

(2) Kato, H. "Spontaneous Sister Chromatid Exchanges Detected by a BudR-Labeling Method." *Nature*, 251:70-72 (1974).

(4) Kligerman, A. D., *et al.* "Sister Chromatid Exchange Analysis in Lung and Peripheral Blood Lymphocytes of Mice Exposed to Methyl Isocyanate by Inhalation." *Environmental Mutagenesis* 9:29-36 (1987).

(5) Kligerman, A.D., *et al.*, "Cytogenetic Studies of Rodents Exposed to Styrene by Inhalation", IARC Monographs no. 127 "Butadiene and Styrene: Assessment of Health Hazards" (Sorsa, *et al.*, eds), pp 217-224, 1993.

(6) Kligerman, A., *et al.*, "Cytogenetic Studies of Mice Exposed to Styrene by Inhalation.", *Mutation Research*, 280:35-43, 1992.

(7) Wolff, S., and P. Perry. "Differential Giemsa Staining of Sister Chromatids and the Study of Sister Chromatid Exchanges Without Autoradiography." *Chromosoma* 48: 341-53 (1974).

§ 79.66 Neuropathology assessment.

(a) *Purpose.* (1) The histopathological and biochemical techniques in this guideline are designed to develop data in animals on morphologic changes in the nervous system associated with repeated inhalation exposures to motor vehicle emissions. These tests are not intended to provide a detailed evaluation of neurotoxicity. Neuropathological evaluation should be complemented by other neurotoxicity studies, e.g. behavioral and neurophysiological studies and/or general toxicity testing, to more completely assess the neurotoxic potential of an exposure.

(2) [Reserved]

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

(c) *Principle of the test method.* (1) Laboratory rodents are exposed to one of several concentration levels of a test atmosphere for at least six hours daily over a period of 90 days. At the end of the exposure period, the animals are anaesthetized, perfused *in situ* with fixative, 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-observed-adverse-effect level is determined. In cases where light microscopy has revealed neuropathology, the NOAEL may be confirmed by electron microscopy.

(2) 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. Specifically, this assay may be combined with a subchronic toxicity study, pursuant to provisions in § 79.62.

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

(e) *Test procedures—(1) Animal selection—(i) Species and strain.* Testing shall be performed in the species being used in other NTX tests. A standard strain of laboratory rat is recommended. 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 at least ten weeks of age at the start of exposure.

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

(2) *Number of Animals.* A minimum of ten animals per group shall be used. The tissues from each animal shall be examined separately.

(3) *Control Groups.* (i) A concurrent control group, exposed to clean, filtered air only, is required.

(ii) The laboratory performing the testing shall provide positive control data, e.g., results from repeated acrylamide exposure, as evidence of the ability of their histology procedures to detect neurotoxic endpoints. Positive control data shall be collected at the time of the test study unless the laboratory can demonstrate the adequacy of historical data for the planned study.

(iii) A satellite group of 10 female and 10 male test subjects shall be treated with the highest concentration level for the duration of the exposure and observed thereafter for reversibility, persistence, or delayed occurrence of toxic effects during a post-treatment period of not less than 28 days.

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

(ii) The general conduct of this study shall be in accordance with the vehicle emissions inhalation exposure guideline in § 79.61.

(5) *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) The following is a minimal list of measures that shall be noted:

(A) Body weight;

(B) Subject's reactivity to general stimuli such as removal from the cage or handling;

(C) Description, incidence, and severity of any convulsions, tremors, or abnormal motor movements in the home cage;

(D) Descriptions and incidence of posture and gait abnormalities observed in the home cage; and

(E) 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.

(iii) *Sacrifice of animals*—(A) *General.* The goal of the techniques outlined for sacrifice of animals and preparation of tissues is preservation of tissue mor-

phology 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), Hayat (1970), and Spencer and Schaumburg (1980) under paragraph (g) of this section. A more sophisticated technique is described by Palay and Chan-Palay (1974) under paragraph (g) of this section. In addition, the lungs shall be instilled with fixative via the trachea during the fixation process in order to preserve the lungs and achieve whole-body fixation.

(C) *Removal of brain and cord.* After perfusion, the bony structure (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 (g) 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.* Cross-sections of the following areas shall be examined: The forebrain, the center of the cerebrum, the midbrain, the cerebellum, and the medulla oblongata; the spinal cord at the cervical swelling (C₃-C₆), and proximal sciatic nerve (mid-thigh and sciatic notch) or tibial nerve (at knee). Other sites and tissue elements (e.g., gastrocnemius muscle) shall be examined if deemed necessary. Any observable gross changes shall be recorded.

(iv) *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 glutaraldehyde 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.

(v) *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*. 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) *Peripheral nerve teasing*. Peripheral nerve fiber teasing shall be used. Detailed staining methodology is available in standard histotechnological manuals such as AFIP (1968), Ralis *et al.* (1973), and Chang (1979) under paragraph (g) of this section. The nerve fiber teasing technique is discussed in Spencer and Schaumberg (1980) under paragraph (g) 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.

(F) *Examination*. 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.

(f) Data collection, reporting, and evaluation. In addition to information meeting the requirements stated under 40 CFR 79.60 and 79.61, the following specific information shall be reported:

(1) *Description of test system and test methods*. (i) 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; and

(ii) Positive control data from the laboratory performing the test that demonstrate the sensitivity of the procedures being used. Historical data may be used if all essential aspects of the experimental protocol are the same.

(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, shall 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; and

(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 atmosphere and the frequency and severity of any lesions observed; and

(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 shall include, where applicable, an assessment in conjunction with any other neurotoxicity studies, electrophysiological, behavioral, or neurochemical, which may be relevant to this study.

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

(1) 40 CFR 798.6400, Neuropathology.

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

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

(4) Dunnick, J.K., et.al. Thirteen-week Toxicity Study of N-Hexane in B6C3F1 Mice After Inhalation Exposure (1989) Toxicology, 57, 163-172.

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

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

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

(8) Sette, W. "Pesticide Assessment Guidelines, Subdivision F, Neurotoxicity Test Guidelines." Report No. 540/09-91-123 U.S. Environmental Protection Agency 1991 (NTIS # PB91-154617).

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

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

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§ 79.67 Glial fibrillary acidic protein assay.

(a) *Purpose.* Chemical-induced injury of the nervous system, *i.e.*, the brain, is associated with astrocytic hypertrophy at the site of damage (see O'Callaghan, 1988 in paragraph (e)(3) in this section). Assays of glial fibrillary acidic protein (GFAP), the major intermediate filament protein of astrocytes, can be used to document this response. To date, a diverse variety of chemical insults known to be injurious to the central nervous system have been shown to increase GFAP. Moreover, increases in GFAP can be seen at concentrations below those necessary to produce cytopathology as determined by routine Nissl stains (standard neuropathology). Thus it appears that assays of GFAP represent a sensitive approach for documenting the existence and location of chemical-induced injury of the central nervous system. Additional functional, histopathological, and biochemical tests are necessary to assess completely the neurotoxic potential of any chemical. This biochemical test is intended to be used in conjunction with neurohistopathological studies.