

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).

[59 FR 33093, June 27, 1994, as amended at 63 FR 63793, Nov. 17, 1999]

§ 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.

(b) *Principle of the test method.* (1) This guideline describes the conduct of a radioimmunoassay for measurement of the amount of GFAP in the brain of vehicle emission-exposed and unexposed control animals. It is based on modifications (O'Callaghan & Miller 1985 in paragraph (e)(5), O'Callaghan 1987 in paragraph (e)(1) of this section) of the dot-immunobinding procedure described by Jahn *et al.* (1984) in paragraph (e)(2) of this section. Briefly, brain tissue samples from study animals are assayed for total protein, diluted in dot-immunobinding buffer, and applied to nitrocellulose sheets. The spotted sheets are then fixed, blocked, washed and incubated in anti-GFAP antibody and [I^{125}] Protein A. Bound protein A is then quantified by gamma spectrometry. In lieu of purified protein standards, standard curves are constructed from dilution of a single control sample. By comparing the immunoreactivity of individual samples (both control and exposed groups) with that of the sample used to generate the standard curve, the relative immunoreactivity of each sample is obtained. The immunoreactivity of the control groups is normalized to 100 percent and all data are expressed as a percentage of control. A variation on this radioimmunoassay procedure has been proposed (O'Callaghan 1991 in paragraph (e)(4) of this section) which uses a "sandwich" of GFAP, anti-GFAP, and a chromophore in a microtiter plate format enzyme-link immunosorbent assay (ELISA). The use of this variation shall be justified.

(2) This assay may be done separately or in combination with the subchronic toxicity study, pursuant to the provisions of § 79.62.

(c) *Test procedure*—(1) *Animal selection*—(i) *Species and strain.* Test shall be performed on the species being used in concurrent testing for neurotoxic or other health effect endpoints. This will generally be a species of laboratory rat. The use of other rodent or non-rodent species shall be justified.

(ii) *Age.* Based on other concurrent testing, young adult rats shall be used. Study rodents shall not be older than ten weeks at the start of exposures.

(iii) *Number of animals.* A minimum of ten animals per group shall be used.

The tissues from each animal shall be examined separately.

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

(2) *Materials.* The materials necessary to perform this study are [I^{125}] Protein A (2–10 μ Ci/ μ g), Anti-sera to GFAP, nitrocellulose paper (0.1 or 0.2 μ m pore size), sample application template (optional; e.g., "Minifold II", Schleicher & Schuell, Keene, NH), plastic sheet incubation trays.

(3) *Study conduct.* (i) All data developed within this study shall be in accordance with good laboratory practice provisions under § 79.60.

(ii) *Tissue Preparation.* Animals are euthanized 24 hours after the last exposure and the brain is excised from the skull. On a cold dissecting platform, the following six regions are dissected freehand: cerebellum; cerebral cortex; hippocampus; striatum; thalamus/hypothalamus; and the rest of the brain. Each region is then weighed and homogenized in 10 volumes of hot (70–90 °C) 1 percent (w/v) sodium dodecyl sulfate (SDS). Homogenization is best achieved through sonic disruption. A motor driven pestle inserted into a tissue grinding vessel is a suitable alternative. The homogenized samples can then be stored frozen at –70 °C for at least 4 years without loss of GFAP content.

(iii) *Total Protein Assay.* Aliquots of the tissue samples are assayed for total protein using the method of Smith *et al.* (1985) in paragraph (e)(7) of this section. This assay may be purchased in kit form (e.g., Pierce Chemical Company, Rockford, IL).

(iv) *Sample Preparation.* Dilute tissue samples in sample buffer (120 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$), 5 mM Hepes, pH 7.4, 0.7 percent Triton X-100) to a final concentration of 0.25 mg total protein per ml (5 μ g/20 μ l).

(v) *Preparation of Standard Curve.* Dilute a single control sample in sample buffer to give at least five standards, between 1 and 10 μ g total protein per 20 μ l. The suggested values of total protein per 20 μ l sample buffer are 1.25, 2.50, 3.25, 5.0, 6.25, 7.5, 8.75, and 10.0 μ g.

(vi) *Preparation of Nitrocellulose Sheets.* Nitrocellulose sheets of 0.1 or

0.2 micron pore size are rinsed by immersion in distilled water for 5 minutes and then air dried.

(vii) *Sample Application.* Samples can be spotted onto the nitrocellulose sheets free-hand or with the aid of a template. For free-hand application, draw a grid of squares approximately 2 centimeters by 2 centimeters (cm) on the nitrocellulose sheets using a soft pencil. Spot 5–10 μ l portions to the center of each square for a total sample volume of 20 μ l. For template aided sample application a washerless microliter capacity sample application manifold is used. Position the nitrocellulose sheet in the sample application device as recommended by the manufacturer and spot a 20 μ l sample in one application. Do not wet the nitrocellulose or any support elements prior to sample application. Do not apply vacuum during or after sample application. After spotting samples (using either method), let the sheets air dry. The sheets can be stored at room temperature for several days after sample application.

(viii) *Standard Incubation Conditions.* These conditions have been described by Jahn *et al.* (1984) in paragraph (e)(2) of this section. All steps are carried out at room temperature on a flat shaking platform (one complete excursion every 2–3 seconds). For best results, do not use rocking or orbital shakers. Perform the following steps in enough solution to cover the nitrocellulose sheets to a depth of 1 cm.

(A) Incubate 20 minutes in fixer (25 percent (v/v) isopropanol, 10 percent (v/v) acetic acid).

(B) Discard fixer, wash several times in deionized water to eliminate the fixer, and then incubate for 5 minutes in Tris-buffered saline (TBS): 200 mM NaCl, 60 mM Tris-HCl to pH 7.4.

(C) Discard TBS and incubate 1 hour in blocking solution (0.5 percent gelatin (w/v)) in TBS.

(D) Discard blocking solution and incubate for 2 hours in antibody solution (anti-GFAP antiserum diluted to the desired dilution in blocking solution containing 0.1 percent Triton X-100). Serum anti-bovine GFAP, which cross reacts with GFAP from rodents and humans, can be obtained commercially

(e.g., Dako Corp.) and used at a dilution of 1:500.

(E) Discard antibody solution, and wash in 4 changes of TBS for 5 minutes each time. Then wash in TBS for 10 minutes.

(F) Discard TBS and incubate in blocking solution for 30 minutes.

(G) Discard blocking solution and incubate for 1 hour in Protein A solution ($[^{125}\text{I}]$ -labeled Protein A diluted in blocking solution containing 0.1 percent Triton X-100, sufficient to produce 2000 counts per minute (cpm) per 10 μ l of Protein A solution).

(H) Remove Protein A solution (it may be reused once). Wash in 0.1 percent Triton X-100 in TBS (TBSTX) for 5 minutes, 4 times. Then wash in TBSTX for 2–3 hours for 4 additional times. An overnight wash in a larger volume can be used to replace the last 4 washes.

(I) Hang sheets to air-dry. Cut out squares or spots and count radioactivity in a gamma counter.

(ix) *Expression of data.* Compare radioactivity counts for samples obtained from control and treated animals with counts obtained from the standard curve. By comparing the immunoreactivity (counts) of each sample with that of the standard curve, the relative amount of GFAP in each sample can be determined and expressed as a percent of control.

(d) *Data Reporting and Evaluation*—(1) *Test Report.* In addition to information meeting the requirements stated under 40 CFR 79.60, the following specific information shall be reported:

(i) Body weight and brain region weights at time of sacrifice for each subject tested;

(ii) Indication of whether each subject survived to sacrifice or time of death;

(iii) Data from control animals and blank samples; and

(iv) Statistical evaluation of results;

(2) *Evaluation of Results.* (i) Results shall be evaluated in terms of the extent of change in the amount of GFAP as a function of treatment and dose. GFAP assays (of any brain region) from a minimum of 6 samples typically will result in a standard error of the mean of ± 5 percent. In this case, a chemically-induced increase in GFAP

of 115 percent of control is likely to be statistically significant.

(ii) The results of this assay shall be compared to and evaluated with any relevant behavioral and histopathological data.

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

(1) Brock, T.O and O'Callaghan, J.P. 1987. Quantitative changes in the synaptic vesicle proteins, synapsin I and p38 and the astrocyte specific protein, glial fibrillary acidic protein, are associated with chemical-induced injury to the rat central nervous system, *J. Neurosci.* 7:931-942.

(2) Jahn, R., Schiebler, W. Greengard, P. 1984. A quantitative dot-immunobinding assay for protein using nitrocellulose membrane filters. *Proc. Natl. Acad. Sci. U.S.A.* 81:1684-1687.

(3) O'Callaghan, J.P. 1988. Neurotypic and gliotypic protein as biochemical markers of neurotoxicity. *Neurotoxicol. Teratol.* 10:445-452.

(4) O'Callaghan, J.P. 1991. Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA. *Neurotoxicol. Teratol.* 13:275-281.

(5) O'Callaghan, J.P. and Miller, D.B. 1985. Cerebellar hypoplasia in the Gunn rat is associated with quantitative changes in neurotypic and gliotypic proteins. *J. Pharmacol. Exp. Ther.* 234:522-532.

(6) Sette, W.F. "Pesticide Assessment Guidelines, Subdivision 'F', Hazard Evaluation: Human and Domestic Animals, Addendum 10, Neurotoxicity, Series 81, 82, and 83" US-EPA, Office of Pesticide Programs, EPA-540/09-91-123, March 1991.

(7) Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Annal. Biochem.* 150:76-85.

§ 79.68 *Salmonella typhimurium* reverse mutation assay.

(a) *Purpose.* The *Salmonella typhimurium* histidine (his) reversion system is a microbial assay which measures his⁻ → his⁺ reversion induced

by chemicals which cause base changes or frameshift mutations in the genome of the microorganism *Salmonella typhimurium*.

(b) *Definitions.* For the purposes of this section, the following definitions apply:

Base pair mutagen means an agent which causes a base change in DNA. In a reversion assay, this change may occur at the site of the original mutation or at a second site in the chromosome.

Frameshift mutagen is an agent which causes the addition or deletion of single or multiple base pairs in the DNA molecule.

Salmonella typhimurium reverse mutation assay detects mutation in a gene of a histidine-requiring strain to produce a histidine independent strain of this organism.

(c) *Reference substances.* These may include, but need not be limited to, sodium azide, 2-nitrofluorene, 9-aminoacridine, 2-aminoanthracene, congo red, benzopurpurin 4B, trypan blue or direct blue 1.

(d) *Test method.*—(1) *Principle.* Motor vehicle combustion emissions from fuel or additive/base fuel mixtures are, first, filtered to trap particulate matter and, then, passed through a sorbent resin to trap semi-volatile gases. Bacteria are separately exposed to the extract from both the filtered particulates and the resin-trapped organics. Assays are conducted using both test mixtures with and without a metabolic activation system and exposed cells are plated onto minimal medium. After a suitable period of incubation, revertant colonies are counted in test cultures and compared to the number of spontaneous revertants in unexposed control cultures.

(2) *Description.* Several methods for performing the test have been described. The procedures described here are for the direct plate incorporation method and the azo-reduction method. Among those used are:

- (i) Direct plate incorporation method;
- (ii) Preincubation method;
- (iii) Azo-reduction method;
- (iv) Microsuspension method; and
- (v) Spiral assay.