(2) *Results.* The following information must be arranged by test group (dose level).

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

(A) Its identification number.

(B) Body weight, total session activity counts, and intrasession subtotals for each date measured.

(ii) Group summary data should also be reported.

(3) *Evaluation of data.* An evaluation of the test results (including statistical analysis comparing total activity counts at the end of exposure of treatment vs control animals must be made and supplied. This submission must include dose-effect curves for motor activity expressed as activity counts.

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

(1) Dixon, W.J., Massey, E.J. *Introduction to Statistical Analysis* 2nd Ed. (New York: McGraw-Hill, 1957).

(2) Finger, F.W. ''Measuring behavioral activity,'' *Methods in Psychobiology* Vol. 2. Ed. R.D. Myers (New York: Academic, 1972) pp. 1–19.

(3) Jensen, D.R. ''Some simultaneous multivariate procedures using Hotelling's $T^2$ Statistics,'' *Biometrics*, 28:39–53 (1972).

(4) Kinnard, E.J. and Watzman, N. ''Techniques utilized in the evaluation of psychotropic drugs on animals activity,'' Journal of Pharmaceutical Sciences, 55:995–1012 (1966).

(5) Neter, J. and Wasserman, W. *Applied Linear Statistical Models.* Homewood, Richard D. Irwin, Inc., 1974.

(6) Reiter, L.E. ''Use of activity measures in behavioral toxicology,'' *Environmental Health Perspectives*, 26:9–20 (1978).

(7) Reiter, L.W. and MacPhail, R.C. ''Motor Activity: A survey of methods with potential use in toxicity testing,'' *Neurobehavioral Toxicology*, 1: Suppl. 1, 53–66 (1979).

(8) Robbins, T.W. ''A critique of the methods available for the measurement of spontaneous motor activity,'' *Handbook of Psychopharmacology*. Vol. 7. Eds. Iversen, L.L., Iversen, D.S., Snyder, S.H. (New York: Plenum, 1977) pp. 37–82.

(9) Sokal, R.P. and Rohlf, E.J. *Biometry*. (San Francisco: W.H. Freeman and Co., 1969).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19082, May 20, 1987]

## § 798.6400 Neuropathology.

(a) *Purpose.* The techniques in this guideline are designed to develop data on morphologic changes in the nervous system for chemical substances and mixtures subject to such testing under the Toxic Substances Control Act. The data will detect and characterize morphologic changes, if and when they occur, and determine a no-effect level for such changes. Neuropathological evaluation should be complemented by other neurotoxicity studies, e.g. behavioral and neurophysiological studies. Neuropathological evaluation may be done following acute, subchronic or chronic exposure.

(b) *Definition.* Neurotoxicity or a neurotoxic effect is an adverse change in the structure or function of the nervous system following exposure to a chemical agent.

(c) *Principle of the test method.* The test substance is administered to several groups of experimental animals, one dose being used per group. The animals are sacrificed and tissues in the nervous system are examined grossly and prepared for microscopic examination. Starting with the highest dosage level, tissues are examined under the light microscope for morphologic changes, until a no effect level is determined. In cases where light microscopy has revealed neuropathology, the no effect level may be confirmed by electron microscopy.

(d) *Test procedure*—(1) *Animal selection*—(i) *Species and strain.* Testing shall be performed in the species being used in other tests for neurotoxicity. This will generally be the laboratory rat. The choice of species shall take into consideration such factors as the comparative metabolism of the chemical and species sensitivity to the toxic effects of the test substance, as evidenced by the results of other studies, the potential for combined studies, and the availability of other toxicity data for the species.

(ii) *Age.* Animals shall be young adults (150–200 gm for rats) at the start of exposure.

(iii) *Sex.* Both sexes shall be used unless it is demonstrated that one sex is refractory to the effects.

(2) *Number of animals.* A minimum of six animals per group shall be used. The tissues from each animal shall be examined separately. It is recomse (iv)mended that ten animals per group be used.

(3) *Control groups.* (i) A concurrent control group(s) is (are) required. This group must be an untreated control group or, if a vehicle is used in administering the test substance, a vehicle control group. If the vehicle used has a known or potential toxic property, both untreated and vehicle control groups are required.

(ii) A satellite group of animals may be treated with the high level for 90 days and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate length; normally not less than 28 days.

(4) *Dose levels and dose selection.* At least 3 doses, equally spaced on a log scale (e.g., ½ log units) 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 two dose levels or (B) no effects at two dose levels, respectively.

(5) *Duration of testing.* The exposure duration will be specified in the test rule. This will generally be 90 days exposure.

(6) *Route of administration.* The test substance shall be administered by a route specified in the test rule. This will generally be the route most closely approximating the route of human exposure. The exposure protocol shall conform to that outlined in the appropriate acute or subchronic toxicity guideline.

(7) *Combined protocol.* The tests described herein may be combined with any other toxicity study, as long as none of the requirements of either are violated by the combination.

(8) *Study conduct*—(i) *Observation of animals.* All toxicological (e.g., weight loss) and neurological signs (e.g., motor disturbance) shall be recorded frequently enough to observe any abnormality, and not less than weekly.

(ii) *Sacrifice of animals*—(A) *General.* The goal of the techniques outlined for sacrifice of animals and preparation of tissues is preservation of tissues morphology to simulate the living state of the cell.

(B) *Perfusion technique.* Animals shall be perfused *in situ* by a generally recognized technique. For fixation suitable for light or electronic microscopy, saline solution followed by buffered 2.5 percent glutaraldehyde or buffered 4.0 percent paraformaldehyde, is recommended. While some minor modifications or variations in procedures are used in different laboratories, a detailed and standard procedure for vascular perfusion may be found in the text by Zeman and Innes (1963) under paragraph (f)(7) of this section, Hayat (1970) under paragraph (f)(3) of this section, and by Spencer and Schaumburg (1980) under paragraph (f)(6) of this section. A more sophisticated technique is described by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section.

(C) *Removal of brain and cord.* After perfusion, the bonystructure (cranium and vertebral column) shall be exposed. Animals shall then be stored in fixative-filled bags at 4 °C for 8-12 hours. The cranium and vertebral column shall be removed carefully by trained technicians without physical damage of the brain and cord. Detailed dissection procedures may be found in the text by Palay and Chan-Palay (1974) under paragraph (f)(4) of this section. After removal, simple measurement of the size (length and width) and weight of the whole brain (cerebrum, cerebellum, pons-medulla) shall be made. Any abnormal coloration or discoloration of the brain and cord shall also be noted and recorded.

(D) *Sampling.* Unless a given test rule specifies otherwise, cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum, the midbrain, the cerebellum

and pons, and the medulla oblongata; the spinal cord at cervical and lumbar swelling ($C_3$–$C_6$ and $L_1$–$L_4$); Gasserian ganglia, dorsal root ganglia ($C_3$–$C_6$, $L_1$–$L_4$), dorsal and ventral root fibers ($C_3$–$C_6$, $L^1$–$L_4$), proximal sciatic nerve (mid-thigh and sciatic notch), sural nerve (at knee), and tibial nerve (at knee). Other sites and tissue elements (e.g., gastrocnemius muscle) should be examined if deemed necessary. Any observable gross changes shall be recorded.

(iii) *Specimen storage.* Tissue samples from both the central and peripheral nervous system shall be further immersion fixed and stored in appropriate fixative (e.g., 10 percent buffered formalin for light microscopy; 2.5 percent buffered gluteraldehyde or 4.0 percent buffered paraformaldehyde for electron microscopy) for future examination. The volume of fixative versus the volume of tissues in a specimen jar shall be no less than 25:1. All stored tissues shall be washed with buffer for at least 2 hours prior to further tissue processing.

(iv) *Histopathology examination.* (A) *Fixation.* Tissue specimens stored in 10 percent buffered formalin may be used for this purpose. All tissues must be immersion fixed in fixative for at least 48 hours prior to further tissue processing.

(B) *Dehydration.* All tissue specimens shall be washed for at least 1 hour with water or buffer, prior to dehydration. (A longer washing time is needed if the specimens have been stored in fixative for a prolonged period of time.) Dehydration can be performed with increasing concentration of graded ethanols up to absolute alcohol.

(C) *Clearing and embedding.* After dehydration, tissue specimens shall be cleared with xylene and embedded in paraffin or paraplast. Multiple tissue specimens (e.g. brain, cord, ganglia) may be embedded together in one single block for sectioning. All tissue blocks shall be labelled showing at least the experiment number, animal number, and specimens embedded.

(D) *Sectioning.* Tissue sections, 5 to 6 microns in thickness, shall be prepared from the tissue blocks and mounted on standard glass slides. It is recommended that several additional sections be made from each block at this time for possible future needs for special stainings. All tissue blocks and slides shall be filed and stored in properly labeled files or boxes.

(E) *Histopathological techniques.* Although the information available for a given chemical substance may dictate test-rule specific changes, the following general testing sequence is proposed for gathering histopathological data:

(*1*) *General staining.* A general staining procedure shall be performed on all tissue specimens in the highest treatment group. Hematoxylin and eosin (H&E) shall be used for this purpose. The staining shall be differentiated properly to achieve bluish nuclei with pinkish background.

(*2*) *Special stains.* Based on the results of the general staining, selected sites and cellular components shall be further evaluated by the use of specific techniques. If H&E screening does not provide such information, a battery of stains shall be used to assess the following components in all appropriate required samples: neuronal body (e.g., Einarson's gallocyanin), axon (e.g., Bodian), myelin sheath (e.g., Kluver's Luxol Fast Blue) and neurofibrils (e.g., Bielchosky). In addition, peripheral nerve fiber teasing shall be used. Detailed staining methodology is available in standard histotechnological manuals such as AFIP (1968) under paragraph (f)(1) of this section, Ralis et al. (1973) under paragraph (f)(5) of this section, and Chang (1979) under paragraph (f)(2) of this section. The nerve fiber teasing technique is discussed in Spencer and Schaumberg (1980) under paragraph (f)(6) of this section. A section of normal tissue shall be included in each staining to assure that adequate staining has occurred. Any changes shall be noted and representative photographs shall be taken. If a lesion(s) is observed, the special techniques shall be repeated in the next lower treatment group until no further lesion is detectable.

(*3*) *Alternative technique.* If the anatomical locus of expected neuro-pathology is well-defined, epoxy-embedded sections stained with toluidine blue may be used for small sized tissue samples. This technique obviates the need

for special stains for cellular components. Detailed methodology is available in Spencer and Schaumberg (1980) under paragraph (f)(6) of this section.

(*4*) *Electron microscopy.* Based on the results of light microscopic evaluation, specific tissue sites which reveal a lesion(s) shall be further evaluated by electron microscopy in the highest treatment group which does not reveal any light microscopic lesion. If a lesion is observed, the next lower treatment group shall be evaluated until no significant lesion is found. Detailed methodology is available in Hayat (1970) under paragraph (f)(3) of this section.

(F) *Examination—*(*1*) *General.* All stained microscopic slides shall be examined with a standard research microscope. Examples of cellular alterations (e.g., neuronal vacuolation, degeneration, and necrosis) and tissue changes (e.g., gliosis, leukocytic infiltration, and cystic formation) shall be recorded and photographed.

(*2*) *Electron microscopy.* Since the size of the tissue samples that can be examined is very small, at least 3 to 4 tissue blocks from each sampling site must be examined. Tissue sections must be examined with a transmission electron microscope. Three main categories of structural changes must be considered:

(*i*) *Neuronal body.* The shape and position of the nucleus and nucleolus as well as any change in the chromatin patterns shall be noted. Within the neuronal cytoplasm, cytoplasmic organelles such as mitochondria, lysosomes, neurotubules, neurofilaments, microfilaments, endoplasmic reticulum and polyribosomes (Nissl substance), Golgi complex, and secretory granules shall be examined.

(*ii*) *Neuronal processes.* The structural integrity or alterations of dendrites, axons (myelinated and unmyelinated), myelin sheaths, and synapses shall be noted.

(*iii*) *Supporting cells.* Attention must also be paid to the number and structural integrity of the neuroglial elements (oligodendrocytes, astrocytes, and microglia) of the central nervous system, and the Schwann cells, satellite cells, and capsule cells of the peripheral nervous system. Any changes in the endothelial cells and ependymal lining cells shall also be noted whenever possible. The nature, severity, and frequency of each type of lesion in each specimen must be recorded. Representative lesions must be photographed and labeled appropriately.

(e) *Data collection, reporting, and evaluation.* In addition to information meeting the requirements stated under 40 CFR part 792 subpart J, the following specific information shall be reported:

(1) *Description of test system and test methods.* A description of the general design of the experiment shall be provided. This shall include a short justification explaining any decisions where professional judgment is involved such as fixation technique and choice of stains.

(2) *Results.* All observations shall be recorded and arranged by test groups. This data may be presented in the following recommended format:

(i) *Description of signs and lesions for each animal.* For each animal, data must be submitted showing its identification (animal number, treatment, dose, duration), neurologic signs, location(s) nature of, frequency, and severity of lesion(s). A commonly-used scale such as 1+, 2+, 3+, and 4+ for degree of severity ranging from very slight to extensive may be used. Any diagnoses derived from neurologic signs and lesions including naturally occurring diseases or conditions, should also be recorded.

(ii) *Counts and incidence of lesions, by test group.* Data shall be tabulated to show:

(A) The number of animals used in each group, the number of animals displaying specific neurologic signs, and the number of animals in which any lesion was found;

(B) The number of animals affected by each different type of lesion, the average grade of each type of lesion, and the frequency of each different type and/or location of lesion.

(iii) *Evaluation of data.* (A) An evaluation of the data based on gross necropsy findings and microscopic pathology observations shall be made and supplied. The evaluation shall include the relationship, if any, between the animal's exposure to the test substance and the frequency and severity of the lesions observed.

(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).

[50 FR 39397, Sept. 27, 1985, as amended at 52 FR 19082, May 20, 1987]

## § 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