

(iv) A copy of the final report and of any amendment to it shall be maintained by the sponsor and the test facility.

(2) *Storage and retrieval of records and data.* (i) All raw data, documentation, records, protocols, specimens, and final reports generated as a result of a study shall be retained. Specimens obtained from mutagenicity tests, wet specimens of blood, urine, feces, and biological fluids, do not need to be retained after quality assurance verification. Correspondence and other documents relating to interpretation and evaluation of data, other than those documents contained in the final report, also shall be retained.

(ii) All raw data, documentation, protocols, specimens, and interim and final reports shall be archived for orderly storage and expedient retrieval. Conditions of storage shall minimize deterioration of the documents or specimens in accordance with the requirements for the time period of their retention and the nature of the documents of specimens. A testing facility may contract with commercial archives to provide a repository for all material to be retained. Raw data and specimens may be retained elsewhere provided that the archives have specific reference to those other locations.

(iii) An individual shall be identified as responsible for the archiving of records.

(iv) Access to archived material shall require authorization and documentation.

(v) Archived material shall be indexed to permit expedient retrieval.

(3) *Retention of records.* (i) Record retention requirements set forth in this section do not supersede the record retention requirements of any other regulations in this subchapter.

(ii) Except as provided in paragraph (h)(3)(iii) of this section, documentation records, raw data, and specimens pertaining to a study and required to be retained by this part shall be archived for a period of at least ten years following the completion of the study.

(iii) Wet specimens, samples of test fuel, additive/base fuel mixtures, or reference substances, and specially prepared material which are relatively

fragile and differ markedly in stability and quality during storage, shall be retained only as long as the quality of the preparation affords evaluation. Specimens obtained from mutagenicity tests, wet specimens of blood, urine, feces, biological fluids, do not need to be retained after quality assurance verification. In no case shall retention be required for a longer period than that set forth in paragraph (h)(3)(ii) of this section.

(iv) The master schedule sheet, copies of protocols, and records of quality assurance inspections, as required by § 79.60(b)(4)(iii) shall be maintained by the quality assurance unit as an easily accessible system of records for the period of time specified in paragraph (h)(3)(ii) of this section.

(v) Summaries of training and experience and job descriptions required to be maintained by § 79.60(b)(1)(ii) may be retained along with all other testing facility employment records for the length of time specified in paragraph (h)(3)(ii) of this section.

(vi) Records and reports of the maintenance and calibration and inspection of equipment, as required by § 79.60(d)(2) (ii) and (iii), shall be retained for the length of time specified in paragraph (h)(3)(ii) of this section.

(vii) If a facility conducting testing or an archive contracting facility goes out of business, all raw data, documentation, and other material specified in this section shall be transferred to the sponsor of the study for archival.

(viii) Records required by this section may be retained either as original records or as true copies such as photocopies, microfilm, microfiche, or other accurate reproductions of the original records.

#### **§ 79.61 Vehicle emissions inhalation exposure guideline.**

(a) *Purpose.* This guideline provides additional information on methodologies required to conduct health effects tests involving inhalation exposures to vehicle combustion emissions from fuels or fuel/additive mixtures. Where this guideline and the other health effects testing guidelines in 40 CFR 79.62 through 79.68 specify differing values

for the same test parameter, the specifications in the individual health test guideline shall prevail for that health effect endpoint.

(b) *Definitions.* For the purposes of this section the following definitions apply.

*Acute inhalation study* means a short-term toxicity test characterized by a single exposure by inhalation over a short period of time (at least 4 hours and less than 24 hours), followed by at least 14 days of observation.

*Aerodynamic diameter* means the diameter of a sphere of unit density that has the same settling velocity as the particle of the test substance. It is used to compare particles of different sizes, densities and shapes, and to predict where in the respiratory tract such particles may be deposited. It applies to the size of aerosol particles.

*Chronic inhalation study* means a prolonged and repeated exposure by inhalation for the life span of the test animal; technically, two years in the rat.

*Concentration* means an exposure level. Exposure is expressed as weight or volume of test aerosol/substance per volume of air, usually mg/m<sup>3</sup> or as parts per million (ppm) over a given time period. Micrograms per cubic meter (µg/m<sup>3</sup>) or parts per billion may be appropriate, as well.

*Cumulative toxicity* means the adverse effects of repeated exposures occurring as a result of prolonged action or increased concentration of the administered test substance or its metabolites in the susceptible tissues.

*Inhalable diameter* means that aerodynamic diameter of a particle which is considered to be inhalable for the organism. It is used to refer to particles which are capable of being inhaled and may be deposited anywhere within the respiratory tract from the trachea to the alveoli.

*Mass median aerodynamic diameter (MMAD)* means the calculated aerodynamic diameter, which divides the particles of an aerosol in half based on the mass of the particles. Fifty percent of the particles in mass will be larger than the median diameter, and fifty percent will be smaller than the median diameter. MMAD describes the particle distribution of any aerosol based on the weight and size of the par-

ticles. MMAD and the geometric standard deviation describe the particle-size distribution.

*Material safety data sheet (MSDS)* means documentation or information on the physical, chemical, and hazardous characteristics of a given chemical, usually provided by the product's manufacturer.

*Reynolds number* means a dimensionless number that is proportional to the ratio of inertial forces to frictional forces acting on a fluid. It quantitatively provides a measure of whether flow is laminar or turbulent. A fluid traveling through a pipe is fully developed into a laminar flow for a Reynolds number less than 2000, and fully developed into a turbulent flow for a Reynolds number greater than 4000.

*Subacute inhalation toxicity* means the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by inhalation for part (less than 10 percent) of a lifespan; generally, less than 90 days.

*Subchronic inhalation study* means a repeated exposure by inhalation for part (approximately 10 percent) of a life span of the exposed test animal.

*Toxic effect* means an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) *Principles and design criteria of inhalation exposure systems.* Proper conduct of inhalation toxicity studies of the emissions of fuels and additive/fuel mixtures requires that the exposure system be designed to ensure the controlled generation of the exposure atmosphere, the adequate dilution of the test emissions, delivery of the diluted exposure atmosphere to the test animals, and use of appropriate exposure chamber systems selected to meet criteria for a given exposure study.

(1) *Emissions generation.* Emissions shall be generated according to the specifications in 40 CFR 79.57.

(2) *Dilution and delivery systems.* (i) The delivery system is the means used to transport the emissions from the generation system to the exposure system. The dilution system is generally a component of the delivery system.

(ii) Dilution provides control of the emissions concentration delivered to

the exposure system, serving the function of diluting the associated combustion gases, such as carbon monoxide, carbon dioxide, nitrogen oxides, sulfur dioxide and other noxious gases and vapors, to levels that will ensure that there are no significant or measurable responses in the test animals as a result of exposure to the combustion gases. The formation of particle species is strongly dependent on the dilution rate, as well.

(iii) The engine exhaust system shall connect to the first-stage-dilution section at 90° to the axis of the dilution section. This is then connected to a right angle elbow on the center line of the dilution section. Engine emissions are injected through the elbow so that exhaust flow is concurrent to dilution flow.

(iv) *Materials.* In designing the dilution and delivery systems, the use of plastic, e.g., PVC and similar materials, copper, brass, and aluminum pipe and tubing shall be avoided if there exists a possibility of chemical reaction occurring between emissions and tubing. Stainless steel pipe and tubing is recommended as the best choice for most emission dilution and delivery applications, although glass and teflon may be appropriate, as well.

(v) *Flow requirements.* (A) Conduit for dilute raw emissions shall be of such dimensions as to provide residence times for the emissions on the order of less than one second to several seconds before the emissions are further diluted and introduced to the test chambers. With the high flow rates in the dilute raw emissions conduit, it will be necessary to sample various portions of the dilute emissions for delivering differing concentrations to the test chambers. The unused portions of the emissions stream are normally exhausted to the atmosphere outside of the exposure facility.

(B) Dimensions of the dilute raw exhaust conduit shall be such that, at a minimum, the flow Reynolds number is 70,000 or greater (see Mokler, *et al.*, 1984 in paragraph (f)(13) of this section). This will maintain highly turbulent flow conditions so that there is more complete mixing of the exhaust emissions.

(C) *Wall losses.* The delivery system shall be designed to minimize wall losses. This can be done by sizing the tubing or pipe to maintain laminar flow of the diluted emissions to the exposure chamber. A flow Reynolds number of 1000-3000 will ensure minimal wall losses. Also, the length of and number and degree of bends in the delivery lines to the exposure chamber system shall be minimized.

(D) Whole-body exposure vs. nose-only exposure delivery systems. Flow rates through whole-body chamber systems are of the order of 100 liters per minute to 500 liters per minute. Nose-only systems are on the order of less than 50 liters per minute. To maintain laminar flow conditions, the principles described in paragraph (c)(2)(v)(C) of this section apply to both systems.

(vi) *Dilution requirements.* (A) To maintain the water vapor, and dissolved organic compounds, in the raw exhaust emissions stream, a manufacturer/tester will initially dilute one part emissions with a minimum of five parts clean, filtered air (see Hinners, *et al.*, 1979 in paragraph (f)(11) of this section). Depending on the water vapor content of a particular fuel/additive mixture's combustion emissions and the humidity of the dilution air, initial exhaust dilutions as high as 1:15 or 1:20 may be necessary to maintain the general character of the exhaust as it cools, e.g., M100. At this point, it is expected that the exhaust stream would be further diluted to more appropriate levels for rodent health effects testing.

(B) A maximum concentration (minimum dilution) of the raw exhaust going into the test animal cages is anticipated to lie in the range between 1:5 and 1:50 exhaust emissions to clean, filtered air. The minimum concentration (maximum dilution) of raw exhaust for health effects testing is anticipated to be in range between 1:100 and 1:150. Individual manufacturers will treat these ranges as approximations only and will determine the optimum range of emission concentrations to elicit effects in Tier 2 health testing for their particular fuel/fuel additive mixture.

(3) *Exposure chamber systems—(i) Referenced Guidelines.* (A) The U.S. Department of Health and Human Services

“Guide for the Care and Use of Laboratory Animals” (*Guide*), 1985 cited in paragraph (c)(3)(ii)(A)(4), and in paragraphs (d)(2)(i), (d)(2)(ii), (d)(2)(iii), (d)(4)(ii), and (d)(4)(iii) of this section, has been incorporated by reference.

(B) This incorporation by reference was approved by the Director of the Federal Register in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies may be purchased from the Superintendent of Documents, U.S. Government Printing Office, Washington, DC 20402. Copies may be inspected at U.S. EPA, OAR, 401 M Street SW, Washington, DC 20460 or at the Office of the Federal Register, 800 North Capitol Street NW., suite 700, Washington, DC.

(ii) *Exposure chambers.* There are two basic types of dynamic inhalation exposure chambers, whole-body chambers and nose-/head-only exposure chambers (see Cheng and Moss, 1989 in paragraph (f)(8) of this section).

(A) *Whole-body chambers.* (1) The flow rate through a chamber shall be maintained at 15 air changes per hour.

(2) The chambers are usually maintained at a slightly negative pressure (0.5 to 1.5 inch of water) to prevent leakage of test substance into the exposure room.

(3) The exposure chamber shall be designed in such a way as to provide uniform distribution of exposure concentrations in all compartments (see Cheng *et al.*, 1989 in paragraph (f)(7) of this section).

(4) Animals are housed in separate compartments inside the chamber, where the whole surface area of an animal is exposed to the test material. The spaces required for different animal species shall follow the *Guide*. In general, the volume of animal bodies occupy less than 5 percent of the chamber volume.

(B) *Head/nose-only exposure chambers.* (1) In head/nose-only exposure chambers, only the head (oronasal) portion of the animal is exposed to the test material.

(2) The chamber volume and flow rates are much less than in the whole-body exposure chambers because the subjects are usually restrained in a tube holder where the animal's breathing can be easily monitored. The head/nose-only exposure chamber is suitable

for short-term exposures or when use of a small amount of test material is required.

(iii) Since whole-body exposure appears to be the least stressful mode of exposure, it is the preferred method. In general, head/nose only exposure, which is sometimes used to avoid concurrent exposure by the dermal or oral routes, *i.e.*, grooming, is not recommended because of the stress accompanying the restraining of the animals. However, there may be specific instances where it may be more appropriate than whole-body exposure. The tester shall provide justification for its selection.

(d) *Inhalation exposure procedures—(1) Animal selection.* (i) The rat is the preferred species for vehicle emission inhalation health effects testing. Commonly used laboratory strains shall be used. Any rodent species may be used, but the tester shall provide justification for the choice of that species.

(ii) Young adult animals, approximately ten weeks of age for the rat, shall be used. At the commencement of the study, the weight variation of animals used shall not exceed  $\pm 20$  percent of the mean weight for each sex. Animals shall be randomly assigned to treatment and control groups according to their weight.

(iii) An equal number of male and female rodents shall be used at each concentration level. Situations may arise where use of a single sex may be appropriate. Females, in general, shall be nulliparous and nonpregnant.

(iv) The number of animals used at each concentration level and in the control group(s) depends on the type of study, number of biological end points used in the toxicity evaluation, the pre-determined sensitivity of detection and power of significance of the study, and the animal species. For an acute study, at least five animals of each sex shall be used in each test group. For both the subacute and subchronic studies, at least 10 rodents of each sex shall be used in each test group. For a chronic study, at least 20 male and 20 female rodents shall be used in each test group.

(A) If interim sacrifices are planned, the number of animals shall be increased by the number of animals

scheduled to be sacrificed during the course of the study.

(B) For a chronic study, the number of animals at the termination of the study must be adequate for a meaningful and valid statistical evaluation of chronic effects.

(v) A concurrent control group is required. This group shall be exposed to clean, filtered air under conditions identical to those used for the group exposed to the test atmosphere.

(vi) The same species/strain shall be used to make comparisons between fuel-only and fuel/additive mixture studies. If another species/strain is used, the tester shall provide justification for its selection.

(2) *Animal handling and care.* (i) A key element in the conduct of inhalation exposure studies is the proper handling and care of the test animal population. Therefore, the exposure conditions must conform strictly with the conditions for housing and animal care and use set forth in the *Guide*.

(ii) In whole-body exposure chambers, animals shall be housed in individual caging. The minimum cage size per animal will be in accordance with instructions set forth in the *Guide*.

(iii) Chambers shall be cleaned and maintained in accordance with recommendations and schedules set forth in the *Guide*.

(A) Observations shall be made daily with appropriate actions taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of animals found dead and isolation or sacrifice of weak or moribund animals). Exposure systems using head/nose-only exposure chambers require no special daily chamber maintenance. Chambers shall be inspected to ensure that they are clean, and that there are no obstructions in the chamber which would restrict air flow to the animals. Whole-body exposure chambers will be inspected on a minimum of twice daily, once before exposures and once after exposures.

(B) Signs of toxicity shall be recorded as they are observed, including the time of onset, degree, and duration.

(C) Cage-side observations shall include, but are not limited to: changes in skin, fur, eye and mucous membranes, respiratory, autonomic, and

central nervous systems, somatomotor activity, and behavioral patterns. Particular attention shall be directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma.

(iv) Food and water will be withheld from animals for head/nose-only exposure systems. For whole-body-exposure systems, water only may be provided. When the exposure generation system is not operating, food will be available *ad libitum*. During operation of the generation system, food will be withheld to avoid possible contamination by emissions.

(v) At the end of the study period, all survivors in the main study population shall be sacrificed. Moribund animals shall be removed and sacrificed when observed.

(3) *Concentration levels and selection.*

(i) In acute and subacute toxicity tests, at least three exposure concentrations and a control group shall be used and spaced appropriately to produce test groups with a range of toxic effects and mortality rates. The data shall be sufficient to produce a concentration-response curve and permit an acceptable estimation of the median lethal concentration.

(ii) In subchronic and chronic toxicity tests, testers shall use at least three different concentration levels, with a control exposure group, to determine a concentration-response relationship. Concentrations shall be spaced appropriately to produce test groups with a range of toxic effects. The concentration-response data may also be sufficient to determine a NOAEL, unless the result of a limit test precludes such findings. The criteria for selecting concentration levels has been published (40 CFR 798.2450 and 798.3260).

(A) The highest concentration shall result in toxic effects but not produce an incidence of fatalities which would prevent a meaningful evaluation of the study.

(B) The lowest concentration shall not produce toxic effects which are directly attributable to the test exposure. Where there is a useful estimation of human exposure, the lowest concentration shall exceed this.

(C) The intermediate concentration level(s) shall produce minimal observable toxic effects. If more than one intermediate concentration level is used, the concentrations shall be spaced to produce a gradation of toxic effects.

(D) In the low, intermediate, and control exposure groups, the incidence of fatalities shall be low to absent, so as not to preclude a meaningful evaluation of the results.

(4) Exposure chamber environmental conditions. The following environmental conditions in the exposure chamber are critical to the maintenance of the test animals: flow; temperature; relative humidity; lighting; and noise.

(i) Filtered and conditioned air shall be used during exposure, to dilute the exhaust emissions, and during non-exposure periods to maintain environmental conditions that are free of trace gases, dusts, and microorganisms on the test animals. Twelve to fifteen air changes per hour will be provided at all times to whole-body-exposure chambers. The minimum air flow rate for head/nose-only exposure chambers will be a function of the number of animals and the average minute volume of the animals:

$$Q_{\text{minimum}}(\text{L}/\text{min}) = 2 \times \text{number of animals} \times \text{average minute volume}$$

(see Cheng and Moss, 1989 in paragraph (f)(8) of this section).

(ii) Recommended ranges of temperature for various species are given in the *Guide*. The recommended temperature ranges will be used for establishing temperature conditions of whole-body-exposure chambers. For rodents in whole-body-exposure chambers, the recommended temperature is  $22\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$  and for rabbits, it is  $20\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$ . Temperature ranges have not been established for head/nose-only tubes; however, recommended maximum temperature limits have been established at the Inhalation Toxicology Research Institute (see Barr, 1988 in paragraph (f)(1) of this section). Maximum temperature for rats and mice in head/nose-only tubes is  $23\text{ }^{\circ}\text{C}$ .

(iii) *Relative humidity*. The relative humidity in the chamber air is important for heat balance and shall be maintained between 40 percent and 60 percent, but in certain instances, this

may not be practicable. Testers shall follow *Guide* recommends for a 30 percent to 70 percent relative humidity range for rodents in exposure chambers.

(iv) *Lighting*. Light intensity of 30 foot candles at 3 ft. from the floor of the exposure facility is recommended (see Rao, 1986 in paragraph (f)(16) of this section).

(5) *Exposure conditions*. Unless precluded by the requirements of a particular test protocol, animal subjects shall be exposed to the test atmosphere based on a nominal 5-day-per-week regimen, subject to the following rules:

(i) Each daily exposure must be at least 6 hours plus the time necessary to build the chamber atmosphere to 90 percent of the target exposure atmosphere. Interruptions of daily exposures caused by technical difficulties, if infrequent in occurrence and limited in duration, may be made up the same day by adding equivalent exposure time after the technical problem has been corrected and the exposure atmosphere restored to the required level.

(ii) Normally, no more than two non-exposure days may occur consecutively during the test period. However, if a third consecutive non-exposure day should occur due to circumstances beyond the tester's control, it may be remedied by adding a supplementary exposure day. Federal and other holidays do not constitute such circumstances. Whenever possible, a make-up day should be taken at the first opportunity, i.e., on the next day which would otherwise have been an intentional non-exposure day. If a compensatory day must be scheduled at the end of the standard test period, then it may occur either:

(A) Immediately following the last standard exposure day, with no intervening non-exposure days; or

(B) With up to two intervening non-exposure days, provided that no fewer than two consecutive compensatory exposure days are completed before the test is terminated and the animals sacrificed.

(iii) Except as allowed in paragraph (d)(5)(ii)(B) of this section, in no case shall there be fewer than four exposure days per week at any time during the test period.

(iv) A nominal 90-day (13-week) sub-chronic test period shall include no fewer than 63 total exposure days.

(6) *Exposure atmosphere.* (i) The exposure atmosphere shall be held as constant as is practicable and must be monitored continuously or intermittently, depending on the method of analysis, to ensure that exposure levels are at the target values or within stated limits during the exposure period. Sampling methodology will be determined based on the type of generation system and the type of exposure chamber system specified for the exposure study.

(A) Integrated samples of test atmosphere aerosol shall be taken daily during the exposure period from a single representative sample port in the chamber near the breathing zone of the animals. Gas samples shall be taken daily to determine concentrations (ppm) of the major vapor components of the test atmosphere including CO, CO<sub>2</sub>, NO<sub>x</sub>, SO<sub>2</sub>, and total hydrocarbons.

(B) To ensure that animals in different locations of the chamber receive a similar exposure atmosphere, distribution of an aerosol or vapor concentration in exposure chambers can be determined without animals during the developmental phase of the study, or it can be determined with animals early in the study. For head/nose-only exposure chambers, it may not be possible to monitor the chamber distribution during the exposure, because the exposure port contains the animal.

(C) During the development of the emissions generation system, particle size analysis shall be performed to establish the stability of an aerosol concentration with respect to particle size. Over the course of the exposure, analysis shall be conducted as often as is necessary to determine the consistency of particle size distribution.

(D) *Chamber rise and fall times.* The rise time required for the exposure concentration to reach 90 percent of the stable concentration after the generator is turned on, and the fall time when the chamber concentration decreases to 10 percent of the stable concentration after the generation system is stopped shall be determined in the developmental phase of the study. Time-integrated samples collected for

calculating exposure concentrations shall be taken after the rise time. The daily exposure time is exclusive of the rise or the fall time.

(ii) Instrumentation used for a given study will be determined based on the type of generation system and the type of exposure chamber system specified for the exposure study.

(A) For exhaust studies, combustion gases shall be sampled by collecting exposure air in bags and then analyzing the collected air sample to determine major components of the combustion gas using gas analyzers. Exposure chambers can also be connected to gas analyzers directly by using sampling lines and switching valves. Samples can be taken more frequently using the latter method. Aerosol instruments, such as photometers, or time-integrated gravimetric determination may be used to determine the stability of any aerosol concentration in the chamber.

(B) For evaporative emission studies, concentration of fuel vapors can usually be determined by using a gas chromatograph (GC) and/or infrared (IR) spectrometry. Grab samples for intermittent sampling can be taken from the chamber by using bubble samplers with the appropriate solvent to collect the vapors, or by collecting a small volume of air in a syringe. Intermediate or continuous monitoring of the chamber concentration is also possible by connecting the chamber with a GC or IR detector.

(7) Monitoring chamber environmental conditions may be performed by a computer system or by exposure system operating personnel.

(i) The flow-metering device used for the exposure chambers must be a continuous monitoring device, and actual flow measurements must be recorded at least every 30 minutes. Accuracy must be  $\pm 5$  percent of full scale range. Measurement of air flow through the exposure chamber may be accomplished using any device that has sufficient range to accurately measure the air flow for the given chamber. Types of flow metering devices include rotameters, orifice meters, venturi meters, critical orifices, and turbinometers (see Benedict, 1984 in

paragraph (f)(4) and Spitzer, 1984 in paragraph (f)(17) of this section).

(ii) *Pressure.* Pressure measurement may be accomplished using manometers, electronic pressure transducers, magnehelics, or similar devices (see Gillum, 1982 in paragraph (f)(10) of this section). Accuracy of the pressure device must be  $\pm 5$  percent of full scale range. Pressure measurements must be continuous and recorded at least every 30 minutes.

(iii) *Temperature.* The temperature of exposure chambers must be monitored continuously and recorded at least every 30 minutes. Temperature may be measured using thermometers, RTD's, thermocouples, thermistors, or other devices (see Benedict, 1984 in paragraph (f)(4) of this section). It is necessary to incorporate an alarm system into the temperature monitoring system. The exposure operators must be notified by the alarm system when the chamber temperature exceeds 26.7 °C (80 °F). The exposure must be discontinued and emergency procedures enacted to immediately reduce temperatures or remove test animals from high temperature environment when chamber temperatures exceed 29 °C. Accuracy of the temperature monitoring device will be  $\pm 1$  °C for the temperature range of 20-30 °C.

(iv) *Relative humidity.* The relative humidity of exposure chambers must be monitored continuously and recorded at least every 30 minutes. Relative humidity may be measured using various devices (see Chaddock, 1985 in paragraph (f)(6) of this section).

(v) Lighting shall be measured quarterly, or once at the beginning, middle, and end of the study for shorter studies.

(vi) Noise level in the exposure chamber(s) shall be measured quarterly, or once at the beginning, middle, and end of the study for shorter studies.

(vii) Oxygen content is critical, especially in nose-only chamber systems, and shall be greater than or equal to 19 percent in the test cages. An oxygen sensor shall be located at a single position in the test chamber and a lower alarm limit of 18 percent shall be used to activate an alarm system.

(8) *Safety procedures and requirements.* In the case of potentially explosive test

substance concentrations, care shall be taken to avoid generating explosive atmospheres.

(i) It is mandatory that the upper explosive limit (UEL) and lower explosive limit (LEL) for the fuel and/or fuel additive(s) that are being tested be determined. These limits can be found in the material safety data sheets (MSDS) for each substance and in various reference texts. The air concentration of the fuel or additive-base fuel mixture in the generation system, dilution/delivery system, and the exposure chamber system shall be calculated to ensure that explosive limits are not present.

(ii) Storage, handling, and use of fuels or fuel/additive mixtures shall follow guidelines given in 29 CFR 1910.106.

(iii) Monitoring for carbon monoxide (CO) levels is mandatory for combustion systems. CO shall be continuously monitored in the immediate area of the engine/vehicle system and in the exposure chamber(s).

(iv) Air samples shall be taken quarterly in the immediate area of the vapor generation system and the exposure chamber system, or once at the beginning, middle, and end of the study for shorter studies. These samples shall be analyzed by methods described in paragraph (d)(6)(ii)(B) of this section.

(v) With the presence of fuels and/or fuel additives, all electrical and electronic equipment must be grounded. Also, the dilution/delivery system and chamber exposure system must be grounded. Guidelines for grounding are given in 29 CFR 1910.304.

(9) *Quality control and quality assurance procedures—*(i) *Standard operating procedures (SOPs).* SOPs for exposure operations, sampling instruments, animal handling, and analytical methods shall be written during the developmental phase of the study.

(ii) Technicians/operators shall be trained in exposure operation, maintenance, and documentation, as appropriate, and their training shall be documented.

(iii) Flow meters, sampling instruments, and balances used in the inhalation experiments shall be calibrated with standards during the developmental phase to determine their sensitivity, detection limits, and linearity.

During the exposure period, instruments shall be checked for calibration and documented to ensure that each instrument still functions properly.

(iv) The mean exposure concentration shall be within 10 percent of the target concentration on 90 percent or more of exposure days. The coefficient of variation shall be within 25 percent of target on 90 percent or more of exposure days. For example, a manufacturer might determine a mean exposure concentration of its product's exposure emissions by identifying "marker" compound(s) typical of the emissions of the fuel or fuel/additive mixture under study as a surrogate for the total of individual compounds in those exposure emissions. The manufacturer would note any concentration changes in the level of the "marker" compound(s) in the sample's daily emissions for biological testing.

(v) The spatial variation of the chamber concentration shall be 10 percent, or less. If a higher spatial variation is observed during the developmental phase, then air mixing in the chamber shall be increased. In any case, animals shall be rotated among the various cages in the exposure chamber(s) to insure each animal's uniform exposure during the study.

(e) *Data and reporting.* Data shall be summarized in tabular form, showing for each group the number of animals at the start of the test, the number of animals showing lesions, the types of lesions, and the percentage of animals displaying each type of lesion.

(1) *Treatment of results.* All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statistical methods shall be selected during the design of the study.

(2) *Evaluation of results.* The findings of an inhalation toxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the observed toxic effects and the necropsy and histopathological findings. The evaluation will include the relationship between the concentration of the test atmosphere and the duration of exposure, and the severity of abnormalities, gross lesions, identified target organs,

body weight changes, effects on mortality and any other general or specific toxic effects.

(3) *Test conditions.* (i) The exposure apparatus shall be described, including:

(A) The vehicle/engine design and type, the dynamometer, the cooling system, if any, the computer control system, and the dilution system for exhaust emission generation;

(B) The evaporative emissions generator model, type, or design and its dilution system; and

(C) Other test conditions, such as the source and quality of mixing air, fuel or fuel/additive mixture used, treatment of exhaust air, design of exposure chamber and the method of housing animals in a test chamber shall be described.

(ii) The equipment for measuring temperature, humidity, particulate aerosol concentrations and size distribution, gas analyzers, fuel vapor concentrations, chamber distribution, and rise and fall time shall be described.

(iii) *Daily exposure results.* The daily record shall document the date, the start and stop times of the exposure, number of samples taken during the day, daily concentrations determined, calibration of instruments, and problems encountered during the exposure. The daily exposure data shall be signed by the exposure operator and reviewed and signed by the exposure supervisor responsible for the study.

(4) Exposure data shall be tabulated and presented with mean values and a measure of variability (e.g., standard deviation), and shall include:

(i) Airflow rates through the inhalation equipment;

(ii) Temperature and humidity of air;

(iii) Chamber concentrations in the chamber breathing zone;

(iv) Concentration of combustion exhaust gases in the chamber breathing zone;

(v) Particle size distribution (e.g., mass median aerodynamic diameter and geometric standard deviation from the mean);

(vi) Rise and fall time;

(vii) Chamber concentrations during the non-exposure period; and

(viii) Distribution of test substance in the chamber.

(5) *Animal data.* Tabulation of toxic response data by species, strain, sex and exposure level for:

- (i) Number of animals exposed;
- (ii) Number of animals showing signs of toxicity; and
- (iii) Number of animals dying.

(f) *References.* For additional background information on this exposure guideline, the following references should be consulted.

(1) Barr, E.B. (1988) Operational Limits for Temperature and Percent Oxygen During HM Nose-Only Exposures—Emergency Procedures [interoffice memorandum]. Albuquerque, NM: Lovelace Inhalation Toxicology Research Institute; May 13.

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## Environmental Protection Agency

## § 79.62

(20) FEDERAL REGISTER, 42 FR 26748, May 25, 1977.

[59 FR 33093, June 27, 1994, as amended at 61 FR 58746, Nov. 18, 1996; 61 FR 36512, July 11, 1996]

### § 79.62 Subchronic toxicity study with specific health effect assessments.

(a) *Purpose*—(1) *General toxicity*. This subchronic inhalation study is designed to determine a concentration-response relationship for potential toxic effects in rats resulting from continuous or repeated inhalation exposure to vehicle/engine emissions over a period of 90 days. A subgroup of perfusion-fixed animals is required, in addition to the main study population, for more exacting organ and tissue histology. This test will provide screening information on target organ toxicities and on concentration levels useful for running chronic studies and establishing exposure criteria. Initial information on effective concentrations/exposures of the test atmosphere may be determined from the literature of previous studies or through concentration range-finding trials prior to starting this study. This health effects screening test is not capable of directly determining those effects which have a long latency period for development (e.g., carcinogenicity and life-shortening), though it may permit the determination of a no-observed-adverse-effect level, or NOAEL.

(2) *Specific health effects assessments (HEAs)*. These supplemental studies are designed to determine the potential for reproductive/teratologic, carcinogenic, mutagenic, and neurotoxic health effect outcomes from vehicle/engine emission exposures. They are done in combination with the subchronic toxicity study and paragraph (c) of this section or may be done separately as outlined by the appropriate test guideline.

(i) *Fertility assessment/teratology*. The fertility assessment is an *in vivo* study designed to provide information on potential health hazards to the fetus arising from the mother's repeated exposure to vehicle/engine emissions before and during her pregnancy. By including a mating of test animals, the study provides preliminary data on the effects of repeated vehicle/engine emissions exposure on gonadal function,

conception, and fertility. The fertility assessment/teratology guideline is found in § 79.63.

(ii) *Micronucleus (MN) Assay*. The MN assay is an *in vivo* cytogenetic test which gives information on potential carcinogenic and/or mutagenic effects of exposure to vehicle/engine emissions. The MN assay detects damage to the chromosomes or mitotic apparatus of cells in the tissues of a test subject exposed repeatedly to vehicle/engine emissions. The assay is based on an increase in the frequency of micronucleated erythrocytes found in bone marrow from treated animals compared to that of control animals. The guideline for the MN assay is found in § 79.64.

(iii) *Sister Chromatid Exchange (SCE) Assay*. The SCE assay is an *in vivo* analysis which gives information on potential mutagenic and/or carcinogenic effects of exposure to vehicle/engine emissions. The assay detects the ability of a chemical to enhance the exchange of DNA between two sister chromatids of a duplicating chromosome. This assay uses peripheral blood lymphocytes isolated from an exposed rodent test species and grown to confluence in cell culture. The guideline for the SCE assay is found in § 79.65.

(iv) *Neurotoxicity (NTX) measures*. NTX measures include (A) histopathology of specified central and peripheral nervous system tissues taken from emission-exposed rodents, and (B) an assay of brain tissue levels of glial fibrillary acidic protein (GFAP), a major filament protein of astrocytes, from emission-exposed rodents. The guidelines for the neurohistopathology and GFAP studies are found in § 79.66 and § 79.67, respectively.

(b) *Definitions*. For the purposes of this section, the following definitions apply:

*No-observed-adverse-effect-level (NOAEL)* means the maximum concentration used in a test which produces no observed adverse effects. A NOAEL is expressed in terms of weight or volume of test substance given daily per unit volume of air ( $\mu\text{g}/\text{L}$  or ppm).