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#### § 799.9380 TSCA reproduction and fertility effects.

(a) *Scope*. This section is intended to meet the testing requirements under section 4 of the TSCA. This section is for two-generation reproduction testing and is designed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the estrous cycle, mating behavior, conception, gestation, parturition, lactation, and weaning, and on the growth and development of the offspring. The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, target organs in the offspring, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. Additionally, since the study design in-

cludes *in utero* as well as postnatal exposure, this study provides the opportunity to examine the susceptibility of the immature/neonatal animal.

(b) *Source*. The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.3800 (February 1996 Public Draft). This source is available at the address in paragraph (g) of this section.

(c) *Good laboratory practice standards*. The study shall be conducted in compliance with 40 CFR part 792—Good Laboratory Practice Standards.

(d) *Principle of the test method*. The test substance is administered to parental (P) animals prior to and during their mating, during the resultant pregnancies, and through the weaning of their F1 offspring. The substance is then administered to selected F1 offspring during their growth into adulthood, mating, and production of an F2 generation, until the F2 generation is weaned.

(e) *Test procedures*—(1) *Animal selection*—(i) *Species and strain*. The rat is the most commonly used species for testing. If another mammalian species is used, the tester shall provide justification/reasoning for its selection, and appropriate modifications will be necessary. Healthy parental animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used. Strains of low fecundity shall not be used.

(ii) *Age*. Parental (P) animals shall be 5 to 9 weeks old at the start of dosing. The animals of all test groups should be of uniform weight, age, and parity as nearly as practicable, and should be representative of the species and strain under study.

(iii) *Sex*. (A) For an adequate assessment of fertility, both males and females shall be studied.

(B) The females shall be nulliparous and nonpregnant.

(iv) *Number of animals*. Each control group shall contain a sufficient number of mating pairs to yield approximately 20 pregnant females. Each test group shall contain a similar number of mating pairs.

(v) *Identification of animals.* Each animal shall be assigned a unique identification number. For the P generation, this should be done before dosing starts. For the F1 generation, this should be done for animals selected for mating; in addition, records indicating the litter of origin shall be maintained for all selected F1 animals.

(2) *Administration of test and control substances*—(i) *Dose levels and dose selection.* (A) At least three-dose levels and a concurrent control shall be used. Healthy animals should be randomly assigned to the control and treatment groups, in a manner which results in comparable mean body weight values among all groups. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose should be chosen with the aim to induce some reproductive and/or systemic toxicity but not death or severe suffering. In the case of parental mortality, this should not be more than approximately 10%. The intermediate dose levels should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either systemic or reproductive toxicity (i.e., the no-observed-adverse-effect level, NOAEL) or should be at or near the limit of detection for the most sensitive endpoint. Two- or four-fold intervals are frequently optimal for spacing the dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e.g., more than a factor of 10) between dosages.

(B) It is desirable that additional information on metabolism and pharmacokinetics of the test substance be available to demonstrate the adequacy of the dosing regimen. This information should be available prior to testing.

(C) The highest dose tested should not exceed 1,000 mg/kg/day (or 20,000 ppm in the diet), unless potential human exposure data indicate the need for higher doses. If a test performed at the limit dose level, using the procedures described for this study, produces no observable toxicity and if an effect would not be expected based upon data from structurally related compounds,

then a full study using three dose levels may not be considered necessary.

(ii) *Control group.* (A) A concurrent control group shall be used. This group shall be an untreated or sham treated group or a vehicle-control group if a vehicle is used in administering the test substance.

(B) If a vehicle is used in administering the test substance, the control group shall receive the vehicle in the highest volume used.

(C) If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: Effects on the absorption, distribution, metabolism, or retention of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals.

(D) If a test substance is administered in the diet and causes reduced dietary intake or utilization, the use of a pair-fed control group may be considered necessary.

(iii) *Route of administration.* (A) The test substance is usually administered by the oral route (diet, drinking water, or gavage).

(B) If administered by gavage or dermal application, the dosage administered to each animal prior to mating and during gestation and lactation shall be based on the individual animal body weight and adjusted weekly at a minimum.

(C) If another route of administration is used, for example, when the route of administration is based upon the principal route of potential human exposure, the tester should provide justification and reasoning for its selection, and appropriate modifications may be necessary. Care should be taken to minimize stress on the maternal animals and their litters during gestation and lactation.

(D) All animals should be dosed by the same method during the appropriate experimental period.

(iv) *Dosing schedule.* (A) The animals should be dosed with the test substance on a 7-days-a-week basis.

(B) Daily dosing of the parental (P) males and females shall begin when they are 5 to 9 weeks old. Daily dosing

of the F1 males and females shall begin at weaning. For both sexes (P and F1), dosing shall be continued for at least 10 weeks before the mating period.

(C) Daily dosing of the P and F1 males and females shall continue until termination.

(3) *Mating procedure*—(i) *Parental*. (A) For each mating, each female shall be placed with a single randomly selected male from the same dose level (1:1 mating) until evidence of copulation is observed or either 3 estrous periods or 2 weeks has elapsed. Animals should be separated as soon as possible after evidence of copulation is observed. If mating has not occurred after 2 weeks or 3 estrous periods, the animals should be separated without further opportunity for mating. Mating pairs should be clearly identified in the data.

(B) Vaginal smears shall be collected daily and examined for all females during mating, until evidence of copulation is observed.

(C) Each day, the females shall be examined for presence of sperm or vaginal plugs. Day 0 of pregnancy is defined as the day a vaginal plug or sperm are found.

(ii) *F1 mating*. For mating the F1 offspring, at least one male and one female should be randomly selected from each litter for mating with another pup of the same dose level but different litter, to produce the F2 generation.

(iii) *Second mating*. In certain instances, such as poor reproductive performance in the controls, or in the event of treatment-related alterations in litter size, the adults may be remated to produce an F1b or F2b litter. If production of a second litter is deemed necessary in either generation, the dams should be remated approximately 1–2 weeks following weaning of the last F1a or F2a litter.

(iv) *Special housing*. After evidence of copulation, animals that are presumed to be pregnant shall be caged separately in delivery or maternity cages. Pregnant animals shall be provided with nesting materials when parturition is near.

(v) *Standardization of litter sizes*. (A) Animals should be allowed to litter normally and rear their offspring to weaning. Standardization of litter sizes is optional.

(B) If standardization is performed, the following procedure should be used. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four males and four females per litter or five males and five females per litter. Selective elimination of pups, i.e. based upon body weight, is not appropriate. Whenever the number of male or female pups prevents having four (or five) of each sex per litter, partial adjustment (for example, five males and three females, or four males and six females) is acceptable. Adjustments are not appropriate for litters of eight pups or less.

(4) *Observation of animals*—(i) *Parental*. (A) Throughout the test period, each animal shall be observed at least once daily, considering the peak period of anticipated effects after dosing. Mortality, moribundity, pertinent behavioral changes, signs of difficult or prolonged parturition, and all signs of overt toxicity shall be recorded at this cageside examination. In addition, thorough physical examinations should be conducted weekly on each animal.

(B) Parental animals (P and F1) shall be weighed on the first day of dosing and weekly thereafter. Parental females (P and F1) should be weighed at a minimum on approximately gestation days 0, 7, 14, and 21, and during lactation on the same days as the weighing of litters.

(C) During the premating and gestation periods, food consumption shall be measured weekly at a minimum. Water consumption should be measured weekly at a minimum if the test substance is administered in the water.

(D) Estrous cycle length and pattern should be evaluated by vaginal smears for all P and F1 females during a minimum of 3 weeks prior to mating and throughout cohabitation; care should be taken to prevent the induction of pseudopregnancy.

(E) For all P and F1 males at termination, sperm from one testis and one epididymis shall be collected for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, sperm from the cauda epididymis (or vas deferens) should be collected for

evaluation of sperm motility and sperm morphology.

(1) The total number of homogenization-resistant testicular sperm and cauda epididymal sperm should be enumerated. The method described in the reference under paragraph (g)(8) of this section may be used. Cauda sperm reserves can be derived from the concentration and volume of sperm in the suspension used to complete the qualitative evaluations, and the number of sperm recovered by subsequent mincing and/or homogenizing of the remaining cauda tissue. Enumeration in only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated.

(2) An evaluation of epididymal (or vas deferens) sperm motility should be performed. Sperm should be recovered while minimizing damage (the evaluation techniques as described in the reference under paragraph (g)(8) of this section may be used), and the percentage of progressively motile sperm should be determined either subjectively or objectively. For objective evaluations, an acceptable counting chamber of sufficient depth can be used to effectively combine the assessment of motility with sperm count and sperm morphology. When computer-assisted motion analysis is performed, the derivation of progressive motility relies on user-defined thresholds for average path velocity and straightness or linear index. If samples are videotaped, or images otherwise recorded, at the time of necropsy, subsequent analysis of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated. In the absence of a video or digital image, all samples in all treatment groups should be analyzed at necropsy.

(3) A morphological evaluation of an epididymal (or vas deferens) sperm sample shall be performed. Sperm (at least 200 per sample) should be examined as fixed, wet preparations (the techniques for such examinations is described in the references under paragraphs (g)(4) and (g)(8) of this section may be used) and classified as either

normal (both head and midpiece/tail appear normal) or abnormal. Examples of morphologic sperm abnormalities would include fusion, isolated heads, and misshapen heads and/or tails. Evaluation of only control and high-dose P and F1 males may be performed unless treatment-related effects are observed; in that case, the lower dose groups should also be evaluated.

(ii) *Offspring.* (A) Each litter should be examined as soon as possible after delivery (lactation day 0) to establish the number and sex of pups, stillbirths, live births, and the presence of gross anomalies. Pups found dead on day 0 should be examined for possible defects and cause of death.

(B) Live pups should be counted, sexed, and weighed individually at birth, or soon thereafter, at least on days 4, 7, 14, and 21 of lactation, at the time of vaginal patency or balanopreputial separation, and at termination.

(C) The age of vaginal opening and preputial separation should be determined for F1 weanlings selected for mating. If there is a treatment-related effect in F1 sex ratio or sexual maturation, anogenital distance should be measured on day 0 for all F2 pups.

(5) *Termination schedule.* (i) All P and F1 adult males and females should be terminated when they are no longer needed for assessment of reproductive effects.

(ii) F1 offspring not selected for mating and all F2 offspring should be terminated at comparable ages after weaning.

(6) *Gross necropsy.* (i) At the time of termination or death during the study, all parental animals (P and F1) and when litter size permits at least three pups per sex per litter from the unselected F1 weanlings and the F2 weanlings shall be examined macroscopically for any structural abnormalities or pathological changes. Special attention shall be paid to the organs of the reproductive system.

(ii) Dead pups or pups that are terminated in a moribund condition should be examined for possible defects and/or cause of death.

(iii) At the time of necropsy, a vaginal smear should be examined to determine the stage of the estrous cycle.

The uteri of all cohabited females should be examined, in a manner which does not compromise histopathological evaluation, for the presence and number of implantation sites.

(7) *Organ weights.* (i) At the time of termination, the following organs of all P and F1 parental animals shall be weighed:

(A) Uterus (with oviducts and cervix), ovaries.

(B) Testes, epididymides (total weights for both and cauda weight for either one or both), seminal vesicles (with coagulating glands and their fluids), and prostate.

(C) Brain, pituitary, liver, kidneys, adrenal glands, spleen, and known target organs.

(ii) For F1 and F2 weanlings that are examined macroscopically, the following organs shall be weighed for one randomly selected pup per sex per litter.

(A) Brain.

(B) Spleen and thymus.

(8) *Tissue preservation.* The following organs and tissues, or representative samples thereof, shall be fixed and stored in a suitable medium for histopathological examination.

(i) For the parental (P and F1) animals:

(A) Vagina, uterus with oviducts, cervix, and ovaries.

(B) One testis (preserved in Bouins fixative or comparable preservative), one epididymis, seminal vesicles, prostate, and coagulating gland.

(C) Pituitary and adrenal glands.

(D) Target organs, when previously identified, from all P and F1 animals selected for mating.

(E) Grossly abnormal tissue.

(ii) For F1 and F2 weanlings selected for macroscopic examination: Grossly abnormal tissue and target organs, when known.

(9) *Histopathology*—(i) *Parental animals.* Full histopathology of the organs listed in paragraph (e)(8)(i) of this section shall be performed for ten randomly chosen high dose and control P and F1 animals per sex, for those animals that were selected for mating. Organs demonstrating treatment-related changes shall also be examined for the remainder of the high-dose and control animals and for all parental animals in

the low- and mid-dose groups. Additionally, reproductive organs of the low- and mid-dose animals suspected of reduced fertility, e.g., those that failed to mate, conceive, sire, or deliver healthy offspring, or for which estrous cyclicity or sperm number, motility, or morphology were affected, shall be subjected to histopathological evaluation. Besides gross lesions such as atrophy or tumors, testicular histopathological examination should be conducted in order to identify treatment-related effects such as retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen. Examination of the intact epididymis should include the caput, corpus, and cauda, which can be accomplished by evaluation of a longitudinal section, and should be conducted in order to identify such lesions as sperm granulomas, leukocytic infiltration (inflammation), aberrant cell types within the lumen, or the absence of clear cells in the cauda epididymal epithelium. The postlactational ovary should contain primordial and growing follicles as well as the large corpora lutea of lactation. Histopathological examination should detect qualitative depletion of the primordial follicle population. A quantitative evaluation of primordial follicles should be conducted for all F1 females; the number of animals, ovarian section selection, and section sample size should be statistically appropriate for the evaluation procedure used. Examination should include enumeration of the number of primordial follicles, which can be combined with small growing follicles (see paragraphs (g)(1) and (g)(2) of this section), for comparison of treated and control ovaries.

(ii) *Weanling.* For F1 and F2 weanlings, histopathological examination of treatment-related abnormalities noted in macroscopic examination should be considered, if such evaluation were deemed appropriate and would contribute to the interpretation of the study data.

(f) *Data and reporting*—(1) *Treatment of results.* Data shall be reported individually and summarized in tabular form, showing for each test group the

types of change and the number of animals displaying each type of change.

(2) *Evaluation of study results.* (i) An evaluation of test results, including the statistical analysis, shall be provided. This should include an evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all abnormalities.

(ii) When appropriate, historical control data should be used to enhance interpretation of study results. Historical data, when used, should be compiled, presented, and analyzed in an appropriate and relevant manner. In order to justify its use as an analytical tool, information such as the dates of study conduct, the strain and source of the animals, and the vehicle and route of administration should be included.

(iii) Statistical analysis of the study findings should include sufficient information on the method of analysis, so that an independent reviewer/statistician can reevaluate and reconstruct the analysis.

(iv) In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

(3) *Test report.* In addition to the reporting requirements as specified under 40 CFR part 792, subpart J, the following specific information shall be reported. Both individual and summary data should be presented.

(i) Species and strain.

(ii) Toxic response data by sex and dose, including indices of mating, fertility, gestation, birth, viability, and lactation; offspring sex ratio; precoital interval, including the number of days until mating and the number of estrous periods until mating; and duration of gestation calculated from day 0 of pregnancy. The report should provide the numbers used in calculating all indices.

(iii) Day (week) of death during the study or whether animals survived to termination; date (age) of litter termination.

(iv) Toxic or other effects on reproduction, offspring, or postnatal growth.

(v) Developmental milestone data (mean age of vaginal opening and

preputial separation, and mean anogenital distance, when measured).

(vi) Number of P and F1 females cycling pattern and mean estrous cycle length.

(vii) Day (week) of observation of each abnormal sign and its subsequent course.

(viii) Body weight and body weight change data by sex for P, F1, and F2 animals.

(ix) Food (and water, if applicable) consumption, food efficiency (body weight gain per gram of food consumed), and test material consumption for P and F1 animals, except for the period of cohabitation.

(x) Total cauda epididymal sperm number, homogenization-resistant testis spermatid number, number and percent of progressively motile sperm, number and percent of morphologically normal sperm, and number and percent of sperm with each identified anomaly.

(xi) Stage of the estrous cycle at the time of termination for P and F1 parental females.

(xii) Necropsy findings.

(xiii) Implantation data and postimplantation loss calculations for P and F1 parental females.

(xiv) Absolute and adjusted organ weight data.

(xv) Detailed description of all histopathological findings.

(xvi) Adequate statistical treatment of results.

(xvii) A copy of the study protocol and any amendments should be included.

(g) *References.* For additional background information on this test guideline, the following references should be consulted. These references are available for inspection at the TSCA Nonconfidential Information Center, Rm. NE-B607, Environmental Protection Agency, 401 M St., SW., Washington, DC, 12 noon to 4 p.m., Monday through Friday, except legal holidays.

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number in mice as a screen of ovarian toxicity. *Growth Factors and the Ovary* (Plenum, NY, 1989) pp. 421-426.

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(12) Zenick, H. *et al.* Ed. Hayes, A.W. Assessment of male reproductive toxicity: a risk assessment approach. *Principles and Methods of Toxicology* (Raven, NY, 1994).

[62 FR 43824, Aug. 15, 1997, as amended at 64 FR 35078, June 30, 1999]

#### § 799.9410 TSCA chronic toxicity.

(a) *Scope*—(1) *Applicability*. This section is intended to meet the testing requirement of the Toxic Substances Control Act (TSCA) (15 U.S.C. 2601).

(2) *Source*. The source material used in developing this TSCA test guideline is the Office of Prevention, Pesticides and Toxic Substances (OPPTS) harmonized test guideline 870.4100 (August 1998, final guidelines). This source is

available at the address in paragraph (h) of this section

(b) *Purpose*. The objective of a chronic toxicity study is to determine the effects of a substance in a mammalian species following prolonged and repeated exposure. A chronic toxicity study should generate data from which to identify the majority of chronic effects and to define long-term dose-response relationships. The design and conduct of chronic toxicity tests should allow for the detection of general toxic effects, including neurological, physiological, biochemical, and hematological effects and exposure-related morphological (pathological) effects.

(c) *Definitions*. The definitions in section 3 of TSCA and in 40 CFR Part 792—Good Laboratory Practice Standards apply to this section. The following definitions also apply to this section.

*Chronic toxicity* is the adverse effects occurring as a result of the repeated daily exposure of experimental animals to a chemical by the oral, dermal, or inhalation routes of exposure.

*Cumulative toxicity* is the adverse effects of repeated doses occurring as a result of prolonged action on, or increased concentration of, the administered test substance or its metabolites in susceptible tissue.

*Dose in a chronic toxicity study* is the amount of test substance administered daily via the oral, dermal or inhalation routes for a period of at least 12 months. Dose is expressed as weight of the test substance (grams, milligrams) per unit body weight of test animal (milligram per kilogram), or as weight of the test substance in parts per million (ppm) in food or drinking water per day. For inhalation exposure, dose is expressed as weight of the test substance per unit volume of air (milligrams per liter) or as parts per million per day. For dermal exposure, dose is expressed as weight of the test substance (grams, milligrams) per unit body weight of the test animal (milligrams per kilogram) or as weight of the substance per unit of surface area (milligrams per square centimeter) per day.

*No-observed-effects level (NOEL)* is the maximum dose used in a study which produces no adverse effects. The NOEL