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Common Mechanism of Toxicity: A Case Study of Organophosphorus Pesticides

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1. ABSTRACT

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The Food Quality Protection Act of 1996 (FQPA) requires the EPA to consider "available information concerning the cumulative effects of such residues and other substances that have a common mechanism of toxicity . . . in establishing, modifying, leaving in effect, or revoking a tolerance for a pesticide chemical residue." This directive raises a number of scientific questions to be answered before the FQPA can be implemented. Among these questions is: What constitutes a common mechanism of toxicity? The ILSI Risk Science Institute (RSI) convened a group of experts to examine this and other scientific questions using the organophosphorus (OP) pesticides as the case study. OP pesticides share some characteristics attributed to compounds that act by a common mechanism, but produce a variety of clinical signs of toxicity not identical for all OP pesticides. The Working Group generated a testable hypothesis, anticholinesterase OP pesticides act by a common mechanism of toxicity, and generated alternative hypotheses that, if true, would cause rejection of the initial hypothesis and provide criteria for subgrouping OP compounds. Some of the alternate hypotheses were rejected outright and the rest were not supported by adequate data. The Working Group concluded that OP pesticides act by a common mechanism of toxicity if they inhibit acetylcholinesterase by phosphorylation and elicit any spectrum of cholinergic effects. An approach similar to that developed for OP pesticides could be used to determine if other classes or groups of pesticides that share structural and toxicological characteristics act by a common mechanism of toxicity or by distinct mechanisms. © 1998 Society of Toxicology.

2. INTRODUCTION

Human health risk assessments are conducted to derive "acceptable" levels of exposure to chemicals that may exist as contaminants in food, drinking water, air, or the environment. Human health risk assessments are conducted by many organizations, including the U.S. Environmental Protection Agency (EPA). The EPA derives acceptable levels of human exposure to compounds, known as reference doses (RfD) and reference concentrations (RfC). The RfD is an estimate (with uncertainty spanning perhaps an order of magnitude) of the daily oral exposure to the general human population, including sensitive subgroups, that is likely to be without appreciable risk of deleterious effects during a lifetime of exposure, and the RfC is the corresponding estimate of the concentration in air that is likely to be without appreciable risk. RfDs and RfCs are derived for individual chemicals and are based on noncarcinogenic effects.

RfDs and RfCs are used as guidelines to determine the safety of an exposure. The EPA Office of Pesticide Programs (OPP) relies on RfDs in the process they use to derive levels of pesticide residues that will be allowed on a food crop. The allowable levels of pesticides on a food crop are known as tolerances, and in the past tolerances have been based on potential human exposure to a single pesticide via multiple food sources. The tolerance-setting process has not included consideration of concurrent exposure to more than one pesticide.

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A change in the process of setting tolerances used by the EPA was mandated by the U.S. Congress in the Food Quality Protection Act of 1996 (FQPA). The FQPA requires the EPA to consider "available information concerning the cumulative effects of such residues and other substances that have a common mechanism of toxicity, in establishing, modifying, leaving in effect, or revoking a tolerance for a pesticide chemical residue." This simple-sounding directive has far-reaching implications and raises a number of scientific questions to be answered before the FQPA can be implemented. Among the questions the EPA has to consider for implementation of the FQPA are: What constitutes a common mechanism of toxicity? What criteria should be used to determine if two or more chemicals induce toxicity by a common mechanism of toxicity?

The ILSI Risk Science Institute (RSI), in a cooperative agreement with the EPA Office of Pesticide Programs and Office of Water, convened a Working Group of experts from government, academia, and industry to examine these and other issues using the organophosphorus (OP) class of pesticides as the test case series. The OP pesticides were selected as the case study because there is an extensive database available for OP compounds, and OP pesticides are of primary importance to the EPA in implementation of the FQPA.

RSI convened a Steering Committee for the project, charged with refining the scope and direction of the consideration of a "common mechanism of toxicity" for the OP pesticides and assisting in selection of members for the expert Working Group. The Steering Committee developed a mission statement and generated guidelines for the Working Group of experts.

The Mission Statement developed by the Steering Committee for the expert Working Group was:

> *Risk assessments traditionally are conducted on individual chemicals; however, humans are exposed to multiple chemicals in daily life, and some of these may act via a common mechanism of toxicity. The potential cumulative effects of substances that may act through a common mechanism of toxicity should be considered in risk assessments. The charge to the Working Group is to develop a comprehensive approach for grouping chemicals by a common mechanism of toxicity using OP pesticides as a case study. The Working Group will focus on the OP pesticides, keeping in mind the basic questions: What constitutes a common mechanism of toxicity? What criteria should be used to determine if two or more chemicals induce toxicity by a common mechanism of toxicity? The Working Group will also be asked to address specific questions related to OP pesticides.*

The charge focused the topic to be considered and also limited the scope of the project. For example, the charge did not direct the Working Group to consider non-OP anticholinesterase agents (such as carbamates) or to describe how to conduct a risk assessment of compounds that act by a common mechanism of toxicity or when and how one might be exposed to compounds that act by a common mechanism of toxicity.

3. DEVELOPMENT OF GENERAL PRINCIPLES TO DETERMINE A COMMON MECHANISM OF TOXICITY

The Working Group members agreed to begin their discussion using the following definition of a mechanism of toxicity drafted by the EPA, with the understanding that it could be modified as necessary. A mechanism of toxicity is described as the major steps leading to an adverse health effect following interaction of a pesticide with biological targets. An understanding of all steps leading to an effect is not necessary, but identification of the crucial events following chemical interaction is required to describe a mechanism of toxicity (U.S EPA, 1997).

The decision to combine risks due to exposure to multiple chemicals that act via a common mechanism of toxicity involves consideration of difficult issues, including the basic question the Working Group was asked to address: What constitutes a common mechanism of toxicity? To stimulate thinking about a common mechanism in the context of risk assessment, RSI staff developed some hypothetical scenarios of exposure to two compounds and asked: Which compounds should be combined in a cumulative risk assessment? The three hypothetical scenarios of combined exposure to two compounds are listed below. The Working Group as a whole discussed the scenarios and outlined a rationale for treatment of each scenario. The goal of the exercise was to agree upon sets of compounds that should either be combined or considered separately for risk assessment and to provide the rationale for each decision. The rationale for each decision would be the basis for general principles developed by the Working Group to help them determine what constitutes a common mechanism of toxicity and when chemicals should be grouped based on a common mechanism of toxicity.

A number of assumptions and simplifications were made in the following scenarios in order to clarify the exercise. Some of the assumptions were: the critical effects were as described, the mechanism of action of relevance was as described, and the exposures result in action of the ultimate toxicants on the target site at the same time. The conclusions below are based only on the information provided and may not hold true after consideration of additional information.

Scenario 1

Two compounds cause the same effect and induce toxicity by the same molecular mechanism.

Compound 1

Compound 1 was carbon monoxide (CO).

Critical effect. The critical effect was decreased time to exercise-induced angina in a sensitive population.

Mechanism. The mechanism was direct binding of CO to hemoglobin in the blood resulting in formation of carboxyhe-

moglobin (COHb) and decreased oxygen delivery to heart muscle.

Compound 2

Compound 2 was methylene chloride (CH_2Cl_2).

Critical effect. The critical effect was decreased time to exercise-induced angina in a sensitive population.

Mechanism. The mechanism was metabolism of CH_2Cl_2 to CO in the liver and subsequent binding of CO to hemoglobin in the blood, formation of COHb, and decreased oxygen delivery to heart muscle.

Conclusion and rationale. CH_2Cl_2 and CO act by a common mechanism of toxicity and should be considered together in a risk assessment, based on the following:

1. The two compounds share an identical toxic intermediate (CO);
2. The two compounds bind to the same target molecule and act by the same molecular mechanism of action, that is CO binding to hemoglobin in the blood; and
3. The two compounds cause the same critical toxic effect of exercise-induced angina.

Scenario 2

Two compounds cause different toxic effects and induce toxicity via the same molecular mechanism.

Compound 1

Compound 1 was 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).

Critical effect. The critical effect was degeneration of the dopamine neurons in the substantia nigra of the brain, causing Parkinsonism.

Mechanism. The mechanism was inhibition of ATP synthesis via inhibition of electron transport at NADH–coenzyme Q reductase.

Specificity of effect. MPTP is nonpolar and readily crosses the blood–brain barrier. MPTP is metabolized in the brain by monoamine oxidase B in the astrocytes. The metabolite autooxidizes to form MPP+ which is selectively transported into the neurons of the substantia nigra by the neuronal dopamine transporter. MPP+ is the ultimate toxicant which may inhibit electron transport at NADH–coenzyme Q reductase.

Compound 2

Compound 2 was rotenone.

Critical effect. The critical effects were an anesthetic-like effect on nerves, respiratory stimulation, and depression.

Mechanism. The mechanism was inhibition of ATP synthesis via inhibition of electron transport at NADH–coenzyme Q reductase.

Specificity of effect. Rotenone is not selectively taken up by the dopamine transporter and so does not accumulate in the substantia nigra of the brain.

Conclusion and rationale. MPTP and rotenone do not act by a common mechanism of toxicity and should not be considered together in a risk assessment. MPP+ and rotenone may bind to the same target molecule and act by the same molecular mechanism of action, but do not cause the same critical toxic response. The two toxicants do not cause the same critical toxic effect because their distribution in the body is different.

Scenario 3

Two compounds cause the same toxic effect and induce toxicity by different molecular mechanisms.

Compound 1

Compound 1 was *n*-hexane.

Critical effect. The critical effect was central–peripheral distal axonopathy characterized by a "stocking and glove distribution" of sensory and motor deficits.

Mechanism. *n*-Hexane is metabolized to 2,5-hexanedione, which binds to amino groups in all tissues to form pyrroles. A pyrrole formation in the neuron is thought to cause development of neurofilament aggregates in the distal axon, forming massive swellings, followed by distal axon degeneration.

Compound 2

Compound 2 was pyridinethione.

Critical effect. The critical effect was central–peripheral distal axonopathy.

Mechanism. The molecular mechanism has not been completely elucidated. Pyridinethione interferes with axonal transport systems. The result is accumulation of tubulovesicular material in the distal axon, forming massive swellings, followed by distal axon degeneration.

Conclusion and rationale. The information presented is inadequate to determine whether or not the two compounds should be considered together or separately in a risk assessment. Both compounds cause accumulation of axonal material in the distal axon causing massive swelling and distal axonal degeneration, but it is not clear from the information presented if the critical effects are identical. The Working Group agreed that it is possible for two compounds to cause the same critical toxic effect and induce toxicity by different molecular mechanisms, but it is important to be very precise in the definition of a critical toxic effect. A definition of the critical toxic effect in this case might include exactly which neurons are affected by each compound. If both compounds target the same sensory and motor neurons, the two compounds do cause the same critical toxic effect initiated by accumulation of neurofibrils in the distal axon and should be considered together in a risk

assessment. If different groups or types of neurons are targeted by the two compounds, the solution is less clear and additional information is needed.

Guidance derived from scenarios. Clearly the three scenarios presented represent only a few of the variety of potential examples of compound pairs that may or may not act by a common mechanism of toxicity. The conclusions and rationales from the three scenarios were combined into a set of generalizations to be used as a starting point for discussion of a common mechanism of toxicity relative to the OP pesticides. Based on the three simple scenarios, two or more chemicals may act via a common mechanism of toxicity if they:

a. cause the same critical effect;
b. act on the same molecular target at the same target tissue; and
c. act by the same biochemical mechanism of action, possibly sharing a common toxic intermediate.

The Working Group agreed that these three points are useful to apply to chemicals that may act by a common mechanism of toxicity, but did not agree whether or not all three principles should be fulfilled in order for compounds to share a common mechanism of toxicity.

The scenarios described contained background information and assumptions necessary to decide if the two compounds act by a common mechanism of toxicity. This information included an understanding of the biological actions of the compounds; characterization of the adverse effects due to exposure; knowledge of the pharmacokinetics of the compounds, particularly distribution and metabolism; and characterization of the pharmacodynamics of the compounds. In addition, molecular structure–activity relationships among compounds can provide supporting evidence for similar actions of compounds that possess similar structures. A brief summary of chemical and toxicological information relevant to consideration of common mechanisms of toxicity of OP pesticides is presented.

4. CHEMICAL AND TOXICOLOGICAL CHARACTERISTICS OF OP PESTICIDES

a. Chemical Characteristics of OP Pesticides

OP compounds are a structurally diverse group of chemicals, and OP pesticides may be classified based on any number of structural similarities and differences. The classification system adopted here is a method commonly used, based on the identity of the atoms bound to the phosphorus atom (P) (Holmstedt, 1959, 1963; Ballantyne and Marrs, 1992; Chambers, 1992). Other classification systems are based on the characteristics of the side chains attached to the P (Gallo and Lawryk, 1991). The P of OP pesticides is pentavalent and tetracoordinate. Three of the substituents are bound to the P by single bonds, and the bond between the P and the fourth substituent is usually represented as a double bond (actually, a coordinate covalent bond; Chambers, 1992). The phosphates have four oxygen atoms bound to the P. Examples of phosphate pesticides include mevinphos and naled. Many OP pesticides in use today belong to the phosphorothionate group, in which P is bound to three oxygens and one sulfur (the double bond). Phosphorothionates include chlorpyrifos, parathion, and tebupirimphos. Compounds in the phosphorodithioate group are like the phosphorothionates but with one of the oxygens replaced by sulfur. Phosphorodithioates include malathion, disulfoton, azinphos-methyl, sulprofos, and dimethoate. The atoms bound to the P of phosphoroamidothiolates are nitrogen, sulfur, and two oxygens; the double bond is to an oxygen. Examples of phosphoroamidothiolates are acephate and methamidophos. For review of additional structures, see Chambers (1992).

The reactivity of OP compounds varies depending upon the chemical structure. Electrophilicity of the P is crucial for the biological actions of OP compounds. OP compounds that have a double bond between P and O are highly electrophilic at the P atom and are highly reactive. Groups that enhance the reactivity of the P are nitro, cyano, halogen, ketone, and carboxylic ester. Deactivating groups include hydroxyl and carboxylic acid.

b. OP Pesticide Actions on Biological Systems

The primary molecular mechanism of action of the OP pesticides is inhibition of acetylcholinesterase (AChE), a widely distributed serine esterase (for review see Ecobichon, 1996). AChE occurs throughout the central and peripheral nervous system of vertebrates, and its normal physiological action is to hydrolyze the neurotransmitter acetylcholine (ACh) so that activation of cholinergic receptors is transient. Inhibition of AChE results in accumulation of ACh and signs of cholinergic toxicity. There are a few OP pesticides that do not inhibit AChE or produce cholinergic signs of toxicity, including the fungicide fosetyl-Al, the plant growth regulator ethephon, and some herbicides including glyphosate. The Working Group decided that these OP pesticides do not share a common mechanism of toxicity with OP compounds that inhibit AChE and produce cholinergic signs of toxicity. Only the anticholinesterase OP pesticides were included in this consideration of common mechanism of toxicity, with the goal of deciding whether this group could be further subdivided based on common mechanisms.

The OP pesticides or their active metabolites are electrophilic compounds with moderate to high potency for phosphorylating the serine hydroxyl group located in the active site of AChE. This phosphorylation occurs by the loss of the "leaving group" of the OP compound and the establishment of a covalent bond with AChE through the serine hydroxyl. The resultant phosphorylated AChE is typically very stable and is only slowly reactivated by spontaneous hydrolysis of the phosphate ester. While the AChE remains phosphorylated, its enzyme

activity is inhibited and therefore ACh accumulates in the synapses and neuromuscular junctions, leading to overstimulation of cholinergic receptors. This phosphorylation may persist for hours to days or even weeks if "aging" has occurred. Aging involves dealkylation of the bound inhibitor and strengthening of the phosphorus–enzyme bond. The rate of aging varies depending upon the OP compound. Phosphorylated AChE is reactivated by the highly nucleophilic oximes (e.g., pralidoxime); however, aged phosphorylated AChE is not reactivated by oximes.

Inhibition of AChE results in accumulation of the neurotransmitter, ACh, throughout the body. ACh binds to, and stimulates, two types of cholinergic receptors, designated muscarinic and nicotinic receptors. The spectrum of effects caused by excess ACh depends upon the distribution of the OP pesticide in the body and the receptor type with which ACh interacts.

Accumulation of ACh alters the function of the autonomic nervous system, the somatic motor neurons, and the brain by action on nicotinic and muscarinic receptors (for review see Watanabe, 1989). The autonomic nervous system controls the visceral functions of the body and is divided into the parasympathetic and sympathetic divisions (for review see Guyton, 1981; Hardmon and Limbird, 1996; Karczmar, 1993). The parasympathetic division stimulates activities associated with conservation and restoration of energy stores of the organism. The sympathetic division stimulates activities that expend energy stores in emergency and stress situations, known as the "fight or flight" reactions. Parasympathetic and sympathetic nerve fibers, or axons, emerge from the spinal cord and brain and release ACh at junctions with ganglionic neurons that express nicotinic and muscarinic receptors; thus, both the parasympathetic and sympathetic divisions of the nervous system may be stimulated by increased ACh. The postganglionic neurons in the parasympathetic division release ACh that acts on muscarinic receptors on effector organs, such as the heart, eyes, glands, gastrointestinal tract, and respiratory system. The postganglionic neurons in the sympathetic division release, at the same effector organs, a different neurotransmitter, norepinephrine. The effects of norepinephrine on effector organs are often opposite to the effects of ACh.

Somatic motor neurons control voluntary functions, including locomotion, respiration, and posture (for review see Guyton, 1981). Somatic motor neuron axons emerge from the spinal cord and directly innervate muscle cells at the neuromuscular junction, releasing ACh to act on nicotinic receptors. The brain and spinal cord contain both muscarinic and nicotinic receptors; the brain is relatively richer in muscarinic receptors, while the spinal cord contains relatively more nicotinic sites (Watanabe, 1989). Cholinergic pathways in the brain are associated with a wide variety of human and animal behavior or function, including hunger and thirst, thermoregulation, respiration, aggression, and cognition (Karczmar, 1990).

In addition to the inhibition of AChE described above, some OP compounds clearly have additional actions on mammals. These actions include inhibition of other esterases in blood and tissue, such as butyrylcholinesterase (BuChE, also known as pseudocholinesterase) and carboxylesterase (CaE). Inhibition of these enzymes has not been linked to any particular physiological effects, but because BuChE and CaE stoichiometrically detoxify OP compounds they are considered a protective buffer for AChE. Collectively, AChE and other cholinesterases may be described as "cholinesterases" (ChE). A subset of OP compounds binds to neuropathy target esterase (NTE, or neurotoxic esterase) present in neural tissue. The normal physiological substrate or function of NTE has not been elucidated. Aging of the OP–NTE complex is associated with central–peripheral distal axonopathy that begins to appear 1 to 3 weeks after exposure (Johnson, 1969; Abou-Donia, 1981; Richardson, 1992, 1995).

Experimental evidence indicates that some OP pesticides may have a direct action on muscarinic and nicotinic receptors, binding to and modulating the function of these receptors (Eldefrawi *et al.*, 1988, 1992; Bakry *et al.*, 1988; Silveira *et al.*, 1990; Huff *et al.*, 1994; Ward and Mundy, 1996). Some OP pesticides bind directly to these receptors with high affinity, and others bind with low affinity. Those that bind with high affinity may be important in modulating the toxicity of OP pesticides (Chaudhuri *et al.*, 1993; Pope *et al.*, 1995). Specific binding of an OP compound to cholinergic receptors does not necessarily produce predictable effects. For example, some OP pesticides bind to muscarinic receptors and activate them, while others may bind to and inhibit the action of muscarinic receptors. Furthermore, some muscarinic receptors may be inhibitory in themselves, and activation by an OP compound may then cause inhibitory actions. OP pesticides may bind to allosteric sites on nicotinic receptors. OP pesticide binding to nicotinic receptors stimulates receptor desensitization (Katz *et al.*, 1997). The combination of excess ACh binding to nicotinic and muscarinic receptors and direct interaction of an OP pesticide with these receptors to either increase or decrease the activity of the receptor may modify the toxicity of some OP compounds. The experimental studies that have been done to characterize the binding affinity of muscarinic and nicotinic receptors for OP compounds were conducted on tissue *in vitro*, and direct extrapolation of the results to the whole animal is not possible at this time.

Some OP compounds are in the active form in the pesticide formulation and others require metabolic activation to confer the capacity to inhibit AChE (Eto, 1974; Neal, 1980; Maxwell and Lenz, 1992; Ecobichon, 1996). Phosphorothionates are activated by oxidative desulfuration mediated by cytochrome P450 isoforms (P450), resulting in an oxygen analog of the parent compound. Phosphoramidates are activated by N-oxidation, and phosphorothiolates are activated by metabolic S-oxidation. Oxygen analogs, or oxons, can be readily deactivated by hydrolases such as carboxylesterases and by A-esterases such as paraoxonase found in mammalian tissues

(Mazur, 1946; Aldridge, 1953; Maxwell, 1992). OP compounds undergo other transformations mediated by cytochrome P450 that do not result in production of an active metabolite, including oxidative dealkylation and dearylation. OP compounds may also be transformed by enzymatic action on the side chains, including aromatic ring hydroxylation, thioether oxidation, and deamination (Ecobichon, 1996).

Activation of some parent OP compounds may also be mediated by flavin-containing monooxygenase (FMO) enzymes (Levi and Hodgson, 1992). Most of the reactions of FMO enzymes do not involve the P=S sulfur but rather a thioether sulfur (CH_2–S–CH_2) or a nitrogen atom in the leaving group. There is one significant exception to this. Phosphonates such as fonofos are activated to their oxons through attack on the phosphorus atom by FMO, in a reaction that involves different stereospecificity from the P450 attack on sulfur. Thus, the isoform specificities for FMO versus P450 may have toxicological significance in the case of the phosphonates.

OP compounds and/or their metabolites may interact with biological target molecules unrelated to cholinesterase. For example, during P450-dependent activation of OP compounds the highly reactive sulfur released inhibits the P450 by interaction with the heme iron. Thus, OP compounds may function as suicide inhibitors of P450 (Neal, 1980). In addition, there is evidence that OP compounds phosphorylate many serine hydrolases of the B type (Aldridge, 1953). For example, diisopropyl fluorophosphate (DFP) is considered a prototypic serine protease inhibitor and will phosphorylate a myriad of brain proteins (Carrington and Abou-Donia, 1985; Pope and Padilla, 1989). Although information is scarce regarding the interaction of OP pesticides with serine hydrolases other than the cholinesterases, this area of study should not be ignored. Since the P450 isoforms that activate OP compounds are readily induced by drugs and other xenobiotics, interactions affecting OP pesticide toxicity are possible.

c. Adverse Effects

Signs of toxicity due to excess ACh accumulation in the parasympathetic nervous system may be mediated by muscarinic receptors on the effector organs. Muscarinic receptors are located on effector organs including the salivary glands, heart, eye, respiratory system, gastrointestinal tract, and blood. Signs of toxicity generally associated with muscarinic receptor stimulation include increased lacrimation and salivation, bronchoconstriction, bronchosecretion, meiosis (constriction of the pupil of the eye), gastrointestinal cramps, diarrhea, urination, and bradycardia.

Nicotinic receptors are located in the ganglia of the sympathetic and parasympathetic divisions of the autonomic nervous system and skeletal neuromuscular junctions. Excess ACh accumulation at nicotinic receptors results in stimulation and subsequent desensitization of nicotinic receptors. Signs of toxicity mediated by nicotinic receptors in the autonomic and somatic systems include tachycardia, hypertension, muscle fasciculations (particularly the eyelids and facial muscles), tremors, and muscle weakness or flaccid paralysis (Wills, 1970; for review see Watanabe, 1989).

Both muscarinic and nicotinic receptors are located in the central nervous system (CNS). Effects of stimulation of nicotinic receptors in the CNS include an alerting action at low concentrations, followed by tremor, emesis, and stimulation of the respiratory center at higher doses. At very high concentrations stimulation of nicotinic receptors causes convulsions. Combined effects reported due to ACh accumulation at both muscarinic and nicotinic receptors in the CNS following exposure to OP compounds include restlessness, emotional lability, ataxia, lethargy, mental confusion, loss of memory, generalized weakness, convulsion, cyanosis, coma, and depression of respiratory centers (for review see Ecobichon, 1996). Signs of cholinergic toxicity include all those mentioned above.

Many of the signs and symptoms of cholinergic toxicity are in direct opposition to one another, such as bradycardia produced by activation of the parasympathetic nervous system and tachycardia via the sympathetic system. OP pesticides may cause bradycardia by increasing the release of ACh from the vagus nerve and inhibiting ACh hydrolysis, thereby activating the inhibitory muscarinic receptors in cardiac muscle. Tachycardia is produced by cholinergic activation of nicotinic receptors on the ganglia, stimulating the release of norepinephrine in cardiac muscle that activates adrenergic receptors. In addition to cholinergic activation causing opposing effects, the same effect may be produced by action on two different parts of the nervous system, such as muscle weakness due to excess stimulation and desensitization of the nicotinic receptors at the neuromuscular junction and generalized weakness due to an effect on the CNS. Multiple causes of cholinergic signs make it difficult to attribute particular effects to OP pesticide action at specific target sites.

Persistent inhibition of AChE following aging of the phosphorylated enzyme can lead to aberrant synaptic function for an extended period of time. AChE inhibition persisted for days in animals treated with several OP compounds following a single exposure, and the inhibition was even more prolonged when the OP compound required bioactivation which extended the time of entry of the active metabolite into the circulation (Chambers and Carr, 1993). This persistent inhibition prolongs the biological impact of OP anticholinesterases even when the relatively labile OP compounds have been metabolized and cleared from the body. Subsequent exposure to an anticholinesterase could exert additional biological effects on the same molecular target.

Persistent deficits in memory and neurophysiological function have been reported in humans exposed to nerve agents (reviewed in Karczmar, 1984) or to OP pesticides (Savage *et al.*, 1988; Rosenstock *et al.*, 1991; Steenland *et al.*, 1994; Stephens *et al.*, 1995). These effects are manifest months to years after the documented exposure, usually following ob-

served cholinergic toxicity. Although animal studies have been conducted to characterize these long-term effects, many questions remain about the cause and nature of these effects (Annau, 1992). Some of the most important questions concern whether low-level repeated exposure to OP pesticides will cause permanent neurological deficits and whether initial AChE inhibition is a prerequisite for these effects.

A small group of OP pesticides has been associated with visual toxicity in laboratory animals and in humans suffering from a syndrome known as "Saku disease" (ILSI, 1994; Boyes and Dyer, 1983). Visual toxicity may result from degeneration of the retina and the optic nerve that may arise following apparent recovery from earlier exposure to an OP. In tests to examine OP pesticide effects on the visual system rats treated with some, but not all, OP compounds exhibit AChE inhibition initially. After 3 months AChE levels recover, but there is pathology in the retina and muscarinic receptor function is impaired. In this case, muscarinic receptor density remains the same, but less inositol phosphate second messenger is released upon stimulation (Tandon *et al.*, 1994). Effects on the visual system have not been observed in laboratory experiments in the absence of ChE inhibition.

As mentioned earlier, a subset of OP compounds binds to NTE in neural tissue, an action that is associated with organophosphate-induced delayed neurotoxicity (OPIDN). OPIDN is characterized by locomotor ataxia beginning 1 to 3 weeks following exposure, resulting from degeneration of the long axons of neurons in the central and peripheral nervous systems. OPIDN may be caused by some OP pesticides that are currently used in the United States, but this occurs only at high doses compared to the dose that inhibits AChE. Causation of OPIDN was not considered in this evaluation of common mechanism of toxicity because the dose of the pesticide required to elicit OPIDN is well above the dose that causes cholinergic toxicity (Richardson, 1992). It is recognized that the subset of OP compounds that bind to NTE shares a common mechanism of toxicity separate from inhibition of AChE.

Liver vacuolization and necrosis have been reported in experimental animals exposed chronically to some OP pesticides including dichlorvos and phosmet at exposure levels that cause inhibition of ChE (IRIS, 1997). The mechanism responsible for this effect on the liver is not known for certain. These effects do not appear to be related to AChE inhibition and were not considered in detail by the Working Group.

The Working Group developed an overview statement describing the adverse effects of anticholinesterase OP pesticides:

"Organophosphorus insecticides share a common action of inhibiting acetylcholinesterase; the resulting excess acetylcholine accumulation underlies the principal mechanism of toxicity, the spectrum of effects being determined by the specific targets and modulated by various pharmacokinetic and pharmacodynamic factors."

5. POTENTIAL METHODS FOR GROUPING OP PESTICIDES

The Working Group developed an approach to determine whether the OP pesticides act by a common mechanism of toxicity or if they can be divided into subgroups based on several distinct mechanisms of toxicity. The Working Group agreed to begin with the hypothesis that the anticholinesterase OP pesticides act by a common mechanism of toxicity. To test this hypothesis, they generated a number of alternate hypotheses that, if true, would cause them to reject the initial hypothesis. The alternate hypotheses evaluated were:

1. OP pesticides can be separated into subgroups based on whether or not they require metabolic activation to confer anticholinesterase activity;
2. OP pesticides can be separated into subgroups based on toxicological actions operating instead of, or in addition to, inhibition of AChE;
3. OP pesticides can be separated into subgroups based on activation or deactivation by distinct enzymes located in different parts of the body;
4. OP pesticides can be separated into subgroups based on differential action on muscarinic versus nicotinic receptors;
5. OP pesticides can be separated into subgroups based on differential distribution in the body and consequent action on different target tissues; and
6. OP pesticides can be separated into subgroups of OP compounds based on action solely on the peripheral nervous system versus action solely on the CNS.

A number of these alternate hypotheses were rejected after a quick consideration, and others were evaluated in more detail. The types of data available to support or reject the hypotheses are summarized in the next section, and evaluation of the alternate hypotheses using the data follows.

6. DATA AVAILABLE TO GROUP OP PESTICIDES

Data from similar studies on a variety of OP pesticides are needed to identify similarities and differences in toxicity among OP compounds. Potential sources of data include results from laboratory studies conducted in support of pesticide registrations and research articles published in the peer-reviewed literature. Toxicity data have been submitted to the EPA by pesticide manufacturers in support of pesticide registration applications for the past 25 years. Data submitted to the EPA include reports from acute, subchronic, and chronic exposure studies conducted on laboratory animals and reports from studies of absorption and elimination of metabolites of OP pesticides.

Acute toxicity studies are conducted to evaluate the toxicity that may result from a single, high exposure to the chemical (Eaton and Klaasson, 1996). Data from these studies are used to define the spectrum of toxic effects that may occur and to

determine the acute median lethal dose (LD50). Observation data from these studies frequently provide the first indications of AChE inhibition through the appearance of cholinergic signs.

Subchronic studies are conducted to evaluate the toxicity that may result from a lower level, longer term (less than lifetime) exposure such as may result in an occupational setting (Moseberg and Hayes, 1989). Data collected in these toxicity studies on OP pesticides include periodic measurements of plasma and erythrocyte cholinesterase activity, reports from observation of clinical signs, histopathological evaluation of target tissues, and brain AChE activity at study termination. From these data, the no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) for adverse effects due to subchronic exposure are derived.

Chronic studies are conducted to evaluate the toxicity that may result from a lifetime exposure. The data generally available from these studies with OP pesticides are estimates of ChE inhibition in plasma, erythrocytes, and brain tissue; a description of the appearance of the animal including clinical signs of cholinergic toxicity; body and organ weights; and histological analysis of target tissues. From these data, the NOAEL and LOAEL due to chronic exposure are derived. These levels are used in risk assessment to estimate acceptable levels of human exposure. The chronic studies rarely provide mechanistic information.

Acute and subchronic neurotoxicity screening studies are also conducted using OP pesticides. These studies include additional observations and specific tests to evaluate effects on spontaneous activity, sensory and neuromuscular performance (known as a functional observational battery), and an acute dosing regimen in hens assayed for NTE to detect delayed neurotoxicity (Sette, 1991). Specialized histopathological techniques are employed to evaluate effects on the nervous system. The acute studies provide additional information on time-to-peak effect and measures of ChE inhibition in blood and brain tissue.

Data from studies on metabolism of OP pesticides are also submitted to the EPA in support of pesticide registration applications. Data from these studies generally include the percentage of the administered dose eliminated in the urine and feces during a given time period after dosing, the percentage of the parent OP eliminated unchanged in the urine, the biological half-life of the compound, the percentage of a dermal dose absorbed over time, tissues containing the highest residues at a given time after exposure, and identification of active and inactive metabolites of the parent compound.

Research articles published in the peer-reviewed literature reveal considerable information about the toxicology of OP pesticides. The understanding of pharmacokinetics and pharmacodynamics of OP compounds is largely due to the contributions of basic researchers. The goal of basic research is generally to gain understanding of a particular aspect of the pharmacology or toxicology of an OP compound rather than to produce data immediately or directly useful for risk assessment. Studies performed in basic research laboratories may be conducted under a variety of conditions and using different methods; direct comparison or combination of data from multiple labs must be undertaken with caution.

7. EVALUATION OF HYPOTHESES FOR GROUPING OP PESTICIDES

The alternate hypotheses outlined above that would cause the rejection of the initial hypothesis and provide criteria for subgrouping OP pesticides based on potential for several distinct mechanisms of toxicity among OP pesticides are reiterated below. Following each hypothesis is a summary of the findings of the Working Group.

1. OP Pesticides Can Be Separated into Subgroups Based on Whether or Not They Require Metabolic Activation to Confer Anticholinesterase Activity

One assumption behind this hypothesis is that the process of metabolic activation may delay the onset of inhibition of AChE, compared to the immediate action of active parent compounds. This may well be the case, but is important only if the exposures to multiple OP pesticides are simultaneous. Exposure to OP pesticides may occur via many routes and throughout days and weeks, rather than simultaneous exposure to two or more OP compounds followed by no exposure. Another line of thinking behind this alternate hypothesis was that a compound requiring metabolic activation would be subject to biotransformation-mediated interactions that might alter the dose–response characteristics of the compound. While this is true, metabolic interactions are not expected to alter the mechanism of toxicity of the OP pesticide or its active metabolite. The distinction between OP compounds that require metabolic activation to confer anticholinesterase activity and those that do not may be very useful in a case-specific risk assessment when exposure is well characterized, but is not useful for separating OP pesticides into subgroups that share a common mechanism of toxicity. This alternative hypothesis was rejected.

2. OP Pesticides Can Be Separated into Subgroups Based on Toxicological Actions Operating Instead of, or in Addition to, Inhibition of AChE

This hypothesis could be tested by looking for indicators that inhibition of AChE does not correlate with toxicity as might be expected. Divergences in the relationship between potency to inhibit AChE and toxicity may be detected using pharmacokinetic data. While different relationships between *in vitro* potency to inhibit AChE and toxicity could be explained by differences in metabolic activation and deactivation of OP compounds, these data may also indicate involvement of additional mechanisms of action. Divergence in potency and

toxicity might allow preliminary subgrouping of the OP pesticides, and additional data could be used to determine if compounds in the preliminary subgroups share a mechanism of toxicity in addition to inhibition of AChE. Pharmacokinetic data that might be used initially to test this hypothesis include concentration of the OP compound that inhibits 50% of AChE activity *in vitro* under specified conditions (IC50), the bimolecular inhibition rate constant (k_i), the spontaneous reactivation rate of phosphorylated AChE, the dose that will cause a given functional change in 50% of intact animals (ED50), the median lethal dose in intact animals (LD50), and the first-order rate constant for aging (k_a).

A divergence in the relationship between potency and toxicity may be characterized by an OP compound of low potency for inhibition of AChE *in vitro* (high IC50), but very toxic to the whole animal at low exposures (low ED50). Alternately, an OP pesticide of high potency *in vitro* (low IC50) and low toxicity in an animal (high ED50) may represent the opposite divergence from an expected correlation. Another measure of discrepancy between potency and anticipated toxicity is illustrated in studies on intact animals by an OP pesticide with a relatively low ED50 and a very high LD50 or maximum tolerated dose (MTD). For example, the chlorpyrifos ED50 for inhibition of brain AChE in rats was approximately 16% of the chlorpyrifos MTD, while the parathion ED50 was approximately 38% of the parathion MTD (Pope and Chakraborti, 1992). In addition, fewer signs of toxicity were noted in chlorpyrifos-treated rats than parathion-treated, even though chlorpyrifos treatment caused greater inhibition of brain AChE, suggesting that additional toxicological actions may modulate the toxicity of OP AChE inhibitors (Chaudhuri *et al.*, 1993).

The end point of the ED50 might be inhibition of AChE in target tissue or a behavioral or functional sign of cholinergic toxicity. If the ED50 end point considered is a functional or behavioral sign of neurotoxicity, there are additional questions to be addressed. The dose of an OP that causes signs of cholinergic toxicity in an animal varies depending upon the dosing regimen. Animals are more sensitive to a single dose of an OP than they are following the same single dose after repeated exposure, because the animals become "tolerant" to the OP during repeated exposure. The question arises as to which effects are most appropriate to consider: those observed in the animal following a single exposure or those observed after repeated exposures?

Discrepancies in the expected correlation between AChE inhibition and acute toxicity of some OP pesticides might be explained by a number of mechanisms including differences in metabolism, differences in interactions with esterases other than AChE (e.g., BuChE or CaE), and/or selective direct interactions with cholinergic receptors. Studies have shown that both muscarinic and nicotinic receptor subtypes can be directly activated, inhibited, or modulated by some OP pesticides (Silviera *et al.*, 1990; Jett *et al.*, 1990; Eldefrawi *et al.*, 1992; Chaudhuri *et al.*, 1993; Huff *et al.*, 1994; Ward and Mundy, 1996). These receptor subtypes differ in function, affinity for toxicants, and regional and cellular distribution (Eldefrawi and Eldefrawi, 1997). For example, a muscarinic receptor subtype most sensitive to direct binding by some OP pesticides appears to be located primarily in the presynaptic terminal (Watson *et al.*, 1986; Jett *et al.*, 1990) and acts as an autoreceptor controlling ACh release (Pope *et al.*, 1995). Direct activation of these receptors would decrease the release of ACh and moderate the consequences of AChE inhibition. Additional data are needed to characterize the role that OP pesticide interaction with cholinergic receptors plays in toxicity.

Working Group members searched company records and the peer-reviewed literature for ED50 data and IC50 and/or k_i data for a list of 39 OP pesticides approved for use in the United States. These data could be used to detect discrepancies between potency and toxicity. Limited data on these 39 compounds were located, and the data set was inconsistent in species studied, tissue assayed, and assay conditions. Species studied included birds, rats, and cows; tissues assayed were erythrocytes, liver, and brain; assay conditions that varied were temperature, pH, and incubation duration. LD50 data are available for all of the OP pesticides, but they are variable and insensitive measures of toxicity. The resulting data set was not particularly useful, and this approach to subgrouping the OP pesticides was rejected due to lack of data.

3. OP Pesticides Can Be Separated into Subgroups Based on Activation or Deactivation by Distinct Types of Enzymes Located in Different Parts of the Body

OP pesticides are activated and deactivated by the cytochrome P450 enzyme system. There are many forms of cytochromes P450, each differing in structure and in the specificity of the reactions they catalyze. Cytochromes P450 are divided into 30 or more families and further divided into subfamilies, based on amino acid sequences of the gene products. Isoforms of cytochromes P450 are differentially distributed throughout the organs and tissues of animals, which may confer differential activation or deactivation capacities on different organs. The isoforms of cytochrome P450 involved in metabolism of OP compounds in humans have not been elucidated, so that until additional information is available, differential activation and/or deactivation of OP pesticides cannot be documented.

The OP pesticides are also substrates for FMO enzymes, a group of enzymes with substrate specificities that overlap each other and P450. In addition, A-esterases have the potential to hydrolyze phosphates or oxon metabolites and thereby destroy them, and serine esterases such as carboxylesterases can be phosphorylated by phosphates and thereby stoichiometrically destroy them. Action of these enzymes on OP compounds may also contribute to differential metabolism in different tissues, but not enough data are available to subgroup OP pesticides on this basis.

4. OP Pesticides Can Be Separated into Subgroups Based on Differential Action on Muscarinic versus Nicotinic Receptors

Inhibition of AChE may cause a spectrum of cholinergic signs of toxicity in animals exposed to OP pesticides, encompassing signs attributed to stimulation of muscarinic receptors and signs that are similar to those caused by nicotine. Differential action of an OP pesticide on muscarinic and nicotinic receptors may be the result of direct interaction of the OP compound with one receptor type (or subtype) or other unknown actions. The hypothesis above was supported by an example of domestic cats poisoned by OP pesticides that had been applied to them. Cats poisoned by diazinon present with signs of muscarinic receptor stimulation, such as salivation and respiratory problems, whereas cats poisoned by chlorpyrifos exhibit tremors and flaccid paralysis, signs normally associated with activation and desensitization of nicotinic receptors.

The data available to evaluate potential action on one or the other receptor type might be from behavioral studies, in which the behavior or physical effects observed following OP exposure have already been associated with a particular receptor type. Researchers have conducted studies on rats exposed to an OP pesticide via the diet for 13 weeks and report the degree of inhibition of ChE in the plasma, erythrocyte, and brain and the results of a behavioral neurotoxicity screen, described as a "functional observational battery" (FOB) of tests (Sette, 1991).

The functional observational analysis of behavior is a neurotoxicity screening tool consisting of noninvasive procedures designed to detect gross functional deficits in animals exposed to toxicants. The screening battery includes evaluation of motor activity, autonomic nervous system function, and grip performance. Motor activity is measured in an automated activity recording device. Autonomic function is assessed by evaluation of lacrimation and salivation, frequency of urination, presence or absence of diarrhea, and constriction of the pupil of the eye in response to light or a measure of pupil size. Other measures include forelimb and hindlimb grip strength, observation of muscle fasciculations and tremors, and decrements in aerial righting.

A study reporting the results of a functional observational battery of tests on six OP insecticides was recently published by Sheets *et al.* (1997). This study is particularly useful because the exposure paradigms, the biochemical measures and the behavioral end points, are consistent and may be compared directly. The authors report cholinesterase inhibition and results of the FOB for each of six structurally diverse OP insecticides that represent a range of lipophilicity (sulprofos, tebupirimphos, disulfoton, azinphos-methyl, trichlorfon, and methamidophos). The Working Group looked for evidence in the article by Sheets *et al.* (1997) that behaviors exhibited by the OP pesticide-treated rats could be categorized as purely or primarily nicotinic or muscarinic in nature. Rats treated with five of the six OP pesticides exhibited muscle fasciculations or tremors, signs of action on nicotinic receptors. Rats treated with the sixth OP were impaired in righting reflex, attributed to effects on the neuromusculature, presumably via nicotinic receptors. All six OPs caused perineal staining, taken as a reflection of increased urination or defecation due to muscarinic receptor stimulation. Thus, both nicotinic and muscarinic actions were observed following exposure to each OP pesticide. The data in the article by Sheets *et al.* (1997) reflect effects observed in laboratory animals following exposure to many other OP compounds and are used here as an example. The data did not support subgrouping of OP pesticides based on differential action on muscarinic and nicotinic receptors.

5. OP Pesticides Can Be Separated into Subgroups Based on Differential Distribution in the Body and Consequent Action on Different Target Tissues

Data to support or reject this hypothesis are scant. Limited data indicate that there may be some differential distribution of OP pesticides following exposure. Studies supporting pesticide registrations may report the concentration of an OP compound in different organs at a given time after exposure. These reports are gross estimates of pesticide distribution at a single time point; the problem is that the dosing regimen, time after dose, and other variables are inconsistent among studies. Thus, these data are insufficient to determine if OP pesticides are distributed differently throughout the body following exposure, but the extreme differential distribution necessary for independent mechanisms of action makes this unlikely. The data are not available to support differential distribution of OP pesticides in the body as a method for subgrouping OP pesticides.

6. OP Pesticides Can Be Separated into Subgroups of OP Pesticides Based on Action Solely on the Peripheral Nervous System versus Action Solely on the CNS

There is the potential for an OP pesticide to act exclusively on AChE (and other cholinesterases) in peripheral tissue and not the brain if the OP pesticide does not cross the blood–brain barrier. Alternatively, an OP could inhibit AChE in the brain more than the periphery if there is a mechanism for preferential distribution of the OP pesticide to the brain. OP pesticides are generally lipophilic and, with a few exceptions, are fully distributed to all parts of the body, including the central nervous system (Watanabe, 1989). Echothiophate (once used in ophthalmic preparations) is an exception that does not cross the blood–brain barrier.

The Working Group again referred to the FOB data from the article by Sheets *et al.* (1997) to try to subgroup OP pesticides based on expression of behaviors attributable to central action or peripheral action of the OP compound. One difficulty in this task is that effects due to central actions and peripheral actions may be very similar; for example, muscle weakness may be due to action at the skeletal muscle or a sign of general

weakness due to CNS effects. A second difficulty is that the FOB was not designed to attribute behavior to the CNS or peripheral nervous system. The behavioral test designed to reflect effects on the CNS is motor activity, but a decrease in motor activity may be the result of effects on the skeletal muscles as well. The data did not support subgrouping the OP pesticides based on primary or exclusive effects on the brain or the peripheral nervous system.

8. SUMMARY AND CONCLUSIONS

Compounds that act by a common mechanism of toxicity may cause the same critical effect, act on the same molecular target at the same target tissue, act by the same biochemical mechanism of action, and share a common toxic intermediate. These principles were derived from the scenarios in Section 3 and were used to consider whether the anticholinesterase OP pesticides act by a common mechanism of toxicity. The anticholinesterase OP pesticides are a group of structurally related compounds that share certain characteristic toxicologic actions, specifically inhibition of AChE by phosphorylation, and subsequent accumulation of ACh in the nervous systems of animals. Anticholinesterase OP pesticides clearly share some of the above-mentioned characteristics, but they produce a variety of clinical signs of neurotoxicity that are not identical for all OP compounds. Therefore, the Working Group evaluated the hypothesis that anticholinesterase OP pesticides act by a common mechanism of toxicity. Should this hypothesis be rejected, one would conclude that it is possible to divide the OP pesticides into subgroups based on several distinct mechanisms of toxicity.

To test the hypothesis that OP pesticides act by a common mechanism of toxicity, several alternative hypotheses were generated. These alternate hypotheses were based on potential differences in OP pesticide metabolism, distribution, and molecular targets. The alternative hypotheses were:

1. OP pesticides can be separated into subgroups based on whether or not they require metabolic activation to confer anticholinesterase activity;
2. OP pesticides can be separated into subgroups based on toxicological actions operating instead of, or in addition to, inhibition of AChE;
3. OP pesticides can be separated into subgroups based on activation or deactivation by distinct enzymes located in different parts of the body;
4. OP pesticides can be separated into subgroups based on differential action on muscarinic versus nicotinic receptors;
5. OP pesticides can be separated into subgroups based on differential distribution in the body and consequent action on different target tissues; and
6. OP pesticides can be separated into subgroups of OP compounds based on action solely on the peripheral nervous system versus action solely on the CNS.

These alternate hypotheses were evaluated by the Working Group using pharmacologic and toxicologic data. Some of the alternate hypotheses were rejected outright, and others were rejected based on a lack of adequate data. After rejecting all the alternate hypotheses, the Working Group accepted the original hypothesis and concluded that OP pesticides should be considered to act via a common mechanism of toxicity if they inhibit AChE by phosphorylation and elicit any spectrum of cholinergic effects.

The charge to the Working Group was "to develop a comprehensive approach for grouping chemicals by a common mechanism of toxicity using the OP pesticides as a case study." The approach developed by the Working Group to determine if the OP pesticides act by a common mechanism of toxicity could be applied to other pesticides that share structural characteristics and biochemical or toxicologic actions. The first step in this approach is to consider carefully the most appropriate end point to select and describe the cascade of events following exposure that led to an adverse effect. The events may include metabolic activation and deactivation of the compounds, reversibility or irreversibility of reactions, or the potential for differential distribution of the active metabolites. If appropriate, this information may be used to formulate a hypothesis that members of a given class of pesticides act by a common mechanism of toxicity. The hypothesis may be tested by developing alternative hypotheses and evaluating the alternatives based on available data, as was done for the OP pesticides. This approach may work well for groups or classes of pesticides that have adequate data available to support the initial hypothesis. If the hypothesis is not supported by data, this approach is not appropriate.

9. EPILOGUE

The charge to the Working Group was extremely narrow, and many topics touched on in consideration of the common mechanism of toxicity were not elaborated on in that context, but should be considered before a cumulative risk assessment is performed. A risk assessment of combined exposure to multiple OP pesticides will be more complicated than a risk assessment considering exposure to a single chemical. The exposure assessment may include consideration of issues that are not part of a risk assessment on an individual agent. The following factors should be considered when conducting the aggregate exposure assessment for a cumulative risk assessment of OP pesticides:

- "Combined exposure" has not been adequately defined, given that exposure to OP pesticides may result in persistent inhibition of AChE;
- The OP pesticides that people are exposed to in combination are not documented; and
- Methods for assessment of exposure to multiple chemicals via multiple sources and routes of exposure are not well developed.

All available information on pharmacokinetics and pharmacodynamics of OP compounds should be considered for use in a risk assessment. A few studies describing toxicity of combinations of OP pesticides in laboratory animals exposed to high doses have been done (see National Research Council, 1989, for review), but studies investigating the toxicity of low-dose exposures to combinations of OP pesticides have not been published. Estimation of the dose–response characteristics of a combination of OP pesticides may be difficult because the following possibilities have not been investigated sufficiently:

- Effects due to exposure to multiple OP pesticides may be additive;
- Effects due to exposure to multiple OP pesticides may be due to the sum of toxicologically equivalent doses;
- Effects due to exposure to multiple OP pesticides may be greater than additive (synergistic) due to interactions between the OP pesticides and receptors or enzymes that activate or detoxify the OP pesticides; and
- Effects due to exposure to multiple OP pesticides may be less than additive (antagonistic) due to interactions analogous to those described for synergism.

The Working Group cautioned that there may be subpopulations of individuals who are particularly sensitive to the effects of OP pesticides. A risk assessment of combined exposure to multiple OP pesticides should specifically consider subpopulations (e.g., children, the elderly, and genetically susceptible) that may be at higher risk.

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REFERENCES

Abou-Donia, M. B. (1981). Organophosphorus ester-induced neurotoxicity. *Annu. Rev. Pharmacol. Toxicol.* **21,** 511–548.

Aldridge, W. N. (1953). Serum esterases: Two types of esterase (A and B) hydrolysing *p*-nitrophenyl acetate, proprionate, and butyrate, and a method for their determination. *Biochem. J.* **53,** 110–117.

Annau, Z. (1992). Neurobehavioral effects of organophosphorus compounds. In *Organophosphates—Chemistry, Fate, and Effects* (J. E. Chambers and P. E. Levi, Eds.), pp. 419–432. Academic Press, San Diego.

Bakry, N. M. S., El-Rashidy, A. H., Eldefrawi, A. T., and Eldefrawi, M. E. (1988). Direct actions of organophosphate anticholinesterase on nicotinic and muscarinic actelycholine receptors. *J. Biochem. Toxicol.* **3,** 23–259.

Ballantyne, B., and Marrs, T. C. (1992). *Clinical and Experimental Toxicology of Organophosphates and Carbamates.* Butterworth-Heinemann, Oxford, England.

Boyes W. K., and Dyer, R. S., (1983). Pattern reversal visual evoked potentials in awake rats. *Brain Res. Bull.* **10,** 817–823.

Carrington, C. D., and Abou-Donia, M. (1985). Characterization of [^{3}H] di-isopropyl phosphorofluoridate-binding proteins in hen brain. *Biochem. J.* **228,** 537–544.

Chambers, J. E., and Carr, R. L. (1993). Inhibition patterns of brain acetylcholinesterase and hepatic and plasma alieserases following exposures to three phosphorothionate insecticides and their oxons in rats. *Fundam. Appl. Toxicol.* **21,** 111–119.

Chambers, H. W. (1992). Organophosphorus compounds An overview. In *Organophosphates—Chemistry, Fate, and Effects* (J. E. Chambers and P. E. Levi, Eds.), pp. 3–17. Academic Press, San Diego.

Chaudhuri, J., Chakraborti, T. K., Chanda, S., and Pope, C. N. (1993). Differential modulation of organophosphorus-sensitive muscarinic receptors in rat brain by parathion and chlorpyrifos. *J. Biochem. Toxicol.* **8,** 207–216.

Eaton, D , and Klaassen, C. D. (1996). Principles of toxicology. In *Casarett & Doull's Toxicology* (C. D Klaassen, M. O. Amdur, and J. Doull, Eds.), pp. 13–33. McGraw-Hill, New York.

Ecobichon, D. J. (1996). Toxic effects of pesticides. In *Casarett & Doull's Toxicology* (C. D. Klaassen, M. O. Amdur, and J Doull, Eds.), pp. 643–689. McGraw-Hill, New York.

Eldefrawi, A. T., and Eldefrawi, M. E. (1997). Neurotransmitter receptors. In *Nervous System and Behavioral Toxicology* (K. R. Reuhl and H. E. Lowndes, Eds.), Vol. 11, pp. 139–153. Elsevier, Cambridge Univ. Press.

Eldefrawi, A. T., Jett, D., and Eldefrawi, M E. (1992). Direct actions of organophosphorus anticholinesterases on muscarinic receptors. In *Organophosphates—Chemistry, Fate, and Effects* (J. E. Chambers and P. E. Levi, Eds.), pp. 258–270. Academic Press, San Diego.

Eldefrawi, M. E., Schweizer, G., Bakry, N M. S , and Valdes, J. J. (1988). Desensitization of the nicotinic acetylcholine receptor by diisopropylfluorophosphate. *J. Biochem Toxicol.* **3,** 21–32.

Eto, M. (1974). *Organophosphorus Pesticides: Organic and Biological Chemistry.* CRC Press, Cleveland.

Food Quality Protection Act (1996) Public Law No. 104-170.

Gallo, M. A., and Lawryk, N. J. (1991) Organic phosphorus pesticides. In *Handbook of Pesticide Toxicology* (W. J. Hayes, Jr., and E. R. Laws, Jr., Eds.), Vol. 2, pp. 917–1123. Academic Press, San Diego.

Guyton, A. C. (1981). The autonomic nervous system: The adrenal medulla. In *Textbook of Medical Physiology* (A. C. Guyton, Ed.), pp. 710–722 Saunders, Philadelphia.

Hardmon, J. G., and Limbird, L. E. (1996). *The Pharmacological Basis of Therapeutics.* McGraw-Hill, New York.

Hodgson, E., and Levi, P. E. (1996). Pesticides: An important but underused model for the environmental health sciences. *Environ. Health Perspect.* **104**(Suppl. 1), 97–106.

Holmstedt, B. (1959). Pharmacology of organophosphorus cholinesterase inhibitors. *Pharmacol. Rev.* **11,** 567–688.

Holmstedt, B. (1963). Structure–activity relationships of the organophosphorus anticholinesterase agents. In *Cholinesterases and Anticholinesterase Agents* (G. B. Koelle, Ed.), pp. 428–485. Springer-Verlag, Berlin.

Huff, R. A., Corcoran, J. J., Anderson, J. K., and Abou-Donia, M. B. (1994). Chlorpyrifos oxon binds directly to muscarinic receptors and inhibits cAMP accumulation in rat striatum. *J. Pharmacol. Exp. Ther.* **269,** 329–335.

ILSI Risk Science Institute (1994). Report: Evaluation of Organophosphorus

Compounds for Potential Toxicity of the Visual System International Life Sciences Institute, Washington, DC

Integrated Risk Information System Online (IRIS) (1997). U S. EPA, Office of Health and Environmental Assessment, Cincinnati, OH.

Jett, D. A., Abdallah, E. E., El—Fakahany, E. E., Eldefrawi, M. E., and Eldefrawi, A. T. (1990). High affinity activation by paraoxon of a muscarinic receptor subtype in rat brain striatum. *Pestic. Biochem. Physiol.* **39,** 149–157.

Johnson, M. K. (1969). The delayed neurotoxic effect of some organophosphorus compounds. Identification of the phosphorylation site as an esterase. *Biochem. J.* **114,** 711–717.

Karczmar, A. G (1990). Physiological cholinergic functions in the CNS. In *Cholinergic Neurotransmission: Functional and Clinical Aspects* (S. Aquilonius and P. Gillberg, Eds.), pp. 437–466. Elsevier, Amsterdam

Karczmar, A. G. (1993). Brief presentation of the story and present status of studies of the vertebrate cholinergic system. *Neuropsychopharmacology* **9**(3), 181–199.

Karczmar, A. G. (1984) Acute and long lasting central actions of organophosphorus agents. *Fundam. Appl. Toxicol.* **4,** S1–S17.

Katz, E. J., Cortes, V. I., Eldefrawi, M. E , and Eldefrawi, A. T. (1997). Chlorpyrifos, parathion and their oxons bind to and desensitize a nicotinic acetylcholine receptor: Relevance to their toxicities. *Toxicol. Appl Pharmacol.* **146,** 227–236.

Levi, P. E., and Hodgson, E. (1992). Metabolism of organophosphorus compounds by the flavin-containing monooxygenase. In *Organophosphates—Chemistry, Fate, and Effects* (J E. Chambers and P. E Levi, Eds), pp. 141–154. Academic Press, San Diego

Maxwell, D. M. (1992). Detoxication of organophosphorus compounds by carboxylesterase. In *Organophosphates—Chemistry, Fate, and Effects* (J. E. Chambers and P. E. Levi, Eds.), pp. 183–199. Academic Press, San Diego.

Maxwell, D. M., and Lenz, D. E. (1992). Structure–activity relationships and anticholinesterase activity. In *Clinical and Experimental Toxicology of Organophosphates and Carbamates* (B. Ballantyne and T. C. Marrs, Eds.), pp. 47–58. Buttersworth & Heinemann, Oxford, England.

Mazur, A. (1946). An enzyme in animal tissues capable of hydrolyzing the phosphorus–fluorine bond of alkyl fluorophosphates. *J. Biol. Chem.* **164,** 271–289.

Mosberg, A. T., and Hayes, A. W. (1989). Subchronic toxicity testing. In *Principles and Methods of Toxicology* (A. W. Hayes, Ed.), pp. 221–236. Raven Press, New York.

National Research Council (1989). Acetylcholinesterase inhibitors: Case study of mixtures of contaminants with similar biologic effects. In *Drinking Water and Health,* Vol. 9, pp 146–161. National Academy Press, Washington, DC.

Neal, R. A. (1980). Microsomal metabolism of the thionosulfur compounds: Mechanisms and toxicological significance. In *Reviews in Biochemical Toxicology* (E. H. Hodgson, J. R. Bend, and R M. Philpot, Eds.), Vol. 2, pp. 131–171. Elsevier/North-Holland, New York.

Pope, C. N. C. J , and Chakraborti, T. K. (1995). Organophosphorus-sensitive cholinergic receptors. Possible role in modulation of anticholinesterase-induced toxicity. In *Enzymes of the Cholinesterase Family* (A S. Balasuberamian, B. T. Doctor, P. Taylor, and D. M. Quinn, Eds.), pp. 305–312. Plenum, New York.

Pope, C. N., and Chakraborti, T. K. (1992). Dose-related inhibition of brain and plasma cholinesterase in neonatal and adult rats following sublethal organophosphate exposures. *Toxicology* **73,** 35–43

Pope, C. N., and Padilla, S. (1989). Chromatographic characterization of neurotoxic esterase. *Biochem. Pharmacol.* **38,** 181–188.

Richardson, R. J (1992). Interactions of organophosphorus compounds with neurotoxic esterase. In *Organophosphates—Chemistry, Fate, and Effects* (J. E. Chambers and P. E. Levi, Eds.), pp. 299–323. Academic Press, San Diego.

Richardson, R. J. (1995). Assessment of the neurotoxic potential of chlorpyrifos relative to other organophosphorus compounds: A critical review of the literature. *J. Toxicol. Environ. Health* **44,** 135–165.

Rosenstock, L., Keifer, M., Daniell, W. E., McConnell, R., and Claypoole, K. (1991). Chronic central nervous system effects of acute organophosphate pesticide intoxication. *Lancet* **338,** 223–227.

Savage, E. P , Keefe, T J., Mounce, L. M., Heaton, R. K., Lewis, J. A., and Burcar, P. J. (1988). Chronic neurological sequelae of acute organophosphate pesticide poisoning. *Arch. Environ. Health* **43,** 38–45.

Sette, W. F. (1991). Pesticide Assessment Guidelines, Subdivision F Hazard Evaluation: Human and Domestic Animals, Addendum 10. Series 81, 82, 83. U.S. EPA, Washington, DC.

Sheets, L. P., Hamilton, B. F., Sangha, G. K., and Thyssen, J. H. (1997) Subchronic neurotoxicity screening studies with six organophosphate insecticides. An assessment of behavior and morphology relative to cholinesterase inhibition. *Fundam. Appl. Toxicol.* **35,** 101–119.

Silveira, C. P., Eldefrawi, A. T., and Eldefrawi, M. E. (1990). Putative M2 muscarinic receptors of rat heart have high affinity for organophosphorus anticholinesterases. *Toxicol. Appl. Pharmacol.* **103,** 474–481.

Steenland, K., Jenkins, B , Ames, R. G., O'Malley, M., Chrislip, D., and Russo, J. (1994). Chronic neurological sequelae to organophosphate pesticide poisoning. *Am. J. Pub. Health* **84,** 731–736.

Stephens, R., Spurgeon, A., Calvert, I. A., Beach, J., Levy, L. S., and Harrington, J. M. (1995). Neuropsychological effects of long-term exposure to organophosphates in sheep dip. *Lancet* **345,** 1135–1139.

Tandon, P., Padilla, S., Barone, S., Jr., Pope, C. N., and Tilson, H. A (1994). Fenthion produces a persistent decrease in muscarinic receptor function in the adult rat retina. *Toxicol. Appl. Pharmacol.* **125,** 271–280.

U.S. EPA (1997). Issues paper: Guidance for establishing a common mechanism of toxicity for use in combined risk assessments. Dated February 11, presented to the FIFRA Scientific Advisory Panel March 20, 1997. U S. EPA, Washington, DC.

Ward, T. R., and Mundy, W. R. (1996). Organophosphorus compounds preferentially affect second messenger systems coupled to M2/M4 receptors in rat frontal cortex. *Brain Res. Bull.* **39,** 49–55.

Watanable, A. M. (1989). Cholinoceptor-activating drugs. In *Basic and Clinical Pharmacology* (B G. Katzung, Ed), pp. 70–105. Appleton & Lange, East Norwalk.

Watson, M , Roeske, W. R., Vickroy, T. W., Smith, T. L., Akiyama, K., Gulya, D., Duckles, S. P., Serra, M., Adem, A., Nordberg, A , Gehlert, D. R., Wamsley, J. K., and Yamamura, H. I. (1986). Biochemical and functional basis of putative muscarinic receptor subtypes and its implications. *Trends Pharmacol. Sci.* **7**(Suppl.), 46–55.

Wills, J. H. (1970). Toxicity of anticholinesterases and treatment of poisoning. In *Anticholinesterase Agents* (A. G. Karczmar, Ed.), pp. 355–469. Pergamon Press, Oxford.