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ORGANOPHOSPHORUS PESTICIDES: DO THEY ALL HAVE THE SAME MECHANISM OF TOXICITY?

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Organophosphorus (OP) pesticides are used extensively to control agricultural, household and structural pests. These pesticides constitute a diverse group of chemical structures exhibiting a wide range of physicochemical properties, with their primary toxicological action arising from inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7). Historically, risk characterizations for these toxicants have been based on hazard and exposure data pertaining to individual chemicals. The Food Quality Protection Act of 1996 now requires, however, that combined risk assessments be performed with pesticides having a common mechanism of toxicity. It is therefore critical to consider whether OP pesticides all exert toxicity through a common mechanism. This brief review evaluates the comparative toxicity of the 38 OP AChE inhibitors currently registered for use as pesticides in the United States and examines the data which suggest that some OP pesticides have toxicologically relevant sites of action in addition to AChE. It is concluded that all OP anticholinesterases potentially have a mechanism of toxicity in common, that is, phosphorylation of AChE causing accumulation of acetylcholine, overstimulation of cholinergic receptors, and consequent clinical signs of cholinergic toxicity. Additional macromolecular targets for some OP pesticides, however, may alter the cascade of events following AChE phosphorylation and thereby modify that common mechanism. Furthermore, other macromolecular targets of some OP pesticides appear capable of altering noncholinergic neurochemical processes. These additional actions may contribute to qualitative and quantitative differences in toxicity sometimes noted in the presence of similar levels of AChE inhibition induced by different OP pesticides. Further investigation of these additional sites of action may allow subclassification and influence the decision to perform combined risk assessments on this class of pesticides based on common mechanism of toxicity.

Organophosphorus (OP) compounds have been utilized as therapeutic agents, agricultural chemicals, plasticizers, lubricants, flame retardants, fuel additives, and, most notoriously, as chemical warfare agents (for review see Ehrich, 1998). The acute toxicity of most of these compounds is initiated by inhibition of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) in the central and peripheral nervous systems. AChE normally

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rapidly degrades acetylcholine in the synapse; thus inhibition of AChE allows accumulation of acetylcholine with subsequent excessive stimulation of acetylcholine receptors in associated postsynaptic cells and/or end organs. It is generally considered that some degree of AChE inhibition can be tolerated without substantial alteration of cholinergic transmission. With more extensive inhibition (>50–60%) of AChE, however, signs of toxicity are elicited including autonomic dysfunction (e.g., excessive secretions of the airways, excretory systems, salivary glands, and lacrimal glands), involuntary movements (e.g., tremors, convulsions), muscle fasciculations, and ultimately respiratory depression (Nostrandt et al., 1997).

While OP compounds have been used for a wide variety of applications, their most widespread use is as pesticides for the control of insects (for review see Ecobichon, 1996). With the restrictions on use of most of the persistent organochlorine insecticides imposed in the 1970s, the less persistent but highly effective OP agents became the insecticides of choice. Currently, 38 different OP pesticides registered and approved for use in the United States are capable of inhibiting AChE either directly or following biotransformation (see Table 1).

TABLE 1. Organophosphorus Anticholinesterase Pesticides Currently Used in the United States

Derivative (class)	Chemical name	Direct-acting	LD50	(Ref.)
Phosphoric acid				
Dichlorvos	Dimethyl 2,2-dichlorovinyl phosphate	Yes	80	(G3)
Dicrotophos	Dimethyl 3-dimethylamino-1-methyl-3-oxo-1-propenyl phosphate	Yes	21	(G3)
Naled	Dimethyl 1,2-dibromo-2,2-dichloroethyl phosphate	Yes	250	(G3)
Tetrachlorvinphos	Dimethyl 2-chloro-1-(2,4,5-trichlorophenyl)vinyl phosphate	Yes	4000	(M)
Phosphonic acid				
Trichlorfon	Dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate	No	650	(G3)
Dithiophosphoric acid, phosphoryl-type				
Ethoprop	*O*-Ethyl *S,S*-dipropyl phosphorodithioate	Yes	34	(E)
Dithiophosphoric acid, thiophosphoryl-type				
Azinphos methyl	*O,O*-Dimethyl *S*-(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)-methyl phosphorodithioate	No	13	(G1)
Bensulide	*O,O*-Bis(1-methylethyl) *S*-2-[(phenylsulfonyl)amino]-ethyl phosphorodithioate	No	770	(E)
Dimethoate	*O,O*-Dimethyl *S*-2-(methylamino)-2-oxoethyl phosphorodithioate	No	215	(G3)
Disulfoton	*O,O*-Diethyl *S*-2-(ethylthio)ethyl phosphorodithioate	No	2	(E)
Ethion	*O,O,O′,O′*-Tetraethyl *S,S′*-methylene bis-(phosphorodithioate)	No	13	(E)
Malathion	*O,O*-Dimethyl *S*-1,2-bis(carbethoxyethyl) phosphorodithioate	No	1375	(G1)
Methidathion	*O,O*-Dimethyl *S*-(2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-yl)methyl phosphorodithioate	No	31	(G4)

TABLE 1. Organophosphorus Anticholinesterase Pesticides Currently Used in the United States (*continued*)

Derivative (class)	Chemical name	Direct-acting	LD50	(Ref.)
Dithiophosphoric acid, thiophosphoryl-type (*continued*)				
Phorate	*O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate	No	2	(G1)
Phosmet	*O,O*-Dimethyl *S*-(1,3-dihydro-1,3-dioxoisoindol-2(2*H*)-yl)methyl phosphorodithioate	No	147	(E)
Sulfopros	*O*-Ethyl *O*-4-(methylthio)phenyl *S*-propyl phosphorodithioate	No	65	(E)
Temephos	*O,O,O′,O′*-Tetramethyl *O,O′*-[sulfane-bis-(4-phenylene)] bis(phosphorothioate)	No	8600	(G2)
Terbufos	*O,O*-Diethyl *S*-[(1,1-dimethylethyl)thio]methyl phosphorodithioate	No	2	(E)
Dithiophosphonic acid				
Fonofos	*O*-Ethyl *S*-phenyl ethylphosphonodithioate	No	3	(E)
Thiophosphoric acid, phosphoryl-type				
Oxydemeton-methyl	*O,O*-Dimethyl *S*-2-(ethylsulfinyl)ethyl phosphorothioate	Yes	47	(G3)
Profenofos	*O*-Ethyl *O*-(4-bromo-2-chlorophenyl) *S*-propyl phosphorothioate	Yes	358	(M)
Thiophosphoric acid, thiophosphoryl-type				
Chlorethoxyfos	*O,O*-Diethyl *O*-1,2,2,2-tetrachoroethyl phosphorothioate	No	5	(M)
Chlorpyrifos	*O,O*-Diethyl *O*-3,5,6-trichloro-2-pyridinyl phosphorothioate	No	155	(G3)
Coumaphos	*O,O*-Diethyl *O*-(3-chloro-4-methyl-7-coumarinyl) phosphorothioate	No	41	(G3)
Diazinon	*O,O*-Diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate	No	250	(G3)
Fenitrothion	*O,O*-Dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate	No	740	(G3)
Fenthion	*O,O*-Dimethyl *O*-(4-methylmercapto-3-methylphenyl) phosphorothioate	No	215	(G3)
Methyl chlorpyrifos	*O,O*-Dimethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate	No	1500	(Et)
Methyl parathion	*O,O*-Dimethyl *O*-4-nitrophenyl phosphorothioate	No	14	(G1)
Parathion	*O,O*-Diethyl *O*-4-nitrophenyl phosphorothioate	No	13	(G1)
Pirimiphos methyl	*O,O*-Dimethyl *O*-(2-diethylamino-6-methyl-4-pyrimidinyl) phosphorothioate	No	1450	(H)
Sulfotepp	*O,O,O′,O′*-Tetraethyl diphosphorothioate	No	5	(E)
Tebupirimphos	*O*-Ethyl *O*-propyl *O*-[2-(1,1-dimethylethyl)-5-pyrimidinyl] phosphorothioate	No	2	(M)
Phosphoramidic acid				
Fenamiphos	*O*-Ethyl *O*-[3-methyl-4-(methylthio)phenyl] *N*-(1-methylethyl)phosphoramidate	Yes	8	(E)
Thiophosphoramidic acid, phosphoryl-type				
Acephate	*O,S*-Dimethyl *N*-acetylphosphoramidothioate	No	700	(E)
Methamidophos	*O,S*-dimethyl phosphoramidothioate	Yes	31	(G4)

(*Table continues on next page*)

TABLE 1. Organophosphorus Anticholinesterase Pesticides Currently Used in the United States (*continued*)

Derivative (class)	Chemical name	Direct-acting	LD50	(Ref.)
Thiophosphoramidic acid, thiophosphoryl-type				
Isofenfos	*O*-Ethyl *O*-[2-[(1-methylethyl)oxycarbonyl]phenyl] *N*-(1-methylethyl)phosphoramidothioate	No	28	(E)
Propetamphos	*O*-Methyl *O*-2-[(1-methylethyl)oxycarbonyl]-1-methylethenyl *N*-ethylphosphoramidothioate	No	119	(M)

Note. Direct-acting implies that the OP is capable of inhibiting AChE without biotransformation. The LD50 values tabulated are from male rats exposed orally and are given in mg/kg body weight. The references (Ref.) from which acute toxicity data were derived are abbreviated as follows: E = U.S. EPA (1984); Et = Eto (1979); H = Gallo and Lawryk (1991); G1 = Gaines (1960); G2 = Gaines et al. (1967); G3 = Gaines (1969); G4 = Gaines and Linder (1986); M = Meister (1992).

Risk assessment of pesticides for noncancer endpoints has routinely been performed using the reference dose method (Barnes & Dourson, 1988). Essentially, laboratory animals are exposed to different levels of a particular pesticide and various signs of toxicity are recorded. The most sensitive toxic endpoint is used as the "effect," and the highest dosage at which no adverse response is noted is termed the no-observed-adverse-effect level (NOAEL). Division of the NOAEL by uncertainty factors (generally based on interspecies extrapolation and intraspecies variability) then allows for calculation of exposure levels that over a lifetime would be expected to be safe. Even though individuals may be exposed to more than one pesticide at any given time (e.g., through consumption of multiple pesticide residues on a food product), risk characterization has been based on toxicity of, and exposure to, individual chemicals. With the enactment of the Food Quality Protection Act (FQPA), however, the U.S. Environmental Protection Agency (U.S. EPA) is now required to conduct combined risk assessments for pesticides sharing a *common mechanism of toxicity*. Essentially, FQPA requires consideration of "information concerning the cumulative effects of such residues and other substances that have a common mechanism of toxicity in establishing . . . tolerance for a pesticide chemical residue" (FQPA, 1996). Thus, if all OP pesticides exert toxicity through a common mechanism, toxicity and exposure information for each will have to be considered in future regulatory decisions governing these agents when coexposures are anticipated.

The U.S. Environmental Protection Agency (U.S. EPA, 1997) provided suggestions for guidance on establishing if two or more chemicals act through a common mechanism of toxicity. Mechanism of toxicity was first defined as "the major steps leading to an adverse health effect following interaction of a pesticide with biological targets" (U.S. EPA, 1997). Further, it was proposed that "identification of the crucial events following chemical interaction" was necessary to describe fully the mechanism of toxicity

for a pesticide (U.S. EPA, 1997). Thus, it is recognized that the mechanism of toxicity can only be described by full knowledge of the cascade of effects following interaction of the chemical with its biological target(s).

In the case of OP pesticides, the initial step in their mechanism of toxicity is the interaction between the toxicant and the target enzyme AChE in which the active site serine residue of the enzyme is organophosphorylated. While this is an essential first step in the sequence leading to cholinergic toxicity, it is not sufficient in itself. Following this initial interaction, accumulation of acetylcholine in the synapses due to effective blockade of its enzymatic breakdown must occur. Furthermore, if acetylcholine molecules only accumulated in the synapses following inhibition of AChE without subsequent excessive binding to and activation of postsynaptic cholinergic receptors, no toxic response(s) would ensue. Finally, if excessive receptor stimulation was not coupled to an alteration in postsynaptic cellular function (e.g., if signal transduction processes coupled to cholinergic receptors were disrupted), no signs of toxicity would be elicited. Thus, for OP anticholinesterases, at least four steps have to be involved in a cascade of reactions culminating in overt toxicity: (1) binding to and inhibition of an extensive number of AChE molecules with substantial impairment of acetylcholine degradation, (2) accumulation of acetylcholine in synapses of the central and/or peripheral nervous systems, (3) excessive stimulation of postsynaptic cholinergic receptors, and (4) altered cellular functions in response to excessive stimulation of those receptors. Modulation of any of the neurochemical processes involved in acetylcholine synthesis, acetylcholine release, cholinergic receptor binding, or signal transduction, concurrent with anticholinesterase exposure, could therefore influence the progression of events from target enzyme (AChE) inhibition to expression of toxicity.

Under FQPA, common mechanism of toxicity refers to two or more chemicals producing adverse effects through the same crucial cascade of steps. The 38 OP anticholinesterases currently approved for use in the United States can all initiate the first step of this cascade, that is, phosphorylation and inhibition of AChE. Several studies suggest that some OP pesticides have additional sites of action capable of altering the progression of these subsequent steps following AChE inhibition and culminating in cholinergic toxicity. A decision to perform combined risk assessments on all OP pesticides based on common mechanism of toxicity could potentially restrict the use of some of these, in particular when multiple residues of OP toxicants are found on certain foodstuffs. Use restrictions could be even further imposed when aggregate exposures from nondietary routes are considered, as required by FQPA. It is therefore critical to determine if OP pesticides all have a common mechanism of toxicity or if sufficient differences in toxic mechanism exist in some cases as to allow subclassification.

Recently, a panel of toxicologists (including the author) considered

basic concepts and issues pertaining to common mechanism of toxicity using OP pesticides as a case study (Mileson et al., 1998). Six hypotheses for subclassifying OP pesticides were proposed; that is, OP pesticides had different mechanisms of toxicity because (1) some required metabolic bioactivation and others did not, (2) some had toxicological effects instead of or in addition to AChE inhibition, (3) some were differentially biotransformed in different tissues, (4) some preferentially affected either muscarinic or nicotinic-mediated functions, (5) some were differentially distributed into tissues with consequent differential toxicity, and (6) some affected only one division of the nervous system (i.e., central or peripheral). Essentially, hypotheses 1, 4, and 6 were rejected by the panel after discussion of the relevant literature. In the other cases, however, it was generally agreed that insufficient data were available to support the hypotheses. For the purpose of this review, hypothesis 2 (that some OP pesticides can be subclassified because of toxicological effects instead of or in addition to AChE inhibition) is reexamined, with information provided to suggest that it may be tenable and worthy of further consideration.

The following is a brief review of the comparative toxicity and putative additional targets of some OP anticholinesterases. Knowledge of molecular sites of action for some OP pesticides in addition to AChE that could potentially modulate neuronal function may impact the decision to perform combined risk assessments on this class of pesticides as a whole.

COMPARATIVE TOXICITY OF OP PESTICIDES

Table 1 shows the chemical class, chemical name, direct or indirect anticholinesterase activity, and estimates of acute toxicity (LD50s) for the 38 OP anticholinesterases currently registered by the U.S. Environmental Protection Agency for use in the United States. As can be seen in this table, the OP pesticides consist of a wide variety of chemical structures and display a remarkable range (over 1000-fold) of acute toxicity. Some of the OP compounds are direct-acting AChE inhibitors, whereas most require biotransformation to the ultimate toxicant. In some cases, requirement for activation as well as reactivity with detoxification systems contributes to the wide range of acute toxicity with these toxicants. Differences in biotransformation of this group of chemicals do not appear to explain completely the wide range of acute toxicity noted with this class of toxicants, however.

Studies to evaluate common mechanism of toxicity for different chemicals should be comparative in design; that is, the same study should evaluate the effects of different chemicals at the same time using the same toxic endpoints. Unfortunately, relatively few studies have compared the acute toxicity of OP toxicants in such manner. In an often-cited study, Holmstedt (1963) compared the acute toxicity (LD50) and in vitro anticholinesterase potency of a group of direct-acting OP anticholinesterases.

In general, a good correlation (r = .7) was noted between inhibitory potency and acute toxicity. None of these OP toxicants are listed among the 38 compounds currently registered for pesticide use, however.

Chambers and co-workers (1990) compared the in vitro anticholinesterase potency of the oxons of five OP pesticides, parathion, leptophos, EPN, chlorpyrifos, and methyl chlorpyrifos (three of which are still currently registered pesticides), with published acute LD50 values for the parent compounds. Within this series of chemicals, the ratio of acute toxicity to inhibitory potency ranged from about 2 (with parathion) to over 1600 (with methyl chlorpyrifos). Interestingly, while methyl chlorpyrifos was the least potent acute toxicant (LD50 about 3 g/kg), its oxon was the most potent anticholinesterase in vitro. From these studies, it is apparent that the relationship between anticholinesterase potency in vitro and acute toxicity proposed by Holmstedt (1963) does not hold up with some commonly used OP pesticides. If relative toxicity is based only on acute lethality, however, the differences between in vitro and in vivo potencies of these six toxicants could merely reflect differential rates of activation and deactivation, contributing to different levels of the ultimate toxicant (oxon) reaching the target sites. Knowledge of the comparative AChE inhibition in vivo is critical for interpretation of the relative roles of dispositional versus toxicodynamic factors in differential toxicity.

Moser (1995) evaluated the dose-related comparative toxicity of four OP pesticides (chlorpyrifos, parathion, diazinon, and fenthion) using a functional observational battery. In general, the OP pesticides all induced signs characteristic of cholinergic toxicity at some levels of exposure. Notable exceptions were reported in the types of signs elicited by the different OP pesticides, however. For example, it was noted that fenthion reduced motor activity without altering the tail-pinch response, whereas the opposite was observed with parathion exposure. While the levels of AChE activity in the different groups were not measured in this study, these findings suggest that different toxic responses can occur following acute exposure to different OP pesticides.

A study by Sheets and co-workers (1997) reported the comparative toxicity of six currently registered OP pesticides (sulfopros, tebupirimphos, disulfoton, azinophos-methyl, trichlorfon, and methamidophos) following 13-wk dietary exposures. Functional signs of toxicity were evaluated by a detailed clinical examination, a functional observational battery, and by automated motor activity assessment throughout the dosing period. Acetylcholinesterase inhibition in whole brain, erythrocytes, and plasma was determined at the termination of the exposure period. As expected, the six OP pesticides exhibited remarkable differences in potencies, with NOAELs ranging from 1 mg/kg/d for disulfoton and methamidophos to 500 mg/kg/d for trichlorfon. Acetylcholinesterase inhibition in the various tissues was dose related and correlated roughly with the incidence of functional toxicity across OP toxicants. Differences in toxic response were evident across

OP pesticides, however. For example, while similar levels of AChE inhibition were noted following either azinphos-methyl or tebupirimphos exposures in male rats (AChE inhibition of about 77 and 82% in brain, 85 and 69% in plasma, and 84 and 95% in erythrocytes for tebupirimphos and azinphos-methyl, respectively), muscle fasciculations and changes in forelimb grip strength and aerial righting were observed with tebupirimphos but not azinphos-methyl exposures. Some possible autonomic signs, that is, soft stool and increased defecation, were also noted following tebupirimphos but not azinphos-methyl exposures. In contrast, lacrimation was noted in animals exposed to azinphos-methyl but not following tebupirimphos exposures. Thus, some characteristic endpoints of anticholinesterase toxicity appeared to be differentially elicited in rats exposed to two different OP pesticides causing relatively similar levels of AChE inhibition. It should be noted that AChE measurements were only made at one time point (i.e., after the end of dosing) and that differences in the rate of AChE inhibition during dosing could influence the expression of toxicity. These data illustrate the fact, however, that OP pesticide exposures ultimately causing similar levels of AChE inhibition may induce a different spectrum of toxic signs. One requirement for demonstration of common mechanism of toxicity for a group of compounds should be that the chemicals in question induce the same signs of toxicity. Thus, based on elicitation of different functional changes following azinphos-methyl and tebupirimphos exposures in the study by Sheets and co-workers (1997) and with other OP toxicants in the study by Moser (1995) cited earlier, one could argue that these compounds must have some crucial steps in their mechanisms of toxicity that are different.

Our laboratory has been studying the comparative toxicity of parathion and chlorpyrifos, two OP pesticides currently registered for use in the United States (see Table 1). The active metabolites of these OP pesticides have very similar in vitro AChE inhibitory potencies with IC50s (15–30 min, 37°C) in the low nanomolar range (Chambers et al., 1990; Mortensen et al., 1998). According to the relationship suggested by Holmstedt (1963), one might expect very similar LD50 values following acute exposure to the parent compounds. As noted in Table 1, however, remarkable differences in acute toxicity (about 75-fold) are noted with these two OP pesticides.

As previously mentioned, some of the differences in acute toxicity following exposure to these OP pesticides reside in differential rates of biotransformation (Costa et al., 1990; Pond et al., 1995). However, metabolic differences cannot completely explain the differential toxicity of these OP agents. When adult male rats were treated with equi-inhibitory dosages of either OP toxicant (i.e., 18 mg/kg parathion and 279 mg/kg chlorpyrifos, sc), similar rates and maximal degrees of brain, plasma, and erythrocyte AChE activity were noted (Pope et al., 1991). Parathion-treated rats consistently exhibited more extensive body weight reductions (Pope et al., 1991; Chaudhuri et al., 1993) and signs of toxicity (Chaudhuri et al., 1993;

Pope et al., 1995) than rats given chlorpyrifos, however. In adult female rats, similar findings were noted following acute exposure to either parathion or chlorpyrifos, that is, at dosages causing similar rates and maximal degrees of brain AChE inhibition, more extensive signs of toxicity were noted following parathion than after chlorpyrifos exposure (Liu & Pope, 1998). Thus, even though metabolic factors can contribute to the differential in vivo potencies of OP pesticides, the similar anticholinesterase actions but differential degrees of toxicity induced by parathion and chlorpyrifos in these studies argue against a purely dispositional mechanism for differential toxicity.

If extensive AChE inhibition with any OP toxicant directly leads to toxicity, one might expect a consistent relationship between in vivo anticholinesterase potency and an index of acute toxicity across different OP pesticides. For example, if the ED50 for brain AChE inhibition was about one-half the LD50 with one OP compound, the same general relationship between anticholinesterase potency and acute lethality might be expected with other OP toxicants. When the relationship between in vivo anticholinesterase potency and acute toxicity (calculated as the ratio between brain AChE ED50 and maximum tolerated dosage or ED50/MTD) was examined in adult male rats exposed to either methyl parathion or parathion, values of about 0.4–0.5 were obtained. In contrast, with higher dosages of chlorpyrifos approaching the MTD, brain AChE inhibition reached a plateau such that the brain AChE ED50 was low relative to the MTD (ED50/MTD ratio = 0.16; Pope & Chakraborti, 1992). We proposed that other factors (i.e., additional actions) could reduce the toxicity of extensive brain AChE inhibition by higher dosages of chlorpyrifos (Pope and Liu, 1997). These findings, along with data from in vitro studies (described later) on OP toxicant interactions with other target molecules, support the hypothesis (Bakry et al., 1988; Katz & Marquis, 1989; Chaudhuri et al., 1993; Ward et al., 1993; Pope et al., 1995) that some OP pesticides have additional sites of action that can influence the toxic consequences of AChE inhibition.

NONCHOLINESTERASE TARGETS FOR OP PESTICIDES

Organophosphorus pesticides act by phosphorylating the active site serine residue on AChE and thereby inhibiting the catalytic degradation of acetylcholine (for review see Gallo & Lawryk, 1991). Many other enzymes contain active site serine residues and are known to be inhibited by some OP compounds (Mounter et al., 1963). A study by Pruett and co-workers (1994) examined the relative inhibitory potencies of seven phosphates, three phosphonates, four phosphorothioates, four phosphinates, and two cyclic phosphates toward AChE, neurotoxic esterase, and trypsin. As would be expected, substantial differences were noted in potency and selectivity of these compounds, but some were found to be potent in-

hibitors of all three enzymes. Thus, in a comparative study examining a relatively small number of OP agents and their interaction with three different target enzymes, multiple macromolecular sites of action were detected for some OP toxicants. These data illustrate the potential for OP compounds to interact with a variety of target enzymes and alter various functions.

As noted earlier, neurotoxic esterase is an additional molecular target for some OP toxicants (for review see Richardson, 1992). Extensive inhibition of this enzyme in nervous tissues has been correlated with initiation of organophosphorus–induced delayed neurotoxicity (OPIDN). None of the 38 OP anticholinesterases currently used in the United States are potent NTE inhibitors, however, nor are they thought to be capable of causing OPIDN without excessive exposures (Richardson, 1992). Some OP pesticides have also been reported to induce visual deficits in laboratory animals and humans (ILSI, 1994) as well as to induce the intermediate syndrome, a condition associated with muscular weakness primarily of the neck flexors, proximal limb muscles, muscles innervated by motor cranial nerves, and muscles of respiration (Senanayake & Karalliedde, 1987; He et al., 1998). The fact that other forms of OP compound-induced neurotoxicity exist, with at least one (OPIDN) known to be completely independent of AChE inhibition, is further evidence that OP toxicants can interact with other toxicologically relevant targets in the body.

While the study of toxic mechanisms of OP pesticides has historically focused on their interaction with and irreversible inhibition of enzymes (in particular esterases), acetylcholine receptors have more recently been evaluated as targets for OP compounds. Acetylcholine receptors have traditionally been subclassified into two major types, muscarinic and nicotinic, based on their sensitivity to two naturally occurring toxicants (muscarine and nicotine, respectively). Muscarinic receptors are members of the G-protein receptor family coupled to second messenger transduction processes, whereas nicotinic receptors are pentameric structures with ionotropic functions. These two major types of cholinergic receptors have each been subsequently classified into multiple subtypes. With muscarinic cholinergic receptors, five subtypes (m1–m5) have been identified by molecular techniques (for review see Bonner, 1989). A multitude of nicotinic receptors have also been described based on subunit heterogeneity (for a review see Albuquerque et al., 1997).

An early study by Eldefrawi and Eldefrawi (1983) reported that several OP pesticides including monocrotophos, dicrotophos, azinphos-methyl, and dichlorvos at high concentrations (100 μ*M*) could bind in vitro to nicotinic receptors of the electric organ of *Torpedo*. This same report indicated that these OP pesticides had little effect on total muscarinic receptor ([^{3}H]quinuclidinyl benzilate, QNB) binding in insect or mammalian brain. Later work from this same laboratory (Bakry et al., 1988) showed that other OP toxicants including echothiophate and the nerve

agent VX could also bind directly to neuromuscular nicotinic receptors at high concentrations. Thus, these early studies suggested that nicotinic cholinergic receptors may be bound by some OP compounds but apparently only at concentrations exceeding those required to inhibit AChE.

Katz and co-workers (1997) recently reported that chlorpyrifos, chlorpyrifos oxon, parathion, and paraoxon all decreased agonist-stimulated binding of [^{3}H]thienylcyclohexylpiperidine (TCP, an analog of phencyclidine) to nicotinic receptors in *Torpedo* membranes in a concentration-dependent and reversible manner (IC50 values from 5 to 300 μ*M*). In contrast, all four OP toxicants increased TCP binding in the absence of agonist. While neither of these OP agents had an effect on equilibrium binding to [α-^{125}I]bungarotoxin in *Torpedo* membranes in vitro, they increased the apparent affinity of the membranes for the agonist carbachol. Similar effects of the prototypic OP toxicant diisopropylphosphorofluoridate on *Torpedo* nicotinic receptors (i.e., inhibition of agonist-stimulated [^{3}H]phencyclidine binding) were previously reported (Eldefrawi et al., 1988). Taken together, these data indicate that some OP toxicants can bind directly to and desensitize nicotinic receptors, and that under some circumstances these interactions could theoretically contribute to the toxicity associated with OP pesticide exposure. Again, however, the concentrations required to affect nicotinic receptor binding appear considerably higher than would be expected to occur in vivo.

Most OP pesticides or their oxons are very potent anticholinesterases in vitro, with IC50s (15–30 min incubation, 37°C) in the low nanomolar range (Benke & Murphy, 1975; Chambers et al., 1990; Huff et al., 1994). While it is difficult to compare relative potencies between reversible (i.e., receptor binding) and irreversible (i.e., AChE phosphorylation) interactions, the extremely low concentrations of OP agents required to inhibit AChE compared to those necessary for interaction with nicotinic receptors suggest that such additional actions on nicotinic receptors may have, under most conditions, little practical relevance.

In contrast to studies evaluating direct interaction with nicotinic receptors by some OP agents at relatively high concentrations, several studies have reported that some OP compounds can interact directly with muscarinic receptor subtypes at much lower concentrations. Volpe and co-workers (1985) showed that paraoxon, dichlorvos, and tetraethylpyrophosphate could inhibit QNB binding in bovine caudate membranes at low (5–50 n*M*) concentrations. Katz and Marquis (1989) reported that paraoxon at extremely low levels (as low as 10^{-15} *M*) could apparently block radioligand binding to muscarinic m2 and m3 receptors and proposed that such interactions might be important in modulating acetylcholine release at presynaptic autoreceptors.

Studies by Eldefrawi and Eldefrawi (1983), Volpe and co-workers (1985), and Katz and Marquis (1989) evaluating the direct interactions of OP toxicants with muscarinic receptors all utilized the radioligand QNB, a mus-

carinic antagonist that binds to all known subtypes of muscarinic receptors with equal affinities. Bakry and co-workers (1988) first evaluated the effects of OP agents on muscarinic receptor binding using the putative subtype-selective muscarinic agonist [^{3}H]-*cis*-dioxolane (CD). It was subsequently shown that CD labels predominantly the m2 receptor subtype (Huff & Abou-Donia, 1994). Echothiophate and VX were shown to be extremely potent at blocking binding to CD. Echothiophate, paraoxon, and the nerve agents VX, soman, and tabun were shown to block CD binding to rat cardiac tissues in vitro with submicromolar concentrations (Silveira et al., 1990).

Jett and co-workers (1991) reported that paraoxon could displace CD binding and inhibit cAMP formation in an atropine-sensitive manner in rat striatal cells, whereas the parent compound, parathion, was 180-fold less potent. Ward and co-workers (1993) compared the in vitro anticholinesterase potency of a series of OP agents with their potencies at displacing CD binding in rodent brain membranes. It was observed that paraoxon and malaoxon were relatively potent blockers of specific CD binding to both hippocampal and cortical membranes, whereas the parent compounds parathion and malathion were much less potent. Huff and coworkers (1994) reported that chlorpyrifos oxon was a potent displacer of specific CD binding in rat striatum and could inhibit adenylyl cyclase but in an apparently atropine-insensitive manner.

In a more recent study, Ward and Mundy (1996) investigated the abilities of paraoxon, malaoxon, and chlorpyrifos oxon to alter muscarinic receptor-mediated phosphoinositide turnover and cAMP formation in rat frontal cortex. All three OP toxicants were displacers of CD binding and inhibited cAMP formation in a concentration-dependent manner (potencies: chlorpyrifos oxon > paraoxon > malaoxon). In contrast, neither of the OP agents affected either basal or carbachol-stimulated phosphoinositide turnover. These findings extended those of Jett and co-workers (1991) and Huff and colleagues (1994), indicating that some OP compounds can bind directly to muscarinic receptors, presumably either m2 or m4, inhibit adenylyl cyclase, and in turn reduce cAMP formation. Furthermore, the lack of any modulation of phosphoinositide turnover under these same conditions eliminated the possibility of indirect activation of these muscarinic receptors by endogenous acetylcholine (through AChE inhibition).

As noted before, CD is an agonist at muscarinic receptors, presumably the m2 subtype (Huff & Abou-Donia, 1994). Recent findings suggest that binding to the muscarinic agonist oxotremorine is also sensitive to relatively low concentrations of some OP agents (van den Beukel et al., 1997). Paraoxon was reported to be a potent displacer of radiolabeled oxotremorine binding to rat brain membranes but, as reported by others using *cis*-dioxolane as the ligand (Jett et al., 1991; Ward et al., 1993), the parent compound parathion was much less potent. Thus, the studies on interac-

tions of OP toxicants with cholinergic receptors generally suggest that muscarinic receptor subtypes, in particular those labeled with the agonists [^{3}H]-*cis*-dioxolane or [^{3}H]oxotremorine, are more sensitive than nicotinic receptors to lower concentrations of a variety of OP agents. Furthermore, the results taken together indicate that such direct OP compound–muscarinic receptor interactions can modulate the levels of second messengers in either presynaptic or postsynaptic cells and thereby alter cellular function at potentially relevant concentrations.

The preceding studies evaluated in vitro actions of OP toxicants on muscarinic receptor binding or muscarinic receptor-mediated biochemical processes. Is there any evidence that indicates OP pesticides have direct actions with cholinergic receptors in vivo? As noted earlier, the selected high dosages of parathion and chlorpyrifos caused similar rates and maximal degrees of brain AChE inhibition (Pope et al., 1991) but markedly different degrees of acute toxicity; that is, parathion-treated rats exhibited more extensive signs of toxicity (Chaudhuri et al., 1993; Pope et al., 1995). Similar levels of AChE inhibition were noted between the OP pesticide treatment groups in all brain regions, and QNB binding was also extensively reduced following exposure to either OP pesticide. Interestingly, binding to the muscarinic agonist CD (reportedly sensitive to direct binding by some OP pesticides; Jett et al., 1991; Ward & Mundy, 1996) was qualitatively different between the two OP pesticide groups. In essence, brain CD binding was reduced following parathion (PS) exposure but was increased following chlorpyrifos (CPF) administration (Chaudhuri et al., 1993). As similar levels of AChE inhibition were noted between the OP pesticide treatment groups, the differential up- and downregulation of CD-labeled muscarinic receptors may be an indication of differential direct actions of the OP pesticides on these receptors contributing to differential toxicity. Apparent upregulation of brain CD binding sites was also reported in female rats following CPF exposure (Liu & Pope, 1996).

It had been previously proposed (Katz & Marquis, 1989) that the muscarinic receptors sensitive to direct binding by paraoxon could be presynaptic autoreceptors responsible for regulating acetylcholine release in the brain. Under conditions of high synaptic acetylcholine levels, such presynaptic receptors may inhibit further transmitter release by a negative feedback loop (Weiler, 1989; Feuerstein et al., 1992). Watson and co-workers (1986) had previously reported that muscarinic receptors labeled with CD were principally located in the presynaptic region. Thus, if some OP pesticides can selectively interact with presynaptic muscarinic autoreceptors, regulation of acetylcholine release into the synapse may be selectively modified by those OP agents. Specifically, if some OP pesticides act as agonists at muscarinic autoreceptors at physiologically relevant concentrations, inhibition of acetylcholine release could diminish the toxic consequences of AChE inhibition.

Pope and co-workers (1995) treated rats with the MTD of either PS

(18 mg/kg, sc) or CPF (279 mg/kg, sc) and then evaluated acetylcholine release and its regulation by muscarinic autoreceptors ex vivo. Muscarinic autoreceptor function was found to be impaired in a time-dependent manner by both OP pesticides. Of importance, however, autoreceptor function was inhibited within 2 d after parathion exposure but was still functional at that time point following chlorpyrifos administration, that is, at the time of peak incidence of toxicity (Pope et al., 1995). These findings suggested that some OP pesticides could affect presynaptic muscarinic autoreceptor function in a selective manner, and that changes in the regulation of transmitter release could contribute to differential toxicity.

More recent studies suggest that selective modulation of acetylcholine synthesis, another cholinergic presynaptic process, may play a more important role in the differential toxicity of parathion and chlorpyrifos (Liu & Pope, 1998). High-affinity choline uptake, the rate-limiting step in acetylcholine synthesis, is differentially reduced in a time-dependent manner by chlorpyrifos and parathion. Some recent reports (Cancela et al., 1995; Vogelsberg et al., 1997) suggest that choline uptake is regulated by presynaptic cAMP levels, which in turn can be affected by activation of muscarinic m2 and/or m4 receptors. If some OP pesticides can selectively activate these subtypes of muscarinic receptors, cAMP formation could be reduced with a concomitant reduction in choline uptake and acetylcholine synthesis. The direct activation of m2 or m4 receptors and consequent reduction of cAMP levels by both chlorpyrifos oxon and paraoxon (Ward & Mundy, 1996) could therefore reduce acetylcholine synthesis and indirectly limit the amount of acetylcholine accumulating in the synapse following AChE inhibition, with chlorpyrifos oxon being more potent at this limiting action.

Together, these studies indicate that nicotinic and muscarinic receptors are additional sites of action for some OP agents. Studies to date comparing relative concentrations of OP toxicants required to affect receptor binding and second messenger production suggest that muscarinic receptors are more biologically relevant targets for OP agents than nicotinic receptors. While few comparative studies have evaluated neurotoxicity of OP agents under conditions of similar levels of AChE inhibition, the studies with parathion and chlorpyrifos cited (Chaudhuri et al., 1993; Pope et al., 1995; Liu & Pope, 1998) suggest that modulation of presynaptic muscarinic receptor-mediated functions may contribute to the differential neurotoxicity noted with these OP agents.

Figure 1 shows a diagrammatic representation of the cholinergic synapse and possible sites of action for OP pesticides through which the toxic consequences of AChE inhibition could be modulated (adapted from Cooper et al., 1991). Under normal conditions, acetylcholine in the synapse is efficiently degraded by AChE releasing choline and acetate. This step (1) is the primary site of action for most OP pesticides. Choline released from the hydrolytic action of AChE is taken back into the presynaptic terminal

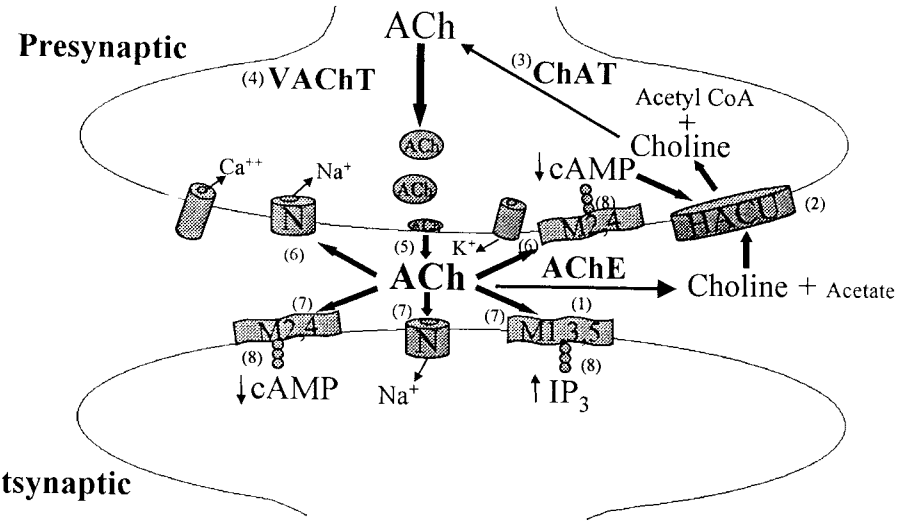

FIGURE 1. The cholinergic synapse and possible additional sites of action for OP pesticides. Acetylcholinesterase (AChE,1) the primary site of action for most OP pesticides, hydrolyzes the neurotransmitter acetylcholine (ACh). The choline released is transported into the presynaptic neuron by the high-affinity choline uptake (HACU) system (2). Acetylcholine is synthesized by choline acetyltransferase (ChAT, 3) using acetyl coenzyme A as a cofactor. Acetylcholine is transported into synaptic vesicles by the vesicular ACh transporter (VAChT, 4). Upon depolarization, the synaptic vesicles fuse with the plasma membrane and ACh is released into the synapse (5). Acetylcholine can bind to either presynaptic (6) or postsynaptic (7) muscarinic or nicotinic receptors to alter cellular function. Muscarinic receptors (m2 or m4: M2,4) are coupled to inhibition of adenylyl cyclase and reduction in cAMP or (m1, m3, or m5: M1,3,5) to activation of phosphoinositide-specific phospholipase C and production of inositol triphosphate (IP3), all through coupling with G-proteins (8). Nicotinic receptors (N) are ligand-gated sodium channels. Sites 1, 2, 5, 6, 7, and 8 have all been reported to be direct targets of, or modulated by, some OP pesticides.

by the sodium-dependent high-affinity choline transporter (2). This step is rate-limiting for acetylcholine synthesis (Murrin et al., 1977). Acetylcholine is synthesized in the cytosol through the action of choline acetyltransferase (ChAT) using choline and acetylcoenzyme A (3). Cytosolic acetylcholine is then transported into small synaptic vesicles by the vesicular acetylcholine transporter, VAChT (4). Upon depolarization of the terminal, voltage-sensitive calcium channels allow influx of calcium and in turn fusion of synaptic vesicles with the plasma membrane and release of acetylcholine into the synapse (5). Synaptic acetylcholine can activate either presynaptic (6) or postsynaptic (7) muscarinic (M) or nicotinic (N) receptors to affect presynaptic or postsynaptic cellular function. As noted before, all muscarinic receptor subtypes are metabotropic and are coupled to effector systems through a G-protein (8).

Activation of muscarinic m2 or m4 receptors (M2,4) generally reduces the activity of adenylyl cyclase and the production of cAMP, whereas activation of muscarinic m1, m3, or m5 receptors (M1,3,5) typically increases

phosphoinositide-specific phospholipase C activity and the release of the second messenger inositol triphosphate (IP_3). In contrast, presynaptic and postsynaptic nicotinic receptors are ligand-gated ion channels that allow sodium ion influx upon activation. Activation of presynaptic muscarinic receptors may inhibit acetylcholine synthesis by reducing cAMP formation and consequently choline uptake (Cancela et al., 1995; Vogelsberg et al., 1997). In addition, presynaptic muscarinic autoreceptors inhibit acetylcholine release through mechanisms unrelated to adenylyl cyclase and cAMP (Allgaier et al., 1993), possibly through potassium channel activation. Presynaptically, nicotinic receptor activation could increase sodium influx, enhance opening of the voltage-sensitive calcium channels and increase transmitter release. Obviously, selective interaction at any of these possible sites of action (2–8) could theoretically modulate the toxic consequences of extensive AChE inhibition.

Recent studies have also reported differential actions of some OP pesticides, apparently outside the cholinergic synapse. Paraoxon (0.3–3 μ*M*), in the presence of tetrodotoxin and sufficient atropine (1 μ*M*) to block muscarinic receptors, altered the frequency of miniature postsynaptic currents (MPCs) in cultured hippocampal cells induced by gamma-aminobutyric acid (GABA), without affecting their magnitude or decay (Rocha et al., 1996). In contrast, at higher concentrations (i.e., at concentrations far exceeding that necessary for inhibition of AChE) of paraoxon (30–300 μ*M*), frequency, decay times, and peak amplitudes of the GABA-mediated MPCs were substantially reduced. Similarly, paraoxon (300 n*M*) increased glutamate-stimulated MPCs over 300%. In this same cellular system, nicotinic agonists (including acetylcholine, 1 m*M*) and antagonists were without effect on either GABA or glutamate-mediated MPCs. At micromolar concentrations, paraoxon also inhibited in a noncompetitive and reversible manner $GABA_A$, glycine, NMDA, and nicotinic receptors, apparently acting as an open-channel blocker. The authors interpreted these findings to indicate that paraoxon at submicromolar concentrations enhances the frequency of neurotransmitter (GABA and glutamate) release in hippocampal neurons and at higher concentrations blocks multiple types of postsynaptic receptors. Furthermore, because of the lack of effects of acetylcholine on MPCs induced by either GABA or glutamate, the possibility that paraoxon alters transmitter release indirectly through AChE inhibition and subsequent activation of nicotinic receptors by acetylcholine was eliminated.

More recently, this same laboratory (Camara et al., 1997) investigated the neurochemical actions of another common OP pesticide, methamidophos. Methamidophos inhibited AChE in a concentration-dependent manner in rat muscle and brain and in frog muscle homogenates. Methamidophos (1–100 μ*M*) reversed the tubocurarine-induced blockade of neuromuscular transmission, and increased the amplitude and prolonged the decay times of spontaneous and nerve-evoked miniature endplate potentials in the rat phrenic nerve–hemidiaphragm muscle preparation, all

consistent with an anticholinesterase mode of action. In contrast to the findings with paraoxon, however, methamidophos (0.1–100 μ*M*) had no effect on the frequency of either GABA- or glutamate-mediated MPCs in cultured hippocampal neurons. These findings indicated that methamidophos had no effect on spontaneous GABA or glutamate release and further, had no significant blockade of postsynaptic receptors for these transmitters, as observed with paraoxon. In addition, methamidophos appeared to have no effect on acetylcholine release in the rat phrenic nerve–hemidiaphragm muscle or the frog sciatic nerve–sartorius muscle preparation. Together, the data suggested that methamidophos primarily acts through inhibition of AChE activity with little evidence of other presynaptic or postsynaptic actions.

Collectively, the studies by Rocha and co-workers (1996) and Camara and colleagues (1997) suggest that the molecular sites of action of paraoxon and methamidophos are different. While both are direct-acting anticholinesterases (methamidophos may, however, be activated to a more potent AChE inhibitor in vivo; Mahajna & Casida, 1998), paraoxon appears to enhance neurotransmitter release at low concentrations and to block a variety of transmitter receptors at higher concentrations. In contrast, methamidophos appears to act primarily through inhibition of AChE and consequent elevation of synaptic acetylcholine levels.

Finally, chlorpyrifos has been shown to affect macromolecular synthesis in the cerebellum of the developing brain (Whitney et al., 1995; Campbell et al., 1997), a brain region with minimal cholinergic innervation and little susceptibility to effects of AChE inhibition. Furthermore, Song and coworkers (1997) reported that multiple steps in the adenylyl cyclase signaling pathway were disrupted by postnatal chlorpyrifos exposures, such as, reduction in adenylyl cyclase activity and alterations in G-protein function, even in noncholinergic (adrenergic) systems. Taken together, these studies illustrate the possible importance of multiple macromolecular targets, even outside the cholinergic synapse, in selectively modulating the neurotoxicity of OP pesticides.

There is thus considerable evidence to suggest that some OP pesticides can interact with multiple targets in the central and/or peripheral nervous systems in addition to AChE. These additional sites of action may affect the cascade of events leading to toxicity following extensive AChE inhibition, as well as alter noncholinergic neurochemical processes, and thereby modify the "common" mechanism of toxicity for OP pesticides.

CONCLUSIONS

The mechanism of toxicity for any compound is a complex sequence of biochemical events starting with interaction between a chemical and its biological target and culminating in the overt expression of toxicity. In essentially all cases, the initial interaction between the chemical and its

cellular target is essential but not sufficient to elicit the characteristic signs of toxicity warranting concern for exposure to that chemical. So it is with the OP pesticides; the initial interaction between the pesticide and the macromolecular target (AChE) leads to a cascade of events resulting in a variety of possible toxic sequelae or cholinergic signs.

There is increasing information to suggest that some OP pesticides interact with other relevant biological targets in addition to AChE. Furthermore, interaction with these additional sites may under some conditions modulate the progression of events, leading to overt toxicity following AChE inhibition. The 38 OP anticholinesterases registered for use in the United States have a diverse chemical makeup, with considerable differences in physicochemical characteristics and toxicity profiles. In some cases, differential potencies of anticholinesterases may merely be a reflection of differences in biotransformation, with resultant differences in the amount of anticholinesterase reaching the target tissues. When differences in toxicity can be demonstrated in the presence of similar rates and levels of AChE inhibition, however, a dispositional basis seems unlikely. Based on the available information, the 38 OP anticholinesterases listed could be considered to have a mechanism of toxicity in common, via the crucial cascade of steps from AChE inhibition to functional toxicity described earlier. It is proposed, however, that these toxicants do not all act exclusively by the same mechanism of toxicity. The selective modulation of biochemical steps in the cascade from AChE phosphorylation to toxic response (e.g., inhibition of acetylcholine synthesis), as well as other noncholinergic neurochemical processes, by some OP anticholinesterases appears likely to contribute to differential toxicity of these toxicants. There has been a half century of research on the anticholinesterase-mediated toxicity of OP agents since the first demonstration that parathion exerted toxicity through this cascade (DuBois et al., 1949). In contrast, only in the last 10–15 yr have additional sites of action for these toxicants been investigated. A detailed evaluation of additional sites of action for anticholinesterases may be warranted and that information considered if combined risk assessments for OP pesticides are performed.

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