

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).

Subchronic inhalation toxicity means the adverse effects occurring as a result of the continuous or repeated daily exposure of experimental animals to a chemical by inhalation for part (approximately 10 percent) of a life span.

(c) *Principle of the test method.* As long as none of the requirements of any study are violated by the combination, one or more HEAs may be combined with the general toxicity study through concurrent exposures of their study populations and/or by sharing the analysis of the same animal subjects. Requirements duplicated in combined studies need not be repeated. Guidelines for combining HEAs with the general toxicity study are as follows.

(1) *Fertility assessment.* (i) The number of study animals in the test population is increased when the fertility assessment is run concurrently with the 90-day toxicity study. A minimum of 40 females per test group shall undergo vaginal lavage daily for two weeks before the start of the exposure period. The resulting wet smears are examined to cull those animals which are acyclic. Twenty-five females shall be randomly assigned to a for-breeding group with the balance of females assigned to a group for histopathologic examination.

(ii) All test groups are exposed over a period of 90 days to various concentrations of the test atmosphere for a minimum of six hours per day. After seven weeks of exposures, analysis of vaginal cell smears shall resume on a daily basis for the 25 for-breeding females and shall continue for a period of four weeks or until each female in the group is confirmed pregnant. Following the ninth week of exposures, each for-breeding female is housed overnight with a single study male. Matings shall continue for as long as two weeks, or until pregnancy is confirmed (pregnancy day 0). Pregnant females are only exposed through day 15 of their pregnancy while daily exposures continue throughout the course of the study for non-pregnant females and study males.

(iii) On pregnancy day 20, pregnant females are sacrificed and their uteri are examined. Pregnancy status and fetal effects are recorded as described

in § 79.63. At the end of the exposure period, all males and non-pregnant females are sacrificed and necropsied. Testes and epididymal tissue samples are taken from five perfusion-fixed test subjects and histopathological examinations are carried out on the remainder of the non-pregnant females and study males.

(2) *Carcinogenicity/mutagenicity (C/M) assessment.* When combined with the subchronic toxicity study, the main study population is used to perform both the *in vivo* MN and SCE assays. Because of the constant turnover of the cells to be analyzed in these assays, a separate study population may be used for this assessment. A study population needs only to be exposed a minimum of four weeks. At exposure's end, ten animals per exposure and control groups are anaesthetized and heart punctures are performed on all members. After separating blood components, individual lymphocyte cell cultures are set up for SCE analysis. One femur from each study subject is also removed and the marrow extracted. The marrow is smeared onto a glass slide, and stained for analysis of micronuclei in erythrocytes.

(3) *Neurotoxicity (NTX) measures.* (i) When combined with this subchronic toxicity study, test animals designated for whole-body perfusion fixation/lung histology and exposed as part of the main animal population are used to perform the neurohistology portion of these measures. After the last exposure period, a minimum of ten animals from each exposure group shall be preserved *in situ* with fixative. Sections of brain, spinal cord, and proximal sciatic or tibial nerve are then cut, processed further in formalin, and mounted for viewing under a light microscope. Fibers from the sciatic or tibial nerve sample are teased apart for further analysis under the microscope.

(ii) *GFAP assay.* After the last exposure period, a minimum of ten rodents from each exposure group shall be sacrificed, and their brains excised and divided into regions. The tissue samples are then applied to filter paper, washed with anti-GFAP antibody, and visualized with a radio-labelled Protein A. The filters are quantified for degree of

immunoreactivity between the antibody and GFAP in the tissue samples. A non-radioactive ELISA format is also referenced in the GFAP guideline cited in paragraph (a)(2)(iv) of this section. Note: Because the GFAP assay requires fresh, *i.e.*, non-preserved, brain tissue, the number of test animals may need to be increased to provide an adequate number of test subjects to complete the histopathology requirements of both the GFAP and the general toxicity portion of the 90-day inhalation study.

(iii) The start of the exposure period for the NTX measures study population may be staggered from that of the main study group to more evenly distribute the analytical work required in both study populations. The exposures would remain the same in all other respects.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and sex*. The rat is the recommended species. If another rodent species is used, the tester shall provide justification for its selection. Both sexes shall be used in any assessment unless it is demonstrated that one sex is refractory to the effects of exposure.

(ii) *Age and number*. Rats shall be at least ten weeks of age at the beginning of the study exposure. The number of animals necessary for individual health effect outcomes is as follows:

(A) Thirty rodents per concentration level/group, fifteen of each sex, shall be used to satisfy the reporting requirements of the 90-day toxicity study. Ten animals per concentration level/group shall be designated for whole body perfusion with fixative (by gravity) for lung studies, and neurohistology and testes studies, as appropriate.

(B) Thirty-five rodents, 25 females and ten males, shall be added for each test concentration or control group when combining a 90-day toxicity study with a fertility assessment.

(C) The tester shall provide a group of 10 animals (five animals per sex per experimental/control groups) in addition to the main test population when performing the GFAP neurotoxicity HEA.

(2) *Recovery group*. The manufacturer shall include a group of 20 animals (10 animals per sex) in the test population,

exposing them to the highest concentration level for the entire length of the study's exposure period. This group shall then be observed for reversibility, persistence, or delayed occurrence of toxic effects during a post-exposure period of not less than 28 days.

(3) *Inhalation exposure*. (i) All data developed within this study shall be in accordance with good laboratory practice provisions under § 79.60.

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in § 79.61.

(4) *Observation of animals*. (i) All toxicological (e.g., weight loss) and neurological signs (e.g., motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly for all study animals. Animals shall be weighed weekly.

(ii) The following is a minimal list of measures that shall be noted:

(A) Body weight;

(B) Subject's reactivity to general stimuli such as removal from the cage or handling;

(C) Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements in the home cage;

(D) Descriptions and incidence of posture and gait abnormalities observed in the home cage;

(E) Description and incidence of any unusual or abnormal behaviors, excessive or repetitive actions (stereotypies), emaciation, dehydration, hypotonia or hypertonia, altered fur appearance, red or crusty deposits around the eyes, nose, or mouth, and any other observations that may facilitate interpretation of the data.

(iii) Any animal which dies during the test is necropsied as soon as possible after discovery.

(5) *Clinical examinations*. (i) The following examinations shall be performed on the twenty animals designated as the 90-day study population, exclusive of pregnant dams and those study animals targeted for perfusion by gravity:

(A) The following hematology determinations shall be carried out at least two times during the test period (after 30 days of exposure and just prior to

terminal sacrifice at the end of the exposure period): hematocrit, hemoglobin concentration, erythrocyte count, total and differential leukocyte count, and a measure of clotting potential such as prothrombin time, thromboplastin time, or platelet count.

(B) Clinical biochemistry determinations on blood shall be carried out at least two times during the test period, after 30 days of exposure and just prior to terminal sacrifice at the end of the exposure period, on all groups of animals including concurrent controls. Clinical biochemical testing shall include assessment of electrolyte balance, carbohydrate metabolism, and liver and kidney function. The selection of specific tests will be influenced by observations on the mode of action of the substance. In the absence of more specific tests, the following determinations may be made: calcium, phosphorus, chloride, sodium, potassium, fasting glucose (with period of fasting appropriate to the species), serum alanine aminotransferase, serum aspartate aminotransferase, sorbitol dehydrogenase, gamma glutamyl transpeptidase, urea nitrogen, albumen, blood creatinine, methemoglobin, bile acids, total bilirubin, and total serum protein measurements. Additional clinical biochemistry shall be employed, where necessary, to extend the investigation of observed effects, e.g., analyses of lipids, hormones, acid/base balance, and cholinesterase activity.

(ii) The following examinations shall initially be performed on the high concentration and control groups only:

(A) Ophthalmological examination, using an ophthalmoscope or equivalent suitable equipment, shall be made prior to exposure to the test substance and at the termination of the study. If changes in the eyes are detected, all animals shall be examined.

(B) Urinalysis is not required on a routine basis, but shall be done when there is an indication based on expected and/or observed toxicity.

(iii) Preservation by whole-body perfusion of fixative into the anaesthetized animal for lung histology of ten animals from the 90-day study population for each experimental and control group.

(6) *Gross pathology.* With the exception of the whole body perfusion-fixed test animals cited in paragraph (d)(1)(ii)(A) of this section, all rodents shall be subjected to a full gross necropsy which includes examination of the external surface of the body, all orifices and the cranial, thoracic, and abdominal cavities and their contents. Gross pathology shall be performed on the following organs and tissues:

(i) The liver, kidneys, lungs, adrenals, brain, and gonads, including uterus, ovaries, testes, epididymides, seminal vesicles (with coagulating glands), and prostate, constitute the group of target organs for histology and shall be weighed as soon as possible after dissection to avoid drying. In addition, for other than rodent test species, the thyroid with parathyroids, when present, shall also be weighed as soon as possible after dissection to avoid drying.

(ii) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for possible future histopathological examination: All gross lesions; lungs—which shall be removed intact, weighed, and treated with a suitable fixative to ensure that lung structure is maintained (perfusion with the fixative is considered to be an effective procedure); nasopharyngeal tissues; brain—including sections of medulla/pons, cerebellar cortex, and cerebral cortex; pituitary; thyroid/parathyroid; thymus; trachea; heart; sternum with bone marrow; salivary glands; liver; spleen; kidneys; adrenals; pancreas; reproductive organs: uterus; cervix; ovaries; vagina; testes; epididymides; prostate; and, if present, seminal vesicles; aorta; (skin); gall bladder (if present); esophagus; stomach; duodenum; jejunum; ileum; cecum; colon; rectum; urinary bladder; representative lymph node; (mammary gland); (thigh musculature); peripheral nerve/tissue; (eyes); (femur—including articular surface); (spinal cord at three levels—cervical, midthoracic, and lumbar); and (zygomatic and exorbital lachrymal glands).

(7) *Histopathology.* Histopathology shall be performed on the following organs and tissues from all rodents:

(i) All gross lesions.

(ii) Respiratory tract and other organs and tissues, listed in paragraph (d)(6)(ii) of this section (except organs/tissues in parentheses), of all animals in the control and high dose groups.

(iii) The tissues mentioned in parentheses, listed in paragraph (d)(6)(ii) of this section, if indicated by signs of toxicity or target organ involvement.

(iv) Lungs of animals in the low and intermediate dose groups shall also be subjected to histopathological examination, primarily for evidence of infection since this provides a convenient assessment of the state of health of the animals.

(v) Lungs and trachea of the whole-body perfusion-fixed test animals cited in paragraph (d)(1)(ii)(A) of this section are examined for inhaled particle distribution.

(e) Interpretation of results. All observed results, quantitative and incidental, shall be evaluated by an appropriate statistical method. The specific methods, including consideration of statistical power, shall be selected during the design of the study.

(f) *Test report.* In addition to the reporting requirements as specified under §§ 79.60 and 79.61(e), the following individual animal data information shall be reported:

(1) Date of death during the study or whether animals survived to termination.

(2) Date of observation of each abnormal sign and its subsequent course.

(3) Individual body weight data, and group average body weight data vs. time.

(4) Feed consumption data, when collected.

(5) Hematological tests employed and all results.

(6) Clinical biochemistry tests employed and all results.

(7) Necropsy findings.

(8) Type of stain/fixative and procedures used in preparing tissue samples.

(9) Detailed description of all histopathological findings.

(10) Statistical treatment of the study results, where appropriate.

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

(1) 40 CFR 798.2450, Inhalation toxicity.

(2) 40 CFR 798.2675, Oral Toxicity with Satellite Reproduction and Fertility Study.

(3) General Statement of Work for the Conduct of Toxicity and Carcinogenicity Studies in Laboratory Animals (revised April, 1987/modifications through January, 1990) appendix G, National Toxicology Program—U.S. Dept. of Health and Human Services (Public Health Service), P.O. Box 12233, Research Triangle Park, NC 27709.

[59 FR 33093, June 27, 1994, as amended at 63 FR 63793, Nov. 17, 1998]

§ 79.63 Fertility assessment/teratology.

(a) *Purpose.* Fertility assessment/teratology is an *in vivo* study designed to provide information on potential health hazards to the fetus arising from the mother's repeated inhalation 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. Since this is a one-generation test that ends with examination of full-term fetuses, but not of live pups, it is not capable of determining effects on reproductive development which would only be detected in viable offspring of treated parents.

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

Developmental toxicity means the ability of an agent to induce *in utero* death, structural or functional abnormalities, or growth retardation after contact with the pregnant animal.

Estrous cycle means the periodic recurrence of the biological phases of the female reproductive system which prepare the animal for conception and the development of offspring. The phases of the estrous cycle for a particular animal can be characterized by the general condition of the cells present in the vagina and the presence or absence of various cell types.