

(B) The evaluation of dose-response, if existent, for various groups shall be given, and a description of statistical method must be presented. The evaluation of neuropathology data should include, where applicable, an assessment in conjunction with other neurotoxicity studies performed (eg. electrophysiological, behavioral, neurochemical).

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

(1) AFIP. *Manual of Histologic Staining Methods.* (New York: McGraw-Hill (1968).

(2) Chang, L.W. *A Color Atlas and Manual for Applied Histochemistry.* (Springfield, IL: Charles C. Thomas, 1979).

(3) Hayat, M.A. "Vol. 1. Biological applications," *Principles and techniques of electron microscopy.* (New York: Van Nostrand Reinhold, 1970)

(4) Palay S.L., Chan-Palay, V. *Cerebellar Cortex: Cytology and Organization.* (New York: Springer-Verlag, 1974).

(5) Ralis, H.M., Beesley, R.A., Ralis, Z.A. *Techniques in Neurohistology.* (London: Butterworths, 1973).

(6) Spencer, P.S., Schaumburg, H.H. (eds). *Experimental and Clinical Neurotoxicology.* (Baltimore: Williams and Wilkins, 1980).

(7) Zeman, W., JRM Innes, J.R.M. *Craigie's Neuroanatomy of the Rat.* (New York: Academic, 1963).

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§ 798.6500 Schedule-controlled operant behavior.

(a) *Purpose.* (1) In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for functional neurotoxic effects. Substances that have been observed to produce neurotoxic signs in other toxicity studies (e.g. CNS depression or stimulation), as well as substances with a structural similarity to known neurotoxicants should be evaluated for these effects.

(2) This guideline defines procedures for conducting studies of schedule-controlled operant behavior, one way of evaluating functional neurotoxic effects (Dews, 1972 under paragraph (f)(1)

of this section; NAS 1975, 1977, 1982 under paragraphs (f)(4), (5) and (6) of this section). Our purpose is to evaluate the effects of acute and repeated exposures on the rate and pattern of responding under schedules of reinforcement. Operant behavior tests may be used to evaluate many other aspects of behavior (Laties, 1978 under paragraph (f)(3) of this section). Additional tests may be necessary to completely assess the behavioral effects of any substance. Behavioral evaluation should be used in conjunction with neuropathologic evaluation and the evaluation of other toxic effects.

(b) *Definitions—(1) Neurotoxicity.* Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent. Behavioral toxicity is an adverse change in the functioning of the organism with respect to its environment following exposure to a chemical agent.

(2) *Operant, operant behavior, operant conditioning.* An operant is a class of behavioral responses which change or operates on the environment in the same way. Operant behavior is further distinguished as behavior which is modified by its consequences. Operant conditioning is the experimental procedure used to modify some class of behavior by reinforcement or punishment.

(3) *Schedule of reinforcement.* A schedule of reinforcement specifies the relation between behavioral responses and the delivery of reinforcers, such as food or water (Ferster and Skinner, 1957 under paragraph (f)(2) of this section). For example, a fixed ratio (FR) schedule requires a fixed number of responses to produce a reinforcer (e.g. FR 30). On a fixed interval (FI) schedule, the first response after a fixed period of time is reinforced (e.g. FI 5 minutes).

(c) *Principle of the test method.* Experimental animals are trained to perform under a schedule of reinforcement and measurements of their operant behavior are made. Several doses of the test substance are then administered according to the experimental design (between groups or within subjects) and the duration of exposure (acute or repeated). Measurements of the operant

behavior are repeated. A descriptive and statistical evaluation of the data is made to evaluate the nature and extent of any changes in behavior in relation to exposures to the test substance. Comparisons are made between any exposures that influence the behavior and exposures that have neuropathological effects or effects on other targets of the chemical.

(d) *Test procedures*—(1) *Experimental design*. These test procedures may be used to evaluate the behavior of experimental animals receiving either acute or repeated exposures. For acute exposure studies, either within-subject or between groups, experimental designs may be used. For repeated exposure studies, between groups designs should be used, but within subject comparisons (pre-exposure and post-exposure) are recommended and encouraged.

(2) *Animal selection*—(i) *Species*. (A) For most studies, the laboratory mouse or rat is recommended. Standard strains should be used.

(B) Under some circumstances other species may be recommended.

(ii) *Age*. Experimental animals should be young adults. Rats or mice should be at least 14 and 6 weeks old, respectively, prior to exposure.

(iii) *Sex*. (A) Approximately equal numbers of male and female animals are required for each dose level and control group.

(B) Virgin females should be used.

(iv) *Experimental history*. Animals should be experimentally and chemically naive.

(3) *Number of animals*. Six to twelve animals should be exposed to each level of the test substance and/or control procedure. If post exposure effects are examined, a separate group, 6 to 12 additional animals not sacrificed for pathology, will be required in subchronic studies.

(4) *Control groups*—(i) *Untreated controls*. A concurrent “sham” exposure or vehicle control group or session (according to the design of the study) is required. The subjects should be treated similarly except that administration of the test substance is omitted.

(ii) *Positive controls*. Positive control data is required to demonstrate that the experimental procedures, under the specific conditions in the testing lab-

oratory, are sensitive to substances known to affect operant behavior. Both increases and decreases in response rate should be demonstrated. Data based on acute exposures will be adequate. Data should be collected according to the same experimental design as that proposed for the test substance. Historical data on the procedure collected in the same species and under the same conditions in the testing laboratory may be acceptable, but the presentation of concurrent control data is strongly encouraged since it provides evidence that the test has remained sensitive.

(5) *Dose levels and dose selection*. At least 3 doses, equally spaced over a log scale (e.g., 10, 30, 100), over a range of at least 1 log unit shall be used in addition to a zero dose or vehicle administration. The data should be sufficient to produce a dose-effect curve.

(i) The highest dose shall produce: (A) Clear behavioral effects; or (B) life-threatening toxicity.

(ii) The data from the lower doses must show either: (A) Graded dose-dependent effects at 2 dose levels; or (B) no effects at 2 dose levels, respectively.

(6) *Duration of exposure*. The duration and frequency of exposure will be specified in the test rule.

(7) *Route of Administration*. The route of administration will also be specified in the test rule and will usually be identical to one of the anticipated or actual routes of human exposure. For some chemicals, another route (e.g. parenteral) may be justified. The exposure protocol should conform to that outlined in the appropriate acute or subchronic toxicity study guideline under subpart B or subpart C of this part.

(8) *Study conduct*—(i) *Apparatus*. Behavioral responses and the delivery of reinforcers shall be controlled and monitored by automated equipment located so that its operation does not provide unintended cues or otherwise interfere with the ongoing behavior. Individual chambers should be sound attenuated to prevent disruptions of behavior by external noise. The response manipulanda, feeders, and any stimulus devices should be tested before each session; these devices should periodically be calibrated.

(ii) *Chamber assignment.* Concurrent treatment groups should be balanced across chambers. Each subject should be tested in the chamber to which it is initially assigned.

(iii) *Deprivation and training.* (A) If a nonpreferred positive reinforcer is used, all subjects should be deprived of food until they reach a fixed percentage (e.g. 80 to 90 percent, commonly) of their ad libitum body weight or for a fixed period (e.g., 18 hours) prior to training. Deprivation should be kept constant throughout the study.

(B) Subjects must be trained until they display demonstrable stability in performance across days prior to exposure. One simple and useful criterion is a minimum number of sessions on the schedule and no systematic trend during the 5 days before exposure.

(C) Cumulative records of cumulative responding over time for each animal should be presented to demonstrate that the pattern of responding is representative of that generated by the schedule of reinforcement.

(iv) *Time, frequency, and duration of testing.*—(A) *Time of testing.* All experimental animals should be tested at the same time of day and with respect to the time of exposure. For acute studies, testing should be performed when effects are estimated to peak, usually shortly after exposure. For subchronic studies, subjects should be tested prior to daily exposure in order to assess cumulative effects.

(B) *Frequency of testing.* The maintenance of stable operant behavior normally will require regular and frequent (e.g., 5 days a week) testing sessions. Animals should be weighed on each test day.

(C) *Duration of testing.* (1) Experimental sessions should be long enough to reasonably see the effects of exposure, but brief enough to be practical. Under most circumstances, a session length of 30–40 minutes should be adequate.

(2) If the nature or duration of effects following cessation of repeated exposure are a concern, animals from the high dose group should be tested following exposure for a suitable period of time.

(v) *Schedule selection.* The schedule of reinforcement chosen should generate

response rates that may increase or decrease as a function of exposure. Many schedules of reinforcement can do this: a single schedule maintaining a moderate response rate; fixed-interval schedules, which engender a variety of response rates in each interval; or multiple schedules, where different components may maintain high and low response rates.

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR part 792, subpart J the final test report should contain the following information:

(1) *Description of system, test methods, experimental design, and control data.* (i) A description of the experimental chamber, programming equipment, data collection devices, and environmental conditions.

(ii) A description of the experimental design including counterbalancing procedures, and the stability criterion.

(iii) A description and statistical evaluation of positive control and other control data, including standard measures of central tendency, variability, coefficient of variation of response rates, and the slope of the dose-effect curve.

(2) *Results.* (i) Data for each animal should be arranged by test group in tabular form including the animal identification number, body weight, pre-exposure rate of responding, changes in response rate produced by the chemical, and group data for the same variables, including standard measures of central tendency, variability and coefficient of variation.

(ii) A description and statistical evaluation of the test results: With particular reference to the overall statistical procedures (e.g., parametric or nonparametric) dose-effect curve, and calculation of slope. Presentation of calculations is encouraged.

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

(1) Dews, P.B. "Assessing the Effects of Drugs," *Methods in Psychobiology*, Vol. 2, Ed., R.D. Myers (New York: Academic Press, 1972) 83–124.

(2) Ferster, C.B. Skinner, B.F. *Schedules of Reinforcement.* (New York: Appleton-Century-Crofts, 1957).

(3) Laties, V.G. "How Operant Conditioning can Contribute to Behavioral Toxicology," *Environmental Health Perspectives*, 28: 29-35 (1978).

(4) National Academy of Science. *Principles for Evaluating Chemicals in the Environment*. (Washington, DC: National Academy of Sciences, 1975).

(5) National Academy of Science. *Principles and Procedures for Evaluating the Toxicity of Household Substances*. (Washington, DC: National Academy of Sciences, 1977).

(6) National Academy of Science. "Strategies to determine needs and priorities for toxicity testing," Appendix 3B. *Reference Protocol Guidelines For Neurobehavioral Toxicity Tests*. 2: 123-129 (1982).

§ 798.6560 Subchronic delayed neurotoxicity of organophosphorus substances.

(a) *Purpose*. In the assessment and evaluation of the toxic characteristics of organophosphorus substances the determination of subchronic delayed neurotoxicity may be carried out, usually after initial information on delayed neurotoxicity has been obtained by acute testing or by the demonstration of inhibition and aging of neurotoxic esterase in hen neural tissue. The subchronic delayed neurotoxicity test provides information on possible health hazards likely to arise from repeated exposures over a limited period of time. It will provide information on dose response and can provide an estimate of a non-effect level which can be of use for establishing safety criteria for exposure.

(b) *Definitions*. Subchronic delayed neurotoxicity is a prolonged, delayed-onset locomotor ataxia resulting from repeated daily administration of the test substance.

(c) *Principle of the test method*. Multiple dose levels of the test substance are administered orally to domestic hens (*Gallus gallus domesticus*) for 90 days. The animals are observed at least daily for behavioral abnormalities, locomotor ataxia and paralysis. Histopathological examination of selected neural tissues is undertaken at the termination of the test period.

(d) *Test procedures*—(1) *Animal selection*. The adult domestic laying hen,

aged 8 to 14 months, is recommended. Standard size breeds and strains should be employed.

(2) *Number of animals*. Ten hens should be used for each treatment and control group.

(3) *Control group*—(i) *General*. A concurrent control group should be used. This group should be treated in a manner identical to the treated group, except that administration of the test substance is omitted.

(ii) *Reference substances*. If a positive control is used, a substance which is known to produce delayed neurotoxicity should be employed. Examples of such substances are triorthocresyl phosphate (TOCP) and leptophos.

(4) *Housing and feeding conditions*. Cages or enclosures which are large enough to permit free mobility of the hens and easy observation of gait should be used. Where the lighting is artificial, the sequence should be 12 hours light, 12 hours dark. Appropriate diets should be administered as well as an unlimited supply of drinking water.

(5) *Dose levels*. At least three dose levels should be used in addition to the control group(s). The highest dose level should result in toxic effects, preferably delayed neurotoxicity, but not produce an incidence of fatalities which would prevent a meaningful evaluation. The lowest dose level should not produce any evidence of toxicity.

(6) *Route of administration*. Oral dosing each day for at least 5 days per week should be carried out, preferably by gavage or administration of gelatine capsules.

(7) *Study conduct*—(i) *General*. Healthy young adult hens free from interfering viral diseases and medication and without abnormalities of gait should be acclimatized to the laboratory conditions for at least 5 days prior to randomization and assignment to treatment and control groups. The test or control substance should be administered and observations begun. All hens should be carefully observed at least once daily throughout the test period. Signs of toxicity should be recorded, including the time of onset, degree and duration. Observations should