National Toxicology Program
U.S. Department of Health and Human Services

FINAL REPORT ON
THE CUMENE (CASRN 98-82-8) GENOTOXICITY STUDIES

[Studies were conducted under NTP Contract N01-ES-34415 at ILS, Inc.]

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National Institute of Environmental Health Sciences
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Abbreviations

PCE, polychromatic erythrocytes
NCE, normochromatic erythrocytes
RET, reticulocytes
MN, micronucleus, micronuclei
MN-PCE, micronucleated polychromatic erythrocytes
MN-NCE, micronucleated normochromatic erythrocytes
MN-RET, micronucleated reticulocytes
DMSO, dimethylsulfoxide

Definitions

PCE, these are reticulocytes identified on stained slides by presence of residual RNA in the cytoplasm.

NCE, these are mature erythrocytes identified on stained slides by absence of residual RNA in the cytoplasm.

RET, these are immature erythrocytes formed in the bone marrow; they mature into erythrocytes within approximately 48 hr. Flow cytometry identifies RET by the presence of an active transferrin (CD71) cell surface receptor. Erythrocytes do not have an active transferrin receptor.

MN, small DNA-containing bodies that resemble a nucleus in all characteristics except for size; they are formed from whole chromosomes or chromosomal fragments that fail to incorporate into daughter nuclei following mitosis. MN are a biomarker of chromosomal damage (numerical or structural).

I. Cumene Chemical Characterization

In June 2012, a procured sample of cumene was sent to the NTP's chemistry contract laboratory for a preliminary one-week characterization to confirm compound identity and purity (>99%, with no impurities over 0.1% observed in 2 chromatographic systems); full characterization was conducted simultaneously with the genetic toxicity testing conducted at ILS, Inc (July 16 – 26, 2012). Results of full characterization (NTP Chemistry Report, Chem 11855, October 20, 2012) confirmed that the current sample of cumene was chemically identical to the one used in the NTP 2-year bioassay in F344 rats and B6C3F1 mice (NTP Technical Report 542).

Cumene, a clear liquid, was tested at the genetic toxicity testing contract laboratory (ILS, Inc.) under code, identified to ILS staff only as Compound Z. The identity of the sample was known only to the dose formulation scientist at ILS. Cumene was stored at room temperature, protected from light.

II. Rationale for Conducting These Genetic Toxicology Investigations with Cumene

These studies were conducted to obtain additional information and clarification regarding the *in vivo* genotoxicity of cumene to help in interpretation of the sporadic positive responses that were reported in the literature for some genetic toxicity assays. In addition, cumene was tested for mutagenicity in bacteria ("Ames Assay") because, although cumene had been tested extensively for bacterial mutagenicity previously, and the weight of evidence indicated that cumene was not mutagenic in bacteria, there were concerns as to whether exposures had been adequate, due to the reported volatility of cumene.

The studies that were conducted to clarify the genotoxicity potential of cumene included:

- Bacterial mutagenicity assay
- Short-term peripheral blood erythrocyte micronucleus (MN) assay in mice and rats
- Short-term Comet assay (single cell gel electrophoresis) for assessment of DNA damage in presumptive target tissues, based on results in the NTP 2-year bioassay in rats and mice

III. Overall Summary of the Studies

Cumene was tested in the bacterial reverse mutation assay using *Salmonella typhimurium* strains TA98 and TA100 and *Escherichia coli* strain WP2 *uvrA* (pKM101) in the pre-incubation method with and without metabolic activation (10% phenobarbital/benzoflavone-induced rat liver S9). This is the standard protocol employed by NTP for bacterial mutagenicity assessment, and these three strains of bacteria permit detection of the vast majority of bacterial mutagens, including chemicals that induce base substitutions, frame-shifts, and oxidative damage (NTP unpublished data). Because volatility was a concern in many of the earlier bacterial mutagenicity studies reported in the literature, pre-incubation was conducted in sealed tubes

to maximize exposure of the bacteria. In dose range finding tests, toxicity was observed at 250 or 500 µg/plate; therefore, the highest dose tested was 250 or 500 µg/plate. The observation of toxicity at 250 or 500 µg/plate confirmed that exposure to cumene had occurred. Under these test conditions, cumene did not induce increases in revertant counts up to dose levels that induced toxicity.

For the *in vivo* studies, male Fisher 344 rats and male and female B6C3F1 mice (6 animals/dose group) were administered vehicle (corn oil), cumene, or the positive control chemical ethyl methanesulfonate (EMS; CAS No. 62-50-0) by gavage, once daily for four consecutive days. Although cumene was tested previously by the NTP in a 2-year rodent bioassay using inhalation as the route of exposure, NTP ADME studies had demonstrated that oral administration of cumene resulted in systemic exposure (NTP Technical Report 542, 2009), and for practical reasons, oral gavage was therefore selected for the short-term genotoxicity studies conducted with cumene. The top doses of cumene used in these studies (800 mg/kg/day for male rats; 1250 and 1000 mg/kg/day for male and female mice, respectively) were selected on the basis of the results of dose setting studies. The final (4^{th}) dose was administered 21 hr following the previous dose; 3 hr later, peripheral blood and liver, lung, and kidney tissues were collected from each animal. Blood samples were prepared for MN analysis, while blood and the tissue samples were processed for the Comet assay. Frequencies of micronucleated polychromatic erythrocytes (MN-PCE; micronucleated reticulocytes (MN-RET)) were measured using flow cytometry. Cells from the kidney, liver, and lung, as well as blood leukocytes, were analyzed for extent of DNA migration (DNA damage) using the alkaline (pH>13) Comet assay. MN and Comet assay data were evaluated by the NTP. Results of the MN tests for cumene in both rats and mice were negative. Results of the Comet assay for all tissues in all species/sexes were negative with two exceptions: results in female mouse lung and male rat liver were judged to be positive based on the presence of a significant trend and a significant increase in DNA damage at the highest dose tested in both tissues, compared with the concurrent vehicle control group.

Based upon the findings from these studies, cumene did not show evidence of bacterial mutagenicity or evidence of chromosomal breakage in proerythrocytes of male and female mice, and male rats. Significant, dose-related increases in DNA damage were observed in male rat liver cells and female mouse lung cells; none of the other tissues sampled in mice and rats showed evidence of DNA damage.

IV. Experimental Design for the Bacterial Mutagenicity Assay

Although there have been numerous bacterial mutagenicity studies reported previously with cumene, there was still some question about the validity of those results, due to the reported volatility of cumene and the potential for having achieved inadequate exposure as a consequence. Therefore, NTP evaluated this well-characterized sample of cumene for bacterial mutagenicity to confirm earlier results.

Cumene was tested as a coded sample in the bacterial reverse mutation assay, using procedures described by Zeiger et al. (1992) with slight modification. A range finder study and two independent mutagenicity assays were conducted. DMSO was used as the solvent. Cumene was incubated with *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvrA* (pKM101) either in buffer or 10% S9 mix (metabolic activation enzymes and cofactors from phenobarbital/benzoflavone-induced male Sprague Dawley rat liver S9). The test compound was serially diluted to produce the range of test doses. Six dose levels were tested in each strain without and with S9. Because of the potential volatility of the compound, dosing solutions were prepared in amber vials that had been flushed with N_2 and capped with lids with septa. Dilutions were made using a Hamilton syringe. Due to observed toxicity in the range finder, nominal doses for the mutagenicity studies ranged from 2.56-500 µg/plate. Direct-acting positive controls were sodium azide (TA100), 2-nitrofluorene (TA98), and 4-nitroquinoline-N-oxide (*E. coli*); indirect-acting positive controls were benzo[a]pyrene (TA100) and 2-aminoanthracene (TA98 and *E. coli*). Exposures were performed in triplicate (mutagenicity studies) and pre-incubated in capped tubes at 37±1°C for 20±1 min. Following pre-incubation, the appropriate agar (histidine/biotin agar for *Salmonella* strains; tryptophan agar for *E. coli*) was added and the mixture poured onto the surface of a minimal glucose agar plate. Plates were incubated at 37±1°C for 48±2 hr. Revertants were counted using the Sorcerer/Ames Study Manager system (Perceptive Instruments, Suffolk, UK), or manually when toxicity interfered with the automatic counts.

The test compound was fully soluble in DMSO at the concentrations tested. No precipitate or color change was observed in the pre-incubation tubes. Indications of toxicity on the test plates included the presence of microcolonies and/or an enhanced lawn and reduced (or 0) revertant counts. Positive and vehicle controls gave the expected responses.

All data were entered into the NTP electronic database for evaluation by NTP staff.

A. Data Evaluation

In this assay, NTP defines a positive response as a reproducible, dose-related increase in histidine- or tryptophan-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose-related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold-increase required for a chemical to be judged positive or weakly positive.

V. Experimental Design for the *In Vivo* Comet and Micronucleus Studies

A. Animals

Male Fischer 344 rats (F344/DuCrl) purchased from Charles River Laboratories, Inc., and male and female B6C3F1 mice purchased from Taconic Farms, Inc. were used in these studies. Each

animal was uniquely identified by an ear punch prior to the start of the study. Rats were 7 weeks of age and mice were 5-6 weeks of age at the time of purchase. Animals were acclimated for at least one week prior to being placed on study. During acclimation, the animals were examined by the facility veterinarian or a designated alternate to assess their health status. At the end of the acclimation period, all animals were re-examined and released from acclimation if healthy; only healthy animals were used in the study. Rats were ~8 weeks of age and mice were ~9-10 weeks at the time they were placed on study.

Male rats and female mice were housed two per cage, and male mice one per cage, in polycarbonate cages with micro-isolator lids and absorbent hardwood bedding (Betachip, Northeastern Products Corp., Warrensbury, NY, USA), in a temperature (20-25°C) and humidity (30-70%) controlled animal room with a 12 hr light/12 hr dark cycle. Animals were transferred to clean cages once weekly. The animals were provided Purina Certified Rodent Chow 5002 (Ralston Purina, St. Louis, MO, USA) *ad libitum*. Feed was analyzed by the producer for nutrients and contaminants (analysis included in complete laboratory report and available upon request). Reverse osmosis treated tap water was provided *ad libitum* using plastic water bottles with stainless steel sipper tubes. Fresh water was supplied twice weekly. Annual analyses of water quality conducted at the source by the City of Durham and at the tap are included in the complete laboratory report and available upon request. There were no known contaminants in the food or water that would be expected to interfere with the conduct of the study.

All animals were observed for morbidity and mortality twice daily during normal workdays. On weekends and holidays, the animals were observed once daily.

Animals were stratified by body weight (BW) and assigned to a dose group such that mean animal weights across dose groups were approximately equal and all animals weighed within ±20% of the weight range (over a 20-30 gm and 150-320 gm weight range for mice and rats, respectively).

B. Preparation of the Dosing Formulation

Corn oil and 0.9% saline were used as the vehicles for cumene and the positive control chemical, EMS, respectively. Dosing occurred within 2 hr of dose formulation. Dose solutions of cumene were prepared daily using sterilized amber vials with septa that had been flushed with N_2. For each concentration prepared, corn oil was weighed into the vial, which was then sealed with a cap and septum. The appropriate volume of cumene was measured using a Hamilton syringe and introduced into the vial through the septum. The mixture was vortexed until a homogeneous solution was obtained.

Cumene and the positive control chemical, EMS, were administered at a dosing volume of 10 mL/kg by oral gavage using a steel gavage needle; doses were administered daily for four consecutive days (24, 48, and 69 hr ±15 min from initial dose). Dosing formulations were stirred for at least 10 min prior to use.

The oral LD50 in mice was reported to be >12,000 mg/kg (Cavender, 1994). Because the LD50 data were considered questionable (much lower lethal doses were reported in ICR BR Swiss mice in an unpublished industry study conducted in 1985), a range finding study using 2 male and 2 female mice per treatment group was designed to identify the dose range to be tested. Doses in the range finding study were 1500, 1250, and 1000 mg/kg/day, administered in corn oil by gavage once daily for 4 days (at times 0, 24, 48, 69 hr), with sacrifice 3 hr after the final dosing (at the 72 hr time point). Any mouse showing clinical signs associated with toxicity or distress was immediately euthanized. Male mice dosed with 1000 and 1250 mg/kg/day cumene appeared normal, but males administered 1500 mg/kg/day cumene were lethargic and had uncoordinated movement at the 4 hr post-dosing check on Days 1 – 4; one of these males was moribund on Day 4 and was euthanized. One female mouse dosed with 1500 mg/kg/day cumene exhibited uncoordinated movement 4 hr after dosing on Days 1-4 but recovered; the second mouse in this group exhibited uncoordinated movement 4 hr after the initial dose and recovered; this mouse was comatose 1 hr following dose administration on Day 2 and was euthanized. Female mice dosed with 1000 mg/kg/day cumene appeared generally normal; however, both animals dosed with 1250 mg/kg/day were observed to have uncoordinated movement on Day 4 at the time of euthanization. Based on the clinical observations in the range finding study, the top dose was set at 1250 mg/kg/day for male mice and 1000 mg/kg/day in female mice, which was consistent with the doses used in the unpublished industry study that assessed genotoxicity of cumene in Swiss mice. All clinical observations are reported in the contract laboratory study report submitted to NTP and are available by request.

In rats, the oral LD50 was reported to be 1400 mg/kg (Wolf et al., 1956). To verify the data in the original report, a range finding study was conducted in male rats, 2 rats per treatment, at 1600, 1200, and 800 mg/kg/day. Any rat showing clinical signs associated with toxicity or distress was immediately euthanized. Both rats in the 1600 mg/kg dose group were euthanized prior to the second treatment on Day 2, and both rats in the 1200 mg/kg group were euthanized approximately 4 hr after the second treatment on Day 2. All rats in the 800 mg/kg group appeared normal for the duration of the range finding study. Based on the clinical observations in the range finding study, the top dose in the definitive study was set at 800 mg/kg/day for male rats. All clinical observations are reported in the contract laboratory study report submitted to NTP.

In the MN/Comet studies, the dose levels of cumene tested were 0, 200, 400, and 800 mg/kg/day in rats (6 rats per treatment group); 0, 312, 625, and 1250 mg/kg/day in male mice (6 per group); and 0, 250, 500, and 1000 mg/kg/day in female mice (6 per group). EMS, the positive control chemical, was administered at 200 mg/kg/day in rats and at 150 mg/kg/day in male and female mice (6 rats and 5 mice per EMS group). Animals were dosed 24, 48, and 69 hr (±15 min) after the initial dosing. Animals were euthanized 3 hr (±15 min) following the 4^{th} administration of the test chemical or positive control.

The dosing schedule permitted evaluation of levels of DNA damage and MN frequencies 24 and 48 hr after dosing, and for DNA damage alone, also after 3-4 hr of exposure (Recio et al., 2010).

C. Collection of Tissues and Cell Samples for the Micronucleus and Comet Assays

Three hr after the final administration of cumene, blood was collected for MN analysis and blood and tissue samples (kidney, lung, liver) were collected for the Comet assay. Blood was collected from the caudal vena cava by heparinized syringe. An aliquot of blood was immediately dispensed into MicroFlow^{PLUS} Kit Anticoagulant/Diluent (heparin) and maintained at room temperature until methanol fixation within 2 hr from the time of collection. The fixed blood samples were stored at -80±10°C for a minimum of 3 days prior to processing for flow cytometry analysis. Additional aliquots of blood were placed into duplicate microcentrifuge tubes containing cold mincing buffer (Mg^{+2}, Ca^{+2}, and phenol red-free Hanks Balanced Salt Solution containing 20 mM EDTA, pH 7.4-7.7, with 10% v/v DMSO added fresh). Lung, liver, and kidney tissues were harvested, rinsed sufficiently with cold mincing buffer to remove residual blood, and then stored on ice until processed (within 3 min of harvesting). The left lobe of the liver was cut longitudinally into two sections; 2-3 mm sections were trimmed from one of these sections. The left lung was cut transversely approximately 2/3 distal to the left bronchus to include tertiary bronchus, bronchioles, and alveoli. The left kidney was trimmed to include a section containing the renal medulla and pelvis near the transition to the medulla. Duplicate single cell suspensions were prepared by mincing the tissues rapidly in cold mincing solution in microcentrifuge tubes and then immediately flash freezing the samples in liquid nitrogen (Recio et al., 2012). For kidney samples, due to limited experience with freezing this tissue, one set of Comet slides was prepared using fresh tissue within one hr following animal sacrifice; frozen samples of kidney were also prepared. All other tubes containing blood and tissue samples were stored in a -80°C freezer until processed later in the Comet assay (Recio et al., 2012). Additional sections of all tissues were fixed in 10% neutral buffered formalin for 18-24 hr and then transferred to 70% histology grade alcohol. The fixed tissues were trimmed, histologically processed, and embedded in paraffin within 5 days for possible histopathology evaluation.

Rationale for species/sex/tissue selection:

- In the NTP bioassay, lung was the site of tumors in male mice, lung and liver the site of tumors in female mice, and kidney the site of tumors in male rats
- Female rats were not included in this study because the only tumor site in the NTP bioassay was nasal epithelium and NTP does not have experience evaluating DNA damage in this tissue
- Blood cells are routinely evaluated for DNA damage in all NTP comet assays

D. Processing of Comet Samples

Thawed cell samples for the Comet assay were kept cold during processing. A portion of the cell suspension was empirically diluted with a fresh aliquot of 0.5% NuSieve GTG low melting point agarose dissolved in Dulbecco's phosphate buffer (Ca^{+2}, Mg^{+2}, and phenol free) at 37-45°C, and layered onto commercially available CometSlides™ (Trevigen, Gaithersburg, MD, USA). The volume of the cell suspension did not decrease the percentage of low melting point agarose by more than 10%. Application of 0.5 % (w/v) low melting point agarose provided a

top layer over the gel-embedded cells. To ensure proper slide quality, slides were prepared in a laboratory with a relative humidity of ≤ 60%. Two slides were made per sample and immersed in chilled (*i.e*. refrigerated at <10°C for ≥30 min) lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton X-100 added fresh) overnight in a refrigerator under a light proof condition. After this incubation period, the slides were rinsed in neutralization solution (0.4 M Trizma base, pH 7.5) to remove residual detergent and salts prior to the alkali-unwinding step. Slides were randomly placed onto the platform of a submarine-type electrophoresis unit and cold (<10°C) electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA; pH>13 as measured just prior to use) was added. The slides were left to unwind at <10°C for 20 min, then electrophoresed at <10°C for 20 min at 25 V (0.7 V/cm), with a current of approximately 300 mA. After electrophoresis, slides were neutralized with 0.4 M Trizma base (pH 7.5) for ≥5 min and then dehydrated by immersion in absolute ethanol (≥99.6%) for ≥5 min and allowed to air dry. Dried slides were stored at room temperature in a desiccator at ≤60% relative humidity until stained and scored; after scoring, stained slides were stored desiccated.

E. Comet Assay Slide Scoring

After staining slides with SYBR® Gold (Invitrogen, Grand Island, NY, USA), 100 cells were scored per sample (50 cells/well of a Trevigen slide) using the Comet IV Image Analysis System (Perceptive Systems, Suffolk, UK). Coded slides were scored without knowledge of their identity. Cells were classified into three categories: scorable, non-scorable, and "hedgehog." Any cells containing ≥90% DNA in the tail were excluded from the analysis. A variety of measurements were captured in the raw data for each scorable cell. 100 scorable cells were evaluated per tissue per animal and % tail DNA (tail fluorescence intensity) was the endpoint upon which the statistical analyses were run. Cells fitting the "hedgehog" criteria were tabulated, but not scored.

Definitions of Comet assay measurements

% Tail DNA:	Intensity of all tail pixels divided by the total intensity of all pixels in the comet, expressed as a percentage.
Hedgehog:	A comet that has a small or no visible head (appears that all of the DNA has migrated), or a comet in which the head and the tail appear separate.

Raw data for the 100 cells scored in fresh kidney and frozen blood, liver, and lung samples for each study animal were provided to Dr. Grace Kissling of the NTP Biostatistics Branch for statistical evaluation.

F. Statistical Methods Used in the Evaluation of Comet Assay Data

The % tail DNA values in each dose group were normally distributed with equal variances, as determined by Levene's test. Linear regression analysis was used to test for linear trend and pairwise differences with the control group were evaluated using Williams' test, after linearizing the data by averaging data points that violated a linear trend. In cases where variances are shown to be unequal, nonparametric methods are used to analyze the data: Jonckheere's test is used to evaluate linear trend and Dunn's test is used to assess the significance of pairwise differences with the control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences from the control group are declared statistically significant if $P \leq 0.025$. The NTP is conservative in its evaluation of Comet assay data and thus uses more stringent P-values than some other organizations.

For a positive call in one cell type and one sex/species, NTP requires both a significant trend and at least one significant dose group. The presence of only one statistically significant indicator of response (either a significant trend or a significantly elevated dose group) results in an equivocal call. In the absence of both a significant trend and a significant dose group, the data set is judged to be negative (i.e., no increase in DNA damage due to chemical exposure).

G. Evaluation of MN in Peripheral Blood of Rats and Mice

A detailed discussion of this assay in mice and in rats is presented by Hayashi et al. (2007), MacGregor et al. (2006), Torous et al. (2005), and Witt et al. (2008). Peripheral blood samples (60 – 120 μL) were obtained from the caudal vena cava from male and female B6C3F1 mice and male F344 rats. Samples were immediately refrigerated until fixation, which was carried out within 4 hr of collection time. Samples were fixed in ultracold methanol (MicroFlow® Basic Kits, Litron Laboratories, Rochester, NY, USA; Dertinger et al., 2004) and stored in a -80° freezer until analysis. Flow cytometric analysis was conducted using a benchtop FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Reticulocytes (RET; PCE) were identified by the presence of an active transferrin receptor (CD71-positive) on the cell surface; mature erythrocytes (normochromatic erythrocytes; NCE) were identified as CD71-negative. For the male rat blood samples, the analysis focused on the youngest RET (i.e., the subpopulation of reticulocytes with the highest CD71 expression), which is the population of RET least altered by the efficient action of the rat spleen in sequestering and destroying micronucleated red blood cells (MacGregor et al., 2006). Using flow cytometry, MN are detected using the DNA staining dye propidium iodide (PI) in conjunction with RNase treatment. Therefore, MN-RET express high levels of CD71 and PI-associated fluorescence, while MN-erythrocytes are negative for CD71 and show PI-associated fluorescence. In mice, both MN-RET and MN-erythrocytes were evaluated, although in a short-term study such as this one, the appropriate population for evaluating MN frequency associated with chemical treatment is the RET population. Damaged erythrocytes do not reach peak levels in the erythrocyte population in mice until around 28 days of repeated daily dosing.

For each peripheral blood sample, 20,000 (±2000) CD71-positive RET were analyzed by flow cytometry to determine the frequency of MN-RET. Cell aggregates were excluded on the basis of forward and side scatter, platelets based on staining with an anti-CD61 antibody, and nucleated leukocytes on the basis of intense propidium iodide staining. More than 10^6 mature erythrocytes (NCE) were enumerated concurrently during MN-RET analysis, allowing for calculation of the percentage of RET (%RET) among total erythrocytes as a measure of bone marrow toxicity.

All data from the flow cytometric evaluation of MN frequencies in peripheral blood RET and erythrocytes were entered directly into the NTP electronic database for statistical evaluation.

H. Data analysis for peripheral blood micronucleus test

In this assay, the animal is the experimental unit and approximately 20,000 RET and/or 1 x 10^6 erythrocytes are evaluated per animal for presence of micronuclei. In addition, the %RET was determined in approximately 1 x 10^6 erythrocytes. The optimum number of cells to score for MN using flow cytometric approaches was determined in earlier studies (Kissling et al., 2007). Data from each treatment group are summarized as the mean frequency of MN-RET per 1000 RET, plus or minus the standard error of the mean. With the large number of cells counted by flow cytometry, it is assumed that the number of micronucleated cells is normally distributed. Levene's test is used to determine if variances among treatment groups are equal. When they are, linear regression analysis is used to test for linear trend and pairwise differences with the control group are evaluated using Williams' test, after linearizing the data by averaging data points that violate a linear trend. When variances are unequal, nonparametric methods are used to analyze the data: Jonckheere's test is used to evaluate linear trend and Dunn's test is used to assess the significance of pairwise differences with the control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences from the control group are declared statistically significant if $P \leq 0.025$.

I. Clinical Observations in the *In Vivo* Studies

Individual clinical observations and records of body weights throughout the 4-day study are included in the appendices of the Contract laboratory report and are available upon request. A summary of the clinical observations follows. In the rat study, two animals in the top dose group (800 mg/kg/day) were observed to be thin or ungroomed on Day 3 of dosing. All other rats appeared normal throughout the entire course of the study.

One male mouse administered cumene at 1250 mg/kg/day exhibited uncoordinated movement and was lethargic on Days 3 and 4; another mouse in this group exhibited similar symptoms only on day 4. A third mouse in the top dose group showed signs of hunched posture, lethargy, piloerection, ungroomed appearance, ocular discharge, and rales on Days 2 and 3; this mouse was moribund and euthanized on Day 4. Results of the necropsy on this mouse determined that gavage error, not the dose of cumene, was the cause of the symptoms. One mouse in the 625 mg/kg/day dose group exhibited an ungroomed appearance on Day 2. All other male mice

appeared normal throughout the study. Three female mice administered cumene at 1000 mg/kg/day exhibited uncoordinated movement 4 hr post dose on Days 1, 3 and 4; one of these mice also appeared lethargic 1 hr post dose on Day 4. All other female mice appeared normal throughout the entire duration of the study.

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VI. NTP Data Evaluation and Interpretation

A. Bacterial Mutagenicity Assays (Ames Tests)

1. Protocol Summary

Care was taken during the bacterial mutagenicity assay to limit the potential reduction in exposure due to the reported volatility of cumene (e.g., exposures occurred in capped tubes). Cumene was incubated with *S. typhimurium* strains TA98 and TA100 and *E. coli* strain WP2 *uvr*A pKM101 either in buffer or S9 mix for 20 minutes at 37°C. Top agar supplemented with L-histidine (for the *S. typhimurium* strains) or tryptophan (for the *E. coli* strain) and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine- and tryptophan-independent mutant colonies arising on these plates were counted following two days incubation at 37°C using an automated colony counter. Each trial consisted of triplicate plates of positive and vehicle (DMSO) controls, and at least 5 dose levels of cumene.

2. NTP Evaluation of the Bacterial Mutagenicity Test Results

In this assay, NTP defines a positive response as a reproducible, dose-related increase in histidine- or tryptophan-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose-related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold-increase required for a chemical to be judged positive or weakly positive.

Based on these evaluation criteria, cumene was judged to be negative in the bacterial mutagenicity assay in all three tester strains, with and without 10% rat liver S9. Adequate exposure of the tester strains was achieved, as evidenced by cytotoxicity at the higher doses.

3. Summary Data Tables for Bacterial Mutagenicity Assays

CASRN: 98-82-8 Chemical: Cumene
Study No.: G1104ZB **Lab:** Integrated Laboratory Systems
Conclusion: Negative

Summary Data - TA100

Dose ug/Plate	1 No Activation (-) Mean ± SEM	2 No Activation (-) Mean ± SEM	1 10% RLI (-) Mean ± SEM	2 10% RLI (-) Mean ± SEM
0	79 ± 5.0	92 ± 7.0	105 ± 9.0	93 ± 14.0
2.56		93 ± 3.0		
6.4		107 ± 2.0		115 ± 24.0
7.8	91 ± 6.0			
15.6	99 ± 6.0		92 ± 9.0	
16		84 ± 13.0		94 ± 3.0
31.2	71 ± 6.0		90 ± 4.0	
40		84 ± 5.0		101 ± 7.0*
62.5	65 ± 5.0		94 ± 4.0	
100		48s ± 5.0		96 ± 6.0
125	7s ± 1.0		78 ± 4.0	
250	8t ± 6.0	13s ± 2.0	63s ± 10.0	67s ± 3.0
500			33s ± 1.0	39s ± 6.0
POSITIVE	801 ± 15.0	687 ± 14.0	825 ± 10.0	700 ± 27.0

Summary Data - TA98

Dose ug/Plate	1 No Activation (-) Mean ± SEM	3 No Activation (-) Mean ± SEM	1 10% RLI (-) Mean ± SEM	2 10% RLI (-) Mean ± SEM
0	18 ± 3.0	13 ± 2.0	10 ± 1.0	23 ± 2.0
2.56				
6.4	19 ± 5.0		7 ± 2.0	
7.8		16 ± 3.0		
15.6		20 ± 2.0		24 ± 1.0
16	18 ± 2.0		13 ± 4.0	
31.2		13 ± 4.0		22 ± 3.0
40	15 ± 2.0		12 ± 2.0	
62.5		15 ± 3.0		24 ± 0.0
100	13 ± 2.0		10 ± 5.0	
125		10s ± 2.0		19 ± 5.0
250	9s ± 3.0	t	8 ± 1.0	11s ± 3.0
500	13s ± 3.0		5 ± 1.0	4s ± 1.0
POSITIVE	746 ± 88.0	805 ± 54.0	515 ± 86.0	1402 ± 111.0

Summary Data - *E. coli* WP2

Dose ug/Plate	1 No Activation (-) Mean ± SEM	2 No Activation (-) Mean ± SEM	1 10% RLI (-) Mean ± SEM	2 10% RLI (-) Mean ± SEM
0	142 ± 15.0	142 ± 8.0	140 ± 10.0	135 ± 5.0
6.4	139 ± 5.0			145 ± 10.0
15.6		119 ± 6.0	138 ± 1.0	
16	123 ± 3.0			129 ± 4.0
31.2		145 ± 10.0	149 ± 15.0	
40	119 ± 20.0			136 ± 7.0
62.5		107 ± 3.0	162 ± 2.0	
100	76s ± 7.0			133 ± 15.0
125		87s ± 11.0	140 ± 5.0	
250	83s ± 14.0	10t ± 5.0	118 ± 10.0	102 ± 7.0
500	t	t	32t ± 4.0	30s ± 5.0
POSITIVE	1455 ± 33.0	1821 ± 20.0	1206 ± 33.0	957 ± 3.0

s = Slight Toxicity; p = Precipitate; x = Slight Toxicity and Precipitate; t = Toxic; c = Contamination

* mean of 2 plates; 3rd plate was contaminated

B. *In Vivo* Rodent Peripheral Blood Micronucleus Tests

1. Protocol Summary

Animals were administered cumene by corn oil gavage once daily for 3 consecutive days at 24 hr intervals and again on the 4th day, 21 hr after the 3rd dose. Animals were humanely euthanized 3-4 hr after the final dosing (CO_2 anesthesia followed by exsanguination). Peripheral blood samples (60 – 120 μL) obtained from male and female B6C3F1 mice and male F344 rats were fixed in ultracold methanol (MicroFlow® Basic Kits; Dertinger et al., 2004); fixed samples were stored in a -80° freezer until analysis by flow cytometry. Using flow cytometry, RET were identified by the presence of an active transferrin receptor (CD71-positive) on the cell surface; mature erythrocytes were identified as CD71-negative. For the male rat blood samples, the analysis was restricted to the youngest RET (i.e., the subpopulation with the highest CD71 expression) to focus on the cell population least altered by the efficient action of the rat spleen in sequestering and destroying micronucleated red blood cells (MacGregor et al., 2006). In mice, both MN-RET and MN-erythrocytes were evaluated, although in a short-term study such as this one, the appropriate population for evaluating MN frequency associated with chemical treatment is the reticulocyte population. Damaged erythrocytes do not reach peak levels in the erythrocyte population in mice until around 28 days of repeated daily dosing.

For all animals, twenty thousand RET were scored for presence of MN, and approximately 1 x 10^6 total erythrocytes were counted concurrently to determine percentage of circulating RET (%RET) as a measure of chemical-induced bone marrow toxicity.

2. Data Analysis for Peripheral Blood Micronucleus Test

The optimum number of cells to score for micronuclei using flow cytometric approaches was determined in earlier studies (Kissling et al., 2007). Data from each treatment group are summarized as the mean frequency of MN-RET per 1000 RET, plus or minus the standard error of the mean. With the large number of cells counted by flow cytometry, it is assumed that the number of micronucleated cells is normally distributed. Levene's test is used to determine if variances among treatment groups were equal. When they are, linear regression analysis is used to test for linear trend and pairwise differences with the control group are evaluated using Williams' test. When variances are unequal, nonparametric methods are used to analyze the data: Jonckheere's test is used to evaluate linear trend and Dunn's test is used to assess the significance of pairwise differences with the control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences from the control group are declared statistically significant if $P \leq 0.025$.

3. NTP Evaluation of the Micronucleus Test Results in Mice and Rats

Based on these evaluation criteria and review of the data by NTP staff, the results of the peripheral blood erythrocyte MN tests in male rats and in male and female mice were judged to be negative. No significant increases in micronucleated RET (PCE) or erythrocytes (NCE) were observed in male rats or either sex of mice. In rats, the percentage of circulating reticulocytes

(% RET or %PCE) was reduced significantly (30% reduction from the control level) at the top dose, confirming exposure of bone marrow cells. However, the degree of reduction was not excessive (OECD Guideline 474 permits a 80% reduction from the control value). The reduction in circulating reticulocytes seen in the rats supports the dose selection based on the results of the range finding test. In male mice, the percentage of circulating reticulocytes increased over the dose range examined, although the increase did not reach the level of statistical significance ($p > 0.025$).

The negative MN test results obtained in rat peripheral blood in this short-term gavage study with cumene contrast with the positive results in an earlier study in rats that used intraperitoneal injection as the route of exposure and a top dose of 1250 mg/kg (NTP, 2009; Appendix Table 1). In the earlier rat study, a 3- to 5-fold increase in MN-PCE was observed in each of two replicate trials. The protocol differences between the two studies, including the route of exposure, the dose levels tested, possible differences in metabolism of cumene following different exposure routes, and the cell populations evaluated (bone marrow versus peripheral blood) may have been factors in the different test outcomes. However, the gavage route of exposure is more relevant to any potential human exposure than is intraperitoneal injection, and flow cytometric evaluation of very early stage reticulocytes in peripheral blood is an accurate method for assessment of MN frequencies in rats (Witt et al., 2008).

The negative MN test results obtained in mouse peripheral blood in this short-term gavage study with cumene are consistent with the negative results in an earlier study in mice exposed to cumene by inhalation (dose range, 62.5 – 1000 ppm for male mice and 62.5 – 500 ppm for female mice) for 13 weeks (NTP, 2009; Appendix Table 2). In that study, female mice were also found to be more sensitive to cumene exposure than male mice, based on the highest dose tested.

4. Summary Data Tables for the Micronucleus Tests

Male Rats

Frequency of Micronucleated Erythrocytes
Following Administration of Compound Z by GAV[a]

Trial Information

Study No	Lab	CASRN	Study Date[b]	Test Type	Tissue	Sample Time	Number of Treatments	Exposure Duration	Species	Strain	Sex
G1104ZC	IL	COMPOUNDZ	07/26/2012	MN	PB	24 Hours	4	4 Days	Rats	F344	M

Group Information

Control	Dose (mg/kg)	Number of Animals	MN-PCE/1000[c]	Pairwise P-value [d2]	Number of Animals	MN-NCE/1000[c]	Pairwise P-value [d2]	Percent PCE[c]	Pairwise P-value [d2]
CRNO[e]	0.0000	6	0.33 ± 0.03		6	0.07 ± 0.01		1.433 ± 0.11	
	200.0000	6	0.27 ± 0.05	0.7758	6	0.07 ± 0.02	0.5486	1.594 ± 0.13	1.0000
	400.0000	6	0.33 ± 0.05	0.8531	6	0.09 ± 0.02	0.2488	1.517 ± 0.13	1.0000
	800.0000	6	0.18 ± 0.06	0.8801	6	0.09 ± 0.01	0.2651	1.001 ± 0.07	0.0042
			P = 0.978 [f2]			P = 0.144 [f2]		P = 0.002 [f2]	
EMS[g]	200.0000	6	4.23 ± 0.66	0.0001	6	0.06 ± 0.01	0.8151	0.190 ± 0.03	0.0000

[a] Study was performed at ILS. The detailed protocol is presented by Witt et al., 2008. "Compound Z" is the coded name for cumene.
[b] The date when animal dosing began.
[c] Mean ± Standard Error, PCE = polychromatic erythrocyte (RET), NCE = normochromatic erythrocyte.
[d1] Pairwise comparison with the Control group; significant at P ≤ 0.025 by Dunn's test.
[d2] Pairwise comparison with the Control group; significant at P ≤ 0.025 by Williams' test.
[e] Vehicle control.
[f1] Dose-related trend; significant at P ≤ 0.025 by Jonckheere's test.
[f2] Dose-related trend; significant at P ≤ 0.025 by linear regression.
[g] Positive control, ethyl methanesulfonate.

Male and Female Mice

Frequency of Micronucleated Erythrocytes Following Administration of Compound Z by GAV[a]

Trial Information

Study No	Lab	CASRN	Study Date[b]	Test Type	Tissue	Sample Time	Number of Treatments	Exposure Duration	Species	Strain	Sex
G1104ZD	IL	COMPOUNDZ	07/19/2012	MN	PB	24 Hours	4	4 Days	Mice	B6C3F1	M

Group Information

Control	Dose (mg/kg)	Number of Animals	MN-PCE/1000[c]	Pairwise P-value [d2]	Number of Animals	MN-NCE/1000[c]	Pairwise P-value [d2]	Percent PCE[c]	Pairwise P-value [d2]
CRNO[e]	0.0000	6	2.75 ± 0.17		6	1.48 ± 0.04		1.255 ± 0.06	
	312.0000	6	2.34 ± 0.11	0.7641	6	1.47 ± 0.04	0.5724	1.433 ± 0.09	0.1698
	625.0000	6	2.90 ± 0.23	0.3552	6	1.47 ± 0.03	0.6588	1.473 ± 0.08	0.1087
	1250.0000	5	3.05 ± 0.29	0.2016	5	1.51 ± 0.03	0.3721	1.582 ± 0.15	0.0350
			P = 0.067 [f2]			P = 0.275 [f2]		P = 0.031 [f2]	
EMS[g]	150.0000	5	8.34 ± 0.69	0.0000	5	1.71 ± 0.01	0.0002	1.310 ± 0.14	0.7775

Trial Information

Study No	Lab	CASRN	Study Date[b]	Test Type	Tissue	Sample Time	Number of Treatments	Exposure Duration	Species	Strain	Sex
G1104ZD	IL	COMPOUNDZ	07/20/2012	MN	PB	24 Hours	4	4 Days	Mice	B6C3F1	F

Group Information

Control	Dose (mg/kg)	Number of Animals	MN-PCE/1000[c]	Pairwise P-value [d2]	Number of Animals	MN-NCE/1000[c]	Pairwise P-value [d2]	Percent PCE[c]	Pairwise P-value [d2]
CRNO[e]	0.0000	6	2.37 ± 0.07		6	1.20 ± 0.02		1.344 ± 0.09	
	250.0000	6	2.23 ± 0.12	0.7604	6	1.17 ± 0.02	0.9076	1.613 ± 0.12	0.1586
	500.0000	6	2.44 ± 0.19	0.8397	6	1.16 ± 0.02	0.9520	1.682 ± 0.10	0.1873
	1000.0000	6	1.89 ± 0.14	0.8685	6	1.12 ± 0.01	0.9666	1.339 ± 0.08	0.1965
			P = 0.985 [f2]			P = 0.996 [f2]		P = 0.704 [f2]	
EMS[g]	150.0000	5	12.45 ± 0.77	0.0000	5	1.42 ± 0.03	0.0001	0.852 ± 0.06	0.0010

[a] Study was performed at ILS. The detailed protocol is presented by Witt et al., 2008. "Compound Z" is the coded name for cumene.

[b] The date when animal dosing began.

[c] Mean ± Standard Error, PCE = polychromatic erythrocyte (RET), NCE = normochromatic erythrocyte.

[d1] Pairwise comparison with the Control group; significant at $P \leq 0.025$ by Dunn's test.

[d2] Pairwise comparison with the Control group; significant at $P \leq 0.025$ by Williams' test.

[e] Vehicle control.

[f1] Dose-related trend; significant at $P \leq 0.025$ by Jonckheere's test.

[f2] Dose-related trend; significant at $P \leq 0.025$ by linear regression.

[g] Positive control, ethyl methanesulfonate.

C. *In Vivo* Rodent Comet Assays

1. Protocol Summary

DNA damage levels were evaluated in multiple cell types obtained from the same rats and mice that were evaluated for the MN endpoint. Cell types that were examined included blood leukocytes, and cells from the liver, lung, and kidney. An OECD Guideline has not yet been established for the Comet assay; one is expected in 2013, based on results from the international Comet assay validation trial overseen by the Japanese Center for the Valuation of Alternative Methods. NTP, through its contract laboratory ILS, Inc., participated in this inter-laboratory validation trial.

Tissue samples were obtained at necropsy, placed into microcentrifuge tubes containing cold mincing buffer, and rapidly minced to create single cell suspensions, with duplicates made for each tissue per animal. Cells were layered onto slides, lysed overnight, DNA was allowed to unwind for 20 minutes, and slides were then subjected to electrophoresis at 7-9°C for 20 min. Following electrophoresis, slides were dehydrated with absolute ethanol, air dried, stained, and scored using an automated image analysis system. All slides were scored blind. 100 cells were evaluated per tissue per animal and % tail DNA (tail fluorescence intensity) was the endpoint upon which the statistical analyses were run. Cells fitting the "hedgehog" criteria were tabulated, but not included in the scoring.

2. Statistical Methods

Data analysis was conducted by Dr. Grace Kissling, Biostatistics Branch, NIEHS/NTP. Normality of the distribution of the % tail DNA values in each dose group was determined by Levene's test. Because the data were all found to be normally distributed with equal variances, linear regression analysis was used to test for linear trend, and differences between each dose group and the control group were evaluated using Williams' test. If variances among treatment groups are found to be unequal, nonparametric methods are used to analyze the data: Jonckheere's test is used to evaluate linear trend and Dunn's test is used to assess the significance of pairwise differences with the control group. To maintain the overall significance level at 0.05, the trend as well as the pairwise differences from the control group are declared statistically significant if $P \leq 0.025$. P values are one-sided.

3. NTP Evaluation of the Comet Assay Results in Mice and Rats

Summary Table of the Comet Assay Results

Species/sex	Cell type	Result
Male rat	Blood	Negative
	Liver	**Positive**
	Lung	Negative
	Kidney	Negative
Male mouse	Blood	Negative
	Liver	Negative
	Lung	Negative
	Kidney	Negative
Female mouse	Blood	Negative
	Liver	Negative
	Lung	**Positive**
	Kidney	Negative

The male rat liver results and the female mouse lung results were judged to be positive, based on a significant trend (P = 0.002; P = 0.008, respectively) and significant increases in DNA damage in the highest dose group for each tissue. For the male rat liver, the pairwise p-value for the top dose group was 0.004, and for female mouse lung, the pairwise p-value for the top dose group was 0.016. The criterion for statistical significance for both the pairwise and the trend tests is $P \leq 0.025$.

The clinical observations observed for mice and rats during the range finding and definitive studies, along with the decreased proportion of circulating reticulocytes observed in male rats in the highest dose group, support a conclusion that systemic exposure to cumene was achieved in these animals following administration via oral gavage. However, it is unknown whether the same relative distribution of cumene and similar metabolic profiles in the target tissues that were identified from the 2-year bioassay that used inhalation as the route of exposure were achieved.

4. Summary Data Tables for the Comet Assay

Female Mice % Tail DNA

	Female mice, Blood				Female mice, Liver				Female mice, Lung				Female mice, Kidney			
mg/kg	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value
0	6	2.097	0.175		6	10.417	1.676		6	6.785	0.324		6	5.646	0.746	
250	6	2.362	0.357	0.468	6	11.182	1.913	0.486	6	7.328	0.551	0.257	6	4.416	0.275	0.880
500	6	1.949	0.210	0.548	6	10.993	0.958	0.566	6	7.787	0.698	0.139	6	4.406	0.436	0.933
1000	6	2.063	0.245	0.582	6	9.303	1.834	0.601	6	8.723	0.660	0.016	6	5.512	0.301	0.699
Trend p-value		0.660				0.732				0.008				0.421		
EMS	5	15.379	0.604	<0.0001	5	24.537	1.218	<0.0001	5	23.693	0.996	<0.0001	5	17.394	0.541	<0.0001

P < 0.025 is significant for pairwise comparisons

P < 0.025 is significant for trends

EMS: 150 mg/kg

Male Mice % Tail DNA

	Male mice, Blood				Male mice, Liver				Male mice, Lung				Male mice, Kidney			
mg/kg	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value
0	6	2.409	0.375		6	7.498	0.784		6	11.875	1.212		6	3.497	0.198	
312	6	2.442	0.248	0.525	6	9.284	0.351	0.176	6	12.145	0.800	0.433	6	4.037	0.456	0.337
625	6	2.580	0.511	0.608	6	7.632	0.546	0.213	6	13.676	1.330	0.221	6	3.385	0.261	0.399
1250	5	2.006	0.274	0.654	5	8.333	1.067	0.241	5	12.983	1.252	0.250	5	3.753	0.483	0.405
Trend p-value		0.770				0.401				0.204				0.457		
EMS	5	14.177	0.407	<0.0001	5	27.973	0.783	<0.0001	5	16.662	1.001	0.008	5	4.847	0.388	0.005

P < 0.025 is significant for pairwise comparisons

P < 0.025 is significant for trends

EMS: 150 mg/kg

Male Rats % Tail DNA

	Male rats, Blood				Male rats, Liver				Male rats, Lung				Male rats, Kidney			
mg/kg	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value	n	Mean	S.E.	p-value
0	6	3.664	0.394		6	5.876	0.616		6	6.374	0.327		6	8.176	0.474	
200	6	3.575	0.195	0.533	6	6.967	0.415	0.110	6	6.344	0.696[N]	0.505	6	7.530	1.005	0.697
400	6	4.188	0.402	0.243	6	7.505	0.637	0.043	6	7.201	1.029	0.240	6	7.681	0.910	0.783
800	6	4.011	0.416	0.260	6	8.465	0.730	0.004	6	7.395	0.450	0.192	6	7.085	0.393	0.816
Trend p-value		0.181				0.002				0.103				0.834		
EMS	6	32.850	0.689	<0.0001	6	37.154	0.570	<0.0001	6	40.608	0.557	<0.0001	6	36.464	2.958	<0.0001

P < 0.025 is significant for pairwise comparisons

P < 0.025 is significant for trends

EMS: 200 mg/kg

VII. Peer Review of Draft Report on the Cumene (CASRN 98-82-8) Genotoxicity Studies

The draft report was peer reviewed by two external reviewers in October 2012. The peer review comments were carefully considered in finalization of the report.

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Appendix: Data Tables from NTP Technical Report 542

Table 1

Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Rats Treated with Cumene by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Male Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	Pairwise P Value[c]	PCE[b] (%)
Trial 1					
Corn oil[d]	0	5	0.50 ± 0.16		50.2 ± 2.9
Cumene	78.13	5	1.20 ± 0.25	0.0447	59.4 ± 5.1
	156.25	5	1.20 ± 0.34	0.0447	64.8 ± 4.2
	312.5	5	1.30 ± 0.54	0.0296	54.6 ± 3.1
	625	5	0.80 ± 0.41	0.2026	45.1 ± 1.7
	1,250	5	2.60 ± 0.29	0.0001	46.6 ± 4.8
	2,500	2[e]	1.25 ± 0.25		49.3 ± 2.8
			P<0.001[f]		
Cyclophosphamide[g]	25	5	17.30 ± 2.32	0.0000	50.3 ± 4.3
Trial 2					
Corn oil	0	5	0.50 ± 0.27		53.2 ± 3.8
Cumene	312	5	1.70 ± 0.20	0.0052	50.2 ± 1.0
	625	5	1.40 ± 0.33	0.0194	47.6 ± 3.1
	1,250	5	1.80 ± 0.34	0.0033	44.5 ± 3.0
	2,500	3	1.50 ± 1.00	0.0192	54.3 ± 2.1
			P=0.085		
Cyclophosphamide	25	5	7.80 ± 1.63	0.0000	38.7 ± 2.7

[a] Study was performed at ILS, Inc. The detailed protocol is presented by Shelby *et al.* (1993).
[b] PCE=polychromatic erythrocyte
[c] Mean ± standard error
[d] Pairwise comparison with the vehicle control; dosed group values are significant at P≤0.005 (trial 1) or P≤0.006 (trial 2); positive control values are significant at P≤0.05 (ILS, 1990)
[e] Vehicle control
[f] Statistical tests not performed due to high mortality
[g] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test, significant at P≤0.025 (ILS, 1990); 2,500 mg/kg group excluded due to high mortality
Positive control

Table 2

Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Treatment with Cumene by Inhalation for 3 Months[a]

Compound	Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P-Value[c]	PCEs[b] (%)
Male					
Air[d]	0	10	2.40 ± 0.69		2.7 ± 0.1
Cumene	62.5	10	2.20 ± 0.66	0.6161	2.6 ± 0.1
	125	10	2.10 ± 0.48	0.6728	2.6 ± 0.1
	250	10	1.80 ± 0.36	0.8230	2.8 ± 0.1
	500	10	2.00 ± 0.26	0.7270	2.9 ± 0.1
	1,000	10	2.20 ± 0.42	0.6161	2.9 ± 0.2
			P=0.553[e]		
Female					
Air	0	10	2.30 ± 0.40		3.3 ± 0.1
Cumene	62.5	9	1.33 ± 0.37	0.9396	2.3 ± 0.1
	125	10	1.70 ± 0.30	0.8289	3.1 ± 0.2
	250	10	2.10 ± 0.53	0.6186	3.3 ± 0.2
	500	10	2.10 ± 0.35	0.6186	3.4 ± 0.1
			P=0.329		

[a] Study was performed at ILS, Inc. The detailed protocol is presented by MacGregor *et al.* (1990).
[b] NCE=normochromatic erythrocyte; PCE=polychromatic erythrocyte
[c] Mean ± standard error
[d] Pairwise comparison with the chamber controls, significant at $P \leq 0.005$ (males) or $P \leq 0.006$ (females) (ILS, 1990)
[e] Chamber control
Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test, significant at $P \leq 0.025$ (ILS, 1990)