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[62 FR 43824, Aug. 15, 1997, as amended at 64 FR 35080, June 30, 1999]

§ 799.9630 TSCA developmental neurotoxicity.

(a) *Scope*—(1) *Applicability*. This section is intended to meet the testing requirements *under* section 4 of the Toxic Substances Control Act (TSCA).

(2) *Source*. The source material used in developing this TSCA test guideline is the OPPTS harmonized test guideline 870.6300 (August 1998).

(b) *Purpose*. In the assessment and evaluation of the toxic characteristics of a chemical substance or mixture (test substance), determination of the potential for developmental neurotoxicity is important. This study is designed to develop data on the potential functional and morphological hazards to the nervous system which may arise in the offspring from exposure of the mother during pregnancy and lactation.

(c) *Principle of the test method*. The test substance is administered to several groups of pregnant animals during gestation and early lactation, one dose level being used per group. Offspring are randomly selected from within litters for neurotoxicity evaluation. The evaluation includes observations to detect gross neurologic and behavioral abnormalities, determination of motor activity, response to auditory startle, assessment of learning, neuropathological evaluation, and brain weights. This protocol may be used as a separate study, as a followup to a standard developmental toxicity and/or adult neurotoxicity study, or as part of a two-generation reproduction study, with assessment of the offspring conducted on the second (F^2) generation.

(d) *Test procedure*—(1) *Animal selection*—(i) *Species and strain*. Testing must be performed in the rat. Because of its differences in timing of developmental events compared to strains that are more commonly tested in other developmental and reproductive toxicity studies, it is preferred that the Fischer 344 strain not be used. If a sponsor wishes to use the Fischer 344 rat or a mammalian species other than the rat, ample justification/reasoning for this selection must be provided.

(ii) *Age*. Young adult (nulliparous females) animals must be used.

(iii) *Sex*. Pregnant female animals must be used at each dose level.

(iv) *Number of animals*. (A) The objective is for a sufficient number of pregnant rats to be exposed to the test substance to ensure that an adequate number of offspring are produced for neurotoxicity evaluation. At least 20 litters are recommended at each dose level.

(B) On postnatal day 4, the size of each litter should be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four male and four females per litter. Whenever the number of pups of either sex prevents having four of each sex per litter, partial adjustment (for example, five males and three females) is permitted. Testing is not appropriate for litters of less than seven pups. Elimination of runts only is not appropriate. Individual pups should be identified uniquely after standardization of litters. A method that may be used for identification can be found under paragraph (f)(1) of this section.

(v) *Assignment of animals for behavioral tests, brain weights, and neuropathological evaluations*. After standardization of litters, one male or

one female from each litter (total of 10 males and 10 females per dose group) must be randomly assigned to one of the following tests: Motor activity, auditory startle, and learning and memory, in weanling and adult animals. On postnatal day 11, either 1 male or 1 female pup from each litter (total of 10 males and 10 females per dose group) must be sacrificed. Brain weights must be measured in all of these pups and, of these pups, six per sex per dose must be selected for neuropathological evaluation. At the termination of the study, either 1 male or 1 female from each litter (total of 10 males and 10 females per dose group) must be sacrificed and brain weights must be measured. An additional group of six animals per sex per dose group (one male or one female per litter) must be sacrificed at the termination of the study for neuropathological evaluation.

(2) *Control group.* A concurrent control group is required. This group must be a sham-treated group or, if a vehicle is used in administering the test substance, a vehicle control group. The vehicle must neither be developmentally toxic nor have effects on reproduction. Animals in the control group must be handled in an identical manner to test group animals.

(3) *Dose levels and dose selection.* (i) At least three dose levels of the test substance plus a control group (vehicle control, if a vehicle is used) must be used.

(ii) If the test substance has been shown to be developmentally toxic either in a standard developmental toxicity study or in a pilot study, the highest dose level must be the maximum dose which will not induce in utero or neonatal death or malformations sufficient to preclude a meaningful evaluation of neurotoxicity.

(iii) If a standard developmental toxicity study has not been conducted, the highest dose level, unless limited by the physicochemical nature or biological properties of the substance, must induce some overt maternal toxicity, but must not result in a reduction in weight gain exceeding 20 percent during gestation and lactation.

(iv) The lowest dose should not produce any grossly observable evidence of either maternal or developmental neurotoxicity.

(v) The intermediate doses must be equally spaced between the highest and lowest doses used.

(4) *Dosing period.* Day 0 of gestation is the day on which a vaginal plug and/or sperm are observed. The dosing period must cover the period from day 6 of gestation through day 10 postnatally. Dosing should not occur on the day of parturition in those animals who have not completely delivered their offspring.

(5) *Administration of the test substance.* The test substance or vehicle must be administered orally. Other routes of administration may be acceptable, on a case-by-case basis, with ample justification/reasoning for this selection. The test substance or vehicle must be administered based on the most recent weight determination.

(6) *Observation of dams.* (i) A gross examination of the dams must be made at least once each day before daily treatment.

(ii) Ten dams per group must be observed outside the home cage at least twice during the gestational dosing period (days 6–21) and twice during the lactational dosing period (days 1–10) for signs of toxicity. The animals must be observed by trained technicians who are unaware of the animals' treatment, using standardized procedures to maximize interobserver reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of interobserver reliability is required.

(iii) During the treatment and observation periods under paragraph (d)(6)(ii) of this section, observations must include:

(A) Assessment of signs of autonomic function, including but not limited to:

(*1*) Ranking of the degree of lacrimation and salivation, with a range of severity scores from none to severe.

(*2*) Presence or absence of piloerection and exophthalmus.

(*3*) Ranking or count of urination and defecation, including polyuria and diarrhea.

(*4*) Pupillary function such as constriction of the pupil in response to light or a measure of pupil size.

(*5*) Degree of palpebral closure, e.g., ptosis.

(B) Description, incidence, and severity of any convulsions, tremors, or abnormal movements.

(C) Description and incidence of posture and gait abnormalities.

(D) 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.

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

(v) Animals must be weighed at least weekly and on the day of delivery and postnatal days 11 and 21 (weaning) and such weights must be recorded.

(vi) The day of delivery of litters must be recorded and considered as postnatal day 0.

(7) *Study conduct*—(i) *Observation of offspring.* (A) All offspring must be examined cage-side at least daily for gross signs of mortality or morbidity.

(B) A total of 10 male offspring and 10 female offspring per dose group must be examined outside the cage for signs of toxicity on days 4, 11, 21, 35, 45, and 60. The offspring must be observed by trained technicians, who are unaware of the treatment being used, using standardized procedures to maximize interobserver reliability. Where possible, it is advisable that the same observer be used to evaluate the animals in a given study. If this is not possible, some demonstration of interobserver reliability is required. At a minimum, the end points outlined in paragraph (d)(6)(iii) of this section must be monitored as appropriate for the developmental stage being observed.

(C) Any gross signs of toxicity in the offspring must be recorded as they are observed, including the time of onset, degree, and duration.

(ii) *Developmental landmarks.* Live pups must be counted and each pup within a litter must be weighed individually at birth or soon thereafter, and on postnatal days 4, 11, 17, and 21 and at least once every 2 weeks thereafter. The age of vaginal opening and preputial separation must be determined. General procedures for these determinations may be found in paragraphs (f)(1) and (f)(11) of this section.

(iii) *Motor activity.* Motor activity must be monitored specifically on postnatal days 13, 17, 21, and 60 (+2 days). Motor activity must be monitored by an automated activity recording apparatus. The device must be capable of detecting both increases and decreases in activity, (i.e., baseline activity as measured by the device must not be so low as to preclude detection of decreases nor so high as to preclude detection of increases in activity). Each device must be tested by standard procedures to ensure, to the extent possible, reliability of operation across devices and across days for any one device. In addition, treatment groups must be balanced across devices. Each animal must be tested individually. The test session must be long enough for motor activity to approach asymptotic levels by the last 20 percent of the session for nontreated control animals. All sessions must have the same duration. Treatment groups must be counter-balanced across test times. Activity counts must be collected in equal time periods of no greater than 10 minutes duration. Efforts must be made to ensure that variations in the test conditions are minimal and are not systematically related to treatment. Among the variables that can affect motor activity are sound level, size and shape of the test cage, temperature, relative humidity, light conditions, odors, use of home cage or novel test cage, and environmental distractions. Additional information on the conduct of a motor activity study may be obtained in § 799.9620.

(iv) *Auditory startle test.* An auditory startle habituation test should be performed on the offspring around the time of weaning and around day 60. Day of testing should be counterbalanced across treated and control groups. Details on the conduct of this testing may be obtained under paragraph (f)(1) of this section. In performing the auditory startle task, the mean response amplitude on each

block of 10 trials (5 blocks of 10 trials per session on each day of testing) must be made. While use of prepulse inhibition is not a requirement, it is highly recommended. Details on the conduct of this test may be obtained in paragraph (f)(10) of this section.

(v) *Learning and memory tests.* A test of associative learning and memory should be conducted around the time of weaning and around day 60. Day of testing should be counterbalanced across treated and control groups. The same or separate tests may be used at these two stages of development. Some flexibility is allowed in the choice of tests for learning and memory in weanling and adult rats. However, the tests must be designed to fulfill two criteria. First, learning must be assessed either as a change across several repeated learning trials or sessions, or, in tests involving a single trial, with reference to a condition that controls for nonassociative effects of the training experience. Second, the tests must include some measure of memory (short-term or long-term) in addition to original learning (acquisition). If the tests of learning and memory reveal an effect of the test compound, it may be in the best interest of the sponsor to conduct additional tests to rule out alternative interpretations based on alterations in sensory, motivational, and/or motor capacities. In addition to the above two criteria, it is recommended that the test of learning and memory be chosen on the basis of its demonstrated sensitivity to the class of compound under investigation, if such information is available in the literature. In the absence of such information, examples of tests that could be made to meet the above criteria include: Delayed-matching-to-position, as described for the adult rat (see paragraph (f)(3) of this section) and for the infant rat (see paragraph (f)(9) of this section); olfactory conditioning, as described in paragraph (f)(13) of this section; and acquisition and retention of schedule-controlled behavior (see paragraphs (f)(4) and (f)(5) of this section). Additional tests for weanling rats are described under paragraphs (f)(20) and (f)(12) of this section, and for adult rats under paragraph (f)(16) of this section.

(vi) *Neuropathology.* Neuropathological evaluation must be conducted on animals on postnatal day 11 and at the termination of the study. At 11 days of age, one male or female pup must be removed from each litter such that equal numbers of male and female offspring are removed from all litters combined. Of these, six male and six female pups per dose group will be sacrificed for neuropathological analysis. The pups will be sacrificed by exposure to carbon dioxide and immediately thereafter the brains should be removed, weighed, and immersion-fixed in an appropriate aldehyde fixative. The remaining animals will be sacrificed in a similar manner and immediately thereafter their brains removed and weighed. At the termination of the study, one male or one female from each litter will be sacrificed by exposure to carbon dioxide and immediately thereafter the brain must be removed and weighed. In addition, six animals per sex per dose group (one male or female per litter) must be sacrificed at the termination of the study for neuropathological evaluation. Neuropathological analysis of animals sacrificed at the termination of the study must be performed in accordance with §799.9620. Neuropathological evaluation of animals sacrificed on postnatal day 11 and at termination of the study must include a qualitative analysis and semiquantitative analysis as well as simple morphometrics.

(A) *Fixation and processing of tissue samples for postnatal day 11 animals.* Immediately following removal, the brain must be weighed and immersion fixed in an appropriate aldehyde fixative. The brains must be postfixed and processed according to standardized published histological protocols such as those discussed in references listed under paragraphs (f)(6), (f)(14), (f)(17), and (f)(21) of this section. Paraffin embedding is acceptable but plastic embedding is preferred and recommended. Tissue blocks and slides must be appropriately identified when stored. Histological sections must be stained for hematoxylin and eosin, or a similar stain according to standard published protocols such as those discussed in references listed under paragraphs (f)(2), (f)(18), and (f)(23) of this section. For

animals sacrificed at the termination of the study, methods for fixation and processing of tissue samples are provided in § 799.9620(e)(7)(iv)(A).

(B) *Qualitative analysis.* The purposes of the qualitative examination are threefold—to identify regions within the nervous system exhibiting evidence of neuropathological alterations, to identify types of neuropathological alterations resulting from exposure to the test substance, and to determine the range of severity of the neuropathological alterations. Representative histological sections from the tissue samples should be examined microscopically by an appropriately trained pathologist for evidence of neuropathological alterations. The following stepwise procedure is recommended for the qualitative analysis. First, sections from the high dose group are compared with those of the control group. If no evidence of neuropathological alterations is found in animals of the high dose group, no further analysis is required. If evidence of neuropathological alterations are found in the high dose group, then animals from the intermediate and low dose group are examined. Subject to professional judgment and the kind of neuropathological alterations observed, it is recommended that additional methods such as Bodian's or Bielchowsky's silver methods and/or immunohistochemistry for glial fibrillary acid protein be used in conjunction with more standard stains to determine the lowest dose level at which neuropathological alterations are observed. Evaluations of postnatal day 11 pups is described in paragraphs (d)(7)(vi)(B)(*1*) and (d)(7)(vi)(B)(*2*) of this section. For animals sacrificed at the termination of the study, the regions to be examined and the types of alterations that must be assessed are identified in § 799.9620(e)(7)(iv)(B).

(*1*) *Regions to be examined.* The brains should be examined for any evidence of treatment-related neuropathological alterations and adequate samples should be taken from all major brain regions (e.g., olfactory bulbs, cerebral cortex, hippocampus, basal ganglia, thalamus, hypothalamus, midbrain (tectum, tegmentum, and cerebral peduncles), brainstem and cerebellum) to ensure a thorough examination.

(*2*) *Types of alterations.* Guidance for neuropathological examination for indications of developmental insult to the brain can be found in paragraphs (f)(8) and (f)(22) of this section. In addition to more typical kinds of cellular alterations (e.g., neuronal vacuolation, degeneration, necrosis) and tissue changes (e.g., astrocytic proliferation, leukocytic infiltration, and cystic formation) particular emphasis should be paid to structural changes indicative of developmental insult including but not restricted to:

(*i*) Gross changes in the size or shape of brain regions such as alterations in the size of the cerebral hemispheres or the normal pattern of foliation of the cerebellum.

(*ii*) The death of neuronal precursors, abnormal proliferation, or abnormal migration, as indicated by pyknotic cells or ectopic neurons, or gross alterations in regions with active proliferative and migratory zones, alterations in transient developmental structures (e.g., the external germinal zone of the cerebellum, see paragraph (f)(15) of this section).

(*iii*) Abnormal differentiation, while more apparent with special stains, may also be indicated by shrunken and malformed cell bodies.

(*iv*) Evidence of hydrocephalus, in particular enlargement of the ventricles, stenosis of the cerebral aqueduct and general thinning of the cerebral hemispheres.

(C) *Subjective diagnosis.* If any evidence of neuropathological alterations is found in the qualitative examination, then a subjective diagnosis will be performed for the purpose of evaluating dose-response relationships. All regions of the brain exhibiting any evidence of neuropathological changes must be included in this analysis. Sections of each region from all dose groups will be coded as to treatment and examined in randomized order. The frequency of each type and the severity of each lesion will be recorded. After all sections from all dose groups including all regions have been rated, the code will be broken and statistical analyses performed to evaluate dose-response relationships. For each type of dose related

lesion observed, examples of different ranges of severity must be described. The examples will serve to illustrate a rating scale, such as 1+, 2+, and 3+ for the degree of severity ranging from very slight to very extensive.

(D) *Simple morphometric analysis.* Since the disruption of developmental processes is sometimes more clearly reflected in the rate or extent of growth of particular brain regions, some form of morphometric analysis must be performed on postnatal day 11 and at the termination of the study to assess the structural development of the brain. At a minimum, this would consist of a reliable estimate of the thickness of major layers at representative locations within the neocortex, hippocampus, and cerebellum. For guidance on such measurements see Rodier and Gramann under paragraph (f)(19) of this section.

(e) *Data collection, reporting, and evaluation.* The following specific information must be reported:

(1) *Description of test system and test methods.* A description of the general design of the experiment should be provided. This must include:

(i) A detailed description of the procedures used to standardize observations and procedures as well as operational definitions for scoring observations.

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. These data do not have to be from studies using prenatal exposures. However, the laboratory must demonstrate competence in evaluation of effects in neonatal animals perinatally exposed to chemicals and establish test norms for the appropriate age group.

(iii) Procedures for calibrating and ensuring the equivalence of devices and the balancing of treatment groups in testing procedures.

(iv) A short justification explaining any decisions involving professional judgement.

(2) *Results.* The following information must be arranged by each treatment and control group:

(i) In tabular form, data for each animal must be provided showing:

(A) Its identification number and the litter from which it came.

(B) Its body weight and score on each developmental landmark at each observation time.

(C) Total session activity counts and intrasession subtotals on each day measured.

(D) Auditory startle response amplitude per session and intrasession amplitudes on each day measured.

(E) Appropriate data for each repeated trial (or session) showing acquisition and retention scores on the tests of learning and memory on each day measured.

(F) Time and cause of death (if appropriate); any neurological signs observed; a list of structures examined as well as the locations, nature, frequency, and extent of lesions; and brain weights.

(ii) The following data should also be provided, as appropriate:

(A) Inclusion of photomicrographs demonstrating typical examples of the type and extent of the neuropathological alterations observed is recommended.

(B) Any diagnoses derived from neurological signs and lesions, including naturally occurring diseases or conditions, should also be recorded.

(iii) Summary data for each treatment and control group must include:

(A) The number of animals at the start of the test.

(B) The body weight of the dams during gestation and lactation.

(C) Litter size and mean weight at birth.

(D) The number of animals showing each abnormal sign at each observation time.

(E) The percentage of animals showing each abnormal sign at each observation time.

(F) The mean and standard deviation for each continuous endpoint at each observation time. These will include body weight, motor activity counts, auditory startle responses, performance in learning and memory tests, regional brain weights and whole brain weights (both absolute and relative).

(G) The number of animals in which any lesion was found.

(H) The number of animals affected by each different type of lesion, the location, frequency and average grade of each type of lesion for each animal.

(I) The values of all morphometric measurements made for each animal listed by treatment group.

(3) *Evaluation of data.* An evaluation of test results must be made. The evaluation must include the relationship between the doses of the test substance and the presence or absence, incidence, and extent of any neurotoxic effect. The evaluation must include appropriate statistical analyses. The choice of analyses must consider tests appropriate to the experimental design and needed adjustments for multiple comparisons. The evaluation must include the relationship, if any, between observed neuropathological and behavioral alterations.

(f) *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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(15) Miale, I. L. and Sidman, R.L. An autoradiographic analysis of histogenesis in the mouse cerebellum. *Experimental Neurology*. 4:277–296 (1961).

(16) Miller, D.B. and Eckerman, D.A. Learning and memory measures. In: *Neurobehavioral Toxicology*, Z. Annau (ed). Johns Hopkins University Press, Baltimore. pp. 94–149 (1986).

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[65 FR 78811, Dec. 15, 2000]

§ 799.9748 TSCA metabolism and pharmacokinetics

(a) *Scope.* (1) This section is intended to meet the testing requirements under section 4 of the Toxic Substances Control Act (TSCA). (1) Testing of the disposition of a test substance is designed to obtain adequate information on its absorption, distribution, biotransformation, and excretion and to aid in understanding the mechanism of toxicity. Basic pharmacokinetic parameters determined from these studies will also provide information on the potential for accumulation of the test substance in tissues and/or organs and the potential for induction of biotransformation as a result of exposure to the test substance. These data can be used to assess the adequacy and relevance of the extrapolation of animal toxicity data (particularly chronic toxicity and/or carcinogenicity data) to human risk assessment.

(2) Metabolism data can also be used to assist in determining whether animal toxicity studies have adequately addressed any toxicity concerns arising from exposure to plant metabolites, and in the setting of tolerances, if any, for those metabolites in raw agricultural commodities.

(b) *Source.* The source material used in developing this TSCA test guideline is the Office of Prevention, Pesticides and Toxic Substances (OPPTS) harmonized test guideline 870.7485 (August 1998, final guideline). This source is available at the address in paragraph (h) of this section.

(c) *Definitions.* The following definitions apply to this section.

Metabolism (biotransformation) is the sum of the processes by which a foreign chemical is subjected to chemical change by living organisms.

LOEL is the lowest observable effects level.

NOEL is the no observable effects level.

Pharmacokinetics is the quantitation and determination of the time course and dose dependency of the absorption, distribution, biotransformation, and excretion of chemicals.

(d) *Good laboratory practice standards.* The pharmacokinetics and metabolism tests outlined in this guideline must conform to the laboratory practices stipulated in 40 CFR Part 792—Good Laboratory Practice Standards.

(e) *Test Procedures.* Test procedures presented below utilize a tier system to minimize the use of resources and to allow flexibility in the conduct of metabolism studies. The proposed tier system consists of a basic data set (Tier 1) and additional studies (Tier 2). These additional studies may be requested based upon the existing toxicology data base and/or the results of Tier 1 testing which are found to impact upon the risk assessment process. For Tier 1 testing, the oral route will typically be required; however, if the use pattern results in other types of exposure, other routes (dermal and/or inhalation) may be required for initial testing of the disposition of a chemical substance. The registrant should justify the route of exposure to the Agency. Complete descriptions of the test procedures for these other routes of exposure can be found in paragraph (i) of