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BAKELITE® Epoxy Resin ERL - 4206

In Vitro Mutagenesis Studies: 3-Test Battery

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Sponsor: Union Carbide Corporation

SUMMARY

Epoxy Resin ERL-4206 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 data indicated that Epoxy Resin ERL-4206 produced significantly positive and dose-related effects in the SCE test and a significantly positive response at only the highest dose level in the CHO test. Although there was no positive effect in the UDS test at any concentration tested, several concentrations produced numerical increases above the values for the concurrent solvent control indicating a low level of activity. Epoxy Resin ERL-4206 was considered an active mutagenic agent based primarily on the significantly positive response in the SCE test and the suggestive, statistically significant production of mutants at the top concentration in the CHO test.

RESULTS AND INTERPRETATION

Epoxy Resin ERL-4206 was selected for mutagenesis testing to validate the sensitivity of our in-house battery of mutagenicity tests for detecting chemicals found to produce a positive effect in lifetime skin carcinogenicity assays with mice (CHF Report #27-152).

Selection of Test Concentrations - Preliminary experiments were performed to select an appropriate range of test concentrations which would allow the survival of at least 10% of the treated cells. A maximum concentration of 0.01% (by volume) was selected as the maximum concentration for testing both with and without metabolic activation. At slightly higher concentrations (eg. 0.03%) fewer than 1% of the treated cells were capable of producing colonies.

CHO Mutation Test - Epoxy Resin ERL-4206 was active only at the highest dose level ($10 \times 10^{-3}\%$) in producing a statistically significant increase of mutant cells when tested without an S9 metabolic activation system. Although we did not observe a dose-related increase in mutants, production of highly statistically significant increases in mutants at the highest dose level prompted a cautious interpretation of the data and the test without S9 activation was con-

sidered questionably-to-weakly positive. The test with an S9 metabolic activation system did not result in a significant increase in the mutant frequency, which suggested that the metabolic activation system may have obscured the weak response obtained without S9.

SCE Test - Epoxy Resin ERL-4206 produced a significant increase in SCE at four of the five dose-levels tested. The observation of a dose-related effect in the tests without S9 metabolic activation provided a clear indication that Epoxy Resin ERL-4206 was active in stimulating SCE in CHO cells. The test without a metabolic activation system indicated that Epoxy Resin ERL-4206 did not require metabolic conversion for activation and the test incorporating an S9 metabolic activation system was not performed.

UDS Test - Epoxy Resin ERL-4206 failed to induce dose-related increases in the UDS detected with either nuclei or DNA. In evaluations over a relatively wide range of concentrations, no concentration of Epoxy Resin ERL-4206 produced statistically significant levels of UDS activity. Production of numerically increased levels of UDS by a few of the concentrations of Epoxy Resin ERL-4206 tested for activity might be an indication of a low level of primary DNA damage. However, the lack of a statistically significant effect indicated that Epoxy Resin ERL-4206 was not active in the induction of UDS in the present test.

Comparative Mutagenicity - The pattern of responses produced by Epoxy Resin ERL-4206 in the 3-test battery of mutagenicity assays indicated that the test agent was highly active in the SCE test and questionably active in the UDS and CHO tests. Because only the highest concentration of Epoxy Resin ERL-4206 produced a significant effect in the CHO test, additional testing of a narrower range of concentrations would be necessary for identification of potential for induction of gene (point) mutations. The definitive production of SCE in a highly statistically significant, dose-related manner indicated that Epoxy Resin ERL-4206 could be considered an active mutagenic agent without additional in vitro testing.

The lack of activity in the UDS test (which uses metabolically competent liver cells) and in the CHO test with liver S9 activation suggested that Epoxy Resin ERL-4206 may be metabolically converted to a non-mutagenic form by the liver activation systems. The production of positive response in mouse dermal carcinogenesis studies (CHF Report #27-152) are consistent with the positive mutagenicity data observed in this study but show that mouse skin cells are affected by the test agent and do not inactivate it following in vivo exposures.

SAMPLE

Quantity: 8 ounces

CHF Sample No.: 42-137

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

Date Received: March 15, 1979

Division: Specialty Chemicals and
Plastics

Identification: Yellow liquid

CAS #: 106-87-6

BAKELITE® Cycloaliphatic Epoxy Resin ERL-4206

In Vitro Mutagenesis Studies: 3-Test Battery

Sponsor: Union Carbide Corporation

OBJECTIVE

The purpose of this study was to evaluate the potential of Epoxy Resin ERL-4206 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-4206 for potential mutagenic activity. A general description of the theoretical basis for each of these three tests is presented in Appendices I, II and III attached to this report.

SAMPLE CHARACTERISTICS

A typical, commercial sample of Epoxy Resin ERL-4206 was received from UCC, South Charleston, WV for testing on March 15, 1979. The 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 tests at the Bushy Run Research Center. Deviations from current Standard Operating Procedures and the appendices attached to this report are noted in the individual test results.

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

- A. Dose Selection - Epoxy Resin ERL-4206 was tested for cytotoxicity to CHO cells at six concentrations from 0.5% to $1 \times 10^{-3}\%$ (by volume) 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 dose levels which would permit survival of approximately 10% of the treated cells. Glass-distilled dimethylsulfoxide (DMSO) was used as the solvent and solvent control; sterile, distilled water (H_2O) was used as the negative control.

To simplify tables and to allow comparisons between different tests, concentrations of Epoxy Resin ERL-4206 in the following sections of the report are given in terms of volume percentages $\times 10^{-3}\%$ to eliminate zeros in the lower concentration values (eg. $0.000625\% = 0.625 \times 10^{-3}\%$).

- B. Mutation - CHO cells were exposed for 5 hours to a minimum of five concentrations of Epoxy Resin ERL-4206 with the addition of an S9 metabolic activation system and to an identical range of concentrations for 16 hours without S9 activation. Dilutions of Epoxy Resin ERL-4206 for testing were prepared in DMSO and various aliquots of the test agent were pipetted into the cell culture media. 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 by exposure to various concentrations of Epoxy Resin ERL-4206 was studied in CHO cells without the incorporation of an S9 metabolic activation system. Selection of dose levels 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-4206 for testing were prepared by direct addition into the culture medium of various aliquots of the test sample diluted in DMSO. For determination of direct mutagenic action, CHO cells were exposed to Epoxy Resin ERL-4206 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 responses obtained in the test without metabolic activation indicated metabolic conversion was not required to detect the action of the test agent. 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 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-4206 for 2 hours in culture medium containing ^3H -thymidine, hydroxyurea and appropriate dilutions of the test agent prepared in DMSO. Determination of UDS activity was performed by analyses of radioactive incorporation 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 4-nitroquinoline oxide (4-NQO) were used as positive controls representing 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 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 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, available analytical data and data used to prepare the final report are stored in the ERRC Archives. Slides are stored in the Genetic Toxicology slide storage area.

RESULTS

SECTION I - CHO MUTATION TEST - Epoxy Resin ERL-4206

- A. Test Dates - Initiated: May 11, 1979
Completed: April 28, 1980

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

CHO cells were exposed to six concentrations of Epoxy Resin ERL-4206 which spanned a concentration range from $500 \times 10^{-3}\%$ to $1 \times 10^{-3}\%$ by volume. The exposure period was for 5 hours with metabolic activation and for 16 hours without metabolic activation. The percentage of cells which survived the exposure, either in the presence or 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 $10 \times 10^{-3}\%$ was selected as the maximum concentration for testing with and without S9 activation; a higher concentration ($30 \times 10^{-3}\%$) allowed survival of fewer than 1% of the cells treated with the test agent either in the presence or absence of a metabolic activation system.

C. Determination of Mutation Induction**1. Survival (Cytotoxicity)**

Table 1 presents the cytotoxicity data for CHO cells treated with Epoxy Resin ERL-4206 in the presence or absence of a liver S9 metabolic activation system. A suggestive dose-related effect with the test agent was apparent for the cytotoxicity values observed for the tests both with or without S9 activation. Although the tests with S9 metabolic activation could have employed slightly higher concentrations, it is likely (based on the test without S9 and from the prescreening data for dose selection) that the highest concentration tested was near the upper limit which would allow sufficient cell survival. Cytotoxicity of Epoxy Resin ERL-4206 was greater in the absence of the S9 metabolic activation system than the response obtained for identical concentrations in the test with S9 activation. The greater toxicity in the absence of S9 was likely due to the longer period of exposure in the test without S9 activation in comparison to the test with S9 [16 hours (-S9) and 5 hours (+S9)]; metabolic inactivation or conversion of the test agent by the S9 homogenate is a possibility which can not be excluded with the present data.

2. Mutation

Table 2 presents the data for induction of mutants by Epoxy Resin ERL-4206 and control agents. No definitive evidence was obtained to indicate the presence of 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 either with or without the presence of an S9 metabolic activation system. However, a single, highly statistically significant increase in the frequency of mutants was produced by $10 \times 10^{-3}\%$ of Epoxy Resin ERL-4206, the highest concentration tested for direct activity without S9 activation. Also, there was a progressive increase in the numerical frequency of mutants produced by three of four of the test concentrations between $1.25 \times 10^{-3}\%$ and $10 \times 10^{-3}\%$. These data

suggested the existence of a dose-related effect of exposure which progressed to a statistically significant level of response only at the top dose level. This single response in the test without S9 complicated the interpretation of the results as clearly positive or negative. Further studies of the existence of a biologically significant effect would require testing at higher doses which would produce very high cytotoxicity levels and survival of substantially less than 10% of the exposed cell population ($10 \times 10^{-3}\%$ allowed only 8.5% cell survival as a percentage of the control). Data produced from testing of dose-levels which produce substantially more than 90% cell killing may also be equivocal unless a comprehensive study over a narrow, effective dose-range can verify the presence or absence of a response.

In consideration of the equivocal and weak responses obtained in this test, the data suggested that this chemical produced a positive mutagenic effect only at high dose-levels which were highly cytotoxic to the target cells. Epoxy Resin ERL-4206 was classified, conservatively, as a questionable-to-weakly active mutagenic agent in the test for direct mutagenic action in the CHO test system.

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. Highly statistically significant mutation frequencies obtained for the DMN and EMS positive controls for both experiments were within the normally expected range of values observed in historical control data and were indicative of an acceptable test.

- D. Deviations from Standard Procedures - Epoxy resin ERL-4206 was tested as a part of the in-house program for development and validation of the battery of mutagenicity assays at our laboratory. Although several minor deviations from the current procedures used for these tests in our laboratory (Appendix II) exist in the Methods section for this test, the single major deviation was the 16-hour exposure period for the test of direct action without S9, rather than 5 hour as stated in the Appendix. A 16-hour exposure was used in the early stages of development of this assay and exposure periods from 2 to 24 hours appear to be acceptable if proper dose ranges are selected for testing.

Unusual differences in the values for the plating efficiencies in single plates from the $5 \times 10^{-3}\%$ and $0.625 \times 10^{-3}\%$ (without S9 activation; Table 2) were not included in the calculation of average plating efficiency. The lack of growth in only one of the four petri dishes was considered atypical and its inclusion would have biased the results toward greater mutants/ 10^6 survivors after correction for plating efficiency. Inclusion of this bias would make the results even more highly significant, thus a conservative interpretation of the results was employed to indicate a questionable, but positive effect.

E. Conclusions

Epoxy Resin ERL-4206 produced a questionable-to-weak response as a mutagenic agent for CHO cells when tested for direct action without an S9 metabolic activation system. Statistically significant increases in the numerical frequency of mutants were obtained at only the highest

concentration of Epoxy Resin ERL-4206 tested without S9 activation. Although a similar positive result was not observed at any other doses, an evaluation of these data in comparison to historical experiences with this test indicated that the data should be considered as a suggestive, weak-positive effect. Other mutation values were not statistically significant from the concurrent solvent control and values were within the expected variation in mutant frequencies observed in historical control data.

SECTION II - SCE TEST - Epoxy Resin ERL-4206

- A. Test Dates - Initiated: January 3, 1980
Completed: March 24, 1980

B. Selection of Test Concentrations

A maximum concentration of $10 \times 10^{-3}\%$ 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. A 16-fold range of Epoxy Resin ERL-4206 concentrations from $10 \times 10^{-3}\%$ to $0.625 \times 10^{-3}\%$ by volume was tested. No experiments with S9 activation were performed because the highly statistically significant responses produced in the test for direct action of the test agent indicated that metabolic conversion was not required for expression of mutagenicity.

C. Determinations of SCE Induction

The data for SCE induction in CHO cells treated with various dose levels of Epoxy Resin ERL-4206 or with appropriate positive, negative or solvent control agents are summarized in Table 3. A highly statistically significant increase in the SCE frequency was produced by four of the five dose-levels of Epoxy Resin ERL-4206 tested for direct action in the absence of a metabolic activation system. Also, the production of SCE by Epoxy Resin ERL-4206 was related in a direct manner to the treatment dose, which is generally considered a definitive indication of mutagenic potential. The test without S9 activation was considered a significant indication of potential direct mutagenic action of the test agent and Epoxy Resin ERL-4206 was classified as a positive mutagenic agent in the SCE test.

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 H₂O solvent and DMSO 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 - None

E. Conclusions

Epoxy Resin ERL-4206 produced a significant increase in the frequency of SCE in CHO cells in tests without addition of an S9 metabolic activation system. Evidence of highly statistically significant and dose-related effects of Epoxy Resin ERL-4206 exposure on the SCE frequency indicated that the test agent was highly active in the present in vitro assay.

SECTION III - UDS TEST - Epoxy Resin ERL-4206

- A. Test Dates - Initiated: August 10, 1979
Completed: August 25, 1980

B. Selection of Test Concentrations

Standard procedures were followed and Epoxy Resin ERL-4206 was tested over a 3-log range of concentrations from $100 \times 10^{-3}\%$ to $0.1 \times 10^{-3}\%$ by volume. 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. These dose levels were considered to be appropriate for testing because uptake and incorporation of ^3H -thymidine into hepatocytes are generally even more sensitive to chemical effects than survival measurements with CHO cells (in which cytotoxicity is measured after a 24 hour recovery period following treatment).

C. Determination of UDS Induction

1. Nuclear-Bound Radioactive Label (Data in Table 4)

The positive control agents, NQO and DMN, induced numerically elevated and highly statistically significant increases in UDS over values obtained with the solvent control. With both positive controls an increase in the amount of UDS in relation to increasing concentration indicated the responsiveness of the test system with measurements using nuclei. However, this response was not linear and not all concentrations of the positive controls produced a response which was statistically significant from the concurrent solvent control. This finding may suggest that the present test with nuclei was less sensitive than necessary to detect weak mutagenic activity.

Induction of "unscheduled" incorporation of radioactive thymidine into nuclei of hepatocytes exposed to Epoxy Resin ERL-4206 or to appropriate positive and negative controls is presented in Table 4. In hepatocytes treated with Epoxy Resin ERL-4206, no concentration tested for potential activity induced a statistically significant increase in the amounts of ^3H -thymidine incorporation. A few numerically elevated increases in the amounts of radioactive incorporation, particularly for concentrations between $1 \times 10^{-3}\%$ to $10 \times 10^{-3}\%$, was a possible indication for a weak level of mutagenic action of the test agent. However, these responses were not statistically above the solvent control values and the test was considered a negative indication of mutagenic action.

2. DNA-Bound Radioactive Label (Data in Table 5)

Analyses of DNA, from aliquots of hepatocyte nuclei used for the UDS studies presented on Table 4, were performed as a second assessment of "unscheduled" incorporation of radioactive thymidine. Values for radioactivity incorporated into the DNA of these hepatocyte nuclei are presented in Table 5.

For hepatocytes treated with Epoxy Resin ERL-4206, none of the test concentrations induced levels of UDS which were statistically significant from the solvent control. More critically, we did not observe a dose-related increase in the levels of UDS; a result consistent with the pattern of negative responses obtained in the assessment of nuclei from cells treated with the same range of concentrations. Moderate numerical increases in the levels of UDS at the $3 \times 10^{-3}\%$ and $10 \times 10^{-3}\%$ dose levels suggested the possibility of a positive response, but these values were below the levels necessary to demonstrate statistical significance. These several considerations were consistent in the classification of Epoxy Resin ERL-4206 as an inactive agent in the induction of DNA damage discernable by UDS activity.

For the positive control agents NQO and DMN, relatively large numerical increases above the concurrent solvent control were produced by the highest concentration of both agents. However, the lowest concentration of NQO and two of three concentrations of DMN did not produce a sufficient response for statistical significance from the solvent control. This result indicated the responsiveness of the test system but suggested that the level of sensitivity of this particular test could be less than necessary to detect weak mutagenic agents.

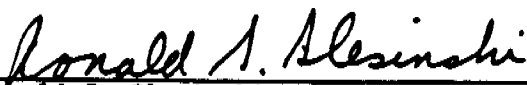
- D. Deviations from Standard Procedures - Testing of Epoxy Resin ERL-4206 was performed as part of the in-house development and validation of the mutagenicity test battery. One previous experiment using an older, less-sensitive method was performed but was not reported because the level of response with the positive controls was not acceptable for a valid test.

E. Conclusion

Epoxy Resin ERL-4206 failed to stimulate significantly the incorporation of radioactive thymidine in cells treated over a 1000-fold range of test concentrations. Although values for the positive controls were low, in the assessment of UDS with precipitated DNA and nuclei, the highest concentrations demonstrated the activity of the positive control agents. Epoxy Resin ERL-4206 was considered inactive in the tests with hepatocytes.

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REFERENCES

A previous study on 30% Epoxy Resin ERL-4206 in acetone (formerly termed UNOX® Epoxide 206) showed that this material was carcinogenic and tumorigenic following lifetime dermal exposures of mice (CHF Report No. 27-152). No other references on Epoxy Resin ERL-4206 were found in a search of the Environmental Mutagen Information Center data file.

Table 1
Chinese Hamster Ovary (CHO) Mutation Assay:
Determination of Toxic Effects of Chemical Treatment

Test Chemicals	Total # Colonies	Total # Cells Plated	% Survival	% of Solvent Control
Without S9 Activation-16 hr exposure				
[Epoxy Resin (ERL-4206)] (X, v/v)				
10.0 x 10 ⁻³	29	800	3.6	8.5
5.0 x 10 ⁻³	147	800	18.4	43.2
2.5 x 10 ⁻³	400	800	50.0	117.6
1.25 x 10 ⁻³	410	800	51.2	120.6
0.625 x 10 ⁻³	346	800	43.2	101.8
Controls				
DMSO (20 ul/ml) - Solvent	340	800	42.5	-
H ₂ O (20 ul/ml) -	333	800	41.6	97.9
EMS (200 ug/ml) -	137	800	17.1	40.3
With S9 Activation-5 hr exposure				
[Epoxy Resin (ERL-4206)] (X, v/v)				
10.0 x 10 ⁻³	301	800	37.6	55.8
5.0 x 10 ⁻³	424	800	53.0	78.7
2.5 x 10 ⁻³	501	800	62.6	92.9
1.25 x 10 ⁻³	509	800	63.6	94.4
0.625 x 10 ⁻³	483	800	60.4	89.6
Controls				
DMSO (20 ul/ml) - Solvent	539	800	67.4	-
H ₂ O (20 ul/ml) -	528	800	66.0	98.0
DMN (3700 ug/ml) -	423	800	52.9	78.5

Abbreviations: H₂O - 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-4206

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-4206)] (X, v/v)						
10.0 x 10 ⁻³	144	400	0.360	14	1 x 10 ⁶	38.9 b
5.0 x 10 ⁻³	73	200	0.365	5	1 x 10 ⁶	13.7
2.5 x 10 ⁻³	136	400	0.408	2	1 x 10 ⁶	4.9
1.25 x 10 ⁻³	210	400	0.525	0	1 x 10 ⁶	0
0.625 x 10 ⁻³	153	300	0.510	12	1 x 10 ⁶	23.5
Controls:						
DMSO (20 ul/ml) - Solvent	238	400	0.595	0	1 x 10 ⁶	0
H ₂ O (20 ul/ml) -	225	400	0.562	1	1 x 10 ⁶	1.8
EMS (200 ug/ml) -	187	400	0.468	92	1 x 10 ⁶	196.8 c
With S9 Activation						
[Epoxy Resin (ERL-4206)] (X, v/v)						
10.0 x 10 ⁻³	358	400	0.895	6	1 x 10 ⁶	6.7
5.0 x 10 ⁻³	405	400	1.012	0	1 x 10 ⁶	0
2.5 x 10 ⁻³	378	400	0.945	0	1 x 10 ⁶	0
1.25 x 10 ⁻³	296	400	0.740	1	1 x 10 ⁶	1.4
0.625 x 10 ⁻³	296	400	0.740	1	1 x 10 ⁶	1.4
Controls:						
DMSO (20 ul/ml) - Solvent	314	400	0.785	2	1 x 10 ⁶	2.5
H ₂ O (20 ul/ml) -	272	300	0.907	0	1 x 10 ⁶	0
DMN (3700 ug/ml) -	299	400	0.748	39	1 x 10 ⁶	52.2 c
¹ Total # mutant colonies per 10 ⁶ cells plated divided by viable fraction. Statistical significance above solvent control: b: 0.01 > p > 0.001; c: p < 0.001. No superscript indicates p > 0.05. Data analyzed by Student's t-test.						

Abbreviations: H₂O - water; S-9 - liver homogenate; DMSO - dimethylsulfoxide; EMS - ethylmethanesulfonate; DMN - dimethylnitrosamine.

Table 3
Sister Chromatid Exchange (SCE) Assay:
Induction of SCE's by Epoxy Resin (ERL-4206) Without S9 Metabolic Activation
5-Hour Treatment

Test Chemicals	Total # of Chromosomes	Total # of SCE	SCE/Cell ¹	Mean Number SCE/Chromo- some ² + S.D.	Significance Above Solvent Contr ³
[Epoxy Resin (ERL-4206)] (X, v/v)					
10.0 x 10 ⁻³	299	833	55.53	2.786 + 0.633	c
5.0 x 10 ⁻³	289	639	42.60	2.206 + 0.492	c
2.5 x 10 ⁻³	294	387	25.80	1.329 + 0.418	c
1.25 x 10 ⁻³	300	293	19.53	0.975 + 0.283	b
0.625 x 10 ⁻³	294	227	15.13	0.768 + 0.207	NS
Contr 1s					
DMSO (5 ul/ml) - Solvent	288	190	12.67	0.665 + 0.212	-
H ₂ O (5 ul/ml) -	296	143	9.53	0.485 + 0.186	NS
EMS (100 ug/ml) -	292	395	26.33	1.347 + 0.284	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: H₂O - water; S9 - liver homogenate; DMSO - dimethylsulfoxide;
EMS - ethylmethanesulfonate; S.D. - standard deviation

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Table 4
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 $\pm$ S.D.	% of Solvent Control $\pm$ S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2%	2070 $\pm$ 256	100.0% $\pm$ 12.3%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	19743 $\pm$ 1572	953.8% $\pm$ 75.9%	c
	1.0 ug/ml	2951 $\pm$ 430	142.6% $\pm$ 20.8%	NS
	0.3 ug/ml	3704 $\pm$ 791	179.0% $\pm$ 38.2%	a
DMN	1000 ug/ml	9079 $\pm$ 1341	438.7% $\pm$ 64.8%	c
	300 ug/ml	2397 $\pm$ 311	115.8% $\pm$ 15.0%	NS
	100 ug/ml	2297 $\pm$ 162	111.0% $\pm$ 7.8%	NS
Test Chemical:				
[Epoxy Resin ERL-4206], (% v/v)	100 x $10^{-3}\%$	1670 $\pm$ 338	80.7% $\pm$ 16.3%	NS
	30 x $10^{-3}\%$	2230 $\pm$ 98	107.7% $\pm$ 4.7%	NS
	10 x $10^{-3}\%$	3004 $\pm$ 203	145.1% $\pm$ 9.8%	NS
	3 x $10^{-3}\%$	2579 $\pm$ 61	124.6% $\pm$ 2.9%	NS
	1 x $10^{-3}\%$	2688 $\pm$ 225	129.9% $\pm$ 10.9%	NS
	0.1 x $10^{-3}\%$	2135 $\pm$ 94	103.2% $\pm$ 4.5%	NS

¹Statistical significance above solvent control: a: $0.05 > p > 0.01$; c: $p < 0.01$; 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

Table 5
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 $\pm$ S.D.	% of Solvent Control $\pm$ S.D.	Significance Above Solvent Control ¹
Solvent - DMSO	2%	1487 $\pm$ 152	100.0% $\pm$ 10.2%	-
Positive Controls:				
4 - NQO	3.0 ug/ml	19886 $\pm$ 1510	1337.0% $\pm$ 101.5%	c
	1.0 ug/ml	8929 $\pm$ 1076	600.4% $\pm$ 72.3%	c
	0.3 ug/ml	2264 $\pm$ 337	152.2% $\pm$ 22.6%	NS
DMN	1000 ug/ml	2908 $\pm$ 783	195.5% $\pm$ 52.6%	a
	300 ug/ml	1770 $\pm$ 366	119.0% $\pm$ 24.6%	NS
	100 ug/ml	1897 $\pm$ 79	127.5% $\pm$ 5.3%	NS
Test Chemical:				
[Epoxy Resin ERL-4206] (% v/v)	100 x $10^{-3}\%$	1241 $\pm$ 373	83.4% $\pm$ 25.1%	NS
	30 x $10^{-3}\%$	1568 $\pm$ 72	105.4% $\pm$ 4.8%	NS
	10 x $10^{-3}\%$	2049 $\pm$ 155	137.7% $\pm$ 10.5%	NS
	3 x $10^{-3}\%$	1994 $\pm$ 270	134.1% $\pm$ 18.2%	NS
	1 x $10^{-3}\%$	1735 $\pm$ 128	116.7% $\pm$ 8.6%	NS
	0.1 x $10^{-3}\%$	1402 $\pm$ 438	94.2% $\pm$ 29.4%	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; 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 AssayTheoretical 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 dimethyl-sulfoxide (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 $3 \times 10^{-4}\%$ (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 ($37^\circ C$) 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, 5×10^5 cells are inoculated 20 to 24 hours prior to treatment into 25 cm² culture flasks containing F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ 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 for 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 5×10^5 cells are subcultured in 100 mm tissue culture dishes in F12-D5 medium and incubated at 37°C in a 5 to 6% CO₂ 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 2.5×10^5 /dish in four culture dishes (1×10^6 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 VitroTheoretical 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 suspected 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 3.0 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 Hsieh 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.

Appendix II

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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 $3 \times 10^{-4}\%$ (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 7- to 8-day incubation period ($37^\circ C$) 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^{-6} to 3×10^{-5} 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 10⁶ cells are plated into 75 cm² culture flasks in F12-D5 medium at least 20 hrs prior to treatment and incubated at 37°C in a 5 to 6% CO₂ 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 3 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 3 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 LiverTheoretical 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 Metofane(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 $\times 10^{-3}\%$ (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 Hapatocytes: 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. Dimethylsulfoxide (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 1.25×10^5 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/10⁶ 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.

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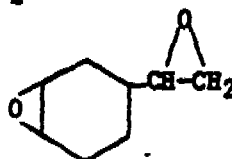
Appendix IV

Physical and Chemical Characteristics of Test Material

CHF Sample No: 42-137
CAS No.: 106-87-6
Chemical Name: 3-(epoxyethyl)-7-oxabicyclo(4.1.0)heptane

Trade Name and/or Synonyms: Bakelite® Cycloaliphatic Epoxy Resin
ERL-4206; vinyl-3-cyclohexene diepoxide;
Epoxyethyl-3, 4-epoxy cyclohexane
Molecular Weight: 140.18
Formula: $C_8H_{12}O_2$

Molecular
Structure:



Specific Gravity (@ 20°C): 1.0986
Boiling Point: 227°C @ 760mm Hg
Solubility in H₂O (% by wt): 18.3 @ 20°C
Purity: Approx. 100%

Vapor Pressure (@ 20°C): <0.1mm Hg
pH: Not Available
Flash Point: 225°F (closed cup)
Stability: Unstable, avoid temperatures > 100°F
Incompatibility: Avoid amines, acids, alkalies
Appearance and Odor: Low viscosity liquid; characteristic odor
Disposal: Small spills can be flushed with water
Larger quantities can be diluted with
an inert solvent and burned.

Protective Measures: Use rubber gloves, goggles and protective
creams on arms and face. An eye bath and safety
shower should be available.

A local exhaust ventilation system is preferable
to reduce exposure. In case of contact flush
eyes or skin with plenty of water.

Health Hazard: Prolonged and repeated contact with liquid or
breathing of vapors or mists may cause delayed
and serious injury. Absorption through skin is
harmful and the liquid causes eye injury. Skin
painting tests in animals caused skin cancers,
but no cancers have been reported in humans.