanine (TG) or 8-azaguanine. Normal cells contain a functional HGPRT enzyme which phosphorylates TG and allows its incorporation into DNA causing the cells to die. Mutant cells with a non-functional HGPRT enzyme are unable to phosphorylate or incorporate TG, thus survive and grow in its presence.

The CHO mutation test is an assay which detects "forward mutations" from TG-sensitivity to TG-resistance caused by a direct loss of the activity of the HGPRT enzyme ($HGPRT^{+} \rightarrow HGPRT^{-}$). An assessment of the ability of several hundred agents to cause gene mutations in vitro indicates that the CHO mutation assay provides a reasonable estimate of the potential genetic activity of the test chemical.

Methods

Cell Culture Procedures: CHO cells used in these studies were obtained from Abraham Hsie at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (or simply CHO for report purposes). Cells are maintained in active growth by subculturing 2 to 3 times/week in antibiotic-free, Ham's Modified F12 Medium supplemented with 10% (v/v) heat-inactivated, fetal bovine sera (F12-10), and lacking in hypoxanthine. For treatment of cells without metabolic activation, F12 medium with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of dialyzed bovine serum (F12-D5) is used. For treatments incorporating an S9 metabolic activation system, identical medium, but without serum, is employed. For determination of mutant frequencies, F12-D5 medium containing 2.0 ug/ml TG (6-thioguanine) is used as a "selective medium." Cell numbers are determined routinely with a Coulter Model F electronic cell counter which is standardized periodically with a pre-counted suspension of latex beads. Presence of Mycoplasma cell contaminants is determined by a microscopic fluorescence assay employing Hoechst 33258 dye. All culture procedures and treatments with test chemicals are performed under aseptic conditions in a laminar-flow, biohazard hood.

Positive and Negative Controls: Sterile water or glass-distilled dimethylsulfoxide (DMSO) are the usual solvents for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) or ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without an S9 metabolic activation system, respectively. Mutation frequencies obtained with concurrent positive and negative controls are used as the basis for monitoring the sensitivity and stability of the CHO mutation test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver, S9 homogenate prepared from Arochlor-1254 induced, Sprague-Dawley, male rats is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for metabolic capability to activate DMN in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 umoles/ml $MgCl_2$, 33 umoles/ml KCl, 5 umoles/ml glucose-6-phosphate, 4 umoles/ml NADP-oxidized (nicotinamide adenine dinucleotide phosphate), 100 umoles/ml Na_2HPO_4, and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity); a volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods:

(1) Clonal assay - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to 3×10^{-4}% (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period (37°C) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).

(2) Growth Inhibition - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by weight or volume, as appropriate.

Dose levels which are moderately toxic but permit survival of at least 10 to 20% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 t 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of cells are assessed for mutation induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment with Test Chemicals: For tests of chemicals which may act directly without incorporation of an S9 metabolic activation system, $5x10^5$ cells are inoculated 20 to 24 hours prior to treatment into 25 cm^2 culture flasks containing F12-D5 medium and incubated at 37°C in a 5 to 6% CO_2 atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and cultures are treated for 5 hr at 37°C. The medium and test agents are removed by suction, cells are rinsed once or twice and fresh F12-D5 medium is added. The cells are allowed a period of 20 to 24 hours of recovery from treatment before survival is determined. Treatment of cells for testing of chemicals which require metabolic activation for mutagenic capacity is performed identically with the procedure above, with the exception that F12 medium without serum and containing 1.0 ml of S9 activation mixture per 4.0 ml of medium is employed.

Determination of Cytotoxicity: The relative survival of treated cells, in comparison to solvent controls, is determined one day after the exposure to the test agents. The level of cytotoxicity is often correlated with the mutation frequencies induced by known chemical mutagens. Thus, excessive cytotoxicity may kill both normal cells and mutants and may depress the actual mutation frequencies; insufficient cytotoxicity may indicate an insufficient concentration of the test agent was employed. The colony-forming potential of 100 to 200 treated cells is used as the measure of treatment-induced cytotoxicity.

Survival values which indicate the cytotoxic effects of the test agents are included in reports in tabular form. Statistical analyses are not performed on these data, since they are only useful to assess whether appropriate doses were employed and are not used to calculate mutation frequencies.

Determination of Mutant Induction: On days 1, 3 and 6 (or alternatively 1, 4 and 6) after treatment with the various test agents, approximately $5x10^5$ cells are subcultured in 100 mm tissue culture dishes in F12-D5 medium and incubated at 37°C in a 5 to 6% CO_2 atmosphere. After a total of 7 days to allow "expression" of the mutant phenotype, cells are dissociated with 0.05 to 0.075% trypsin, counted and plated at a concentration of $2.5x\ 10^5$/dish in four culture dishes ($1x\ 10^6$ total cells) which each contain 5 ml of F12-D5 (TG) selective medium. At this time, cells are diluted and 100 cells/dish are added to four culture plates containing F12-D5 medium (without TG) to assess viability (plating efficiency) of the treated cell population and to determine the surviving fraction. All cultures are then incubated for an additional 6 to 8 days to allow growth of cells; medium is then discarded and colonies are fixed and stained for counting. The number of colonies in selection plates and in the viability test are counted by electronic methods, checked by manual counts and data are recorded both as total mutants, mutants/10^6 total cells and mutants/10^6 viable cells.

Statistical Analyses: Uniform statistical procedures to evaluate in vitro mutation data have not been developed. The distribution of mutation frequencies from historical controls in at least two laboratories indicates that the frequency distribution and variances encountered do not justify the use of parametric analyses unless data is transformed before application of standard parametric tests. Analysis of mutation frequencies in the CHO test follow the procedure of Irr and Snee (Reference 4) which employs the Box-Cox Transformation (Reference 5) to transform data before parametric analyses. The mutation frequency for each plate is increased by 1.0 (to eliminate zeros) and raised to the 0.15 power. Experience with historical negative control data in our laboratory indicates that a normal probability distribution of the data suitable for parametric analyses is achieved by this transformation. Parametric analysis of mutation data by the Student's t-test is performed with the transformed data. The degree of statistical significance for the mutation values are indicative of a difference from the concurrent solvent control, but these statistical indicators must be viewed conservatively until additional historical control data are available.

Interpretation of Data: The criteria for interpretation of the test results as a positive or negative response depend upon both the level of statistical significance from the concurrent control and the evidence of a dose-response following treatment. When a definite dose-response relationship is not evident but one or more marginally significant values are obtained, a careful examination of the data from the concurrent positive and negative controls and comparisons to historical control data are used to evaluate the possible significance of the responses. Historical control data indicate that a spontaneous mutation frequency in CHO cells of approximately 4 to 5 mutants/10^6 viable cells, with a range of 0 to 25 mutants/10^6 viable cells, can be obtained in the absence of mutagenic treatment. Statistical comparisons against unusually high or low spontaneous controls are subjectively scrutinized in respect to the above variability.

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APPENDIX II

Determination of Sister Chromatid Exchange (SCE) Frequencies in Chinese Hamster Ovary (CHO) Cells In Vitro

Theoretical Basis

Exchanges of genetic material between the individual arms of a chromosome (i.e. sister chromatids) are thought to arise from breakage and physical interchanges in the DNA of a cell during cell division. An increase in the frequency of such interchanges between sister chromatids can be observed in cells treated with physical or chemical mutagenic agents, or in cells exposed to many suspect or proven human carcinogens. Thus, analysis of SCE frequencies in cells treated with a test agent has been suggested as a sensitive screening test for potential mutagenic/carcinogenic chemicals.

The method used in our study to visualize SCE's in CHO cells grown in culture is based on the procedure described by Perry and Wolff (1974). A standard concentration of 3.0 ug/ml of bromodeoxyuridine (BrdU) was used in the growth medium to allow a visualization of SCE's after two cell divisions in the presence of BrdU. Staining of chromosomes with 5.0 ug/ml of 33258-Hoechst fluorescent dye, exposure to light and Giemsa staining was used to differentiate chromatids for SCE analysis.

Methods

Cell Culture Procedures: Chinese hamster ovary (CHO) cells were obtained from Abraham Hsie at Oak Ridge National Laboratory with the designation CHO-K1-BH4-D1 (referred to simply as CHO for report purposes). CHO cells are maintained in active growth by 2 to 3 weekly subcultures into fresh antibiotic-free, Ham's F12 (modified) medium fortified with 10% (v/v) of heat-inactivated fetal bovine serum and lacking hypoxanthine and thymidine. Cell concentrations are determined routinely with a Coulter® Model-F electronic cell counter calibrated with a precounted suspension of latex beads. All cell culture procedures prior to final harvesting of cells for chromosome preparations are performed under aseptic conditions in a laminar flow, biohazard hood. Presence of Mycoplasma cell contaminants is determined using a fluorescent microscopic assay employing Hoechst 33258 dye.

For treatments with test chemicals without S9 metabolic activation, modified F12 medium is used with 50 units/ml of penicillin, 50 ug/ml streptomycin and 5% (v/v) of heat-inactivated, dialyzed fetal bovine serum (F12-D5). Identical medium but without serum is used for treatments incorporating an S9 metabolic activation system.

Positive and Negative Controls: Sterile water or glass-distilled dimethyl sulfoxide (DMSO) are the usual solvents used for test chemicals and the respective solvent is tested as a control at the maximum concentration used to add the test agent. Dimethylnitrosamine (DMN) and ethylmethanesulfonate (EMS) are used as positive control mutagens for tests with or without the addition of an S9 metabolic activation system, respectively. Results from treatments with concurrent control agents are used as a basis of comparison and for demonstrating the sensitivity and stability of the SCE test system. Comparison of concurrent control values with historical controls is used to delineate the range of acceptable variations in the test system.

Metabolic Activation: Rat liver S9 homogenate (prepared from Arochlor 1254 induced, Sprague-Dawley, male rats) is purchased from Litton Bionetics, Kensington, MD. Each lot of liver homogenate is prescreened for activity in our laboratory before use in the testing program. The complete S9 metabolic activation system contains the following: 8 umoles/ml $MgCl_2$, 33 umoles/ml KCl, 5 umoles/ml KCl, 5 umoles/ml glucose-6-phosphate, 4 umoles/ml NADP-oxidized form (nicotinamide adenine dinucleotide phosphate), 100 umoles/ml Na_2HPO_4 and between 500 to 4000 ug/ml of S9 protein (depending on metabolic activity). A volume of 1.0 ml of the complete mixture of the above reagents is added to each 4.0 ml of culture medium.

Dose Selection: Toxicity of the test chemical is determined prior to assessment of mutagenic potential to select doses which produce a maximum of 80 to 90% cell killing. Cytotoxicity is determined by either of the following two methods as part of the CHO mutation testing procedure:

(1) Clonal assay - 200 to 400 CHO cells are exposed to a minimum of five dose levels of the test agent at concentrations from 0.1% to 3×10^{-4}% (by weight or volume, as appropriate) with and without the presence of a metabolic activation system. The number of cells which survive the treatment is determined by counting the number of colonies produced after a 7- to 8-day incubation period (37°C) in comparison with the colonies formed by cells treated only with appropriate concentrations of solvent (generally 20 ul/ml).

(2) Growth Inhibition - 5×10^5 cells in 25 cm^2 culture flasks are treated for 5 hours with a minimum of five test concentrations both with and without S9 metabolic activation. Following treatment the cells are rinsed, fresh F12-D5 medium is added and the flasks are incubated for an additional 18 to 24 hours. Cytotoxicity is determined by comparing the relative number of cells in control (untreated cells) and in cells treated with various concentrations of the test agent.

If no cytotoxicity is evident at the highest concentrations in the cytotoxicity tests, the test is either repeated at higher concentrations, or mutation testing is performed with a greater number of treatment flasks starting at higher dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of 3×10^{-4} to 3×10^{-6} percent by volume.

Dose levels which are moderately toxic but permit survival of at least 40 to 50% of the cells, in comparison to the solvent control, are selected as the maximum dose, and at least four additional one-half dilutions are tested for induction of mutations. If cytotoxicity data are equivocal, a total of 5 to 8 one-half dilutions of the selected, maximum concentration are used to treat cells; but only the highest five concentrations which permit survival of a sufficient number of mitotic cells with SCE staining, are evaluated for SCE induction.

Chemical samples are sterilized by membrane filtration when microbiological tests indicate this is required to assure sterility. Liquid test agents are tested on a percentage by volume basis. Solid chemicals are dissolved in an appropriate solvent by making a 10 to 20% stock solution (by weight) and subsequent dilutions are made from this stock on a volume/volume basis.

Treatment With Test Chemicals: Testing of chemicals for direct mutagenic action (without S9 metabolic activation) is performed first. For chemicals with clearly positive mutagenic capabilities by direct action, testing with metabolic activation is generally not performed.

For testing direct acting chemicals for SCE induction, between 1 to 2 x 10^6 cells are plated into 75 cm^2 culture flasks in F12-D5 medium at least 20 hrs prior to treatment and incubated at 37°C in a 5 to 6% CO_2 atmosphere. Appropriate concentrations of the test agent or control chemicals are added to the cells and 3 ug/ml BrdU is added to all flasks. Cells are treated with test agents for 5 hrs, media is then removed by suction, cells are rinsed with buffered, physiological salt solution and fresh medium containing 3 ug/ml BrdU is added for at least 24 hrs of additional incubation at 37°C to allow two rounds of cell division. Cells are harvested and chromosomes are prepared for SCE staining.

Treatment of cells for testing of chemicals which require metabolic activation for mutagenic effectiveness is performed similarly as for treatments without activation, except for three modifications:

1. Before treatment with the test agents, F12-D5 medium is removed and F12 medium without serum is added.

2. S9 metabolic activation mixture is added to each flask (including solvent and positive controls) before addition of test agents.

3. Cells are treated for a total of 2 hrs (rather than 5 hrs) and then incubated for 38 to 42 additional hours before harvest for chromosome preparation.

Preparation of Chromosomes: Colcemid® (0.1 ug/ml) or Colchicine (0.2 ug/ml) is added to culture flasks 1 to 2 hrs prior to harvesting to arrest cells in mitosis. Cells are then removed from flasks, after a brief incubation with 0.01% DIFCO trypsin, suspended in 0.075M KCl (hypotonic) solution and incubated for 15 to 20 min at 37°C. Cells are centrifuged, fixed with 3 or 4 changes of Carnoy's fixative (3:1 methanol acetic acid) and chromosome spreads are prepared from cells suspended in a small volume of fixative. One slide/dose level is prepared, but fixed cells are saved if needed for preparation of additional slides.

Chromosomes are stained for SCE's by treatment with 5.0 ug/ml of Hoechst 33258 dye for 20 min, rinsed in distilled water, immersed in Sorenson's buffer and exposed to a high intensity sunlamp for 15 to 30 min., as required. Irradiated chromosomes are stained in Gurr's giemsa (diluted 1:25 with water), rinsed in water and dried before application of coverslips.

Examination of SCE's: All slides are coded and read in a blind fashion without indication of the specific treatment or concentration of the test agent. The number of chromosomes and the number of SCE's in a minimum of 15 cells are recorded for each dose level. The mean number of SCE/cell and SCE/chromosome are calculated and recorded. Slides are decoded only after examination of all slides in the experiment has been completed.

Statistical Analyses: Data are analyzed by appropriate parametric statistical procedures which follow BRRC standard operating procedures for analyses of data. Significance values and the statistical test employed are shown for data summarized in tabular form.

Interpretation of Data: The criteria for evaluation of a positive or negative response depend both on the level of statistical significance and subjective analyses of concurrent and historical control data. The key determinant is whether a dose-dependent increase in SCE's is induced by the test agent. When no clear dose-response relationship is evident and when one or more responses of marginal statistical significance are obtained, a careful examination of the data in comparison to the concurrent controls and historical data base is necessary. Testing may be repeated to clarify unusual responses, if data for the concurrent positive or negative controls suggest a defect in the original experiment. Overall assessment will also rely on corroborating data from the other tests in the testing battery. Clearly positive responses will include any of the following: (i) Doubling in the SCE frequency at a minimum of two of the five concentrations tested; (ii) Statistically significant responses of $p < 0.05$ at three concentrations or at 2 concentrations if $p < 0.01$; (iii) Induction of a statistically significant, dose-related increase in the number of SCE.

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APPENDIX III

Unscheduled DNA Synthesis (UDS) in Hepatocytes from Rat Liver

Th oretical Basis

Chemicals may interact with both the cellular components and the genetic material of a cell (e.g. DNA and RNA) because of their electrophilic nature or by conversion into reactive electrophiles by the metabolic enzymes of the cell. Damage to the DNA of a cell can result in cell death, mutation or, theoretically, carcinogenic transformation. Studies of agents which are capable of reacting and damaging the cellular DNA have suggested that such methods may be useful as a sensitive screening test for detecting potential mutagenic/carcinogenic chemical properties.

Detection of the relatively small amounts of DNA damage induced by chemical treatment requires a cellular system in which normal, semi-conservative DNA replication, which occurs during cell division, is inhibited. The system employed for the present study uses a suspension culture of primary hepatocyte cells isolated from rat liver according to the general methods of Seglen (1973) and Williams (1976). Hepatocytes do not normally divide in the minimal culture medium employed and stimulation of "unscheduled" incorporation of radioactive DNA precursors can be detected by scintillation spectrometry. The stimulation of incorporation of tritiated thymidine into both purified hepatocyte nuclei and DNA is used as the indicator of chemically induced DNA damage. The amount of unscheduled DNA synthesis (UDS) following treatment is compared with both concurrent positive and negative controls as well as with historical data for similar tests.

Methods

Preparation of Hepatocyte Suspensions: Hilltop-Wistar albino rats are anesthetized with Metafane(R). The abdominal cavity is surgically exposed and 1250 units of heparin is injected intravenously. A catheter is inserted into th portal vein and warm Hanks Balanced Salt Solution (HBSS) is pumped into the vein and through the liver. This first solution contains heparin and EGTA, [ethylene glycol-bis-(beta-aminoethyl-ether)N,N-tetracetic acid], which preferentially chelates calcium; the solution contains no magnesium or calcium. After the liver is blanched, a second solution of HBSS containing 60 units/ml of collagenase is perfused. This solution is pumped through the liver until the liver is digested. The liver is then removed and the cells are freed in cold medium 199 by combing through the lobes with a sterile metal comb. The cell suspension is passed through two nylon meshes to remove cell clumps and the cells are washed once at low centrifugation speed. After resuspension in medium 199, equal volumes of cells and 0.4% trypan blue are mixed together and the cell viability and number of viable cells per ml is determined microscopically.

Preincubation of Hepatocytes: Approximately 2 x 10^6 viable hepatocytes are added to 5 ml medium 199 containing 10 mM hydroxyurea and 30 mM Hepes (N-2-hydroxyethyl piperazine-N-2 ethane sulfonic acid) buffer. After the cells are dispensed into the tubes, they are placed on a rocker platform and are incubated at 37°C for 1 hour. Although hepatocytes do not normally divide in culture, medium 199 which lacks serum and contains hydroxyurea is used to further block semi-conservative DNA synthesis. Thus, any radioactive thymidine in orporated into the nucl i is expected t result from repair or unscheduled DNA synthesis.

Selection of Doses of Test Chemical: Initially, the following concentrations x 10^{-3}% (by volume) are tested: 100, 30, 10, 3, 1, and 0.1. If these concentrations prove to be cytotoxic, or if additional information is available from other in vitro tests as to the proper dose levels, then an appropriate series of concentrations is used over a 3-log range of concentrations.

Treatment of Hepatocytes: After preincubation, 25 microCuries of tritiated thymidine (20 Curies/millimole) is added to each tube. The test chemical and positive controls are diluted in an appropriate solvent and they are then added to each labeled tube. Generally, at least six concentrations of the test chemical over a 3-log range of concentrations are tested and each concentration is run in duplicate. The tubes are returned to the rocker platform for a 2-hour exposure at 37°C.

Positive and Negative Controls: 4-nitroquinoline oxide (NQO), a direct-acting mutagen, which induces UV-type DNA repair and dimethylnitrosamine (DMN), which requires metabolic activation by microsomal enzymes for activity, are run in duplicate as positive control chemicals. The solvent control is run in quadruplicate and consists of 100 to 150 microliters (concentration specified in individual reports) of the solvent used to dilute the sample. Dimethyl-sulfoxide (DMSO) or water are the usual solvents for test chemicals.

Harvest: At the end of incubation with the test agent, the cells are centrifuged from the medium at 200 x g at 5°C. The cells are rinsed once in 5 ml of cold medium 199 and are resuspended in 0.25% Triton X-100, 5% citric acid and 3 mM $MgCl_2$, a lysing solution which liberates the nuclei. The nuclei are rinsed once in this solution and resuspended in 0.25 M sucrose, 2.5% citric acid and 3 mM $MgCl_2$. The nuclei are then centrifuged at 600 x g for 10 min at 5°C and resuspended in 2 ml of the lysing solution.

Determination of Nuclear-Bound Label: To measure the amount of radioactive thymidine incorporated into the nuclei, 0.25 ml of the nuclear suspension is mixed with 1.0 mg of NCS tissue solubilizer in a scintillation vial. Ten ml of Dimilume® scintillation cocktail is added and the radioactive disintegrations per minute (DPM) are determined by counting twice in a scintillation counter for ten minutes. The measured DPM are then used to calculate the DPM/10^6 viable hepatocytes presented on tables.

Determination of DNA-Bound Label: The amount of radioactive thymidine incorporated into DNA is quantitated in DNA isolated and precipitated from 1.00 to 1.25 x 10^5 viable hepatocytes. To 1.25 ml of the nuclear suspension, 2.75 ml of 1% sodium dodecyl sulfate (SDS) and 5 mM Ethylenediaminetetraacetic Acid (EDTA) is added to lyse the nuclei. The DNA is precipitated from this solution with 4 ml of ice-cold 10% trichloroacetic acid (TCA) and the sample tubes are incubated at 0°C for at least 30 minutes. The solution is then poured onto Whatman glass fiber filters under vacuum and the tubes and filters are washed twice with cold 5% TCA. Finally, each filter is rinsed once with methanol, dried and placed in a scintillation vial. The filters are incubated at 50°C for 1 hour with 1 ml of a diluted solution of NCS tissue solubilizer; prepared by adding 1 part solubilizer to 2 parts of Dimilume® cocktail. Dimilume® is then added to each vial and the vials are counted twice in a scintillation counter for ten minutes.

Statistical Analysis: The average DPM is calculated for each dose level and the controls and final results are expressed as DPM/10^6 viable hepatocytes. Data are also expressed as a percent of the solvent control for purposes of comparison. The original data are statistically analyzed by the appropriate parametric test, following the BRRC standard procedures for statistical analyses and the test(s) employed is indicated on the respective tables. Comparison between the mean for each dose level with the 95% confidence limits of the historical solvent control may also be used in some cases to assess the potential biological significance of the data. Testing may be repeated to clarify unusual responses, if data with the concurrent controls suggest a defect in the original experiment.

Interpretation of Results: The classification of a chemical as a positive, active agent depends upon the production of a statistically significant, dose-related increase in the amount of UDS activity. If a definite dose-response relationship is not evident, or when a few increases with marginal statistical significance are obtained, comparison of the responses to historical control data provides a meaningful assessment of the possibility for random variations which may be statistically significant only in relation to the concurrent control. A key determinant of the reliability of the UDS data is th detection of a similar response with both DNA and isolated nuclei determined at two or three consecutive concentrations.

General References

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APPENDIX IV

Physical and Chemical Characteristics of Test Material

BRRC Chemical No:	42-138
CAS No.:	1675-54-3
Chemical Name:	BAKELITE® Liquid Epoxy Resin ERL-2774
Trade Name and/or Synonyms:	Diglycidyl Ether of Bisphenol A
Molecular Weight:	340
Formula:	$C_{21}H_{24}O_4$
Specific Gravity (@ 25°C):	1.15 to 1.17
Boiling Point:	Not applicable
Solubility in H_2O (% by wt):	Insoluble
Purity	Not available
Vapor Pressure (@ 20°C):	Not available
pH:	Not available
Flash Point:	450°F (Cleveland open cup)
Stability:	Stable
Incompatibility:	Avoid high temperatures > 450°F and contamination with acids, amines and H_2O.
Appearance and Odor:	Clear liquid
Disposal:	Incinerate. Spills should be absorbed and burned.

Protective Measures: Use rubber or plastic gloves, safety glasses and mechanical ventilation.

Health Hazard: Appeared to be relatively non-toxic following acute animal exposure tests. Repeated or prolonged contact may cause skin rash. Avoid prolonged or repeated skin contact and breathing of vapors.