levels—cervical, midthoracic, and lumbar); and (zymbal 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.

Vaginal cytology evaluation means the use of wet vaginal cell smears to determine the phase of a test animal's estrous cycle and the potential for adverse exposure effects on the regularity of the animal's cycle. In the rat, common cell types found in the smears correlate well with the various stages of the estrous cycle and to changes occurring in the reproductive tract.

(c) *Principle of the test method.* (1) For a two week period before exposures start, daily vaginal cell smears are examined from a surplus of female test animals to identify and cull those females which are acyclic. After culling, testers shall randomly assign at each exposure concentration (including unexposed) a minimum of twenty-five females for breeding and fifteen non-bred females for later histologic evaluation. Test animals shall be exposed by inhalation to graduated concentrations of the test atmosphere for a minimum of six hours per day over the next 13 weeks. Males and females in both test and control groups are mated after nine weeks of exposure. Exposures for pregnant females continue through gestation day 15, while exposures for males and all non-pregnant females shall continue for the full exposure period.

(2) Beginning two weeks before the start of the mating period, daily vaginal smears resume for all to-be-bred females to characterize their estrous cycles. This will continue for four weeks or until a rat's pregnancy is confirmed, *i.e.*, day 0, by the presence of sperm in the cell smear. On pregnancy day 20, shortly before the expected date of delivery, each pregnant female is sacrificed, her uterus removed, and the contents examined for embryonic or fetal deaths, and live fetuses. At the end of the exposure period, males and all non-pregnant females shall be weighed, and various organs and tissues, as appropriate, shall be removed and weighed, fixed with stain, and sectioned for viewing under a light microscope.

(3) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in § 79.62.

(d) *Limit test.* If a test at one dose level of the highest concentration that can be achieved while maintaining a particle size distribution with a mass median aerodynamic diameter (MMAD) of 4 micrometers (μm) or less, using the procedures described in section 79.60 of this part produces no observable toxic effects and if toxicity would not be expected based upon data of structurally related compounds, then a full study using three dose levels might not be necessary. Expected human exposure though may indicate the need for a higher dose level.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The rat is the preferred species. Strains with low fecundity shall not be used and the candidate species shall be characterized for its sensitivity to developmental toxins. If another rodent species is used, the tester shall provide justification for its selection.

(ii) Animals shall be a minimum of 10 weeks old at the start of the exposure period.

(iii) *Number and sex.* Each test and control group shall have a minimum of 25 males and 40 females. In order to ensure that sufficient pups are produced to permit meaningful evaluation of the potential developmental toxicity of the test substance, twenty pregnant test animals are required for each exposure and control level.

(2) *Observation period.* The observation period shall be 13 weeks, at a minimum.

(3) *Concentration levels and concentration selection.* (i) To select the appropriate concentration levels, a pilot or trial study may be advisable. Since pregnant animals have an increased minute ventilation as compared to non-pregnant animals, it is recommended that the trial study be conducted in pregnant animals. Similarly, since presumably the minute ventilation will vary with progression of pregnancy, the animals should be exposed during the same period of gestation as in the main study. It is not always necessary, though, to carry out a trial study in pregnant animals. Comparisons between the results of a trial study in non-pregnant animals, and the main study in pregnant animals will demonstrate whether or not the test

substance is more toxic in pregnant animals. In the trial study, the concentration producing embryonic or fetal lethalities or maternal toxicity should be determined.

(ii) The highest concentration level shall induce some overt maternal toxicity such as reduced body weight or body weight gain, but not more than 10 percent maternal deaths.

(iii) The lowest concentration level shall not produce any grossly observable evidence of either maternal or developmental toxicity.

(4) *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.

(iii) Pregnant females shall be exposed to the test atmosphere on each and every day between (and including) the first and fifteenth day of gestation.

(f) *Test performance*—(1) *Study conduct.* Directions specific to this study are:

(i) The duration of exposure shall be at least six hours daily, allowing appropriate additional time for chamber equilibrium.

(ii) Where an exposure chamber is used, its design shall minimize crowding of the test animals. This is best accomplished by individual caging.

(iii) Pregnant animals shall not be subjected to beyond the minimum amount of stress. Since whole-body exposure appears to be the least stressful mode of exposure, it is the preferred method. In general oronasal or head-only exposure, which is sometimes used to avoid concurrent exposure by the dermal or oral routes, is not recommended because of the associated 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/reasoning for its selection.

(iv) Measurements shall be made at least every other day of food consumption for all animals in the study. Males and females shall be weighed on the first day of exposure and 2–3 times per week thereafter, except for pregnant dams.

(v) The test animal housing, mating, and exposure chambers shall be operated on a twenty-four hour lighting schedule, with twelve hours of light and twelve hours of darkness. Test animal exposure shall only occur during the light portion of the cycle.

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

(vii) Females showing signs of abortion or premature delivery shall be sacrificed and subjected to a thorough macroscopic examination.

(viii) Animals that die or are euthanized because of morbidity will be necropsied promptly.

(2) *Vaginal cytology.* (i) For a two week period before the mating period starts, each female in the to-be-bred population shall undergo a daily saline vaginal lavage. Two wet cell smears from this lavage shall be examined daily for each subject to determine a baseline pattern of estrus. Testers shall avoid excessive handling and roughness in obtaining the vaginal cell samples, as this may induce a condition of pseudo-pregnancy in the test animals.

(ii) This will continue for four weeks or until day 0 of a rat's pregnancy is confirmed by the presence of sperm in the cell smear.

(3) *Mating and fertility assessment.* (i) Beginning nine weeks after the start of exposure, each exposed and control group female (exclusive of the histology group females) shall be paired during non-exposure hours with a male from the same exposure concentration group. Matings shall continue for a period of two weeks, or until all mated females are determined to be pregnant. Mating pairs shall be clearly identified.

(ii) Each morning, including weekends, cages shall be examined for the presence of a sperm plug. When found, this shall mark gestation day 0 and pregnancy shall be confirmed by the presence of sperm in the day's wet vaginal cell smears.

(iii) Two weeks after mating is begun, or as females are determined to be pregnant, bred animals are returned to pre-mating housing. Daily exposures continues through gestation day 15 for all pregnant females or through the

balance of the exposure period for nonpregnant females and all males.

(iv) Those pairs which fail to mate shall be evaluated in the course of the study to determine the cause of the apparent infertility. This may involve such procedures as additional opportunities to mate with a proven fertile partner, histological examination of the reproductive organs, and, in males, examination of the spermatogenic cycles. The stage of estrus for each nonpregnant female in the breeding group will be determined at the end of the exposure period.

(4) All animals in the histology group shall be subject to histopathologic examination at the end of the study's exposure period.

(g) *Treatment of results.* (1) 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.

(2) Data and reporting. In addition to the reporting requirements specified under §§ 79.60 and 79.61, the final test report must include the following information:

(i) *Gross necropsy.* (A) All animals shall be subjected to a full necropsy which includes examination of the external surface of the body, all orifices, and the cranial, thoracic, and abdominal cavities and their contents. Special attention shall be directed to the organs of the reproductive system.

(B) The liver, kidneys, adrenals, pituitary, uterus, vagina, ovaries, testes, epididymides and seminal vesicles (with coagulating glands), and prostate shall be weighed wet, as soon as possible after dissection, to avoid drying.

(*i*) At the time of sacrifice on gestation day 20 or at death during the study, each dam shall be examined macroscopically for any structural abnormalities or pathological changes which may have influenced the pregnancy.

(*ii*) The contents of the uterus shall be examined for embryonic or fetal deaths and the number of viable fetuses. Gravid uterine weights need not be obtained from dead animals where decomposition has occurred. The degree of resorption shall be described in order to help estimate the relative time of death.

(*iii*) The number of corpora lutea shall be determined in each pregnant dam.

(*iv*) Each fetus shall be weighed, all weights recorded, and mean fetal weights determined.

(*v*) Each fetus shall be examined externally and the sex determined.

(*vi*) One-half of the rat fetuses in each litter shall be examined for skeletal anomalies, and the remaining half shall be examined for soft tissue anomalies, using appropriate methods.

(ii) *Histopathology.* (A) Histopathology on vagina, uterus, ovaries, testes, epididymides, seminal vesicles, and prostate as appropriate for all males and histology group females in the control and high concentration groups and for all animals that died or were euthanized during the study. If abnormalities or equivocal results are seen in any of these organs/tissues, the same organ/tissue from test animals in lower concentration groups shall be examined.

NOTE: Testes, seminal vesicles, epididymides, and ovaries, at a minimum, shall be examined in perfusion-fixed (pressure or gravity method) test subjects, when available.

(B) All gross lesions in all study animals shall be examined.

(C) As noted under mating procedures, reproductive organs of animals suspected of infertility shall be subject to microscopic examination.

(D) The following organs and tissues, or representative samples thereof, shall be preserved in a suitable medium for future histopathological examination: all gross lesions; vagina; uterus; ovaries; testes; epididymides; seminal vesicles; prostate; liver; and kidneys/adrenals.

(3) *Evaluation of results.* (i) The findings of a developmental toxicity study shall be evaluated in terms of the observed effects and the exposure levels producing effects. It is necessary to consider the historical developmental toxicity data on the species/strain tested.

(ii) There are several criteria for determining a positive result for reproductive/teratologic effects; a statistically significant dose-related decrease in the weight of the testes for treated subjects over control subjects, a decrease in neonatal viability, a significant change in the presence of soft tissue or skeletal abnormalities, or an increased rate of embryonic or fetal resorption or death. Other criteria, e.g., lengthening of the estrous cycle or the time spent in any one stage of estrus, changes in the proportion of viable male vs female fetuses or offspring, the number and type of cells in vaginal smears, or pathologic changes found during gross or microscopic examination of male or female reproductive organs may be based upon detection of a reproducible and statistically significant positive response for that evaluation parameter. A positive result indicates that, under the test conditions, the test substance does induce reproductive organ or fetal toxicity in the test species.

(iii) A test substance which does not produce either a statistically significant dose-related change in the reproductive organs or cycle or a statistically significant and reproducible positive response at any one of the test points may not induce reproductive organ toxicity in this test species, but further investigation , e.g., to establish absorption and bioavailability of the test substance, should be considered.

(h) *Test report.* In addition to the reporting requirements as specified under 40 CFR 79.60 and the vehicle emissions inhalation toxicity guideline as published in 40 CFR 79.61, the following specific information shall be reported:

(1) *Individual animal data.* (i) Time of death during the study or whether animals survived to termination.

(ii) Date of onset and duration of each abnormal sign and its subsequent course.

(iii) Feed and body weight data.

(iv) Necropsy findings.

(v) Male test subjects.

(A) Testicle weight, and body weight: testicle weight ratio.

(B) Detailed description of all histopathological findings, especially for the testes and the epididymides.

(vi) Female test subjects.

(A) Uterine weight data.

(B) Beginning and ending collection dates for vaginal cell smears.

(C) Estrous cycle length compared within and between groups including mean cycle length for groups.

(D) Percentage of time spent in each stage of cycle.

(E) Stage of estrus at time of mating/sacrifice and proportion of females in estrus between concentration groups.

(F) Detailed description of all histopathological findings, especially for uterine/ovary samples.

(vii) Pregnancy and litter data. Toxic response data by exposure level, including but not limited to, indices of fertility and time-to-mating, including the number of days until mating and the number of full or partial estrous cycles until mating.

(A) Number of pregnant animals,

(B) Number and percentage of live fetuses, resorptions.

(viii) *Fetal data.* (A) Numbers of each sex.

(B) Number of fetuses with any soft tissue or skeletal abnormalities.

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

(3) Statistical treatment of the study results.

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

(1) 40 CFR 798.2675, Oral Toxicity with Satellite Reproduction and Fertility Study.

(2) 40 CFR 798.4350, Inhalation Developmental Toxicity Study.

(3) Chapin, R.E. and J.J. Heindel (1993) Methods in Toxicology, Vol. 3, Parts A and B: Reproductive Toxicology, Academic Press, Orlando, FL.

(4) Gray, L.E., et al. (1989) "A Dose-Response Analysis of Methoxychlor-Induced Alterations of Reproductive Development and Function in the Rat" Fund. App. Tox. 12, 92–108.

(5) Leblond, C.P. and Y. Clermont (1952) "Definition of the Stages of the Cycle of the Seminiferous Epithelium of the Rat." Ann. N. Y. Acad. Sci. 55:548–73.

(6) Morrissey, R.E., et al. (1988) "Evaluation of Rodent Sperm, Vaginal Cytology, and Reproductive Organ Weight Data from National Toxicology

Program 13-week Studies." Fundam. Appl. Toxicol. 11:343–358.

(7) Russell, L.D., Ettlin, R.A., Sinhattikim, A.P., and Clegg, E.D (1990) Histological and Histopathological Evaluation of the Testes, Cache River Press, Clearwater, FL.

[59 FR 33093, June 27, 1994, as amended at 61 FR 36513, July 11, 1996]

§ 79.64 *In vivo* micronucleus assay.

(a) *Purpose.* The micronucleus assay is an *in vivo* cytogenetic test which uses erythrocytes in the bone marrow of rodents to detect chemical damage to the chromosomes or mitotic apparatus of mammalian cells. As the erythroblast develops into an erythrocyte (red blood cell), its main nucleus is extruded and may leave a micronucleus in the cell body; a few micronuclei form under normal conditions in blood elements. This 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 visualization of micronuclei is facilitated in these cells because they lack a main nucleus.

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

Micronuclei mean small particles consisting of acentric fragments of chromosomes or entire chromosomes, which lag behind at anaphase of cell division. After telophase, these fragments may not be included in the nuclei of daughter cells and form single or multiple micronuclei in the cytoplasm.

Polychromatic erythrocyte (PCE) means an immature red blood cell that, because it contains RNA, can be differentiated by appropriate staining techniques from a normochromatic erythrocyte (NCE), which lacks RNA. In one to two days, a PCE matures into a NCE.

(c) *Test method*—(1) *Principle of the test method.* (i) Groups of rodents are exposed by the inhalation route for a minimum of 6 hours/day over a period of not less than 28 days to three or more concentrations of a test substance in air. Groups of animals are sacrificed at the end of the exposure period and femoral bone marrow is extracted. The bone marrow is then smeared onto glass slides, stained, and PCEs are scored for micronuclei. Researchers may need to run a trial at the highest tolerated concentration of the test atmosphere to optimize the sample collection time for micronucleated cells.

(ii) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions in § 79.62.

(2) *Species and strain.* (i) The rat is the recommended test animal. Other rodent species may be used in this assay, but use of that species will be justified by the tester.

(ii) If a strain of mouse is used in this assay, the tester shall sample peripheral blood from an appropriate site on the test animal, e.g., the tail vein, as a source of normochromatic erythrocytes. Results shall be reported as outlined later in this guideline with "normochromatic" interchanged for "polychromatic", where specified.

(3) *Animal number and sex.* At least five female and five male animals per experimental/sample and control group shall be used. The use of a single sex or a smaller number of animals shall be justified.

(4) *Positive control group.* A single concentration of a compound known to produce micronuclei *in vivo* is adequate as a positive control if it shows a significant response at any one time point; additional concentration levels may be used. To select an appropriate concentration level, a pilot or trial study may be advisable. Initially, one concentration of the test substance may be used, the maximum tolerated dose or that producing some indication of toxicity, e.g., a drop in the ratio of polychromatic to normochromatic erythrocytes. Intraperitoneal injection of 1,2-dimethyl-benz-anthracene or benzene are examples of positive control exposures. A concentration of 50–80 percent of an LD50 may be a suitable guide.

(d) *Test performance*—(1) *Inhalation exposure.* (i) All data developed within this study shall be in accordance with good laboratory practice provisions under § 79.60.