

## Mini-Review Article

# Screening Pesticides for Neuropathogenicity

**John D. Doherty**

*Health Effects Division (7509C), Office of Pesticide Programs, United States Environmental Protection Agency,  
1200 Pennsylvania Avenue NW, Washington DC 20460, USA*

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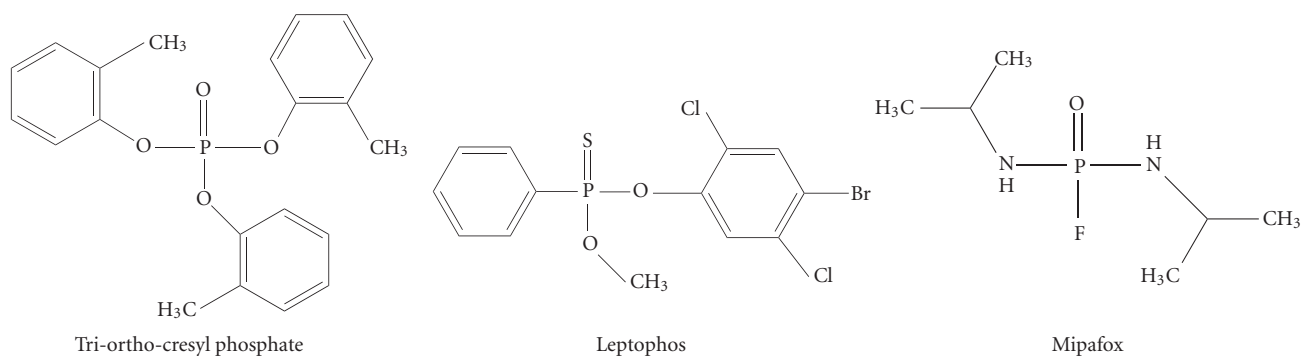
Pesticides are routinely screened in studies that follow specific guidelines for possible neuropathogenicity in laboratory animals. These tests will detect chemicals that are by themselves strong inducers of neuropathogenesis if the tested strain is susceptible relative to the time of administration and methodology of assessment. Organophosphate induced delayed neuropathy (OPIDN) is the only known human neurodegenerative disease associated with pesticides and the existing study guidelines with hens are a standard for predicting the potential for organophosphates to cause OPIDN. Although recent data have led to the suggestion that pesticides may be risk factors for Parkinsonism syndrome, there are no specific protocols to evaluate this syndrome in the existing study guidelines. Ideally additional animal models for human neurodegenerative diseases need to be developed and incorporated into the guidelines to further assure the public that limited exposure to pesticides is not a risk factor for neurodegenerative diseases.

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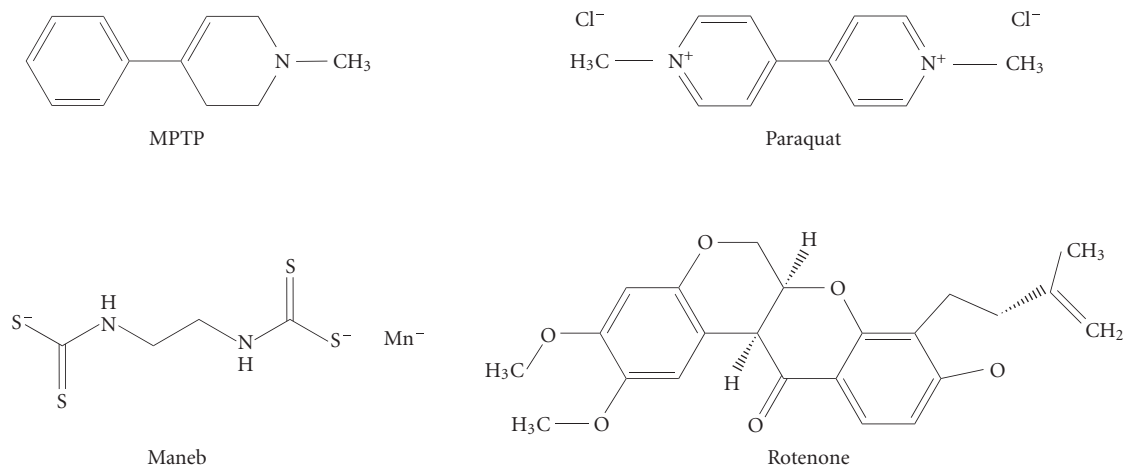
## INTRODUCTION

In the early 1980s, there was an unfortunate human situation in which drug abusers developed Parkinsonism syndrome [1] following exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP, see Figure 1) that is a by-product in an attempt to chemically synthesize heroin. Although earlier researchers sought links between manganese exposure and Parkinsonism [2], the MPTP incident greatly increased the interest in correlating environmental exposure to contaminants and human neurodegenerative diseases. Human exposure to pesticides is essentially unavoidable in modern life both in the developed nations and more increasingly in the developing ones. Worldwide pesticide sales and usage in both 2000 and 2001 were in excess of five billion pounds. In the United States alone there were about 1.2 billion pounds of pesticides used including insecticides, herbicides, fungicides, rodenticides, but not including wood preservatives, special biocides, and chlorine/hydrochlorides [3]. Human exposure to pesticides depends upon many factors and often agricultural workers have the highest rates of exposure as they apply pesticides to crops. Spray drift and migration of the pesticides to potable water as well as residues in food stuffs and residues resulting from home and garden applications are also very significant sources of exposure. Many insecticides are neurotoxic by design with targets being acetylcholinesterase (organophosphates and carbamates), the Na<sup>+</sup> conductance channel (DDT, pyrethrins,

and pyrethroids), the acetylcholine receptor (nicotinic), the GABA receptor (emamectin), Ca<sup>++</sup> channels (ryanodine), and some agents such as rotenone that affects mitochondrial function and also may affect the nervous system. If a poisoned individual recovers from the initial toxicity following a single dose of anticholinesterase inhibitors (with the exception of some organophosphates) or agents that act on transmitter receptors, and when the chemical is rapidly metabolized and excreted, there is usually no established pathological or neurodegenerative change although there are many anecdotal reports of persistent subtle effects (see reference [4]). The trauma of the acute poisoning incident may have some psychological effects that may not actually be related to the neuropharmacology of the agent. The consequences of chronic exposure to pesticides, whether they are designed to act on the nervous system as are insecticides or are herbicides designed to be specific for plants, may be causing effects in humans through their known or yet to be discovered effects in the nervous system. Over the past decade there has been a growing body of literature that suggests pesticides as being risk factors either for possibly initiating or facilitating the progression of neurodegenerative diseases (eg, see Table 1). Theoretically humans may have the initiation of the diseases triggered by exposure to a pesticide or a pesticide in combination with other environmental contaminants. In some cases, it is possible that individuals with a genetic predisposition for a neurodegenerative disease may be at an increased risk to exposure to



(a) Chemicals associated with OPIDN



(b) Chemicals proposed to be associated with Parkinson's syndrome

FIGURE 1: Chemical structures.

pesticides that might initiate the disease. In other cases where the initiating event in either normal or genetically susceptible persons is caused by a spontaneous event or another chemical exposure, the progression following its initiation may be facilitated to various degrees by exposure to pesticides.

The potential toxicity of pesticides is evaluated in laboratory animals prior to registration and updated in the reregistration process in a series of required or conditionally required studies that follow specific guidelines [5]. Partly as a consequence of the discovery that MPTP caused a neurodegenerative disease as well as the interest in the possibility that there is increased susceptibility associated with prenatal and neonatal exposures, there has been increased testing as a part of the registration/reregistration process to attempt to determine the potential effects of pesticides on the nervous system. As a result, a series of special neurotoxicity study guidelines were developed in the early 1990s. These guidelines for special neurotoxicity testing together with other more general study guidelines that also assess for effects on the nervous system are listed in Table 2.

## OVERALL GOAL OF THE STUDY GUIDELINES AND RISK ASSESSMENT

In classical terms, the goal of the study guidelines is to characterize the toxicity of the pesticide and to identify the most sensitive endpoint in the most sensitive species. Once this endpoint is selected from the pesticide's toxicity database including the required studies following the guidelines in Table 2, nonguideline studies that are either conducted at the registrant's own initiative or as recommended by the USEPA as well as studies from the open literature, a risk assessment is performed. Traditionally the risk assessment is based on the no observable adverse effect level (NOAEL) for this endpoint coupled with available or estimated exposure data. The NOAEL is adjusted by uncertainty factors to further assure the safety of the chemical to humans. First, a factor of 10 X for intraspecies variation based on the assumption that within species some individuals may be 10 times more sensitive than the tested group is employed. Another 10 X factor for interspecies variation based on the assumption that humans may be 10 times more sensitive than the most sensitive laboratory animal species is also employed. Another 10 X

TABLE 1: Selected examples of human neurodegenerative and other neurological diseases both demonstrated and possibly attributed to pesticides.

Disease	Pesticide (reference)	Association with humans	Guidelines for assessment
Organophosphate-induced delayed neuropathy (OPIDN)	Organophosphates cholinesterase inhibitors. (8-review)	Strong. Actual association demonstrated	Yes—hen studies
Parkinson's disease	Paraquat [20–25], maneb [26, 27], rotenone [28–30], organochlorines [31–34], also [35]	Not firmly established but circumstantial evidence	No
Alzheimer's disease	No specific pesticide—agricultural workers [36, 37]	One case study—association not proven. Epidemiological study with 68 cases—no association concluded	No learning and memory not assessed in older animals
Amyotrophic lateral sclerosis	2-4-dichlorophenoxy-acetic acid [38, 39]	Report of increased relative risk among employees in manufacturing. Agricultural workers have higher rates	No specific test but several tests would detect neurological and muscular degeneration
Autism	No specific pesticide [22]	Suggestion that impaired metabolism of pesticides may be associated with increased incidence of autism	No, but certain patterns in the DNT study may be an indicator
Psychiatric disorders	Organophosphates [40–42]	Authors claim of positive association in epidemiological studies and EEG changes in humans and monkeys	No

factor may be applied if it is determined that the database is incomplete or there is no NOAEL for the most sensitive endpoint. When there is an evidence of developmental toxicity in fetuses or neonatal animals at lower doses than parental or adult toxicity, an additional FQPA (Food Quality Protection Act) 10 X (reducible to 3 X or 1 X depending upon the circumstances) safety factor is applied to assure the protection of fetuses, newborns, and children. It should be noted that the application of the uncertainty factors is to the NOAEL and the lowest observable adverse effect level (LOAEL) is always higher than the NOAEL. Thus, a total uncertainty factor of 100 applied to an NOAEL is in reality a factor of 300 from a dose where there is an effect when the LOAEL is a dose three times higher than the NOAEL. In order to eliminate or compensate for some of the limitations of the NOAEL and LOAEL approaches, statistical methods have been developed to determine a benchmark dose (BMD) that accounts for gaps in dose spacing or account for a study not showing an NOAEL [6]. The uncertainty factors as described above (except for not having an NOAEL) are applied to the BMD.

A risk assessment for a chemical with an NOAEL based on liver toxicity at the LOAEL that has evidence of neuropathogenicity in laboratory animals at higher doses than

at the LOAEL will be protective against the neuropathogenesis although neuropathogenicity was not the basis for the selection of the NOAEL. The protective nature of both the NOAEL and BMD approaches to risk assessment assumes that most other potential target organs in humans will only be affected at higher doses than the most sensitive endpoint in laboratory animals. If humans are especially susceptible to neuropathogenesis resulting from exposure to a certain pesticide, the endpoint based on animal studies may underestimate the risk to humans. However, the minimum 100 X uncertainty factor plus any additional factors would only in rare cases not be protective against such neuropathogenesis in humans having extreme sensitivity to the chemical. To date, although this may be debatable by some, there is no *known* neuropathological condition caused or facilitated by pesticides that should not be protected against by the current approach to risk assessment as outlined above provided that the pesticide does not interact with other environmental contaminants, drugs, or naturally occurring chemicals to render neuropathogenicity. The principle of selecting the most sensitive endpoint in the most sensitive test animal species and using either the NOAEL or BMD and applying uncertainty factors to drive down exposure is still the basis for risk

TABLE 2: List of guideline studies both general and especially designed to assess for neurotoxicity.

Guideline	Study title	When required	Species [1]	Protocol timing	Nonpathology [2]	Histopathology
General toxicity assessment						
870.1100 870.1200 870.1300	Acute oral, dermal, and inhalation toxicity	For all chemicals (inhalation not required for some)	Rat or rabbit	Acute dose to young adults and observations for 14 days	Clinical signs, body weight and mortality	Gross necropsy only
870.3100 870.3150	Subchronic oral, dermal, and inhalation dosing	For most chemicals	Rat or dog	90 days dosing starting with young adult	Clinical signs, body weight and hematology, clinical chemistry and urinalysis and mortality	Generally hematoxylin and eosin
870.3700	Prenatal developmental	For most chemicals	Rat or rabbit	Gestation days 6–16 for rats and 6–18 for rabbits	Clinical signs, body weight, uterine data and pup data and mortality	Gross dissection for visceral and skeletal assessment of the pups
870.3800	Reproductive	For chemicals with food uses and chronic exposure	Rat	Continuous from pre-mating through gestation, lactation and adults for two generations	Reproductive performance, clinical signs, body weight and mortality, pup growth and development	Necropsy and histopathology of the reproductive organs
870.4100 870.4200 870.4300	Chronic dosing and carcinogenicity	For chemicals with food uses or where chronic exposure is expected	Rat, dog, and mouse	6 months for dogs, 18 months or more for mice and 24 months for rats	Clinical signs and mortality	Generally the same as for subchronic
Special studies for organophosphate-induced delayed neuropathy						
870.6100	Acute delayed neurotoxicity for OPIDN	All organophosphate AChE inhibitors	Hen (8–14 months of age)	Single limit dose of 2 gm/kg or near lethal dose (ie, LD <sub>50</sub> ). Atropine can be used to prevent death by AChE inhibition. Must have 6 survivors. Sacrifice at day 21	Gait assessment neuropathy toxic esterase acetylcholinesterase	Whole body perfusion. Sections of medulla oblongata, spinal cord (rostral cervical, midthoracic, and lumbosacral), and peripheral nerves. “Appropriate” myelin and axon specific stains
870.6100	Subchronic delayed neurotoxicity for OPIDN	For organophosphate AChE inhibitors when the acute study is inconclusive		Limit dose of 1 gm/kg. Three dose levels required. Establish NOAEL and LOAEL		

TABLE 2: Continued.

Guideline	Study title	When required	Species [1]	Protocol timing	Nonpathology [2]	Histopathology
Special neurotoxicity assessments in rats*						
870.6200a	Acute neurotoxicity screen	Conditionally required	Rat	Single dose to young adults with assessments at predosing, optimum time of effect and at days 7 and 14	FOB and Motor Activity at pretest, time to peak effect and days 7 and 14	In situ perfusion with fixative. Paraffin embedding acceptable for CNS but plastic embedding required for peripheral. GFAP immunohistochemistry, Bodian's stain and Bielschowsky's silver methods recommended in addition to standard stains
870.6200b	Subchronic neurotoxicity screen		Rat	Daily dosing with live assessments at predosing, weeks 4, 8 and 13 but histopathology at week 13 only	FOB and Motor activity at pretest, 4th, 8th and 13th weeks	
870.6300	Developmental neurotoxicity		Rat	Gestation and lactational exposure. Optional direct pup gavage exposure. Maternal assessments. Pup assessments before weaning and at day ~21 and day ~60	FOB, motor activity. Learning and memory. Acoustic startle response. Brain weight and morphometric	
870-6500	Schedule-controlled operant behavior	Rarely conducted or required. Recommended for chemicals showing neurotoxicity in other studies that would be further characterized by this special test. Test can be combined with other guideline studies	Rat	Open	Special operant behavior	Not specified
870.6850	Peripheral nerve function		Rat		Peripheral nerve conduction velocity and amplitude	Not specified
870.6855	Neurophysiology: sensory evoked potentials		Pigmented rat strain		Implantation of electrodes in brain followed by visual, auditory or somatosensory stimuli evaluated	Not specified

Most commonly used species.

For organophosphates and carbamates, plasma cholinesterase and RBC and brain acetylcholinesterase are periodically assessed.

\*The guidelines recommend rats to be tested but other species can be tested under special circumstances.

Copies available at <http://www.epa.gov/pesticides> (see under Science and Policy-Test Guidelines).

assessment although some may consider this principle outdated. The toxicity database as generated by the required studies is intended to be a thorough screening process and is not intended to be an in-depth assessment of any organ including the nervous system unless special inclusions are made. However, when there are justifications to believe that the toxicity for a given chemical is being underestimated by the standard set of required toxicity studies, and validated methods for additional testing are available, these additional studies can be recommended to further characterize the toxicity.

An inherent problem with the guidelines for neurotoxicity studies is that the rat, dog, mouse, or rabbit may not provide a model for certain types of neurotoxicity that humans may be especially sensitive to. No matter how much testing is done in animals, such toxicity will not be detected prior to exposure to humans. The case of aplastic anemia is one example of there not being an animal model for prediction of a particular type of toxicity. It is estimated that one person in 30–40,000 is susceptible to the aplastic anemia caused by the antibiotic chloramphenicol [7]. There might also be cases of unusual human susceptibility to a neuropathogen of similar low frequency and there would be no way to detect them using the current battery of studies. Unlike the chloramphenicol model where the dosage was intentional and monitored, exposure to pesticides is much smaller and the actual amounts, times, and frequencies of pesticide exposure are not known.

The guidelines (Table 2) for the more general acute (870.1100 for oral, 870.1200 for dermal, and 870.1300 for inhalation), subchronic (870.3100 for rodents and 870.3150 for nonrodents—usually dogs), prenatal development (870.6300) and reproductive (870.3800) and chronic toxicity in rats and dogs (870.4100) and carcinogenicity in rats and mice (870.4200 or 870.4300) are nonspecific in their description of methods recommended for evaluation of the histopathology of the nervous system. The more obvious neurotoxicity would be detected by observation of the behavior of the animals based on daily cage-side evaluations if the technical staff is appropriately trained to look for and detect changes in behavior. The only instructions in the nonacute studies for histopathology preparation apply to all tissues and are not specific for nerve tissue: “tissues and organs designated for microscopic examination should be fixed in 10 percent buffered formalin or a recognized suitable fixative as soon as necropsy is performed and no less than 48 hours prior to trimming.” No commentary on the special stains to be used is provided. Hematoxylin and eosin are routinely used.

### **SPECIAL STUDIES FOR ASSESSMENT OF NEUROTOXICITY IN RATS**

The studies designed for specific assessment of potential neurotoxicity in rats include the series 870.6200 for acute and subchronic screening in adults and 870.6300 for developmental neurotoxicity (DNT). The latter study includes exposure to pups in utero and during lactation either via lactation or by direct gavage exposure to the pups. These

studies include cage-side observations for the more obvious clinical signs and for functional observational battery (FOB)<sup>1</sup> which assess the animal for motor and sensory effects. The technical staff making these observations is supposed to be especially trained to detect subtle changes in clinical signs indicative of neurotoxicity and typically is unaware whether the animal was dosed with the test material or otherwise. For these studies, the instructions for histopathological evaluation of the nervous system are more specific than for the general screening studies. “Tissues should be prepared for histological analysis using in situ perfusion and paraffin and/or plastic embedding procedures. Paraffin embedding is acceptable for tissues from the central nervous system. Plastic embedding of tissue samples from the central nervous system is encouraged, when feasible. Plastic embedding is required for tissue samples from the peripheral nervous system. Subject to professional judgment and the type of neuropathological alterations observed, it is recommended that additional methods such as Bodian’s and Bielchowsky’s (*sic*) silver methods, and/or glial fibrillary acidic protein (GFAP) immunohistochemistry be used in conjunction with more standard stains to determine the lowest dose level in which neuropathological alterations are observed.”

In the developmental neurotoxicity study (870.6300), pups (11 or 21 day old depending on the length of lactational exposure) and adults (about 62 days old) derived from dams exposed to the pesticide from day 6 of gestation through lactation (at least to day 10 but many laboratories continue dosing up to the time of parturition) via lactation or by direct gavage dosing during the lactation period are examined histologically. In addition to histopathology, an abbreviated FOB assessment, learning and memory and motor activity and acoustic startle responses are all evaluated in the pups at about weaning time and again as young (about 60 days) adults. Histology of pups is different from the 870.6200 studies in that the brain is fixed by immersion rather than in situ. The guidelines for the neurotoxicity screening studies and the developmental neurotoxicity study provide references for more detailed instructions for histopathology and behavior assessments. The laboratory conducting the study is responsible for selecting the techniques and stains to be used.

Positive control studies such as with trimethyl tin or acrylamide for neurohistopathology as well as positive controls such as amphetamine and haloperidol for motor activity and scopolamine for learning and memory are currently recommended to assure the susceptibility of the strain and the competence of the laboratory personnel conducting the study. The argument for also considering nonchemical agents to evaluate the proficiency of a laboratory in the use of a test (eg, memory) has been made [8].

These screening studies should detect alterations of the nervous system that occur within the limited time frames of testing with respect to the age of the rat when tested provided that the relatively pure strains of rat used are susceptible to

<sup>1</sup> The FOB assessment in the series 870.6300 is less detailed than for the series 870.6200. In particular, there is no requirement to include grip strength or landing foot splay.



any neurotoxicity that could be induced by the chemical. Consequently there are limitations with regard to their predictive value for the major neurodegenerative diseases which are associated with older humans such as Parkinson's and Alzheimer's. In particular, only young adults are assessed in the 870.6200 screening studies and exposure to the rats is in utero and up to the first three weeks of life in the series 870.6300 developmental neurotoxicity study. The rats are not kept on the study to determine if the in utero exposure predisposed them to development of neuropathological conditions in the later stages of life or if a challenge by the test chemical would be worse if the rats were not exposed in utero.

Three other studies (870.6500, scheduled operant behavior, 870.6850, peripheral nerve function, and 870.6855, neurophysiology: sensory evoked potentials) are rarely conducted but can be used to further characterize indications of neurotoxicity suggested in either the general or the special neurotoxicity guideline studies or based on the pesticide's structure and predicted activity relationships.

There are no guidelines for studies with monkeys which may have a similar level of susceptibility to neurotoxins that may produce or facilitate human neuropathogenesis. Reasons for this are that studies with monkeys are expensive and only limited numbers of animals can be used.

## **NONGUIDELINE STUDIES**

Studies from the open literature or studies conducted by the pesticide industry that either do not have protocols consistent with the guidelines or that are conducted to address a specific question are grouped together as nonguideline studies. Such nonguideline studies can provide endpoints for risk assessment when peer review determines that they are of acceptable scientific merit. It is, however, difficult to request that companies conduct special nonguideline studies without sufficient justification that the study is validated to render data useful for risk assessment purposes.

A recurring problem with nonguideline studies is that they often use routes of test chemical administration not related to human exposure scenarios. Intraperitoneal, intravenous, and intramuscular modes of administration may be very useful in attempting to determine the mode of action of a chemical. Such data are important in understanding the possible molecular basis for the neuropathogenesis. However, extrapolating data from these routes of administration to human exposure by the dietary, dermal, or inhalation routes is problematic.

The use of nonguideline studies with purposeful dosing of human volunteers to assist in the risk assessment for pesticides is done on a case by case basis following both scientific and ethical review. Such studies with human volunteers are occasionally conducted with pesticides that may cause transitory effects such as cholinesterase inhibition but certainly not to see if a neurodegenerative condition results. Epidemiological studies that attempt to correlate the incidence of certain types of diseases with pesticide exposure to humans derived from surveys of the subjects' personal history provide

insight into the possibility that exposure to the pesticide may be related to the onset and progression of neuropathogenesis. These studies, however, can only suggest a possible relationship because the subjects are also simultaneously exposed to many other chemicals and there is no real way to determine the actual extent to which the subjects were exposed to the suspect pesticide chemical or if exposure occurred during the critical times to affect the onset or progression of the neuropathological condition.

## **STUDY GUIDELINES FOR ASSESSING ORGANOPHOSPHATE-INDUCED DELAYED NEUROPATHY**

The only established neuropathy in humans associated with pesticides is organophosphate-induced delayed neuropathy (OPIDN) caused by certain but not all organophosphate insecticides and some other organophosphates not used as insecticides. A recent review on OPIDN provides more detailed information on the history and development of this model [9]. Documentation that OPIDN affects humans dates back to the early part of the last century when a major incident occurred during the prohibition years in the USA as a result of consumption of a Jamaican ginger alcoholic drink that was later demonstrated to be contaminated with tolyl phosphate esters. It is estimated that some 20,000 persons were affected to various degrees with a syndrome that was called *Ginger jake paralysis* or *jake leg*. The classical work of M. Smith and R.D. Lillie [10] of the US Public Health Service in the 1920s and 30s demonstrated that the phosphate contaminants were responsible for the condition and could reproduce the syndrome in rabbits, dogs, monkeys, and calves. In human exposure to ginger jake, the condition was described as "the initial flaccidity, characterized by muscle weakness in the arms and legs giving rise to a clumsy, shuffling gait, was replaced by spasticity, hypertonicity, hyperreflexia, clonus, and abnormal reflexes, indicative of damage to the pyramidal tracts and a permanent upper-motor neuron syndrome. In many patients, recovery was limited to the arms and hands and damage to the lower extremities (foot drop spasticity and hyperactive reflexes) was permanent, suggesting damage to the spinal cord" [4]. Validation that organophosphate insecticides cause OPIDN in humans comes partly from an incident concerning workers manufacturing the insecticide mipafox following an accident [11]. Domestic animals are also susceptible to OPIDN as indicated by the poisoning of water buffalo in Egypt [12] by the insecticide leptophos. A review of the possible association between leptophos with OPIDN in humans [13] describes problems in distinguishing between leptophos and other contaminants as the cause of OPIDN.

Considerable research on the structure of organophosphate insecticides that can cause this neuropathy has been done [9]. Of the tolyl phosphate contaminants in the ginger product, it was later determined that only one, the ortho-isomer, was responsible for the toxicity, indicating the highly specific chemical structural nature of the induction of this syndrome. Figure 1 presents some of the chemical structures of organophosphates that are known to cause OPIDN.

Research on biochemical approaches has led to the discovery that inhibition of “neuropathy target esterase” (NTE) by the organophosphates that cause the delayed-type neuropathy has provided a basis for screening of new organophosphate candidates for development as insecticides [14]. Chemicals showing higher levels of inhibition of NTE are reported to have a good correlation with development of OPIDN.

The hen was determined to be a relatively very susceptible species but the laboratory rat and mouse were not appreciably susceptible to OPIDN. The hen provides a model for assessing the potential for an organophosphate to cause neurotoxicity and is used in the acute and repeat dose study guidelines (870.6100). It is necessary to use adult domestic hens 8–14 months of age since the chick has a lower sensitivity [15]. In the acute study, a near lethal dose is administered usually by gavage and the hen may be protected by atropine from the inhibitory effects of the organophosphate on acetylcholinesterase. Following dosing, the hens are observed for their gait characteristics including the ability to walk up an incline and after 21 days are sacrificed and examined histologically. The repeat dose study is conducted when there is an evidence of OPIDN in the acute study or when there is an evidence of inhibition of NTE. The focus of the repeat dose study is to determine the NOAEL and LOAEL for OPIDN and it includes control, low, mid, and high (maximum 1 gm/kg) doses. The guidelines provide the following details for histopathological examination of the nervous system. “Tissues should be fixed by whole body perfusion, with a fixative appropriate for the embedding media. Sections should include medulla oblongata, spinal cord, and peripheral nerves. The spinal cord sections should be taken from the rostral cervical, the midthoracic, and the lumbosacral regions. Sections of the proximal regions of both of the tibial nerves and their branches should be taken. Sections should be stained with appropriate myelin- and axon-specific stains.” The guidelines recommend that TOCP (tri-orthocresyl phosphate, Figure 1) be used as a positive control to assure the susceptibility of the hens. Not all hens are equally susceptible to OPIDN [16].

No new organophosphate insecticides have been introduced in recent years and either organophosphate insecticides that were demonstrated to cause OPIDN have been phased out or their uses have been greatly restricted. New organophosphates, however, may in the future be needed for control of certain pests that become resistant to currently registered pesticides. OPIDN is not considered to be related to another known human neurodegenerative disease. However, the OPIDN model may be very useful in studying the progressive degeneration of the nervous system following initiation of the nerve degeneration that may be applied to human neurodegenerative diseases if the underlying mechanisms of OPIDN can be elucidated and compared with human diseases.

## PESTICIDES AND PARKINSONISM SYNDROME

Parkinson’s disease (PD) is regarded as the second most common neurodegenerative disorder in humans and affects

about 2% of the population over the age of 60 years. Clinically, PD is a disorder of motor function characterized by tremor, slow and decreased movement (bradykinesia), muscular rigidity, poor balance, and problems in gait [17]. Pathologically, PD patients show loss of dopaminergic neurons in the substantia nigra pars compacta and frequently have Lewy bodies, eosinophilic intracellular inclusions composed of amyloid-like fibers and  $\alpha$ -synuclein [18]. PD may have a genetic basis for susceptibility for an early onset form but the occurrence of the more prevalent late onset form does not have an established genetic basis [19]. The latter form may result from a multitude of different factors including insults from xenobiotics and an individual’s inherent sensitivity or differences in the metabolism and pharmacokinetics of the xenobiotics. Since the discovery that MPTP [1] could cause PD like syndrome, interest in the herbicide paraquat, which has some structural similarity to MPTP (Figure 1) led to the possibility that this herbicide could be a risk factor in the PD syndrome [20–25]. Factors such as exposure from living in rural areas, farming, drinking water from wells and exposure to agricultural chemicals have been investigated and claimed as support for an association between paraquat and increased PD. Interest in the herbicide maneb as a possible risk factor for PD developed because of its reported effects on dopamine whereas it was demonstrated to enhance the effects of the active metabolite of MPTP or MPP+ [26, 27]. Rotenone, a pesticide that is an inhibitor of mitochondrial Complex I function, has also been implicated for being associated with PD based partly on work that associates the mode of action of MPTP or its principal metabolite MPP+ with an effect on mitochondrial Complex I function [28–30].

Organochlorine insecticides as well as tricyclohexyl and triphenyl tin inhibit various ATPases in nerve membranes including one enzyme species that also shows a bell-shaped curve for activation and then inhibition of activity by  $Mn^{++}$  and it was earlier suggested [31] that inhibition of ATPases might be related to an environmental factor in Parkinson’s disease etiology. DDT and dieldrin persist in the body and once ingested can remain there indefinitely. Mobilization of DDT or dieldrin from fat stores as the body ages to critical areas associated with PD might be a factor in its development or progression. An association between dieldrin presence and PD syndrome [32] was reported based on a small number of patients examined. Heptachlor has also been demonstrated to affect dopamine function [33] in laboratory animals.

The association between PD and pesticides is a controversial issue and the USEPA does not currently consider that pesticides are risk factors in this disease. A recent comprehensive review of this issue, supported in part by industry but published in a peer reviewed journal, led the authors to conclude “that animal and epidemiological data reviewed do not provide sufficient evidence to support a causal association between pesticide exposure and PD” [43].

If a pesticide was causing or affecting a PD like syndrome in susceptible laboratory animals, the signs of tremor, slow and decreased movement, muscular rigidity, problems in gait would be expected to be detected in the screening process if all of the appropriate studies were requested and conducted.



The available studies for paraquat, maneb, or rotenone do not show obvious indications of these signs at least not at their LOAELs in the strains tested. The histopathological effects would probably not be so obvious within the limited assessment for histopathology in the current study guidelines since the substantia nigra is a relatively small section of the brain and would require special assessment to determine if there were test chemical induced changes in the dopamine dependent cells within it. Chronic exposure for paraquat is currently based on “chronic pneumonitis” in dogs with a conventional 100 fold uncertainty factor. Maneb is currently regulated for chronic exposure based on its effects on the thyroid in rats at the LOAEL plus a 1000 fold uncertainty factor including an extra 10 X because of an incomplete database. Endpoints for rotenone are currently being reevaluated and the reports of its association with PD syndrome being considered for future testing but the current LOAEL is not based on indications of neuropathogenesis.

Historically, the rat has limitations as an in vivo model for PD and attempts to study the effects of either  $Mn^{++}$  or MPTP in this species resulted with some but limited data. A detailed review of the development of animal models for PD and other neurodegenerative diseases is beyond the scope of this review and there are no suitable models for incorporation into the guidelines. Reviews of neurotoxicant induced models of PD in the rat have been published recently in 2004 [44] and 2005 [45] and provide comprehensive discussions of the many problems associated with trying to develop an animal model. A review of the development of animal models in mice has also been presented [46] and limitations of this species including genetically engineered strains are discussed [47]. Factors such as the low susceptibility of rodents to PD like syndrome or a narrow or limited vulnerable age or the differences in metabolism and access to the critical sites by the critical form of the toxic agents as well as the cumulative effects and the influence of combinations of chemicals all contribute to problems in developing animal models for predicting a chemical’s potential to be a risk factor for PD.

One important consideration in the development of animal models for PD concerns the question: what is the goal of the model? For example, some models are developed to further understand the neurochemical events associated with the initiation and progression of the disease in order to develop therapy. Other models may have the goal of establishing a basis for risk assessment. One of the criticisms of some of the developing models that they do not mimic the disease in humans closely enough is not necessarily detrimental to the goal of providing data for risk assessment. This is because if the model shows an effect suggestive at all of neuropathogenesis it would be important in the hazard characterization of the chemical. Thus, genetically manipulated mice that spontaneously develop PD like syndrome whether it mimics the human condition exactly or not would be an important addition to the guidelines. The suspect chemicals could be tested in these strains to see if the spontaneous rates of the syndrome are increased, occur at an earlier onset time, or are worse in the presence of the pesticides.

In vitro data using rat or other animal tissue preparations can be very useful for providing data on mechanisms but not always generalize to the in vivo situation. One such example is the effect of paraquat which was suspected as causing PD like syndrome based on its structural similarity to MPTP/MPP+ that does not have the same affinity for the dopamine transporter or cause inhibition of mitochondrial complex I in in vitro studies indicating that paraquat has an effect on dopamine neurons that is unique from rotenone and MPTP [48]. It is still possible that an NTE like model such as for predicting OPIDN could be developed based on in vitro studies. Limitations associated with in vitro models based on animal tissue include that in real life, exposure is not just to the single chemical but to complex mixtures, in vitro studies do not reflect the cumulative effects of the pesticide or the temporal aspects of the initiation or progression of the disease.

Another animal model for induction of PD involves mice and their early exposure and later challenge based on work with paraquat and maneb [49]. The mice exposed as fetuses during pregnancy were reported to be more susceptible to indications of PD when challenged later in life by these pesticides. This implied that an initial injury predisposed the animals to susceptibility in later stages of life. This model is based on the “Barker hypothesis” or its expanded form for Parkinsonism where it is postulated [50] that early exposure to chemicals destroys certain critical cells in the substantia nigra to levels below those needed to sustain function associated with advancing age. In these studies, combinations of paraquat and maneb were used assessing the mutual influence of each. The role of early life environmental risk factors in PD has been independently reviewed [51].

As indicated above, a problem with attempting to assess for the effects of pesticides as risk factors of neuropathogenesis by the study guidelines is that some of the literature reports associating pesticides with PD imply that combinations of pesticides or other agents rather than the individual pesticides are the risk factors. Extensive justification would be needed before studies with combinations of xenobiotics could be requested to provide data for risk assessment. Establishing what combinations of chemicals should be tested, how long the tests should run for and what relative doses of each chemical to be tested would be a task in itself and interpreting the data with regard to which chemical is really the contributing factor would be problematic.

## OTHER NEUROLOGICAL DISORDERS

Table 1 lists Alzheimer’s disease (the most common neurodegenerative disease), amyotrophic lateral sclerosis, autism and psychiatric disorders as possibly being related to pesticide exposure. Tests for learning and memory through the life cycle including the later months near study termination in chronic or cancer studies might be considered for incorporation into the guidelines to attempt to assess for at least some aspects of Alzheimer’s disease. Alzheimer’s disease may have both genetic and environmental factors [52] and animal models of Alzheimer’s disease are being developed [53]

but their usefulness for evaluating risk associated with pesticide exposure has not been established. Many factors may influence the progression of Alzheimer's disease and a very recent report indicates that persons with higher levels of education have faster rates of cognitive decline [54]. Animal models of autism are being developed [55] and the endpoints of (i) lower sensitivity to pain and higher sensitivity to non-painful stimuli, (ii) diminished acoustic prepulse inhibition, (iii) locomotor and repetitive/stereotypic-like hyperactivity combined with lower exploratory activity, and (iv) decreased number of social behaviors and increased latency to social behaviors are considered possible indicators of a drug association with autism based on studies with valproic acid. The developmental neurotoxicity study (DNT, 870.6300) can assess for some of these parameters but there are no inclusions in the current guidelines for DNT studies for assessing social behaviors. If a pesticide caused neurological or muscular degeneration, it could possibly aggravate amyotrophic lateral sclerosis but would be regulated based on its NOAEL to be protective. Although neuropsychiatric disorders may not be strictly within the description of neurodegenerative disease, there has been a continuous debate over the possibility that organophosphate poisoning causes neuropsychiatric sequella [40]. A review of this topic is beyond the scope of this manuscript. Nonguideline studies with monkeys [41] with sarin have been reported to produce long-lasting EEG changes that are claimed to confirm an earlier observation of changes in the human EEG patterns [42] following organophosphate exposure. The persons exposed to sarin (a potent cholinesterase inhibitor) gas in the Tokyo subway incident in the mid 1990s have been assessed periodically and reports indicate possible neurological effects either related to the gas itself or post traumatic stress disorder [56–58].

#### **OVERALL ASSESSMENT OF NEUROTOXICITY STUDY GUIDELINES FOR CHARACTERIZING RISK FOR NEUROPATHOGENICITY**

*Strength of the neurotoxicity study guidelines.* The neurotoxicity study guidelines provide a screening procedure that should detect pesticides that are strong inducers of neuropathogenesis in the animal strains and species tested relative to the time of administration and ages of the tested animals. The guidelines are adaptable and as more and better techniques and models (such as genetically manipulated strains) are developed these models can be incorporated into the guidelines. Humans would have to be inherently especially more sensitive to a neuropathological response to a pesticide or there would have to be other contributing factors from the real world if they were to develop neuropathological conditions as a result of the low level of exposure that is set by the selection of the most sensitive endpoint in the most sensitive species as determined by the battery of required studies and other available data and the application of uncertainty factors to drive down exposure.

*Weaknesses or limitations of the study guidelines.* Several inherent weaknesses in the study guidelines can be identified.

Most of these reflect a disparity between the stringent conditions of laboratory testing and real world exposure. These include the following.

(i) Pesticides are tested individually. Thus, the interaction and cumulative effects of the individual pesticide with the many other pesticides, xenobiotics, drugs, and natural food-stuffs are not assessed.

(ii) Relatively pure strains of standardized laboratory animals are tested meaning that a neuropathological condition will be detected only if that particular strain is sensitive to the chemical. The human population is very diverse with varying degrees of sensitivity to a given chemical. The standardized strains do not have a predisposition to develop neuropathogenesis such that it cannot be assessed if there is a potential for the pesticide to accelerate the progression of a neuropathological condition once started.

(iii) Healthy young animals on diets optimized for their health would be the least susceptible to a toxic insult are used for testing. There is a wide variation in diet and disparity in ages and in the level of health that makes humans possibly more susceptible.

(iv) Temporal conditions are not fully evaluated such as early in utero and fetal exposure affecting the animal to be more sensitive to an insult by the chemical in the later phases of its life.

(v) Neuropathogenesis may result from the destruction of only a very small structure of the brain (ie, the pars compacta of the substantia nigra) and such changes in structure may be missed in routine histopathological assessment of the brain. This is an important concept since the laboratory animal may have a higher tolerance to destruction of the brain area than the human and the animal may not show clinical signs until there is a major destruction but the human may show clinical signs after only minimal or moderate destruction.

(vi) Laboratory animals are not the same as humans. Detection of neuropathogenesis in animals does not mean that the human will develop the same lesion. Conversely, failure of animals to develop a neuropathological condition does not mean that the human will.

All of the factors above for weaknesses or limitations apply in to the study guidelines in general such as for assessing for cancer and developmental toxicity and these limitations are well recognized. In essence, studies conducted following the guidelines, as imperfect as they may be, plus other available data are what risk assessments are based on. Epidemiological data come later.

#### **SUMMARY**

Pesticides are individually tested in a series of studies with established guidelines with laboratory animals to determine if they have the potential for neuropathogenicity. Thus, the neuropathological effects of chemicals that are strong inducers of neuropathogenesis in the species of animals tested and if tested at the critical susceptible times will be detected in the battery of studies required for registration and reregistration. Additional testing in animals can be conducted based

on suggestions of neuropathogenesis from existing studies or based on structure activity relationships to further characterize a neuropathological condition possibly associated with the pesticide. Currently, the endpoints determined by the completed battery of required and other studies and the use of uncertainty factors in risk assessment are designed to provide a reasonable protection against possible neuropathogenesis of pesticides to humans. If humans are uniquely susceptible or the timing for test chemical administration in the animal studies is not appropriate, or if the pesticide must interact with other chemicals, potential effects in humans could be missed but the inclusion of the uncertainty factors is designed to protect against such possibilities by driving down exposure. The discovery that humans are susceptible to OPIDN resulted from accidental exposure to an organophosphate led to the development of the hen model for OPIDN testing which is the only model for neuropathy that is purposefully assessed for in routine screening studies. Had this accident not happened, there might be an occasional incident of persons developing the OPIDN syndrome today without knowing its cause. The OPIDN model is unlike the major human neurodegenerative diseases since OPIDN starts soon after exposure while Parkinson's and Alzheimer's may require long intervals between exposures and onset or they may require a natural onset before pesticides can facilitate their progression. Therefore, the possibility that pesticide exposures can be risk factors for neurodegenerative diseases needs to be considered in epidemiological models, whether alone or in combination with other factors. Development of animal models to more completely assess for possible relationships between pesticide exposure and neurodegeneration in humans need to be developed and validated to render data useful for risk assessment. Animal models with strains genetically engineered to be susceptible to known human neurodegenerative diseases may eventually be developed and validated and be important additions to the guidelines for neurotoxicity assessment.

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