fuged at 137,000*g* (maximum) for 60 minutes, and the sediment (microsomal fraction) is resuspended in 1 ml of 20 m*M* Pipes, *p*H 7.0. Protein is determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, [*J. Biol. Chem.* **193**, 265 (1951)].
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Oxidative Bioactivation of *S*-Alkyl Phosphorothiolate Pesticides: Stereospecificity of Profenofos Insecticide Activation

Abstract. *The mouse liver microsomal mixed-function oxidase system converts several phosphorothiolate pesticides with* S*-ethyl,* S*-propyl, or* S*-butyl groups to more potent inhibitors of acetylcholinesterase. This activation is stereospecific for the chiral isomers of* O*-(4-bromo-2-chlorophenyl)* O*-ethyl* S*-propyl phosphorothiolate (profenofos insecticide); the more toxic (−) isomer becomes a 34-fold better inhibitor of acetylcholinesterase in vitro, whereas the less toxic (+) isomer is deactivated by a factor of 2. Prior treatment of the microsomes with piperonyl butoxide or another mixed-function oxidase inhibitor markedly decreases the activation. Piperonyl butoxide also protects against brain acetylcholinesterase inhibition and cholinergic symptoms in chicks resulting from (−)-profenofos administration, thus establishing the importance of the oxidative bioactivation of* S*-alkyl phosphorothiolate pesticides in vivo.*

The phosphorothiolate (P–S–C) substituent is present in about 15 percent of the commercial insecticides and in many nematocides, herbicides, and fungicides. Insecticides with the phosphorothionate (P = S) substituent, such as parathion, require oxidative bioactivation before inhibiting acetylcholinesterase (AChE) (*1*). The possibility of a comparable bioactivation of phosphorothiolates have eluded experimental verification (*1–3*). Two recent findings with the insecticide profenofos (*4*) [*O*-(4-bromo-2-chlorophenyl) *O*-ethyl *S*-propyl phosphorothiolate] (compound **4** in Table 1) have prompted renewed interest in possible bioactivation of phosphorothiolates. First, it was surprising to find that the (−) isomer of profenofos is the more toxic to mice and each of three insect species assayed, whereas the (+) isomer is the more potent in vitro inhibitor of four esterases including AChE (*5*). This finding suggests differential detoxification of the isomers or, more likely, that the (−) isomer undergoes bioactivation, whereas the (+) isomer is unaltered or detoxified under the same conditions. Second, peracid oxidation converts profenofos into a potent phosphorylating agent, which is proposed to be the phosphorothiolate *S*-oxide (*6*). We now provide definitive evidence—including appropriate stereospecificity for profenofos—for oxidative bioactivation of various phosphorothiolate pesticides (Fig. 1).

A simple detoxification or bioactivation assay for AChE inhibitors (*7*) was modified for the analysis of unstable metabolites formed by the mixed-function oxidase (MFO) system. Activation on NADPH (reduced nicotinamide adenine dinucleotide phosphate) fortification is evident for parathion and the *S*-ethyl, *S*-propyl, and *S*-butyl phosphorothiolates, but not for the *S*-methyl and *S*-benzyl phosphorothiolates (Table 1). Lowering the phosphorothiolate concentrations results in decreased AChE inhibition after activation. The activation of (±)-profenofos and the *S*-ethyl isomer of *O,O*-diethyl *O*-phenyl phosphorothionate (SV-1) is strongly inhibited by piperonyl butoxide (PB) and SV-1 (Table 1), two MFO inhibitors that act by entirely different mechanisms (*8*). Compounds for which activation is not detected at $10^{-4}M$ include methamidophos and Kitazin P (Table 1) and *O,S*-dimethyl acetylphosphoramidothioate (acephate).

The profenofos chiral isomers (*5*) react in an opposite manner in the oxidase system in vitro (Fig. 2). (−)-Profenofos is activated by a factor of 34, whereas (+)-profenofos is deactivated by a factor of 2. Although (+)-profenofos is intrinsically the more potent AChE inhibitor, (−)-profenofos on activation becomes even more effective than (+)-profenofos. These findings are consistent with the greater toxicity of the (−) isomer relative to the (+) isomer (*5*). The reversal of chiral specificity in the bioactivation system may be due to differences in rates of oxidation or, more probably, in the enantiomeric selectivity of AChE inhibition (*1, 9*). Thus AChE reacts preferentially with the nonoxidized form of (+)-profenofos and the oxidized form of (−)-profenofos. This may involve a change in the leaving group, that is, cleavage of the P–O–phenyl linkage for (+)-profenofos and of the activated P–S–propyl linkage for (−)-profenofos (Fig. 1) (*10*).

(−)-Profenofos also undergoes MFO

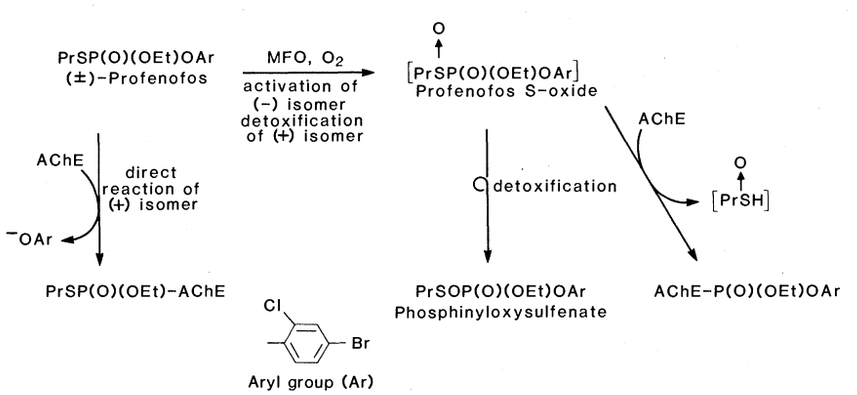

Fig. 1. Proposed mechanism for reversal of chiral specificity for acetylcholinesterase inhibition after oxidation of profenofos enantiomers.

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activation in vivo, as shown by studies with chicks in which PB is used as an oxidase inhibitor. Mammals were not employed for this test because of their relative insensitivity to profenofos (*4, 5*). One-day-old chicks, however, are very sensitive to profenofos; the median lethal dose (LD_{50}) at 24 hours for intraperitoneally administered profenofos was 0.8 and 17 mg/kg for (−)- and (+)-profenofos, respectively, and 2.2 mg/kg for the racemate. The symptoms of poisoning are initial drowsiness, loss of righting response, defecation, salivation, tremors, and finally tonic and clonic convulsions. The LD_{50} for the racemate can be increased fourfold by prior injection of atropine sulfate (50 mg/kg) and HS-6 (*11*) (100 mg/kg), intraperitoneally. These observations are consistent with a cholinergic mechanism of poisoning. As with other AChE sources in vitro (*5*), chick brain AChE is less sensitive to (−)- than to (+)-profenofos in the absence of bioactivation (50 percent inhibition levels with 10-minute reaction times of $6 \times 10^{-6}M$ and $4 \times 10^{-7}M$, respectively). Prior treatment with PB protects chick brain AChE in vivo from inhibition by (−)-profenofos (Fig. 2). Moreover, PB delays the onset of tremors, which also indicates a protective effect.

The ultimate phosphorylating agent of the bioactivated *S*-ethyl, *S*-propyl, and *S*-butyl phosphorothiolates has not yet been identified, but is probably the corresponding *S*-oxide (*6*). Methamidophos, in our studies, did not undergo biological activation (Table 1), in contrast to earlier speculation (*3*); its toxicity may be associated with unusual persistence, allowing eventual AChE inhibition that involves direct cleavage of the P–S–methyl bond (*12*).

As with many bioactivation phenomena, the presumed sulfoxidation of phosphorothiolates is both an activation and a detoxification mechanism, since the phosphorothiolate *S*-oxides readily rearrange to the phosphinyloxysulfenates (Fig. 1), which are probably poor phosphorylating agents (*6*). Thus, some phosphorothiolates appear to be intrinsically toxic, whereas others undergo sulfoxidation leading either to increased or decreased toxicity. These opposing fates are illustrated by the chiral isomers of profenofos.

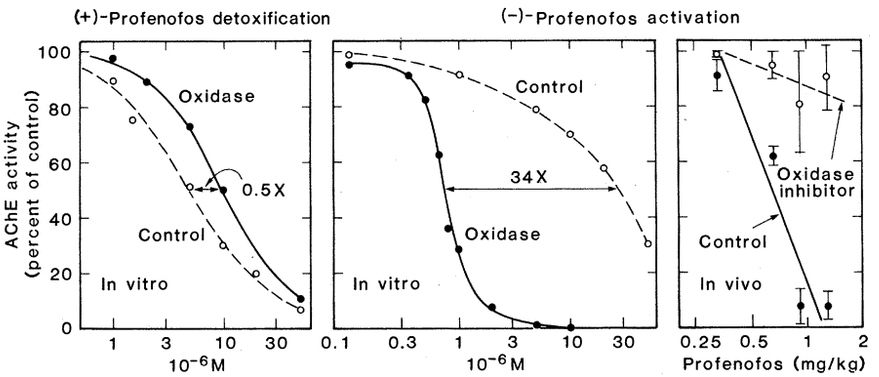

Fig. 2. Oxidative bioactivation of (−)-profenofos in vitro and in vivo and detoxification of (+)-profenofos in vitro. The in vitro data, determined as in Table 1, are means of three experiments with standard deviations less than 10 percent of the mean. The in vivo data were obtained from 1-day-old male White Leghorn chicks (38 ± 1.5 g) treated with the oxidase inhibitor piperonyl butoxide (1.1 g/kg, intraperitoneally) or sham-injected (control) 30 minutes before being challenged with (−)-profenofos administered intraperitoneally in 30 μl of methoxytriglycol (MTG); controls received MTG alone. Brain AChE activity was determined (*13*) 30 minutes after the challenge. Piperonyl butoxide alone did not affect brain AChE levels, although mild sedation was occasionally observed. Data are means ± standard deviation for five chicks given each dose.

Table 1. Oxidative bioactivation of phosphorothiolate pesticides to more potent acetylcholinesterase inhibitors. The pesticides are **1**, methamidophos insecticide; **2**, phosphorothiolate isomer of SV-1; **3**, ethoprop nematocide; **4**, (±)-profenofos insecticide; **5**, DEF cotton defoliant; **6**, Kitazin P fungicide; and **7**, parathion insecticide. Abbreviations: Me, methyl; Et, ethyl; Pr, *n*-propyl; *i*-Pr, isopropyl; Bu, *n*-butyl; Ph, phenyl; and $PhCH_2$, benzyl. The pesticide (> 97 percent pure, based on gas chromatographic analysis) was added in acetone (5 μl) to mouse liver microsomes (1 mg protein) in phosphate buffer (2.5 ml, *p*H 7.4, 50 m*M*) containing AChE (electric eel, 4 units) alone (control) or with added NADPH (2 μmole) (oxidase system). Pesticide concentrations were: **7**, $10^{-6}M$; **4**, $5 \times 10^{-6}M$; **3**, $10^{-5}M$; and for the other compounds, $10^{-4}M$. Oxidase inhibitors in acetone (5 μl) were added to the microsomes for 10 minutes before pesticide addition, with final concentrations of $10^{-3}M$ PB and $10^{-4}M$ SV-1. The oxidase inhibitors alone did not inhibit AChE in the presence or absence of NADPH. At 30 minutes (37°C) after pesticide addition, 100 μl of the mixture was assayed directly for AChE activity (*13*). Controls received acetone alone. Data are means of three experiments ± standard deviation.

Compound	Oxidase inhibitor	AChE inhibition (%)	
		Control	Oxidase
	S-*Alkyl phosphorothiolates*		
1 $MeSP(O)(NH_2)OMe$	None	57 ± 6	57 ± 5
2 EtSP(O)(OEt)OPh	None	14 ± 1	98 ± 1
	PB	28 ± 3	25 ± 2
	SV-1	21 ± 2	30 ± 3
3 PrSP(O)(OEt)SPr	None	3 ± 0	92 ± 1
4 (±)-PrSP(O)(OEt)OPh-Cl-2-Br-4	None	26 ± 8	100 ± 1
	PB	12 ± 1	30 ± 3
	SV-1	26 ± 1	58 ± 4
5 $BuSP(O)(SBu)_2$	None	3 ± 1	100 ± 0
6 $PhCH_2SP(O)(Oi\text{-}Pr)_2$	None	16 ± 1	16 ± 2
	O,O-*Dialkyl phosphorothionate*		
7 $EtOP(S)(OEt)OPh\text{-}NO_2\text{-}4$	None	0	96 ± 4

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MFO system cleaves [^{14}C-*phenyl*]-(±)-profenofos (CIBA-Geigy) at both the P–O–phenyl and P–S–propyl groups, in each case requiring NADPH, as indicated by thin-layer chromatography with authentic standards.
11. The oxime HS-6 [1-(2-hydroxyiminomethylpyridinium)-1-(3-carboxamidopyridinium)-dimethyl ether] is an antidote for organophosphate poisoning acting on the cholinergic system in part by reactivating AChE [J. G. Clement, *Fundam. Appl. Toxicol.* **1**, 193 (1981)].
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Guanosine Triphosphate Activation of Brain Adenylate Cyclase: Enhancement by Long-Term Antidepressant Treatment

Abstract. *Activation of adenylate cyclase by a stable guanosine 5'-triphosphate analog was augmented in brain membrane preparations from rats treated on a long-term basis with tricyclic antidepressants or electroconvulsive shock. These treatments may facilitate cyclase activation by promoting the interaction of the regulatory and catalytic subunits of the enzyme. This finding suggests a possible mechanism for the changes in sensitivity to various neurotransmitters seen after antidepressant administration.*

A clinical response to antidepressant therapy typically does not develop until one or more weeks after the initiation of treatment (*1*). Accordingly, investigators of antidepressant mechanisms have recently focused on biochemical and physiological changes induced by long-term treatment that may be correlated with the delayed therapeutic effect (*2, 3*). Specifically, altered sensitivity of neurotransmitter receptors has been detected in a variety of systems and has been proposed as a common mechanism underlying the clinical response to various antidepressant therapies in humans (*2*).

Much of the current interest in antidepressant-induced receptor changes stems from early observations that both tricyclic antidepressants and electroconvulsive treatment (ECT) can reduce the norepinephrine-stimulated production of adenosine 3',5'-monophosphate (cyclic AMP) in rat brain slices (*4, 5*). Cyclic AMP has been proposed to function in synaptic transmission as a "second messenger," perhaps mediating the action of certain neurohumors at a postreceptor level (*6*). The activation of adenylate cyclase, the plasma membrane–associated enzyme that catalyzes the formation of cyclic AMP from adenosine triphosphate, is regulated by a guanosine 5'-triphosphate (GTP)–binding protein (G unit) that couples the catalytic moiety with neurotransmitter (or other hormone) receptors (*7, 8*). Decreases in β-receptor density after long-term antidepressant treatment have been correlated with decreases in isoproterenol-induced cyclic AMP accumulation (*9*). In several cases (*2*), however, altered receptor binding following antidepressant treatment cannot fully account for changes in hormone-stimulated accumulation of cyclic AMP. Accordingly, it is of interest to examine the effects of antidepressant treatment on the regulation of adenylate cyclase through the G unit. We report that long-term, but not short-term, treatment of rats with tricyclic antidepressants or ECT produces an increase in G unit–dependent activity of adenylate cyclase in the brain. An enhanced physical association between the adenylate cyclase G unit and the catalytic moiety may be responsible for this effect.

Adult male albino rats (Charles River) weighing 225 to 300 g were treated for 15 to 20 days with daily intraperitoneal injections of 10 mg of amitriptyline (Merck Sharpe & Dohme), desipramine (USV Pharamaceuticals), imipramine (Ciba-Geigy), or iprindole (Wyeth) per kilogram. Controls received daily injections of 0.9 percent saline in the same volume (1 ml/kg). Animals receiving long-term treatment were killed 22 to 28 hours after the last injection. Animals receiving single injections were killed 60 minutes later.

Electroconvulsive treatment was administered with sinusoidal current (80 mA, 60 Hz, 0.1 second) through saline-moistened corneal electrodes. This treatment reliably produced convulsions with tonic extension, followed by clonus persisting for 10 to 25 seconds (*10*). One group received ECT on alternate days for a total of five to seven treatments; a second group was given a single treatment, and a third received sham ECT (electrodes applied without current). All

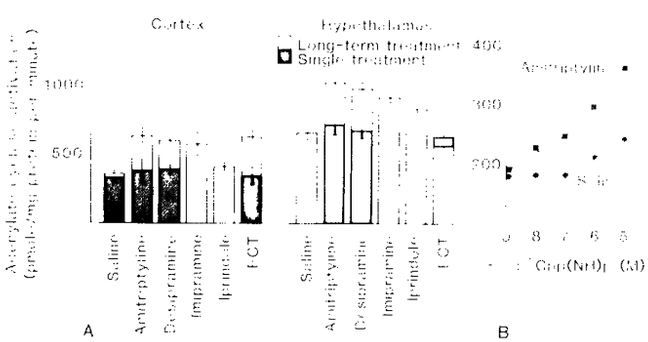

Fig. 1. (A) Effects of long- and short-term antidepressant treatment on Gpp(NH)p activation of adenylate cyclase in membrane particles from the cerebral cortex or hypothalamus. Hypothalamic membranes were prepared as described by Wheeler *et al.* (*29*). Hypothalami were homogenized 1:20 (weight to volume) in ice-cold 2 m*M* tris maleate with 0.8 m*M* EGTA (*p*H 7.4) by a Teflon-on-glass homogenizer at 1000 rev/min. Homogenates were centrifuged for 5 minutes at 600*g* and the pellet was discarded. Supernatants were centrifuged for 10 minutes at 7500*g* and the resulting pellets were resuspended in 0.32*M* sucrose with 50 m*M* tris maleate (*p*H 7.8), 5 m*M* $MgSO_4$, 1 m*M* EDTA, and 1 m*M* dithiothreitol. Following two cycles of centrifugation (7500*g* for 10 minutes) and washing, the pellets were resuspended in the same media to a protein concentration of 1 to 2 mg/ml and stored under liquid nitrogen until assay. Adenylate cyclase assays (*13*) were performed in 50 μl of a reaction mixture containing 80 m*M* tris maleate (*p*H 7.4), 1 m*M* isobutylmethylxanthine, 2 m*M* $MgSO_4$, 0.5 m*M* adenosine triphosphate (ATP) with 60 m*M* phosphenol pyruvate and pyruvate kinase (0.5 mg/ml), 0.5 m*M* dithiothreitol, and 0.4 m*M* EGTA. Hypothalamic membranes (1 to 2 mg of protein per milliliter) were incubated with 5 μ*M* Gpp(NH)p for 10 minutes before the reaction mixture was added. Reactions were allowed to proceed for 10 minutes and were then stopped by boiling for 4 minutes. Cortical membrane particles were prepared in a similar manner (*13*), and the adenylate cyclase assay was identical except for the concentration of Gpp(NH)p, which was 2 μ*M*. Reaction rates in both cases were linear for 20 minutes. In the presence of the ATP-regenerating system, ATP concentrations were unchanged for up to 20 minutes (*13*). Accumulated cyclic AMP was assayed by a modification of the method of Brown *et al.* (*30*) involving a binding protein from rabbit skeletal muscle. Protein content was determined in accordance with the method of Bradford (*31*). Values are means ± standard deviations for one of at least four experiments performed in duplicate (cortex) or triplicate (hypothalamus). (B) Effect of varying the concentration of Gpp(NH)p on the activation of adenylate cyclase in hypothalamic membranes from animals given repeated saline or amitriptyline treatments. Tissue was prepared and assays were carried out as described in (A), except that Gpp(NH)p and the reaction mixture were added to the membranes simultaneously. Standard deviations are less than 10 percent of the means graphed.

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