

(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 include, but not be limited to, behavioral abnormality, locomotor ataxia and paralysis. At least once a week the hens should be taken outside the cages and subjected to a period of forced motor activity, such as ladder climbing, in order to enhance the observation of minimal responses. The hens should be weighed weekly. Any moribund hens should be removed and sacrificed.

(ii) *Pathology*—(A) *Gross necropsy*. In the presence of clinical signs of delayed

neurotoxicity useful information may be provided by gross necropsy.

(B) *Histopathology.* Tissues from all animals should be fixed *in situ*, using perfusion techniques. Sections should include medulla oblongata, spinal cord and peripheral nerves. The spinal cord sections should be taken from the upper cervical bulb, the mid-thoracic and lumbosacral regions. Sections of the proximal region of the tibial nerve and its branches and of the sciatic nerve should be taken. Sections should be stained with appropriate myelin and axon-specific stains. Microscopic examination should be carried out on all hens in the control and high-dose groups. Microscopic examination should also be carried out on hens in the low and intermediate dose groups when there is evidence of effects in the high-dose group.

(e) *Data reporting and evaluation—(1) Test report.* In addition to the reporting requirements specified under 40 CFR part 792, subpart J the final test report must include the following information:

(i) Toxic response data by group with a description of clinical manifestations of nervous system damage; where a grading system is used the criteria should be defined.

(ii) For each animal, time of death during the study or whether it survived to termination.

(iii) The day of observation of each abnormal sign and its subsequent course.

(iv) Body weight data.

(v) Necropsy findings for each animal, when performed.

(vi) A detailed description of all histopathological findings.

(vii) Statistical treatment of results, where appropriate.

(2) *Treatment of results.* (i) Data may be summarized in tabular form, showing for each test group the number of animals at the start of the test, the number of animals showing lesions or effects, the types of lesions or effects and the percentage of animals displaying each type of lesion or effect.

(ii) All observed results should be evaluated by an appropriate statistical method. Any generally accepted statistical method may be used; the statis-

tical methods should be selected during the design of the study.

(3) *Evaluation of results.* The findings of a subchronic delayed neurotoxicity study should be evaluated in conjunction with the findings of preceding studies and considered in terms of the incidence and severity of observed neurotoxic effects and any other observed effects and histopathological findings in the treated and control groups. A properly conducted subchronic test should provide a satisfactory estimation of a no-effect level based on lack of clinical signs and histopathological changes.

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

(1) Abou-Donia, M.B. "Organophosphorus ester-induced delayed neurotoxicity" Annual Review of Pharmacology and Toxicology, 21:511-548 (1981).

(2) Abou-Donia, M.B., Pressing, S.H. "Delayed neurotoxicity from continuous low-dose oral administration of leptophos to hens." *Toxicology and Applied Pharmacology*, 38:595-608 (1976).

(3) Baron, R.L. (ed). "Pesticide Induced Delayed Neurotoxicity," Proceedings of a Conference, February 19-20, 1976, Washington, DC. U.S. Environmental Protection Agency. EPA Report No. 600/1-76-025, Washington, DC (1976).

(4) Cavanaugh, J.B. "Peripheral neuropathy caused by chemical agents" *Critical Reviews of Toxicity*, 2:365-417 CRC Press, Inc. (1973).

(5) Johannsen, F.R., Wright, P.L., Gordon, D.E., Levinskas, G.L., Radue, R.W., Graham, P.R. "Evaluation of delayed neurotoxicity and dose-response relationship of phosphate esters in the adult hen," *Toxicology and Applied Pharmacology*, 41:291-304 (1977).

(6) Johnson, M.K. "Organophosphorus esters causing delayed neurotoxic effects: mechanism of action and structure/activity studies," *Archives of Toxicology*, 34:259-288 (1975).