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Project Report 43-105 (Amendment)

Tel: (412) 327-1020

January 14, 1981

First Amendment to

BAKELITE® Cycloaliphatic Epoxy Resin ERL-4221

In Vitro Mutagenesis Studies: 3-Test Battery

Authors: R. S. Slesinski, M. W. Gaunt, P. J. Guzzie, W. C. Hengler

Sponsor: Union Carbide Corporation

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On page 5, Section 2 (SCE Test), the unit of measure should have been ug/ml, therefore, the second to last sentence should read:

"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."

Reviewed and Approved by:

Ronald S. Slesinski

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BAKELITE® Cycloaliphatic Epoxy Resin ERL-4221

In Vitro Mutagenesis Studies: 3-Test Battery

Authors: R. S. Slesinski, M. W. Gaunt, P. J. Guzzie, W. C. Hengler

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SUMMARY

Epoxy Resin ERL-4221 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-4221 did not produce a strong mutagenic effect typical of known chemical mutagens but it appeared to possess significant mutagenic potential in the sister chromatid exchange test and questionable-to-weak activity in the UDS test. The lack of a definitive response in at least two of the three tests prevented an unequivocal classification of Epoxy Resin ERL-4221 as mutagenic or non-mutagenic. However, the strongly positive and dose-related increase in the SCE frequency in cells treated with Epoxy Resin ERL-4221 suggests that additional testing may be appropriate to investigate the possible biological significance of these results.

RESULTS AND INTERPRETATION

Epoxy Resin ERL-4221 was selected for mutagenesis testing in part to develop and validate the sensitivity of our in-house battery of mutagenicity tests. Epoxy Resin ERL-4221 was found to be inactive in a previous lifetime dermal carcinogenesis study with mice performed at our laboratory (CHF Report #27-6, January, 1964) and it was used for testing the ability of our 3-test battery to discriminate between chemicals found to be either active or inactive in animal studies.

Selection of Test Concentrations - Preliminary experiments were performed to select an appropriate range of concentrations in which the maximum dose level would allow survival of approximately 10% of the treated cells. A maximum concentration of 0.01% (by volume) was chosen as the top dose level for tests both with and without an S9 metabolic activation system. In a second repeat test, necessitated by technical problems in the first test, the maximum dose level tested with S9 activation was increased to 0.02%.

CHO Mutation Test - Epoxy Resin ERL-4221 was not active in stimulating a dose-related increase of mutant cells when tested either with or without the presence of an S9 metabolic activation system. Neither of two experiments provided any indication of a statistically significant mutagenic effect of the test agent. Epoxy Resin ERL-4221 was considered inactive as an agent for inducing mutation of CHO cells in culture.

SCE Test - Epoxy Resin ERL-4221 was highly active in significantly stimulating the induction of SCE in vitro at three of six of the concentrations tested. The indication of a dose-related increase in the number of SCE in tests without S9 metabolic activation provided a convincing indication that Epoxy Resin ERL-4221 was active in stimulating SCE in CHO cells. Tests of SCE production with the addition of a metabolic activation system were not performed because the test without addition of liver homogenate indicated that metabolic conversion was not required for activity of the test chemical.

UDS Test - Epoxy Resin ERL-4221 did not produce dose-related increases in the amount of UDS detected with either nuclei or DNA. However, in evaluations over a relatively wide range of concentrations, the lowest three concentrations f Epoxy Resin ERL-4221 produced highly numerically elevated levels of UDS activity. Because these values were not consistently significant in statistical comparisons to the concurrent solvent control, the results could not be definitively labelled as either positive or negative. The data were considered to be suggestive of a low level of activity and Epoxy Resin ERL-4221 appeared to be questionably-to-weakly active in the present test with the hepatocyte test system.

Comparative Mutagenicity - The pattern of responses produced in the 3-test battery of mutagenicity tests indicated that Epoxy Resin ERL-4221 was not a potent mutagenic agent but that it appeared to possess a low level of activity in the UDS and significant activity in the SCE test. The lack of definitively positive responses in at least two tests of the 3-test battery prevented an unequivocal classification of Epoxy Resin ERL-4221 as either mutagenic or non-mutagenic. However, significantly positive results related to the treatment dose observed in the SCE test should be considered as an indication of unconfirmed but potential biological activity. Additional testing of this chemical using other test systems may be warranted to determine the biological significance of the results of these in vitro studies. In the previous dermal carcinogenesis test of this chemical (CHF Report #27-6), 1 tumor in 15 mice in the effective group was observed but this result was not significantly above the tumor incidence in the control group of animals.

SAMPLE

Quantity: 8 ounces

CHF Sample No.: 42-136

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

Date Received: March 15, 1979

Division: Specialty Chemicals and Plastics

Identification: light yellow, viscous liquid

CAS #: 2386-87-0

BAKELITE® Cycloaliphatic Epoxy Resin ERL-4221

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-4221 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-4221 for potential mutagenic activity. A general description of the theoretical bases of these three tests is presented in Appendices I, II and III (attached to the complete report).

SAMPLE CHARACTERISTICS

A typical, commercial sample of Epoxy Resin ERL-4221 was received for testing on March 15, 1979. The available information from the Toxicology Data Bank or from "Material Safety Data Sheets" for this product is 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 in-house development and validation of the mutagenicity test battery. A copy of the current procedures used for these tests at the Bushy Run Research Center are attached to this report and deviations from there 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-4221 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 the dose level which should permit survival of at least 10% of the treated cells. Glass-distilled dimethylsulfoxide (DMSO) was used as the solvent and solvent control; sterile water (H_2O) was used as the negative control.

To simplify tables and to allow comparisons between different tests, concentrations of Epoxy Resin ERL-4221 in the following sections of the report are given in terms of volume percentages x 10^{-4} to eliminate zeros in the lower concentration values (eg. 0.0003125% = 3.125 x 10^{-4}%).

B. Mutation - In experiment #1, CHO cells were exposed for 16 hours to five concentrations of Epoxy Resin ERL-4221 from 100 x 10^{-4}% to 6.25 x 10^{-4}% (by volume) without the addition of an S9 metabolic activation system and for 5 hours to an identical range of concentrations with S9 activation. A second, repeat experiment was performed at identical concentrations in the test without S9 but concentrations from 200 x 10^{-4}% to 12.5 x 10^{-4}% were tested with S9 activation. Dilutions of Epoxy Resin ERL-4221 were prepared by either direct addition of the test agent into the cell culture media or by making sequential one-half dilutions from the stock solution f r the highest concentration using glass-distilled DMSO. The surviving fraction was determined at 20 to 24 hours after treatment and the mutant fraction was determined after a 7-to 9-day period to allow "expression" of the mutant phenotype. Only the top five concentrations which allowed sufficient cell survival were assessed for survival and induction of mutants. 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-4221 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-4221 for testing, ranging from 100 x 10^{-4}% to 3.125 x 10^{-4}% (by volume), were prepared either by direct addition into the culture medium or by addition of various aliquots of a stock solution prepared in DMSO. For determination of direct mutagenic action, CHO cells were exposed to Epoxy Resin ERL-4221 and appropriate controls for 5 hours without S9 activation. Indirect mutagenic action, requiring metabolic activation by liver S9 homogenate, was not studied because a highly significant positive response was obtained without metabolic activation which indicated a direct-acting mechanism for this test agent. Bromodeoxyuridine (BrdU) required to differentiate between the individual "sister" chromatids by SCE staining, was present at a concentration of 3 g/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, with or without metabolic activation were examined. The number of SCE/cell, mean # of SCE/chromosome and the level of statistical significance of the increases above 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 at a minimum of six dose levels which spanned a 1000-fold range of concentrations. Cells were treated with Epoxy Resin ERL-4221 for 2 hours in culture medium containing ^{3}H-thymidine, hydroxyurea and appropriate dilutions of Epoxy Resin ERL-4221 prepared in DMSO. Determination of UDS activity was performed by analyses of radioactive incorporation into

isolated hepatocyte nuclei and 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 4-nitroquinoline oxide (4-NQO) were used as positive controls for indirect- or 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 contained 38.5 mg/ml protein and had a benzo(a)pyrene hydroxylase activity of 21.6 nmol hydroxybenzpyrene/20 min/mg protein, (assayed by Litton). A concentration of 2400 ug of S9 protein was added to 5 ml of culture media.

6. Statistical Analyses - Data from the SCE and UDS tests were analyzed by appropriate parametric tests following Standard Operating Procedures f r 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 suitable transformation of the mutation frequencies (MF) following the procedure 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 - Epoxy Resin ERL-4221

A. Test Dates - Initiated: April 26, 1979
Completed: May 16, 1980

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

In tests without S9 activation, CHO cells were exposed for sixteen hours to six concentrations of Epoxy Resin ERL-4221 which spanned a concentration range from 0.5% to 5 x 10^{-4}% by volume. An identical range of concentrations was tested for five hours with S9 activation. Th 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. In this prescreening determination of cytotoxicity, n surviving cells produced colonies after treatment with 0.5% of Epoxy Resin ERL-4221, the highest concentration tested. As a percentage of the control values, 3.5% of the cells produced colonies after treatment with 50 x 10^{-4}% of Epoxy Resin ERL-4221 in the presence of an S9 activation system; without S9 activation, 0.8% of the cells treated at this same concentrati n formed colonies. A concentration of 100 x 10^{-4}% was selected as the maximum concentration for testing with and without S9 activation. In a second repeat experiment, the maximum concentration tested with S9 activation was increased to 200 x 10^{-4}% to attain a higher level of cytotoxicity.

C. Determination of Mutation Induction

1. Survival (Cytotoxicity)

Table 1 presents the cytotoxicity data for CHO cells treated with Epoxy Resin ERL-4221 in the absence of a liver S9 metabolic activation system. A steep dose-response effect with the test agent was suggested from the high degree of cytotoxicity observed for the top concentrations (100 x 10^{-4}%) in comparison to the markedly lower cytotoxicity obtained at only one-half the top dose-level (Table 1). The cells treated with the test agent together with an S9 activation system were not assessed for mutant induction because the CO_2 concentration in the incubator used for these plates was abnormally high (due to a malfunction) and this malfunction inhibited or killed the cells. The survival results of the second, repeat experiment are shown on Table 3. These data indicated a similar cytotoxic response at the highest dose level tested without S9 activation in comparison to the value obtained in experiment #1 (Table 1). Also, the slope of the dose-response relationship for the cytotoxicity data in this second experiment suggested a more usual response without the steep drop observed at the highest two doses in experiment #1.

2. Mutation

Table 2 presents the data for induction of mutants by Epoxy Resin ERL-4221 and control agents. Epoxy Resin ERL-4221 did not produce a dose-related increase in the frequency of mutants/10^6 viable cells over the 16-fold range of concentrations tested for potential mutagenic action without the presence of an S9 metabolic activation system. Although no concentration of Epoxy Resin ERL-4221 produced a statistically significant increase in the mutation frequency in the test without S9 activation, the test was repeated because the EMS positive control was also not significantly above the solvent control values. In experiment #2, summarized on Table 4, data were consistent with the negative responses observed in experiment #1 (Table 2). No dose-level of Epoxy Resin ERL-4221 produced an increase which was statistically significant from the solvent control, and there was no indication of a dose-related production of mutants. This second experiment was consistent with the classification of Epoxy Resin ERL-4221 as not active in producing a mutagenic effect detectable in the CHO test system.

Mutation frequencies for the solvent controls for tests in both experiment #1 and #2 without S9 activation were in an acceptable and low range based upon experience with historical control values. The mutation frequency for the solvent control in experiment #2 with S9 activation was numerically higher than our historical control values but similar small increases in the frequency of mutants have been seen in previous experiments in which the S9 liver homogenate itself displays a weak mutagenic activity. Small numerical increases at some treatment levels of Epoxy Resin ERL-4221 were within the range of historical variability encountered for negative and solvent controls using this test.

Highly statistically significant mutation frequencies were obtained for the DMN and EMS positive controls in experiment #2 and these values were within the normally expected range of variation observed in historical control data.

D. Deviations from Standard Procedures - Testing of Epoxy Resin ERL-4221 was performed as part of the development and validation phase for the in-house battery of mutagenicity assays. There are numerous deviati ns from our current SOP in the present test on this chemical but none of these deviations are believed to decrease the sensitivity of the test. The major deviation involved the use of a 16-hour exposure period in the test without S9 rather than 5 hours as stated in Appendix I. A 16-hour exposure period would normally be expected to improve rather than to decrease the sensitivity of the assay; exposure periods between 2 to 24 hours are acceptable if suitable toxic to non-toxic dose ranges are tested. The 100×10^{-4}% dose-level was allowed an expression period of 9 days (rather than 7 days as used with all other dose-levels) because this top concentration produced an extended depression of cell growth and a longer period is thought to be more reliable at such high cytotoxic doses.

E. Conclusions

Epoxy Resin ERL-4221 was consistently inactive as a mutagenic agent for CHO cells when tested with or without an S9 metabolic activation system over a 16-fold range of concentrations. Although small increases in the numerical frequency of mutants were obtained at some test concentrations of Epoxy Resin ERL-4221, these results appeared to be a random effect without statistical or probable biological significance.

SECTION II - SCE TEST - Epoxy Resin ERL-4221

A. Test Dates - Initiated: December 10, 1979
Completed: March 24, 1980

B. Selection of Test Concentrations

A maximum concentration of 100 x 10^{-4}% was chosen as the top dose levels for testing without S9 activation 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, based on experience in other studies. A 32-fold range of concentrations from 100 x 10^{-4}% to 3.125 x 10^{-4}% (by volume) was examined without S9 activation. Because we observed a highly statistically significant and dose-related indication of a direct mutagenic effect of the test agent in this experiment without S9, tests with an S9 activation system were not performed.

C. Determinations of SCE Production

The data for SCE production in CHO cells treated with various dose levels of Epoxy Resin ERL-4221 and with appropriate positive, negative or solvent control agents are summarized in Table 5. Epoxy Resin ERL-4221 produced statistically significant increases in the SCE frequency at three of the six dose-levels tested for direct action in the absence of a metabolic activation system. Also, the increase in the numbers of SCE was dose-dependent. The test without S9 activation was considered an indication of a significant direct mutagenic action of Epoxy Resin ERL-4221.

Induction of SCE 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 H_2O solvent and DMSO controls were also in an acceptable range of values included in the variability encountered in our historical experience with this test.

Testing of Epoxy Resin ERL-4221 with S9 metabolic activation was not performed because the highly positive results obtained without S9 indicated that metabolic activation was not required to express mutagenic activity.

D. Deviations from Standard Procedures

The experiment was repeated three times but only the final, successful study was reported. In the first two experiments cytotoxicity of the test agent reduced the number of mitotic cells and the chromosome preparations were not suitable for scoring.

E. Conclusions

Epoxy Resin ERL-4221 produced highly significant increases in the frequency of SCE when tested over a 32-fold range of concentrations in tests without addition of an S9 metabolic activation system. Evidence of a dose-related effect on the SCE frequency following exposure to Epoxy Resin ERL-4221 indicated that the test agent should be considered significantly active in the present in vitro assay.

SECTION III - UDS TEST - Epoxy Resin ERL-4221

A. Test Dates - Initiated: August 31, 1979
Completed: March 11, 1980

B. Selection of Test Concentrations

Standard procedures were followed and Epoxy Resin ERL-4221 was tested over a 3-log range of concentrations from 1000 x 10^{-4}% to 1.0 x 10^{-4}% by volume. The dose-levels were selected to span a range of cytotoxic to non-cytotoxic concentrations based upon data obtained in the CHO cytotoxicity test.

C. Determination of UDS Induction

1. Nuclear-Bound Radioactive Label (Data in Table 6)

Values for "unscheduled" incorporation of radioactive thymidine into nuclei of hepatocytes exposed to Epoxy Resin ERL-4221 or to appropriate positive and negative controls are presented in Table 6. In hepatocytes treated with Epoxy Resin ERL-4221, only one concentration tested for potential activity induced a statistically significant increase in the amounts of ^{3}H-thymidine incorporation. A gradual decrease in the amounts of radioactive incorporation, over the entire range of concentrations tested, was considered an indication of the cytotoxicity of the test agent. The production of statistically significant levels of UDS at only the lowest concentration may suggest that even lower concentrations should be tested. These data were considered equivocal but suggestive of a questionable-to-weak activity for Epoxy Resin ERL-4221.

Both of the positive control agents, NQO and DMN, induced numerically elevated increases in UDS over values obtained with the solvent control. With DMN, however, only a single concentration produced a sufficiently high level of activity to produce a response which was statistically significant. These data with nuclei indicated that the test system may be less sensitive than desirable for detection of weakly-active mutagenic agents which require metab lic conversion (eg. DMN).

2. DNA-Bound Radioactive Label (Data in Table 7)

Analyses of DNA, from aliquots of hepatocyte nuclei used for the UDS studies presented on Table 6, were performed as a second assessment of "unscheduled" incorporation of ^{3}H-thymidine. Values for radioactivity incorporated into the DNA of these hepatocyte nuclei are presented in Table 7.

For hepatocytes treated with Epoxy Resin ERL-4221, two of the six test concentrations induced levels of UDS which were statistically significant from the solvent control. We also observed a similar pattern of responses as obtained in the assessment of nuclei from cells treated with the same range of concentrations; three of the lowest six concentrations produced numerical elevations in UDS similar in magnitude to values obtained with the DMN positive control. Although there was no indication of a dose-effect relationship due to treatments with the test agent, the UDS values were sufficiently elevated to suggest a very weak level of mutagenic activity. These several considerations were consistent in the classification of Epoxy Resin ERL-4221 as a questionable-to-weakly active agent in the induction of DNA damage in the present test with the hepatocyte test system.

The test employing precipitated DNA for measurement of incorporation of ^{3}H-thymidine was apparently more sensitive than the assay with nuclei, following a comparison of the level of responses produced by the positive control agents NQO and DMN with data from DNA and nuclei. With DMN four of six of the tested concentrations produced a numerical elevation in UDS which was also statistically significant from the solvent control (Table 7). With NQO, only the highest concentration was statistically above the solvent control but a dose-related response was produced which is considered to be a definitive biological indication of a positive mutagenic effect.

D. Deviations from Standard Procedures

Testing of Epoxy Resin ERL-4221 was performed during the development and validation phase of our in-house program of mutagenicity testing. Two prior experiments were performed with methods different from the final procedures developed and described in Appendix III. These previous experiments are not reported because insufficient responses with the positive control agents invalidated the results for an acceptable test. The final experiment, which employed our current procedures, tested several concentrations of DMN (rather than three as stated in the SOP) because we wished to monitor the appropriate response of the cellular activation system for metabolizing such indirect-acting chemicals.

E. Conclusion

Epoxy Resin ERL-4221 appeared to produce a very weak response in the present test with cells treated over a 1000-fold range of test concentrations. Epoxy Resin ERL-4221 was considered questionable-to-weakly active in producing DNA damage in the tests with hepatocytes.

Reviewed and Approved by:

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Contributors:

Chinese Hamster Ovary test	Peggy J. Guzzie, B.S. Master Technologist
Sister Chromatid Exchange test	Michelle W. Gaunt, B.S. Master Technologist
Unscheduled DNA Synthesis Assay	W. Christopher Hengler, M.S. Assistant Scientist

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REFERENCES

A search of the major mutagenesis/carcinogenesis computer data files failed to find any articles pertinent to mutagenic/carcinogenic potential for this test agent. A previous study on dermal carcinogenic potential for mice indicated that Epoxy Resin ERL-4221 did not produce a statistically significant increase in skin tumors (CHF Project Report #27-6; CHF Sample #24-172).

Table 1
Chinese Hamster Ovary (CHO) Mutation Assay:
Determination of Toxic Effects of Chemical Treatment During 16 Hr Mutation Induction Period
Experiment #1

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
		Without S9 Activation		
[Epoxy Resin ERL-4221] (%, v/v)				
100.0×10^{-4}	42	800	5.2	8.4
50.0×10^{-4}	255	800	31.9	50.8
25.0×10^{-4}	419	800	52.4	83.5
12.5×10^{-4}	565	800	70.6	112.5
6.25×10^{-4}	354	800	44.2	70.5
Controls				
DMSO (20 ul/ml) - Solvent	502	800	62.8	-
H_2O (20 ul/ml) -	512	800	64.0	102.0
EMS (200 ug/ml) -	578	800	72.2	115.1

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-4221
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-4221] (%, v/v)						
100.0×10^{-4}	49	300	0.163	0	1×10^6	0
50.0×10^{-4}	82	300	0.273	1	1×10^6	3.7
25.0×10^{-4}	144	300	0.480	0	1×10^6	0
12.5×10^{-4}	144	300	0.480	2	1×10^6	4.2
6.25×10^{-4}	152	300	0.507	0	1×10^6	0
Controls:						
DMSO (20 ul/ml) - Solvent	270	300	0.900	1	1×10^6	1.1
H_2O (20 ul/ml) -	203	300	0.677	1	1×10^6	1.5
Medium	255	300	0.850	0	1×10^6	0
EMS (200 ug/ml) -	180	300	0.600	22	1×10^6	36.7

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

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

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

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% f Solvent Control
		Without S9 Activation		
[Epoxy Resin ERL-4221] (%, v/v)				
100.0×10^{-4}	7	400	1.8	3.0
50.0×10^{-4}	60	800	7.5	12.8
25.0×10^{-4}	123	800	15.4	26.2
12.5×10^{-4}	208	800	26.0	44.3
6.25×10^{-4}	266	800	33.2	56.7
Controls				
DMSO (20 ul/ml) - Solvent	469	800	58.6	-
H_2O (20 ul/ml) -	399	800	49.9	85.1
EMS (200 ug/ml) -	43	600	7.2	12.2
		With S9 Activation		
[Epoxy Resin ERL-4221] (%, v/v)				
200.0×10^{-4}	351	800	43.9	169.6
100.0×10^{-4}	381	800	47.6	184.1
50.0×10^{-4}	326	800	40.8	157.5
25.0×10^{-4}	419	800	52.4	202.4
12.5×10^{-4}	337	800	42.1	162.8
Controls				
DMSO (20 ul/ml) - Solvent	207	800	25.9	-
H_2O (20 ul/ml) -	297	800	37.1	143.5
DMN (3700 ug/ml) -	103	800	12.9	49.8
DMN (740 ug/ml) -	231	800	28.9	111.6

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

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Chinese Hamster Ovary (CHO) Mutation Assay:
Results on Evaluation of Mutant Induction by Epoxy Resin ERL-4221
Experiment #2

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-4221] (%, v/v)						
100.0×10^{-4}	76	400	0.190	0	0.74×10^6	0
50.0×10^{-4}	87	400	0.218	3	1×10^6	13.8
25.0×10^{-4}	137	400	0.342	0	1×10^6	0
12.5×10^{-4}	172	400	0.430	2	1×10^6	4.7
6.25×10^{-4}	186	400	0.465	3	1×10^6	6.5
Controls:						
DMSO (20 ul/ml) - Solvent	211	400	0.528	3	1×10^6	5.7
H_2O (20 ul/ml) -	246	400	0.615	2	1×10^6	3.3
EMS (200 ug/ml) -	180	400	0.450	124	1×10^6	275.6[c]
With S9 Activation						
[Epoxy Resin ERL-4221] (%, v/v)						
200.0×10^{-4}	312	400	0.780	2	1×10^6	2.6
100.0×10^{-4}	333	400	0.832	15	1×10^6	18.0
50.0×10^{-4}	313	400	0.782	15	1×10^6	19.2
25.0×10^{-4}	289	400	0.722	3	1×10^6	4.2
12.5×10^{-4}	353	400	0.882	14	1×10^6	15.9
Controls:						
DMSO (20 ul/ml) - Solvent	362	400	0.905	18	1×10^6	19.9
H_2O (20 ul/ml) -	386	400	0.965	5	1×10^6	5.2
DMN (3700 ug/ml) -	198	400	0.495	63	1×10^6	127.3[c]
DMN (740 ug/ml) -	263	400	0.658	30	1×10^6	45.6[a]

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

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-4221) 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-4221)] (%, v/v)					
100.0×10^{-4}	280	174	11.60	0.626 ± 0.212	NS
50.0×10^{-4}	307	572	38.13	1.869 ± 0.301	c
25.0×10^{-4}	301	415	27.67	1.379 ± 0.184	c
12.5×10^{-4}	303	316	21.07	1.041 ± 0.316	c
6.25×10^{-4}	297	220	14.67	0.743 ± 0.233	NS
3.125×10^{-4}	299	233	15.53	0.779 ± 0.238	NS
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: $p > 0.05$. Data analyzed by Student's t-test.

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

Nuclear-bound label: all DPM values are calculated from nuclei 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 Nuclei Avg. DPM ± S.D.	% of Solvent Control ± S.D.	Significance Above Solvent Control[1]
Solvent - DMSO	3.0%	6995 ± 909	100.0% ± 13.0%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	16791 ± 1307	240.1% ± 18.7%	c
	1.0 ug/ml	10913 ± 4668	156.0% ± 66.7%	a
	ug/ml	9623 ± 456	137.6% ± 6.5%	NS
DMN	1000 ug/ml	9286 ± 748	132.8% ± 10.7%	NS
	300 ug/ml	8234 ± 877	117.7% ± 12.5%	NS
	100 ug/ml	12230 ± 927	174.8% ± 13.3%	b
	30 ug/ml	10570 ± 2484	151.1% ± 35.5%	NS
	10 ug/ml	9390 ± 3683	134.3% ± 52.7%	NS
	1 ug/ml	10679 ± 474	152.7% ± 6.8%	NS
Test Chemical:				
[Epoxy Resin ERL-4221] (%, v/v)	1000 x 10^{-4}%	578 ± 207	8.3% ± 3.0%	NS
	300 x 10^{-4}%	6636 ± 292	94.9% ± 4.2%	NS
	100 x 10^{-4}%	9458 ± 334	135.2% ± 4.8%	NS
	30 x 10^{-4}%	10322 ± 2777	147.6% ± 39.7%	NS
	10 x 10^{-4}%	10547 ± 478	150.8% ± 6.8%	NS
	1.0 x 10^{-4}%	11600 ± 1399	165.8% ± 20.0%	a

[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; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine; DPM - disintegrations per minute; S.D. - standard deviation

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Table 7
Unscheduled DNA Synthesis in Hepatocytes from Rat Liver

DNA-bound label: 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	3.0%	8537 ± 3379	100.0% ± 39.6%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	16720 ± 940	195.0% ± 11.0%	b
	1.0 ug/ml	12011 ± 3654	140.7% ± 42.8%	NS
	0.3 ug/ml	10219 ± 757	119.7% ± 8.9%	NS
DMN	1000 ug/ml	9818 ± 786	115.0% ± 9.2%	NS
	300 ug/ml	14608 ± 1669	171.1% ± 19.5%	a
	100 ug/ml	13195 ± 681	154.6% ± 8.0%	a
	30 ug/ml	13322 ± 4554	156.0% ± 53.3%	a
	10 ug/ml	12198 ± 3117	142.9% ± 36.5%	NS
	1 ug/ml	16243 ± 1690	190.3% ± 19.8%	b
Test Chemical:				
[Epoxy Resin ERL-4221] (%, v/v)	1000 x 10^{-4}%	495 ± 289	5.8% ± 3.4%	NS
	300 x 10^{-4}%	7864 ± 307	92.1% ± 3.6%	NS
	100 x 10^{-4}%	10882 ± 1198	127.5% ± 14.0%	NS
	10 x 10^{-4}%	14760 ± 1453	172.9% ± 17.0%	a
	10 x 10^{-4}%	12172 ± 242	142.6% ± 2.8%	NS
	1.0 x 10^{-4}%	14629 ± 993	171.4% ± 11.6%	a

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

Abbreviations: DMSO - dimethylsulfoxide; 4-NQO - 4-nitroquinoline oxide; DMN - dimethylnitrosamine; DPM - disintegrations per minute; S.D. - standard deviation

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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 lose 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 th 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 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 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 r covery 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 f 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 comparis n and for demonstrating the sensitivity and stability f 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 high r dose levels. If marked toxicity is evident even at the lowest dose, the cytotoxicity test is repeated at a concentration range of $3x10^{-4}$ to $3x10^{-6}$ 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 appropriat solvent by making a 10 t 20% stock soluti n (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 f r 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 3.0 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 f 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 ther tests in the testing battery. Clearly positive responses will include any f 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

Theoretical 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 the 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 2×10^6 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 incorporated into the nuclei is expected to result from repair or unscheduled DNA synthesis.

Selection of Doses of Test Chemical: Initially, the following concentrations x 10^{-3}% (by volume) are teeted: 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 soluti n 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 c unter 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 the detection of a similar response with both DNA and isolated nuclei determined at two or three consecutive concentrations.

General References

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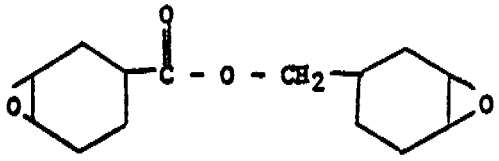
APPENDIX IV

Physical and Chemical Characteristics of the Test Sample

CHF No.:	42-136
CAS No.:	2386-87-0
Chemical Name:	3, 4-Epoxycyclohexylmethyl-3, 4 Epoxycyclohexylcarboxylate
Trade Name and/or Synonyms:	BAKELITE® Cycloaliphatic resin ERL-4221 (previously EP-221)
Molecular Weight:	252.30
Formula:	$C_{14}H_{20}O_4$
Molecular Structure:	
Specific Gravity (@ 20°C):	1.1725
Boiling Point:	354°C (669.2°F)
Solubility in H_2O (% by wt):	0.03 (at 25°C)
Purity:	Not available; commercial sample tested
Vapor Pressure (@ 20°C):	< 0.01 mm Hg
pH:	Not available
Flash Point:	245°F Closed cup
Stability:	Stable, avoid heating over 100°F
Incompatibility:	Avoid acids, amines, strong bases
Appearances and Odor:	Low viscosity liquid; characteristic odor
Disposal:	Dilute with inert solvent and incinerate. Small spills may be flushed with water; larger spills absorbed and burned
Protective Measures:	Use goggles, plastic gloves and exhaust ventilation.
Health Hazard:	Avoid skin and eye contact. No effects of overexposure are currently known.