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Project Summary

Chemistry and Mode of Action of Insecticides: Phase II

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Results gathered for the period February 15, 1976-May 31, 1981 are presented. Progress is reported in areas dealing with the chemistry, metabolism, and mode of action of organophosphorus, carbamate, and organochlorine insecticides. Among the topics included are: insecticide metabolism and mode of action; insecticide selectivity; structure-activity relationships; biological and environmental alteration of insecticides; effect of impurities on the potentiation of organophosphorus insecticides; delayed toxicity of trialkyl phosphorothioates; delayed neurotoxicity of organophosphorus esters; mode of action of phosphoramidothioate esters; gas chromatography of insecticides; and neurophysiological studies on the mode of action of insecticides.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Insecticide Metabolism and Mode of Action

Studies on the Oxidative Conversion of P=S to P=O Esters and the Effect of Chirality on Toxicological Properties

The stereochemistry of P=S to P=O conversion effected by chemical model oxidation systems and mouse liver mixed-function oxidases (MFO), using the resolved isomers of fonofos (*O*-ethyl *S*-phenyl ethylphosphonodithioate) was examined. The stereochemical aspects of the chemistry, metabolism, and mode of action of the chiral isomers of fonofos are presented.

The conversion of $(S)_P$-fonofos to $(R)_P$-fonofos oxon by the action of *m*-chloroperoxybenzoic acid occurred predominantly with retention of configuration of the phosphorus atom. Oxidation to the rearrangement product, $(R)_P$-phenyl ethyl(ethoxy)phosphinyl disulfide took place with predominant inversion of configuration. In the reaction leading to the oxon, the stereochemical course was identical to that observed with mouse liver MFO.

Toxicological data indicated a 2- to 4-fold higher toxicity of $(R)_P$-fonofos to mice and houseflies relative to $(S)_P$-fonofos. Compared to fonofos, the difference in toxicity between the fonofos oxon enantiomers was greater and $(S)_P$-oxon was 2.6- (i.p.) and 12-fold more toxic to white mice and houseflies, respectively, than the $(R)_P$-oxon.

The relative toxicities of $(S)_P$- and $(R)_P$-fonofos oxon to houseflies and white mice were qualitatively in agreement with their relative *in vitro* anticholinesterase activities toward flyhead, mouse-brain, and blood ChE. Quantitatively the differences in anticholinesterase activities between the isomers were substantially greater (49- to 60-fold) than the differences in toxicities to houseflies and white mice. At the same intraperitoneal dosage of oxon isomers (0.6 mg/kg), the level of maximum inhibition of both brain and

blood ChE obtained from mice treated with $(S)_P$-oxon was approximately 3-fold greater than enzymes obtained from mice treated with the $(R)_P$-enantiomer. Maximum inhibition, however, occurred at different time intervals after treatment and the relationship was further complicated by the differences in recovery rates of the inhibited enzymes. Nevertheless, agreement between *in vivo* inhibition of mouse ChE and intraperitoneal toxicity was good.

The relationship between *in vivo* inhibition of housefly-head acetylcholinesterase (HFAChE) and toxicity of houseflies treated with the two isomers was less satisfactory. Maximum reduction of HFAChE in flies treated with 5 $\mu g/g$ $(R)_P$-oxon was about 35% compared to about 80% for flies treated with the same amount of $(S)_P$-oxon, a slightly greater than 2-fold difference, a difference substantially smaller than the 12-fold difference observed for housefly toxicity. Plots show that HFAChE inhibited by $(R)_P$-oxon recovered to a significantly greater extent (compare activities at 12 h), and it is possible that ChE depression over longer time periods should be considered in relating *in vivo* inhibition with toxicity.

Studies on the *in vitro* metabolism of the two isomers in the presence of mouse liver MFO showed that $(R)_P$-fonofos and $(S)_P$-fonofos were converted to the respective $(S)_P$-oxon and $(R)_P$-oxon with 70-80% stereospecificity. Since $(S)_P$-oxon is a stronger anticholinesterase and is more toxic than $(R)_P$-oxon, the order of toxicity of the fonofos isomers is in the right direction. Although metabolic conversion of fonofos to the oxon occurred predominantly with retention of configuration, a significant amount of inversion apparently also took place, leading to partial racemization of the oxon. This result undoubtedly contributed to the smaller toxicity ratios between the fonofos isomers.

Studies on the relative rates of metabolism revealed that while $(R)_P$-fonofos was metabolized at a rate approximately 2-fold greater than $(S)_P$-fonofos, analysis of the products showed that $(R)_P$-fonofos was converted in greater proportion to the detoxication product diphenyl disulfide (DPDS) than its enantiomer. Thus, even though $(R)_P$-fonofos was metabolized by mouse liver MFO at a faster rate, the difference in amounts of fonofos oxon formed from the fonofos isomers was not large.

Rat and mouse serum were effective in degrading fonofos oxon but not fonofos. The *in vitro* degradation of fonofos oxon by rat or mouse serum was relatively slow compared to the oxidative reactions, but results clearly showed that $(S)_P$-oxon was degraded at a faster rate than the $(R)_P$-oxon (approximately 2-fold). The greater susceptibility of $(S)_P$-oxon to enzymatic degradation may account for the smaller difference in toxicities between the fonofos-oxon enantiomers relative to their differences in anticholinesterase activities (50- to 60-fold). Further, since $(R)_P$-fonofos is preferentially metabolized to $(S)_P$-oxon and $(S)_P$-fonofos to $(R)_P$- oxon, the faster rate of degradation of $(S)_P$-oxon may contribute to the even smaller differences in toxicities of the fonofos enantiomers Thus, the smaller difference in toxicities between the fonofos enantiomers compared to the relative toxicities of the oxons and their anticholinesterase activities may be accounted for by the partial racemization in the oxidative desulfuration reaction and faster rate of degradation of $(S)_P$-oxon.

Studies on the Mode of Action of Methamidophos

O,S-Dimethyl phosphoramidothioate (methamidophos), although highly effective with a bimolecular inhibition constant of (k_i) 9.2×10^2 M^{-1} min^{-1} for the inhibition of fly-head AChE, is a relatively weak anticholinesterase. Studies were conducted on the toxicological properties, *in vitro* and *in vivo* anticholinesterase behavior, and metabolism of methamidophos

Evaluation of the kinetics of housefly ChE inhibition showed that its affinity for the enzyme and its phosphorylation and bimolecular inhibition rates are all relatively low. *In vivo* metabolism studies in houseflies provided evidence that it is not activated to a more effective ChE inhibitor and indirect evidence also was obtained for its slow degradation *In vitro* metabolism studies in housefly and mouse tissues provided additional evidence for its lack of activation and slow metabolic degradation. Compared to other effective organophosphorus insecticides, methamidophos was slow in producing acute symptoms of poisoning and ChE inhibition and required the accumulation of comparatively high internal levels for toxic effects. However, *in vivo* ChE inhibition studies provided evidence for the interrelationships of ChE inhibition and toxic effects. Thus, its relative stability and low *in vivo* degradation appeared to be of critical importance in accumulating and maintaining a sufficient internal concentration for a long enough period of time to permit the development of its slowly expressed toxicity.

Insecticide Selectivity

Selective Toxicity of N,N'-Thiodicarbamates

A series of *N*-(alkyl alkylcarbamoylsulfenyl) derivatives of the methylcarbamate insecticides carbofuran, *m*-isopropylphenyl methylcarbamate, propoxur, aldicarb, methomyl, and oxamyl were prepared and examined for toxicity to houseflies, mosquito larvae, and white mice. Compared to the parent methylcarbamate, the derivatives were generally of equal toxicity to the housefly and substantially more toxic to mosquito larvae With the exception of the oxamyl derivatives, all other derivatives were much less acutely toxic to the white mouse The toxicities of the derivatives to mosquito larvae and the white mouse were correlated with their octanol-water partition coefficients

Insecticidal Properties of the N-Sulfonyl Derivatives of Propoxur and Carbofuran

A series of substituted *N*-benzenesulfonyl derivatives of carbofuran and propoxur containing electron-withdrawing substituents of the benzene ring was synthesized and examined for toxicity to houseflies and mosquito larvae The derivatives were generally noninsecticidal when tested alone, but some of the compounds were toxic to houseflies when synergized with piperonyl butoxide. The poor insecticidal activity and low mammalian toxicity of these derivatives are attributable to their high stability and their inability to be converted into the parent methylcarbamate.

Selective Toxicity of N-Arylsulfenyl Derivatives of Methylcarbamate Insecticides

The toxicological properties of a series of *N*-sulfenylated derivatives of commercial methylcarbamate insecticides were studied with special focus on the toxicological response observed in honeybees. Work on the comparative metabolism of one of these derivatives was conducted in the honeybee and housefly to determine the basis for its selective toxicity.

Of the 34 different sulfenylated derivatives of propoxur, carbofuran, and carbaryl evaluated, only three showed a toxicity ratio greater than one although many were safer to bees than the parent methylcarbamate. The most notable exception was the 2-methyl-4-*t*-butylphenylsulfenyl derivative of propoxur (*1*) which was more than 178-fold safer to the honeybee than propoxur Although the 4-*t*-butyl moiety in the ring appeared to introduce the greatest amount of honeybee safety in these derivatives, no obvious relationship between the structure of the carbamate derivative and honeybee toxicity was apparent

The pathways in the honeybee and housefly, while qualitatively similar, contain enough quantitative differences to account for the difference in the toxicity of (*1*) to the two insects. Penetration of (*1*) and its subsequent conversion into propoxur are apparently the rate-limiting steps in the metabolism of (*1*) in the honeybee Rapid conversion of propoxur into secondary metabolites results in its accumulation in a low, steady-state level in the bee Consequently, intoxication does not occur. In houseflies, however, penetration and conversion of (*1*) into propoxur was fast. Intoxicating levels of propoxur accumulated within a short period of time after treatment.

Selective Toxicity of Phosphoramidothioate Esters

A study of the comparative metabolism of the hexanoyl derivatives and the smaller chain acyl derivatives of methamidophos in a mammal and insect was undertaken to establish the basis for their toxicological properties Metabolic and toxicological data gathered for *O,S*-dimethyl propionyl- (*2*) and hexanoylphosphoramidothioate (*3*) show that the metabolism of the two compounds in the white mouse and housefly is qualitatively and quantitatively different.

The major difference in the metabolism of (*2*) and (*3*) in the mouse appears to be in the larger amounts of methamidophos formed from (*3*) compared to (*2*). Methamidophos is highly toxic to the mouse (LD_{50} 14 mg/kg), and is probably the agent responsible for intoxication when the mouse is treated with either (*2*) or (*3*). The small amounts of methamidophos formed from (*2*) readily account for its safety to mice. Similarly, the relatively high toxicity of (*3*) to mice may be attributed to the substantial quantity of methamidophos formed in the mouse treated with (*3*)

Structure-Activity Relationships

Hydrolytic and Toxicological Properties of Ethyl α-Cyanobenzaldoxime Phosphoramidates

The desired order of selectivity demonstrated by phoxim (*O,O*-diethyl *O*-α-cyanobenzaldoxime phosphorothioate) and the limited knowledge of the relationship between chemical structure and biological activity of phosphoramidate esters led to studies on phosphoramidates containing the α-cyanobenzaldoxime leaving group which is present in phoxim.

A series of ethyl alkylphosphoramidate esters of α-cyanobenzaldoximes were, therefore, synthesized and examined for alkaline hydrolysis, anticholinesterase activity, and toxicity to houseflies and mice The compounds showed variable toxicity to both houseflies and mice, and were effective inhibitors of acetylcholinesterase.

Satisfactory correlation was obtained between alkaline hydrolysis rate constants and anticholinesterase activity with Taft's σ^* and E_S values Toxicity to houseflies was related to anticholinesterase activity and Hansch's π constants Several of the compounds were of low toxicity to the white mouse but were highly effective against the housefly

Insecticidal Properties of Phosphonamidothioate Esters and Derivatives

A series of *S*-alkyl methyl- or ethylphosphonamidothioate esters analogous to methamidophos (*O,S*-dimethyl phosphoramidothiate) and acephate (*O,S*-dimethyl *N*-acetylphosphoramidothioate) was synthesized and evaluated for toxicological properties. Although *S*-methyl methyl- and ethylphosphonamidothioate were more effective against the housefly than methamidophos, the acylated derivatives showed variable toxicity but were all substantially less effective than acephate. The acylated derivatives, however, showed improved mouse toxicity. *S*-methyl methyl- and ethylphosphonamidodithioate were less active against the housefly than methamidophos

Insecticidal Activity of DDT Analogs

A series of DDT analogs whose structures were based on a hypothetical model for the DDT receptor site was synthesized and examined for insecticidal activity. Several of these compounds showed activity; one of them, 2,2-bis(*p*-ethoxyphenyl)-1,1-dichloropropane showed activity equal to that of DDT against houseflies and mosquito larvae. This is the first case in which a DDT analog with an α-methyl moiety in place of hydrogen has been shown to have insecticidal activity.

Effect of Ring Substituents on the Inhibition of Cholinesterase by Carbamate Esters

A series of 27 substituted aryl *N*-methoxy-*N*-methyl carbamates was synthesized. The ability of these compounds to reversibly inhibit housefly-head (HFAChE) and bovine erythrocyte acetylcholinesterase (BAChE) and horse serum cholinesterase (HSChE) was determined. All were competitive reversible inhibitors of BAChE, but some showed mixed competitive inhibition against HFAChE and HSChE. Dissociation constants (K_i) as small as 9.9×10^{-9} *M* and as large as 1.4×10^{-4} *M* were observed. Satisfactory correlation between log K_i for the inhibition of fly-head acetylcholinesterase by the *N*-methoxy-*N*-methylcarbamates and -log I_{50} for the inhibition of the same enzyme by the corresponding methylcarbamates was noted.

Biological and Environmental Alteration of Insecticides

Characterization of the Bound Phenthoate Residues in Citrus

The metabolism of phenthoate or *O,O*-dimethyl *S*-[α-(carboethoxy)benzyl] phosphorodithioate (Cidial®) in Valencia orange fruit was examined with emphasis on the characterization of bound phenthoate residues in the fruit peel. Data for the distribution of radioactivity in the surface wash, peel, and pulp after treatment with ^{14}C-phenthoate showed a gradual decline in external fruit surface wash with a gradual increase in percentage of radioactivity recovered in the internal acetone extract and peel residue. Radioactive labeling was also used to indicate the nature and distribution of metabolites from the different fractions of orange fruit. The five principal metabolites obtained were phenthoate oxon, demethyl phenthoate, phenthoate acid, ethyl mandelate, and mandelic acid, along with unchanged phenthoate.

Metabolism of Dibutylaminosulfenyl and Morpholinosulfenyl Carbofuran in Plants, Animals and Soil

Metabolism in Corn and Cotton Plants

The metabolism of 2,3-dihydro-2,2-dimethyl-7-benzofuranyl (di-*n*-butylaminosulfenyl) (methyl)-carbamate (DBSC or dibutylaminosulfenyl carbofuran) and 2,3-dihydro-2,2-dimethyl-7-benzofurenyl(morpholinosulfenyl)(methyl)-carbamate (MSC or morpholinosulfenyl carbofuran), two potentially useful insecticides, in corn and cotton plants was studied.

DBSC metabolites in cotton plants were identified 1, 3, 6, and 10 days following stem injection of ring-^{14}C-DBSC or carbonyl-^{14}C-DBSC and confirmed by chromatography in several solvent systems. Both sets of labeling experiments showed similar results. The principal species found after 1 and 3 days was carbofuran, but 3-hydroxycarbofuran was equally important at 6 and 10 days.

The metabolism of MSC in cotton plants was determined following stem injection of carbonyl-^{14}C-MSC. Overall, results were similar to those found in the metabolism of labeled DBSC.

DBSC metabolites in cotton plants were also identified by treatment of the foliage with carbonyl- and ring-labeled DBSC. Absorption occurred steadily over the 10-day test period; the same metabolites as those identified following stem injection were observed. Overall, the rate of metabolism after foliage treatment appeared more rapid than after stem injection

Stem injection of carbonyl-labeled DBSC was used to identify the various metabolites of DBSC in corn plants. The same metabolites found in cotton were found in corn, although DBSC was converted to carbofuran more rapidly than in cotton, and the carbofuran was converted into subsequent metabolites. The percentage of radioactivity remaining in the residual straw was higher in corn compared to cotton.

Metabolism of DBSC in the Rat

The metabolism of DBSC in the rat was examined to determine the fate of this compound in mammals and to establish the basis for its lower mammalian toxicity relative to carbofuran. Ring-labeled, carbonyl-labeled, or butyl-labeled DBSC was administered in two doses orally in male and female rats The most important means of elimination was via the urine for the ring-labeled and dibutyl-labeled DBSC and via expired carbon dioxide and urine for carbonyl-labeled DBSC. The distribution and identity of the metabolites was determined by calculating percentages of radioactivity recovered.

DBSC is initially metabolized by two major pathways in the rat: via oxidation of the sulfur to give sulfonyl DBSC, and via N-S bond cleavage to give the actual toxicant, carbofuran. Following the initial metabolic degradation of DBSC to carbofuran, carbofuran was detoxified primarily via oxidation at the 3-carbon position of the dihydrofuranyl ring. The primary excreted metabolite was 3-ketocarbofuran phenol, followed by 3-hydroxycarbofuran.

While DBSC is converted to carbofuran in the rat, it appears that the rate of formation of carbofuran is slower than expected from studies on its degradation under different pH conditions. Based on the high levels of DBSC present in the blood 3 h after treatment, it appears that carbofuran is released slowly and this slow release, in part, accounts for the lower mammalian toxicity of DBSC

Metabolism in Soil

The alteration of MSC and DBSC in Cosad® sandy loam soil under aerobic and anaerobic conditions was studied, and the degradation of the major primary metabolites, carbofuran and dibutylamine, investigated.

Metabolism of ring-labeled carbonyl-labeled DBSC occurred rapidly in both types of soil conditions, a half-life of 2-3 days and a first-order thiolysis of DBSC are reported. The major radioactive component during the 30-day test period remained carbofuran, which was either oxidized at the 3-position of the ring, or hydrolyzed at the carbamate ester to form carbofuran phenol Biscarbofuran-disulfide, dibutylamine, and at least 7 unidentified minor compounds were also detected. Phenolic degradation products appeared to be bound to the soil humus by an oxygen-dependent process. Ring cleavage was also oxygen-dependent.

Alteration of DBSC in a Water Environment

A study was undertaken to determine the reactions involved in the degradation of DBSC and the resulting breakdown products under different environmental conditions. The breakdown of DBSC in different buffer solutions (hydrolysis), in buffer solutions containing sulfhydryl reagents (thiolysis), and on the surface of silica gel TLC plates was investigated.

In buffer solution and on thin-layer plates, N-S bond cleavage readily occurred to give carbofuran as a major product, with minor amounts of biscarbofuran-*N,N'*-disulfide and -trisulfide. First-order kinetics was observed for the hydrolysis reaction in buffer. An unknown polar compound was also obtained in these systems in significant amounts. Thiolytic N-S bond cleavage occurred in the presence of excess cysteine and glutathione at pH 7.0 to give carbofuran as the sole identifiable product, and first-order kinetics was observed.

Acid-Catalyzed Alteration of DBSC

During the course of a study on the behavior of DBSC under different solvent conditions, DBSC was found unstable in a dichloromethane-acetic acid (9:1) mixture. In addition to carbofuran, TLC analysis showed that other reaction products were the polysulfide derivatives of DBSC (CF-S_n-NBu_2 where n = 2-4), biscarbofuran-*N,N'*-disulfide (CF-S_2-CF) and polysulfides (CF-S_n-CF where n = 3-5), and dibutylamine

NMR and mass spectral analysis were used to establish the overall structure of each component in CF-S_n-NBu_2 and CF-S_n-CF. It was not possible, however, to determine the exact nature of the polysulfide moiety. Poor insecticidal activity was observed for CF-S_2-CF, a finding that was unexpected in light of the good activity observed for the other derivatives.

Analysis of carbonyl- and dibutylamino-^{14}C-labeled DBSC degradation products showed that the amount of CF-S_n-NBu_2 gradually increased to a steady-state level after 60-72 h, while amounts of CF-S_2-CF and CF-S_n-CF continued to increase throughout the entire reaction period of 120 h This finding suggests that CF-S_n-NBu_2 is an intermediate and is slowly transformed into CF-S_2-CF and CF-S_n-CF.

Silica gel KC_{18} reversed-phase TLC proved to be a simple and convenient method for separation of individual components in the polysulfide mixtures of DBSC and biscarbofuran disulfide Separation of CF-S_n-NBu_2 yielded at least 8 components, and separation of CF-S_n-CF, the biscarbofuran derivatives,

7 components. A general correspondence between R_f values and the number of sulfurations was observed.

Effect of Impurities on the Potentiation of Organophosphorus Insecticides

Identification and Toxicological Properties of Impurities

The major impurities commonly present or those that may be developed during storage in technical malathion and acephate were studied. An evaluation of the impurities' effect on the mammalian toxicity of purified insecticides was made

Impurities present in technical malathion (~95% pure) and technical acephate (~95% pure) were isolated by column and thin-layer chromatography Eleven structures in malathion and seven in acephate were identified by nuclear magnetic resonance, infrared spectroscopy, and mass spectroscopy. Eight of the compounds in technical malathion were examined for their toxicological effects when added to purified malathion. Data show that several of the impurities potentiated rat toxicity. The highest potentiation was observed with *O,S,S*-trimethyl phosphorodithioate and the *S*-methyl isomeride of malathion. Potentiating effects were also decidedly greater in the rat than in the mouse Of the impurities isolated from acephate, *O,O,S*-trimethyl phosphorothioate showed slight potentiation of mouse toxicity when added to purified acephate but an antagonizing effect was observed with *O,O*-dimethyl-*N*-acetylphosphoramidothioate.

Storage of technical malathion for 3 to 6 months at 40°C resulted in materials that were noticeably more toxic to mice. A significant reduction in mouse toxicity was observed after storage of technical acephate under the same conditions.

Mode of Potentiating Action of Impurities Present in Malathion

Results provided *in vitro* and *in vivo* evidence that the impurities described above diminish the activities of serum carboxylesterase and liver carboxylesterase (the enzymes involved in malathion detoxication) and also diminish the activity of serum cholinesterase The compounds tested were, in order of increasing potency, *O,O,S*-trimethyl phosphorodithioate, *O,O,S*-trimethyl phosphorothioate, *O,S,S*-trimethyl phosphorodithioate, and *O,S*-dimethyl *S*-(1,2-dicarboethoxy)ethyl phosphorodithioate. *In vivo*, the diminutions of serum esterase activity caused by these compounds were transient, with the exception of those caused by *O,O,S*-trimethyl phosphorothioate, which persisted for at least 54 h.

The titer of malathion carboxylesterase in liver and sera of rats, three strains of mice, and five groups of pretreated mice was correlated with the malathion LD_{50} values measured in these groups. The equation of a regression line relating enzyme titer to toxicity was used to predict the median lethal dosage of purified malathion to humans. The mean value, 3655 mg/kg, was compared with dosages ingested in actual cases of human malathion poisonings A discrepancy was noted between LD_{50} value predicted in this study and the life-threatening doses of commercial malathion reported in the clinical literature

A soluble, stable, and partially purified preparation of human liver malathion carboxylesterase was obtained by detergent extraction and fractionation of whole-liver homogenate. The activity of this fraction was susceptible to concentration-dependent inactivation by isomalathion, the most potent of the esterase inactivators, but was resistant to three other impurities isolated from technical malathion.

Properties of Rat Malathion Carboxylesterase

Two distinct esterase fractions isolated from rat liver microsomes were examined for enzymatic hydrolysis of [methoxy-^{14}C] malathion with special emphasis on product analysis The effect of *O,S,S*-trimethyl phosphorodithioate and isomalathion as inhibitors of these esterases was also investigated

Two malathion carboxylesterase fractions, designated as esterase fractions A and B, which hydrolyze malathion were purified 13- and 18-fold, respectively. Although the two enzymes could not be distinguished from each other kinetically, fraction A contained at least one electrophoretic species not present in fraction B. The molecular weight of esterase fraction A was estimated at 50,000-60,000, but that of B was about twice this value

Incubation of [methoxy-^{14}C] malathion with either fraction resulted in a mixture of malathion α- and β-monoacids, but the composition of the mixture produced by fraction A (α/β ratio = 1.5) differed from that produced by fraction B (α/β ratio = 0.2), indicating the presence of multiple species of carboxylesterases in mammalian liver microsomes.

Isomalathion was substantially more potent as an inhibitor of both rat liver and rat serum malathion carboxylesterases than *O,S,S*-trimethyl phosphorodithioate. Isomalathion appeared to be equipotent in inhibiting the rat liver carboxylesterase-catalyzed reactions leading to either α- or β-monoacid, while *O,S,S*-trimethyl phosphorodithioate preferentially diminished those reactions. In contrast, the rat serum carboxylesterase-catalyzed reactions leading to either α- or β-monoacid were inhibited to approximately an equal degree by either isomalathion or *O,S,S*-trimethyl phosphorodithioate

Delayed Toxicity of Trialkyl Phosphorothioates

Delayed Toxicity of O,O,S-Trimethyl Phosphorothioate

The delayed toxic effects observed in rodents after treatment with simple trialkyl phosphorothioates and phosphorodithioates were investigated *O,O,S*-Trimethyl phosphorothioate, an impurity present in several technical organophosphorus insecticides, administered orally to rats, caused delayed mortality at single doses as low as 15 mg/kg, with death occurring 4-22 days following treatment. Delayed toxic signs were also observed in mice but in less severity than in rats. *O,O,S*-Triethyl phosphorothioate and *O,S,S*-trimethyl phosphorodithioate induced the same signs of intoxication at slightly higher doses.

Rats treated with *O,O,S*-trimethyl phosphorothioate refused food and water within 24 h after treatment until time of death. Neither injection of nutrient solution nor atropine reduced or blocked intoxication The isomeric *O,O,O*-trimethyl phosphorothioate was, however, a potent antagonist of the toxicity of *O,O,S*-trimethyl phosphorothioate As little as 1% of the *O,O,O*-trimethyl isomer protected rats from the intoxicating effects of the *O,O,S*-trimethyl isomer at doses as high as 200 mg/kg. Rat serum carboxylesterase and ChE were inhibited for prolonged periods following a single oral dose of *O,O,S*-trimethyl phosphorothioate but the duration of inhibition was significantly less when the toxicant contained 1% of the *O,O,O*-trimethyl isomer

Structure-Delayed Toxicity Relationships

Analogs of *O,O,S*-trimethyl phosphorothioate were examined for acute and delayed toxicity to rats as well as for anticholinesterase activity. The *O,O*-dimethyl *S*-alkyl phosphorothioate esters containing small *S*-alkyl moieties (methyl, ethyl, *n*-propyl) were highly toxic to the rat. An increase in the *S*-alkyl chain length beyond propyl, however, caused a precipitous drop in rat toxicity Of the smaller *S*-alkyl *O,O*-dimethyl esters, the *S*-methyl and *S*-ethyl analogs clearly caused delayed toxic effects with death occurring at long time intervals after treatment Rats treated with the *S*-*n*-propyl analog, the most toxic ester, died relatively quickly and it was difficult to differentiate acute from delayed effects.

Anticholinesterase measurements show that the dimethyl esters are inactive as cholinesterase inhibitors, being 10^5- to 10^6-fold less effective than such strong inhibitors as paraoxon and tetraethyl pyrophosphate Bimolecular rate constants (k_i) for the inhibition of either bovine erythrocyte acetylcholinesterase (BAChE) or rat serum cholinesterase (RSChE) were essentially the same for all the straight-chain *O,O*-dimethyl *S*-alkyl esters (CH_3 to *n*-C_6H_{13}), and the range in rat toxicity for these compounds was 12 to $>$ 750 mg/kg. The striking difference between rat toxicity and anticholinesterase activity suggests a noncholinergic mechanism of intoxication.

Delayed Toxicity and Delayed Neurotoxicity of Phosphoro- and Phosphonothioate Esters

Simple dialkyl phenylphosphonothioates such as *O,O*-diethyl phenylphosphonothioate, its corresponding oxon, and *O,O*-diethyl 4-chlorophenylphosphonothioate, impurities present in technical-grade ethyl leptophos [*O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate], caused delayed neurotoxicity in hens The mode of action of delayed neurotoxic compounds is still uncertain but clinical symptoms associated with delayed neurotoxicity are not seen until 8 to 14 days after exposure to the toxic agent The delayed toxic response described in rats treated with the simple trialkyl phosphorothioates suggested the possibility that some of those compounds may cause delayed neurotoxicity in hens. It was also possible that dialkyl phenylphosphonates may cause delayed toxicity in rats. Therefore, trimethyl phosphate, trimethyl phosphorothioates, and a series of methyl and ethyl esters of methyl-, ethyl-, and phenylphosphonates and phosphonothioates were examined for delayed neurotoxicity to hens and delayed toxicity to rats.

Comparison of the toxic properties of those compounds that are delayed-toxic to rats with those that are delayed-neurotoxic to hens suggests that the two types of toxicity are different. At least compounds that cause delayed toxicity in rats are not delayed-neurotoxic to hens and *vice versa* Of the 15 compounds examined in the present study, only *O,O*-diethyl phenylphosphonothioate and its oxon, *O,O*-diethyl phenylphosphonate, were delayed-neurotoxic

O,S-Diethyl ethylphosphonothioate produced cholinergic symptoms in rats and most of the rats lost weight for the first two or three days. As the cholinergic symptoms subsided, rats that lost less than 25% of their initial body weight completely recovered while those that lost more than 25% of their initial weight died Death occurred up to 6 days after treatment when the weight loss was 34 to 37% The symptomology of poisoning appears to indicate a possible similarity between the effects caused by the two types of compounds

Mode of Action of Delayed-Toxic Action of O,O,S-Trimethyl Phosphorothioate

Efforts to determine the mode(s) of action of *O,O,S*-trimethyl phosphorothioate have focused on the following areas the effects of this compound on serum electrolyte; serum enzyme and urinary electrolyte levels, determinations of elimination, respiration, and tissue distribution of radiochemical label in animals treated with ^{14}C-*O,O,S*-trimethyl phosphorothioate (labeled in the OCH_3 moiety), pathological studies of tissue damage following treatment with the compound; and the definition of pharmacokinetic parameters through the use of cannulae implanted in the exterior jugular vein of test animals.

Animals intoxicated with *O,O,S*-trimethyl phosphorothioate showed several physiological and pathological changes, the most important of which are lowered serum K^+ levels, severe hemato-concentration resulting from diarrhea and excessive urination, and damage to the liver and kidneys at moderate-to-high doses of the material

Initial ^{14}C-labeling studies showed rapid uptake and stabilization in the body with slow decline of total radioactivity up to and including 3 h following treatment. Relative levels were much higher following i.p. and i.v. treatments when compared to oral treatment. At 24 h, however, a higher percentage of the initial maximum ^{14}C-level in the blood remained following oral treatment when compared to levels from i.p treatment

Metabolism leads primarily to excretion in the urine with a half-life of 20-24 h. Of the 63% of total applied radioactivity excreted in the urine, only 1.6% was isolated as unchanged material Little of the ^{14}C-label was excreted as CO_2 or in fecal matter

Delayed Neurotoxicity of Organophosphorus Esters

Effect of Structure on the Delayed Neurotoxicity of O-Alkyl O-Aryl Phenylphosphonothioate Analogs Related to Leptophos

A systematic study of pesticidal activity versus delayed-neurotoxic potential of structural analogs of leptophos was conducted. Analogs of leptophos [*O*-(4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate] were examined for acute toxicity to mice and houseflies, and for delayed-neurotoxic activity in adult hens following administration of a single oral dose Development of ataxia after recovery from acute poisoning was the criterion for delayed-neurotoxic activity. All mono- and dichlorophenyl analogs were delayed-neurotoxic, the 2,5-dichlorophenyl analog being the most potent delayed-neurotoxic compound tested. Substitution for the methyl by an ethyl group abolished delayed-neurotoxic activity in both leptophos and the 2,5-dichlorophenyl analog at doses of 1000 mg/kg. The *n*-propyl and *n*-butyl analogs of the latter were also nondelayed-neurotoxic at 500 mg/kg and 333 mg/kg, respectively. Substitution of methyl for ethyl in EPN [*O*-*p*-nitrophenyl *O*-ethyl phenylphosphonothioate] did not alter its delayed neurotoxicity.

Effect of Chirality on the Toxicological Properties of Leptophos and Desbromoleptophos

The resolution and determination of the absolute configuration of the chiral

isomers of leptophos and desbromoleptophos and the toxicological properties of these compounds were investigated

The chiral isomers of *O*-methyl phenylphosphonothioic acid, *O*-(4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate (leptophos), and *O*-(2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate (desbromoleptophos) were prepared and their absolute configurations established by x-ray diffraction analysis of the (-)-α-methylbenzylammonium salt. The absolute configurations of the enantiomers of leptophos and desbromoleptophos were assigned by relating them to the configurations of the corresponding *O*-methyl phenylphosphonothioic acids. The $(R)_P(+)$ isomers were more acutely toxic to the housefly and white mouse, while the $(S)_P(-)$ isomers were more delayed-neurotoxic to the hen

Effect of Impurities on the Delayed Neurotoxicity of Ethyl Leptophos

The delayed neurotoxicity of ethyl leptophos, the effect of organophosphorus (OP) impurities in the technical-grade material on delayed neurotoxicity, the relationship of dose and time on delayed neurotoxicity of technical versus purified material with multiple doses, and the inherent delayed neurotoxicity of significant OP impurities were investigated.

The delayed neurotoxicity of technical and purified *O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate administered orally to hens was compared under different conditions. The technical material was neurotoxic at 750 mg/kg while the purified compound was neurotoxic at 1000-1250 mg/kg. The chemical composition of the technical material was analyzed for organophosphate contaminants. Impurities present as more than 0.1% were examined for delayed-neurotoxic activity. *O,O*-Diethyl phenylphosphonothioate, its oxon analog, and *O,O*-diethyl 4-chlorophenylphosphonothioate were 5-10 times more potent as delayed neurotoxins than the parent compound. These impurities evidently potentiate the delayed neurotoxicity of ethyl leptophos and may potentiate the delayed neurotoxicity of other *O*-ethyl phenylphosphonothioate pesticides as well.

Mode of Action of Phosphoramidothioate Esters

The mode of action of methamidophos (*O,S*-dimethyl phosphoroamidothioate) was studied A poor anticholinesterase agent, it is yet highly toxic to animals, which die of typical cholinergic symptoms of poisoning. The study was designed to determine the nature of the phosphorylating moiety when electric eel acetylcholinesterase (EEAChE) is inhibited by methamidophos

Separation of EEAChE from methamidophos was first established using a Sephadex G-25 column. Enzyme inhibition studies were conducted using both [*O*-methyl ^{14}C]- or [*S*-methyl ^{14}C]-methamidophos. Results provide strong evidence for P-S bond cleavage in the reaction leading to the inhibition of acetylcholinesterase by methamidophos

Gas Chromatography of Insecticides

The development of a novel gas chromatographic column (surface-modified support), obtained by high-temperature conditioning and subsequent exhaustive methanol extraction of 6% Carbowax 20M on HCl-extracted Chromosorb W, was applied to insecticides and extended to other pesticides. An evaluation of other support phases for the gas chromatography of pesticides was also made.

Forty-four stationary phases were prepared as surface-modified supports and evaluated Several of these supports exhibit excellent characteristics for the direct gas chromatography of organophosphorus and carbamate insecticides low bleed, very good peak symmetry and resolution, short retention times at relatively low temperatures, and high surface inactivation with little decomposition of labile compound.

The most effective supports for both phosphorothionates and phosphates were prepared with AN 600, Reoplex 400, SILAR-9CP, NPGS, EGSP-Z, and BDS These columns are effective in resolving fenthion, fenoxon, and their sulfoxides and sulfones at a single isothermal temperature. The SILAR-9CP column is interesting because it shows the greatest retention differences between phosphorothionates and their oxygen analogs The NPGS surface-modified support is the only support examined so far that successfully chromatographs azinphosmethyl oxon, a compound which pyrolyzes on-column above 205°C but requires an oven temperature of at least 185°C for volatilization.

Surface-modified supports were evaluated for the direct gas chromatography of 12 carbamate insecticides of diverse chemical structures and physical properties, and for the temperature-programmed separation of impurities in their technical products. Promising results were achieved with a 2-ft × 2-mm Nickel 200 column containing a surface-modified Carbowax 1540 support Effective isothermal separation of Isolan, aldicarb, 11203, MIPC, and propoxur with sharp symmetrical peaks was obtained at column temperatures of 100-130°C and an injection temperature of 110°C. Less volatile carbamates carbofuran, mexacarbate, 9699, Pyrolan, and carbaryl produced broader, less symmetrical peaks at these temperatures but, except for carbaryl, which showed increased pyrolysis, higher column temperatures appear promising for improving peak shape and symmetry. Temperature-programmed chromatography of the technical insecticides from 50 to 130°C at 10°C/min appears promising for the separation of impurities for further identification studies Triton X-305 and EGSP-Z also show promise for the direct chromatography of a number of carbamate insecticides

Neurophysiological Studies on the Mode of Action of Insecticides

A series of neurophysiological studies on the mode of action of insecticides was conducted. Pyrethroid insecticides were found to poison the nervous system by acting at specific sites on neurons. On motor nerves, all poisoning was confined to presynaptic nerve terminals, in sensory nerves the sensory endings were poisoned.

Picrotoxinin, a natural plant convulsant, was toxic to the desheathed housefly central nervous system (CNS), and was much less toxic to the intact CNS

A series of substituted *N,N'*-thiodicarbamates was found to poison intact houseflies with the same latency from application to disruption of flight motor neuron coordination Evidence suggested that the sulfenylated carbamate analogs were converted very rapidly at the cuticle to the parent carbamate and that this conversion was not rate-limiting.

A housefly strain was obtained containing the kdr factor, which rendered the strain resistant to pyrethroid and

DDT insecticides. The entire nervous system of the kdr housefly was resistant to direct perfusion of pyrethroids and DDT. Both the central and peripheral nervous systems were refractory to compounds. In addition, the perineurial sheath on the CNS made little difference to bioassay responses whether intact or removed, and, therefore, played no role in the resistance. An experimental procedure was outlined which measures quantitatively the amount of kdr present in mosquitoes and houseflies.

"Slow" and "fast" motor units of Dipteran maggots are mediated by different neurotransmitters, that aspartic acid mimics the "slow" neurotransmitter, and glutamic acid the "fast" neurotransmitter However, a major problem was encountered with accepting any amino acid as a neurotransmitter when it was shown that normal, neurally-evoked synaptic transmissions occurred in the presence of slightly elevated calcium concentrations, which also rendered the preparations completely insensitive to perfused or iontophoretically-applied asparate or glutamate under the same conditions.

Attempts were made to distinguish between DDT and pyrethroid actions on the insect nervous system A list of criteria was reported which allowed analogs to be categorized as DDT-like or pyrethroid-like.

The consequences of insecticide poisoning were investigated by comparing the histological appearance of the thoracic ganglion of houseflies which had been treated with doses causing mortality in 50% of a population Lindane treatment was found to cause "vacuoles" in the thoracic ganglion that were correlated in number with the amount of disability measured behaviorally. The disability type was correlated with the vacuolization site.

Examination of CNS activity in the housefly was accomplished by recording the comparing motor unit activity before, during, and after poisoning. Measurements were made without dissection and during performance of uninhibited movement, the only restrictions being a tether and wires inserted through the cuticle in a few sites on the thorax. This approach allowed the first unambiguous description of overt poisoning symptoms based on nervous activity.

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The complete report, entitled "Chemistry and Mode of Action of Insecticides: Phase II," (Order No. PB 83-247 213; Cost: $20.50, subject to change) will be available only from:
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