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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OCT 30 1987

MEMORANDUM

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

SUBJECT: Request For A Neurotoxicity Study On N,N-diethyl-m-toluamide (Deet)

TO: Joseph Tavano
Product Manager
Registration Division (TS-767c)

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THROUGH: Marcia van Gemert, Ph.D. 10/27/87
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Introduction:

Deet is generally regarded as the most effective topical insect repellent. It was estimated that 22% of the general population in American is exposed to pressurized insect repellent containing Deet. The Consumer Union has seen several case reports of acute neurotoxicity in children exposed to Deet contaning insect repellents. The Toxicology Branch has been requested to consider if a neurotoxicity study with an appropriate animal species be required to address this issue.

Discussion and Conclusion:

This reviewer has evaluated the available information on the neurotoxicity of Deet in two journals (JAMA, Sept 18, 1987; 258: No. 11 and Journal of Toxicology and Environmental Health, 18: 503-525, 1986) and the Deet Registration Standard, 1980. The data indicate that several children developed encephelopathies after exposure to "unspecified amounts of Deet ranging from small to massive doses". Neurotoxic effects were also observed in workers exposed to 4 gm or more of Deet per week. In addition, a subchronic oral toxicity study using technical Deet in dogs showed that 0.3 ml/kg of 85% m-Deet with 10% of other isomers caused "slight-to-moderate central nervous system excitation consisting of tremor and hyperactivity". This subchronic dog study was considered as

a supplementary study. At the present, adequately conducted neurotoxicity study with any animals on Deet does not exist.

Based upon the above observations, the Toxicology Branch recommends a neurotoxicity study be carried out according to the study guidelines published in the Federal Register (Vol. 50/No. 188/Sept. 27, 1985)(Attachment). It is recommended that the test animals be orally administered Deet for 14 days and the highest dose in this study be 2,000 mg/kg which has been proven in the oral subchronic toxicity study to cause overt toxicity and not to cause excessive death.

Subpart G—Neurotoxicity

§798.6050 Functional observational battery.

(a) *Purpose.* In the assessment and evaluation of the potential human health effects of substances, it may be necessary to test for neurotoxic effects. Substances that have been observed to cause neurotoxic signs (e.g., convulsions, tremors, ataxia) in other toxicity tests, as well as those having a structural similarity to known neurotoxicants, should be evaluated for neurotoxicity. The functional observational battery is a noninvasive procedure designed to detect gross functional deficits in young adults resulting from exposure to chemicals and to better quantify neurotoxic effects detected in other studies. This battery of tests is not intended to provide a detailed evaluation of neurotoxicity. It is designed to be used in conjunction with neuropathologic evaluation and/or general toxicity testing. Additional functional tests may be necessary to assess completely the neurotoxic potential of a chemical.

(b) *Definitions.* (1) Neurotoxicity is any adverse effect on the structure or function of the central and/or peripheral nervous system related to exposure to a chemical substance.

(2) A toxic effect is an adverse change in the structure or function of an experimental animal as a result of exposure to a chemical substance.

(c) *Principle of the test method.* The material is administered by an appropriate route to laboratory rodents. The animals are observed under carefully standardized conditions with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. Various functions that could be affected by neurotoxicants are assessed during each observation period.

(d) *Test procedures*—(1) *Animal selection*—(i) *Species and strain.* The laboratory rat or mouse is recommended. Although information will generally be lacking, whenever possible the choice of species should 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 should also be considered. Standard strains should be used.

(ii) *Age.* Young adult animals (at least 42 days old for the rat or mouse) should be used.

(iii) *Sex.* (A) Equal numbers of animals of each sex are required for each dose level.

(B) The females should be nulliparous and nonpregnant.

(2) *Number of animals.* All exposed animals should be tested. At least 10 animals of each sex should be used at each dose level. If interim sacrifices are planned, the number should be increased by the number of animals scheduled to be sacrificed before the end of the study. Animals should be randomly assigned to treatment and control groups.

(3) *Control groups.* (i) A concurrent ("sham" exposure or vehicle) control group is required. Subjects should be treated in the same way as for an exposure group except that administration of the test substance is omitted.0, RULES AND REGS) A35AD0 Barrett, Douglas 04942 7-29-85 J. 54-999 File a35ad0.422 Folio 1110-11

(ii) Concurrent or historic data from the laboratory performing the testing should provide evidence of the ability of the procedures used to detect major neurotoxic endpoints such as limb

weakness or paralysis (e.g., acrylamide). CNS stimulation (e.g., β, β'-iminodiproprionitrile) autonomatic signs (e.g., physostigmine).

(iii) A satellite group may be treated with the high dose level for the duration of exposure and observed for reversibility, persistence, or delayed occurrence of toxic effects for a post-treatment period of appropriate duration, 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 2 dose levels or (B) no effects at 2 dose levels, respectively.

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

(6) *Route of exposure.* The test substance shall be administered by the route specified in the test rule. This route will usually be the one most closely approximating the expected route of human exposure. The exposure potocol shall conform to that outlined in the appropriate acute or subchronic toxicity study guideline under Subpart B or Subpart C of this Part.

(7) *Combined protocol.* Subjects used for other toxicity studies may be used if none of the requirements of either study are violated by the combination.

(8) *Study conduct.* (i) All animals in a given study should be observed carefully by the same trained technician who is blind with respect to the animals' treatments. All animals should be observed prior to initiation of exposure. Subsequent observations should be made with sufficient frequency to ensure the detection of behavioral and/or neurologic abnormalities, if present. At minimum, observations at 1 hour, 6 hours, 24 hours, 7 days, and 14 days and monthly thereafter are recommended. In a subchronic study, subsequent to the first exposure, all observations should be made before the daily exposue. The animals should be removed from the home cage to a standard arena for observation. Effort should 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 behavior are sound level, temperature, humidity, lighting, odors, time of day, and environmental distractions. Explicit, operationally defined, scales for each function should be used. The development of objective quantitative measures of the observational endpoints specified is encouraged.

(ii) The following is a minimal list of observations that should be noted:

(A) Any unusual responses with respect to body position, activity level, coordination of movement, and gait.

(B) Any unusual or bizarre behavior including, but not limited to, headflicking, head searching, compulsive biting or licking, self-mutilation, circling, and walking backwards.

(C) The presence of:

(*1*) Convulsions.

(*2*) Tremors.

(*3*) Increased levels of lacrimation and/or red-colored tears.

(*4*) Increased levels of salivation.

(*5*) Piloerection.

(*6*) Pupillary dilation or constriction.

(*7*) Unusual respiration (shallow, labored, dyspneic, gasping, and retching) and/or mouth breathing.

(*8*) Diarrhea.

(*9*) Excessive or diminished urination.

(*10*) Vocalization.

(D) Forelimb/hindlimb grip strength. The procedure described by Meyer et al. (1979), under paragraph (f)(9) of this section is recommended.

(E) Sensory function. A simple assessment of sensory function (vision, audition, pain perception) should be made. Marshall et al. (1971) under paragraph (f)(8) of this section have described a neurologic exam for this purpose; these procedures are also discussed by Deuel (1977), under paragraph (f)(4) of this section. Irwin (1968) under paragraph (f)(7) of this section described a number of reflex tests intended to detect gross sensory deficits, including the visual placing response, Preyer reflex, and tail pinch. Many procedures have been developed for assessing pain perception (e.g., Ankier, 1974 under paragraph (f)(1) of this section; D'Amour and Smith 1941 under paragraph (f)(3) of this section; Evans 1971 under paragraph (f)(6) of this section).

(e) *Data reporting and evaluation.* In addition to the reporting requirements specified under 40 CFR Part 792 Subpart J the final test report must include the following information.

(1) *Description of system and test methods.* (i) A detailed description of the procedures used to standardize observation, including the arena and operational definitions for scoring observations.

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

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

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

(A) Its identification number.

(B) Its body weight and score on each sign at each observation time, the time and cause of death (if appropriate).

(ii) Summary data for each group must include:

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

(B) The number of animals showing each observation score at each observation time.

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

(D) The mean and standard deviation for each continuous endpoint at each observation time.

(3) *Evaluation of data.* The findings of a functional observational battery should be evaluated in the context of preceding and/or concurrent toxicity studies and any correlative histopathological findings. The evaluation should include the relationship between the doses of the test substance and the presence or absence, incidence and severity, of any neurotoxic effects. The evaluation should include appropriate statistical analyses. Choice of analyses should consider tests appropriate to the experimental design and needed adjustments for multiple comparisons.

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

(1) Ankier, S.I. "New hot plate tests to quantify antinociceptic and narcotic antagonist activities," *European Journal of Pharmacology*, 27: 1–4 (1974).

(2) Coughenour, L.L., McLean, J.R. and Parker, R.B. "A new device for the rapid measurement of impaired motor function in mice," *Pharmacology, Biochemistry and Behavior, 6:* 351–353 (1977).

(3) D'Amour, F.E., Smith, D.L. "A method for determining loss of pain sensation," *Journal of Pharmacology and Experimental Therapeutics, 72:* 74–79 (1941).

(4) Deuel, R.K. "Determining sensory deficits in animals," *Methods in Psychobiology* Ed. Myers R.D. (New York: Academic Press, 1977) pp. 99–125.

(5) Edwards, P.M., Parker, V.H. "A simple, sensitive and objective method for early assessment of acrylamide neuropathy in rats," *Toxicology and*

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Applied Pharmacology. 40: 589–591 (1977).

(6) Evans, W.O. "A new technique for the investigation of some analgesic drugs on reflexive behavior in the rat." *Psychopharmacologia. 2:* 318–325 (1961).

(7) Irwin, S. "Comprehensive observational assessment: Ia. A systematic quantitative procedure for assessing the behavioral and physiologic state of the mouse." *Psychopharmacologia. 13:* 222–257 (1968).

(8) Marshall, J.F., Turner, B.H., Teitlbaum, P. "Sensory neglect produced by lateral hypothalamic damage." *Science. 174:* 523–525 (1971).

(9) Meyer, O.A., Tilson, H.A., Byrd, W.C., Riley, M.T. "A method for the routine assessment of fore- and hindlimb grip strength of rats and mice." *Neurobehavioral Toxicology. 1:* 233–236 (1979).

§ 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 should 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 should 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 recommended 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) should be exposed. Animals should 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) should be made. Any abnormal coloration or discoloration of the brain and cord should 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 should 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 should 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 neuropathology 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 should be reported:

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