°C, pouring the residue into several volumes of H_2O, and extracting with several portions of ether. The dried (Na_2SO_4) solution was freed of solvent and distilled to give a quantitative yield (5.25 g) of the methyl ester as a pale yellow liquid, bp 162 °C (1.0 mmHg), bp 210 °C (12 mmHg), n_D^{25} 1.5000. The compound exhibited strong blue fluorescence in UV light.

Ethyl α-eleostearate was similarly prepared in quantitative yield by using ethanol instead of methanol; it showed bp 190 °C (4.0 mmHg), n_D^{25} 1.4900.

Although ethyl α-eleostearate did not deter feeding by the boll weevil (35 punctures; 39 punctures for the control), methyl α-eleostearate was as effective a feeding deterrent as the free acid, permitting only 9 punctures as compared with 60 for the control. Furthermore, the methyl ester is much more stable than the acid, remaining a mobile, active liquid for at least 1 week at room temperature and indefinitely under ordinary refrigeration (10 °C); UV irradiation at room temperature resulted in slow conversion (over 1 week) to the inactive all-trans methyl ester, bp 169 °C (1.0 mmHg), n_D^{25} 1.5037.

The high activity of methyl α-eleostearate and *erythro*-9,10-dihydroxy-1-octadecanol acetate as boll weevil feeding deterrents in laboratory bioassay trials indicates that they have considerable potential for use under practical conditions to prevent damage to cotton plants by this serious pest. However, only actual field testing can confirm this. Such tests are planned on cotton crops in Mississippi and Mexico during the next growing season. In the meantime, patent applications have been filed covering the isolation of α-eleostearic acid and *erythro*-9,10-dihydroxy-1-octadecanol acetate from tung oil and the activity of the latter compound and methyl α-eleostearate as boll weevil feeding deterrents. Toxicological tests with these compounds are also under way.

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Effect of Impurities on the Delayed Neurotoxicity of *O*-(4-Bromo-2,5-dichlorophenyl) *O*-Ethyl Phenylphosphonothioate Administered Orally to Hens

J. Gary Hollingshaus, Takaaki Nishioka, Ralph B. March, and T. Roy Fukuto*

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 analogue, and *O,O*-diethyl (4-chlorophenyl)phosphonothioate 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.

The organophosphorus pesticide *O*-(4-bromo-2,5-dichlorophenyl) *O*-methyl phenylphosphonothioate (leptophos) has been shown to cause delayed neurotoxicity in many species of animals (Report of the Leptophos Advisory Committee, 1976). Its delayed neurotoxic potential was significantly increased by oxidation to its oxon and by photolytic debromination to desbromoleptophos. It was also suggested that differences in the delayed neurotoxic potential of technical leptophos reported from several studies may have been due to potentiating impurities (Sanborn et al., 1977).

In a recent study of the delayed neurotoxicity of structural analogues related to leptophos, it was reported that substitution of the methoxy moiety with a longer chain alkoxy group, e.g., ethoxy or propoxy, abolished

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delayed neurotoxic activity in hens at the highest doses tested (Hollingshaus et al., 1979). The minimum effective dose (MED) for desbromoleptophos, the most potent delayed neurotoxic compound tested, was 25 mg/kg, yet neither ethyl leptophos [*O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate] nor ethyl desbromoleptophos [*O*-(2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate] showed delayed neurotoxic activity at 1000 mg/kg. Against insects ethyl leptophos has been reported to be nearly as effective as leptophos against the bollworm, *Heliothis zea*, and the tobacco budworm, *Heliothis virescens* (Wolfenbarger, 1969).

The above observations suggested a need for a more critical evaluation of the toxicological properties of ethyl leptophos. The present study describes investigations pertaining to (a) the delayed neurotoxicity of ethyl leptophos, (b) the effect of organophosphorus (OP) impurities in the technical-grade material on delayed neurotoxicity, (c) the relationship of dose and time on delayed neurotoxicity of technical vs. purified material with multiple doses, and (d) the inherent delayed neurotoxicity of significant OP impurities.

MATERIALS AND METHODS

General. Silica gel 60 F-254 sheets (E.M. Reagents) of 0.2-mm thickness and plates of 0.25-mm thickness were used for analytical thin-layer chromotography (TLC). Preparative TLC was conducted with silica gel 60 PF-254 (E.M. Reagents) plates of 1.0-mm thickness. Solvent systems used for TLC were hexane, hexane–benzene (9:1), and hexane–benzene (6:4). Spots were located by ultraviolet detection and by 0.5% 2,6-dibromoquinone-4-chloroimide (DBQ) in ether as a spray reagent (Menn et al., 1957). Silicic acid (Mallinckrodt CC-7 Special) was used for column chromatography with a hexane–ethyl acetate solvent gradient.

Analytical gas chromatography–chemical ionization mass spectrometry (CIMS) was conducted by using a Finnigan Model 3300 mass spectrometer equipped with a Finnigan Model 9500 gas chromatograph. Methane was used as the carrier and reagent gas with the gas flow rate adjusted to give a reagent gas pressure of 0.5 torr in the ion source. The ion source temperature was 100 °C and the electron energy of the ion source was 150 eV. Technical samples and synthetic compounds were injected as acetone solutions (~40 mg/mL) with injection volumes of 0.5–8 μL into a 5 ft × 2 mm i.d. glass U-tube column. The column packing was prepared after the surface-modified support methodology of Aue et al. (1973) utilizing 6% EGSP-Z (Applied Science Laboratories, State College, PA) on HCl-extracted 80–100-mesh Chromosorb W, vacuum-coated and fluidized, conditioned for 24 h at 230 °C, and exhaustively extracted with chloroform. The column oven temperature was programmed from 80 to 225 °C at 12 °C/min. CIMS data acquisition and reduction were performed by a System 150 computer (System Industries) at an integration time of 6 s/scan, scan range m/e 90–700, with a 1-s delay between scans.

Quantitative determination of impurities was carried out with a Hewlett-Packard Model 402 gas chromotograph fitted with a 6 ft × 2 mm i.d. column with identical packing as above and equipped with an alkali (KCl) flame ionization detector (AFID). Gas flows for H, He, and air were 40, 36, and 320 mL/min, respectively. Quantitation of overlapping peaks was by total ion current for particular molecular ions by CIMS.

Proton magnetic resonance (^{1}H NMR) spectra were recorded in a Varian EM-390 spectrometer by using tetramethylsilane as the internal standard and chloroform-*d* solvent. Melting and boiling points were uncorrected, and elemental analyses were carried out by C. F. Geiger, Ontario, CA.

Chemicals. Technical ethyl leptophos, 89–90% pure, and analytical standards of *O,O*-bis(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate, *O,O*-diethyl phenylphosphonothioate, *O*-ethyl phenylphosphonochloridothionate, *O,O'*-diethyl *P,P'*-diphenyldithiopyrophosphonate, and 4-bromo-2,5-dichlorophenol were supplied by Velsicol Chemical Corp., Chicago, Il. Purified ethyl leptophos (1), >99% pure, mp 63–64 °C, was obtained by repeated recrystallization of the technical material from hexanes. *O*-(2.5-Dichlorophenyl) *O*-ethyl phenylphosphonothioate (2) and *O,O*-bis(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate (3) were available from a previous study (Hollingshaus et al., 1979).

O,O-Diethyl phenylphosphonate (4) and *O,O*-diethyl phenylphosphonothioate (5) were prepared by dropwise addition of phenylphosphonic dichloride or phenylphosphonothioic dichloride (Aldrich Chemical Co.) to 2 equiv of sodium ethoxide in absolute ethanol. The phosphonate (4), a colorless liquid, was distilled at 95 °C (0.02 mm): n^{23}_D 1.4944. The phosphonothioate (5), also a colorless liquid, was distilled at 80 °C (0.05 mm): n^{23}_D 1.5363.

O,S-Diethyl phenylphosphonothioate (6) was prepared by the dropwise addition of diethyl sulfate (Aldrich) to the sodium salt of *O*-ethyl phenylphosphonothioic acid, obtained by hydrolysis of 4, in acetonitrile. The mixture was refluxed overnight and then purified by column chromatography using hexane–ethyl acetate: n^{23}_D 1.5382; ^{1}H NMR ($CDCl_3$) δ 8.1–7.4 (Ar-*H*, m), 4.16 (–OC*H*$_2$–, m), 2.7 (–SC*H*$_2$–, m), 1.38 (–OCH$_2$C*H*$_3$, t, J = 4.8 Hz), 1.23 (–SCH$_2$C*H*$_3$, t, J = 6.3 Hz).

O-(4-Bromo-2,5-dichlorophenyl) *S*-ethyl phenylphosphonothioate (7) was prepared by first demethylating leptophos with benzenethiol and dicyclohexylamine. The resulting dicyclohexylamine salt was recrystallized from ethanol: mp 187–188 °C (Sanborn et al., 1977). Reaction of this salt with diethyl sulfate gave 7 which was purified by column chromatography using hexane–ether (9:1): n^{23}_D 1.6208; ^{1}H NMR ($CDCl_3$) δ 8.3–7.5 (Ar-*H*, m), 2.8 (–SC*H*$_2$, m), 1.2 (–SCH$_2$C*H*$_3$, t, J = 7.5 *hz*).

O,O-Diethyl (4-chlorophenyl)phosphonothioate (8) was prepared from (4-chlorophenyl)phosphonothioic dichloride and sodium ethoxide as described for 5: n^{23}_D 1.5463; ^{1}H NMR ($CDCl_3$) δ 7.80 (Ar-H_{ortho}, dd, $J_{P,H}$ = 15.3 Hz, J = 8.7 Hz), 7.36 (Ar-H_{meta}, dd, $J_{P,H}$ = 4.7 Hz), 4.15 (–OC*H*$_2$–, m), 1.22 (–OCH$_2$C*H*$_3$, t, J = 7.5 Hz). (4-Chlorophenyl)-phosphonothioic dichloride was prepared by heating at reflux 1 equiv of phosphorus trichloride and chlorobenzene with 1.1 equiv of anhydrous aluminum chloride for 12 h (Buchner and Lockhart, 1963; Michaelis, 1897), followed by careful addition 1 equiv of sulfur. The mixture was refluxed overnight and then 1.1 equiv of phosphorus oxychloride was added. The resulting phosphorus oxychloride–aluminum chloride complex, which quickly precipitated, was removed by filtration, and the product (8) was distilled: bp 88 °C (0.15 mm); n^{23}_D 1.6393.

O-(4-Bromo-2,5-dichlorophenyl) *O*-ethyl (4-chlorophenyl)phosphonothioate (9) was prepared by the addition of *O*-ethyl (4-chlorophenyl)phosphonochloridothioate to equivalent amounts of 4-bromo-2,5-dichlorophenol and pyridine in benzene. The product was purified by preparative TLC and then crystallized from hexane: mp 59–60 °C; ^{1}H NMR ($CDCl_3$) δ 7.96 (PAr-H_{ortho}, m, $J_{P,H}$ = 15.3 Hz, J = 8.7 Hz), 7.64 (OAr-H_{ortho}, s), 7.45 (PAr-H_{meta}, m, $J_{P,H}$ = 4.7 Hz), 7.38 (OAr-H_{meta}, s), 4.33 (–OC*H*$_2$–, m), 1.40

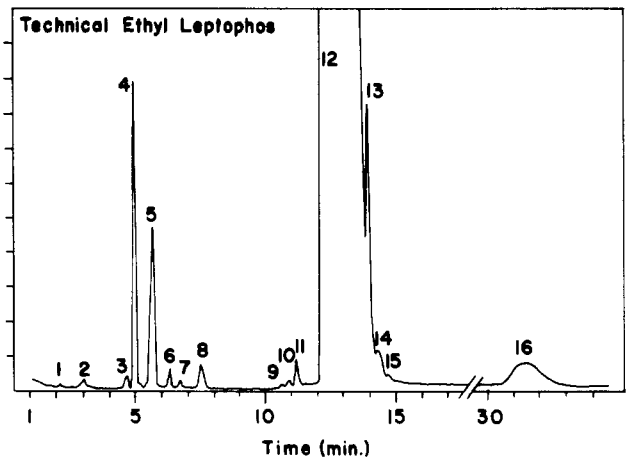


Figure 1. GC/CIMS chromatogram reconstructed from total ion current from m/e 90 to 650 for technical ethyl leptophos. The column was programmed from 80 to 225 °C at 12 °C/min.

($-OCH_2CH_3$, t, J = 6.9 Hz). *O*-Ethyl (4-chlorophenyl)-phosphonochloridothioate was prepared by hydrolysis of 8 with 1 equiv of potassium hydroxide in ethanol. The free acid was converted to the chloridothioate by reaction with phosphorus pentachloride in carbon tetrachloride at −5 °C and then distilled at 91 °C (0.14 mm): n^{23}_D 1.5797.

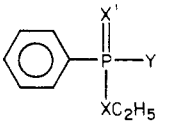
O,O-Bis(4-bromo-2,5-dichlorophenyl) (4-chlorophenyl)-phosphonothioate (10) was prepared by the addition of (4-chlorophenyl)phosphonothioic dichloride to 2 equiv of 4-bromo-2,5-dichlorophenol and pyridine in toluene. The product was recrystallized from ethanol: mp 116–117 °C. The ^1H NMR spectra of these compounds were consistent with the assigned structures.

Effect of Storage on Technical Ethyl Leptophos. Samples (7.0–7.5 g) of technical ethyl leptophos were sealed in glass ampules under an air atmosphere and placed in a Haake Series F constant-temperature bath at 40 °C. The ampules were opened after 4.5 months and examined for content and delayed neurotoxicity to hens.

Delayed Neurotoxicity. Adult White Leghorn hens (AAA Egg Ranch, Lakeview, CA), 1.3–2.4 kg in weight and 24 months of age, were used to determine delayed neurotoxic activity. Toxicants were administered orally to at least three hens at each dose level. Liquid compounds were given by stomach intubation. Crystalline compounds, owing to the large doses, were administered in gelatin capsules, but this may have resulted in some attenuation of the effects due to poor absorption. Birds were housed three per cage in standard laying cages and given standard layer's mash and water ad libitum. Birds were examined 6 times per week for at least 30 days for clinical signs of ataxia (Davies and Holland, 1972; Abou-Donia et al., 1979). The minimum effective dose (MED) was described as the lowest dose administered that produced any visually detectable ataxia. Hens showing symptoms of acute cholinergic poisoning were given intramuscular injections of 20 mg/kg atropine in saline.

RESULTS AND DISCUSSION

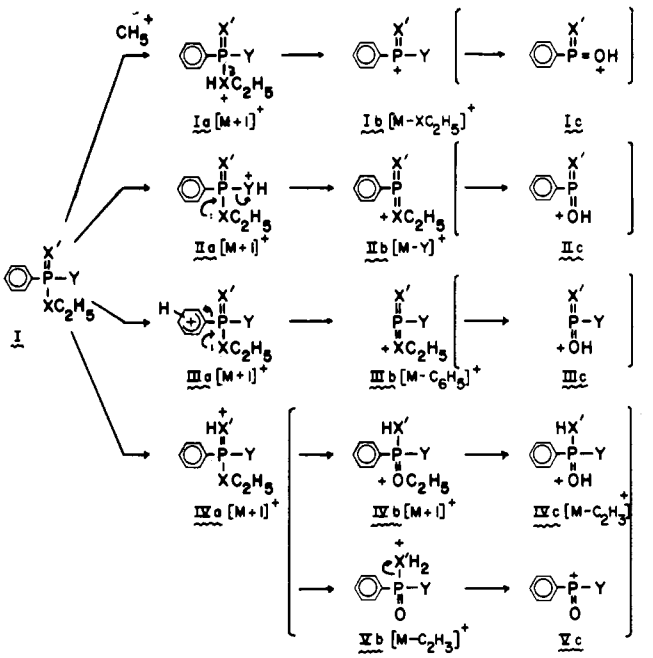
Phosphorus-Containing Impurities in Technical Ethyl Leptophos. A GC profile showing the separation of impurities in technical ethyl leptophos is shown in Figure 1. The gas chromatogram was reconstructed from the total ion current over the range m/e 90–650. Peaks 1, 2, and 3 were missing in gas chromatograms produced under the same analytical conditions when AFID was the means of detection. Nonphosphorus impurities in peaks 1–3 were identified as isomeric dichloroanisoles and bromodichlorophenols were observed at peaks 9–12. Phosphorus-containing impurities appeared at peaks 4–16. They were expected to have the general formula I from



I

the industrial synthetic method and starting materials (Richter, 1969; Hanna, 1971). X and X′ represent either oxygen or sulfur; Y represents either chlorine, *O*- or *S*-ethyl, or *O*- or *S*-aryl substituents. The reactant ions of methane in CIMS function primarily as proton donors to phosphorus esters (Holmstead and Casida, 1974; Saas and Fisher, 1979). The general formula I has four likely sites for protonation, as indicated in Scheme I. Significant quantities of $(M + 41)^+$ and $(M + 29)^+$ ions were observed with a base peak $(M + 1)^+$. Complete CIMS data is given in the supplementary material (see paragraph at end of paper regarding supplementary material). Structures of phosphorus-containing impurities in peaks 4–16 were determined as follows.

Scheme I. CIMS Fragmentation of Phenylphosphonothioates and Phenylphosphonates Present in Technical Ethyl Leptophos



Peak 4. Ions m/e 261 $(M + 41)^+$, 249 $(M + 29)^+$, and 221 $(M + 1)^+$ satisfy the requirement for the molecular ions in methane CIMS. The molecular weight of this compound is 220. The isotopic abundance of molecular ion and fragment m/e 185 $(M - 35)^+$ (IIb in Scheme I) suggested that this compound contained one chlorine atom. *O*-Ethyl and benzene groups in the structure were supported by fragments m/e 193 $(M - C_2H_3)^+$ (IVc), 175 $(M - OC_2H_5)^+$ (Ib), and 143 $(M - C_6H_5)^+$ (IIIb). The major fragment m/e 185 is a characteristic ion to a structure $C_6H_5P(S)(OC_2H_5)$ or $C_6H_5P(O)(SC_2H_5)$. This compound was identified as *O*-ethyl phenylphosphonochloridothioate. This structure consistently explains the other fragment ions.

Peak 5. Ions m/e 271, 259, and 231 indicated a molecular weight of 230. Two major fragment ions m/e 185 $(M - OC_2H_5)^+$ (Ib) and 153 $(M - C_6H_5)^+$ (IIIb) indicated two possible structures, $C_6H_5P(O)(OC_2H_5)(SC_2H_5)$ and $C_6H_5P(S)(OC_2H_5)_2$. The relatively low abundance of m/e

169 $(M - SC_2H_5)^+$ (Vc) and the presence of this isomeric compound as an impurity in peak 8 supported the structure *O,O*-diethyl phenylphosphonothioate.

Peak 6. The molecular weight of this compound is 214. There are three kinds of fragments present: (1) *m/e* 169 $(M - OC_2H_5)^+$ (Ib), (2) *m/e* 137 $(M - C_6H_5)^+$ (IIIb), and (3) all others make pairs by the difference of 28 amu, [169 (Ib), 141 (Ic)], [137 (IIIb), 109 (IIIc)], and [187 (Vb), 159 (Vc)]. These fragments indicate there are only two groups in this compound, C_6H_5 and OC_2H_5. *O,O*-Diethyl phenylphosphonate meets these requirements.

Peak 7. Molecular ions *m/e* 305, 293, and 265 and their isotopic abundance indicated a chlorine-containing compound with molecular weight 264. Intense fragments *m/e* 229 $(M - Cl)^+$ (IIb), 219 $(M - OC_2H_5)^+$ (Ib), and 153 $(M - Cl - C_6H_4)^+$ (IIIb) gave two possible structures: (a) $ClC_6H_4P(OC_2H_5)$ and (b) $C_6H_5P(Cl)(OC_2H_5)$. A trace of *m/e* 187 $(M - C_6H_5)^+$ ion did not rule out structure b. However, (a) was definitely required by the most intense fragment *m/e* 153. This compound was identified as *O,O*-diethyl (4-chlorophenyl)phosphonothioate and is related to the impurity, peak 12c, *O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl (4-chlorophenyl)phosphonothioate.

Peak 8. This compound showed the same ion species, but with different intensities, as peak 5 (*O,O*-diethyl phenylphosphonothioate). The impurity represented by peak 8 should be an isomer of peak 5. Fragment *m/e* 169 $(M - SC_2H_5)^+$ (IIb) was much more intense than fragment *m/e* 185 $(M - OC_2H_5)^+$ (Ib). This compound was identified as *O,S*-diethyl phenylphosphonothioate.

Peaks 9–11. Compounds in these peaks have the same molecular weight, 346. Isotopic abundances of molecular ions indicated they contain two chlorine atoms. Ion *m/e* 185 (IIb) is the most abundant fragment; therefore these compounds contain $C_6H_5P(S)(OC_2H_5)$ or $C_6H_5P(O)(SC_2H_5)$ as a part of their structures. The other part of the molecules appears as a protonated neutral fragment *m/e* 163 which was identified as protonated dichlorophenol from its isotopic abundance. Compounds in peaks 9–11 are isomers of $C_6H_5P(S)(OC_2H_5)(OC_6H_3Cl_2)$.

The quantity of material in peak 9 was so small that other fragment ions necessary to determine its structure were not detected, and its exact structure is therefore unknown.

The compound in peak 10 gave an *m/e* 311 $(M - Cl)^+$ ion which is a specific ion for 2-chlorophenyl esters. From the GC retention time and CIMS of the synthesized material, this peak was identified as *O*-(2,5-dichlorophenyl) *O*-ethyl phenylphosphonothioate.

The compound in peak 11 showed fragments *m/e* 301 $(M - OC_2H_5)$ (Ib) and 269 $(M - C_2H_5)^+$ (IIIb). Fragment peak *m/e* 311 $(M - Cl)^+$ was missing. The substituent position of the two chlorine atoms on the benzene ring was not determined and the exact structure of this compound is unknown.

Peak 12. This largest GC peak corresponds to ethyl leptophos. Gas chromatograms constructed by ion current of fragments *m/e* 387 and 219, which were not observed in CIMS of pure ethyl leptophos, showed that at least two different peaks overlap with peak 12. Figure 2 represents a gas chromatogram reconstructed from currents of ions *m/e* 90–700 analyzed under a constant column temperature of 190 °C. Gas chromatograms reconstructed by ion current of single ions, *m/e* 219, 387, 421, 425, 441, and 503, suggested peaks 12 and 13 contained six different compounds.

Peak 12a. CIMS gave the same fragment species as ethyl leptophos and is an isomeric *O*-(bromodichlorophenyl) ester but its exact structure is unknown.

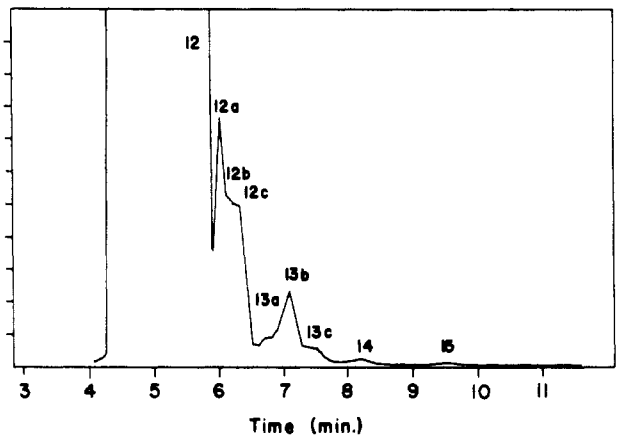


Figure 2. GC/CIMS chromatogram reconstructed from total ion current from *m/e* 90 to 650 for technical ethyl leptophos. The column was operated isothermally at 190 °C.

Peak 12b. This compound has a mass of 386. Fragments *m/e* 341 $(M - OC_2H_5)^+$ (Ib), 309 $(M - C_6H_5)^+$ (IIIb), and 185 (IIb) indicated the presence of $C_6H_5P(S)(OC_2H_5)$. Fragments *m/e* 259, 231, 203, 185, 169, 153, 141, and 125 are common to *O,O*-diethyl and *O,S*-diethyl phenylphosphonothioate. Fragment $(M - SC_2H_5)^+$ was not observed, leaving two possible structures: $[C_6H_5P(S)(OC_2H_5)]_2O$ and its disulfide isomer. From the GC retention time and CIMS of synthetic material, this was identified as *O,O'*-diethyl *P,P'*-diphenyldithiopyrophosphonate.

Peak 12c. Isotopic abundances of molecular ions indicated the presence of Cl_3 and Br_1. Fragment ions, except for *m/e* 347 $(M - Cl - C_6H_4)^+$, corresponded to those of ethyl leptophos by a difference of 34 amu. Characteristic fragment *m/e* 185 for the series of compounds analyzed shifted to *m/e* 219 with an isotopic abundance of one chlorine. The presence of fragments *m/e* 413 $(M - OC_2H_5)^+$ (Ib) and 347 $(M - Cl - C_6H_4)^+$ (IIIb) gave additional evidence that the benzene ring of phenylphosphonothioate had a chlorine substitutent. The position of the chlorine was determined by NMR analysis of a sample purified by preparative TLC. This compound was identified as *O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl (4-chlorophenyl)phosphonothioate.

Peak 13a. Molecular ions *m/e* 461, 449, and 421 were observed. Other fragments overlapped with those of peak 12c such that this compound could not be identified.

Peak 13b. Molecular ions and most fragment ions were the same as those of ethyl leptophos. One of the major fragments was *m/e* 363 $(M - SC_2H_5)^+$ (Ib) which was not observed in ethyl leptophos. The low abundance of fragment *m/e* 379 $(M - OC_2H_5)^+$ (Vc) supported the structure *O*-(4-bromo-2,5-dichlorophenyl) *S*-ethyl phenylphosphonothioate, iso ethyl leptophos.

Peak 13c. Molecular ions and fragment ions, except for the ions *m/e* 379 $(M - SC_2H_5)^+$ (Ib) and 241 $(BrCl_2C_6H_2OH_2)^+$, corresponded to those of ethyl leptophos with a difference of 16 amu. This compound was then identified as *O*-(4-bromo-2,5-dichlorophenyl) *S*-ethyl phenylphosphonodithioate.

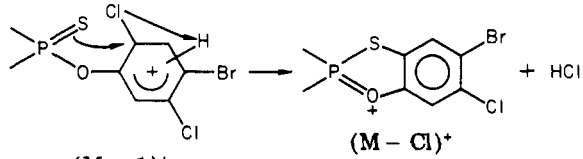
Peak 14. Molecular ions *m/e* 543, 531, and 503 and their isotopic abundances suggested this compound was a bromo derivative of ethyl leptophos. No fragment ions were observed at sufficient intensity to identify the structure, however.

Peak 15. This compound is unknown.

Peak 16. Molecular ions *m/e* 659, 647, and 619 and their isotopic abundances indicated this compound con-

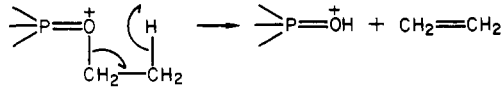
tained Cl_4Br_2 and its molecular weight was 618. Its spectrum showed only four fragments: m/e 583 $(M - Cl)^+$, 541 $(M - C_6H_5)^+$ (IIIb), 379 $(M - BrCl_2C_6H_2O)^+$ (IIb), and 241 $(BrCl_2C_6H_2OH_2)^+$. This compound was identified as *O,O*-bis(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate.

Scheme I consistently explained most fragmentations of the impurities. The protonated molecular ions $(M + 1)^+$ decompose to fragments Ib, IIb, and IIIb with the loss of neutral fragments. Another primary fragmentation was the dechlorination of protonated 4-bromo-2,5-dichlorophenyl esters: ethyl leptophos, *S*-ethyl isomeride of ethyl leptophos, *O,O*-bis(4-bromo-2,5-dichlorophenyl) phenylphosphonothioate, and *O*-(4-bromo-2,5-dichlorophenyl) *O*-ethyl (4-chlorophenyl)phosphonothioate. CIMS of 4-bromo-2,5-dichlorophenol showed only one fragment $(M - Br + 1)^+$ produced by loss of the bromine atom. No debrominated fragment was observed for the phenyl esters. Specific dechlorination can be explained by the stabilization of the fragment ions by neighboring participation of sulfur or oxygen atoms.

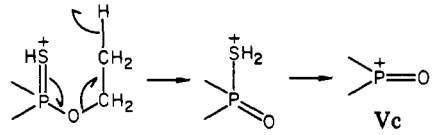


$(M + 1)^+$ $(M - Cl)^+$

Fragment ions Ib, IIb, IIIb, and IVb further decompose to Ic, IIc, IIIc, and IVc, respectively, by dealkylation of an *O*-ethyl group. Holmstead and Casida (1974) utilized methane-d_6 CIMS of *O*-alkyl phosphorus esters and provided evidence that dealkylation of *O*-alkyl groups proceeds by rearrangement. This rearrangement can be explained by the four-membered ring transition state in which the double bond is exocyclic (McLafferty, 1973).



Dealkylation of an *S*-ethyl group was not observed. The formation of fragment $(M - SC_2H_5)^+$ (Vc) was observed with *O,O*-diethyl phenylphosphonothioate and *O,O*-diethyl (4-chlorophenyl)phosphonothioate which have no SC_2H_5 groups. This is explained by dealkylation of an OC_2H_5 group by a sulfenium ion (IVa) and subsequent loss of SH_2 (Vb).



Structures of compounds for which synthetic standards were available gave identical CIMS fragmentation patterns to identified impurities and were further confirmed by GC retention times and TLC cochromatography. The source of the (4-chlorophenyl)phosphonothioate impurities 8 and 9 was determined to be from an impurity in phenylphosphonothioic dichloride. An analytical sample of *O,O*-diethyl phenylphosphonothioate supplied by Velsicol Chemical Co., which was prepared as described previously, contained 8 as an impurity. Its presence can only be explained by contamination by (4-chlorophenyl)-phosphonothioic dichloride.

Effects of Storage on the Chemical Composition of Technical Ethyl Leptophos. Figure 3 is a gas chromatogram reconstructed from the total ion current from m/e 90 to 650 for technical ethyl leptophos stored at 40 °C under an air atmosphere for 4.5 months. A comparison of this chromatogram with Figure 1 shows little difference except for the loss of peak 4, which is *O*-ethyl phenylphosphonochloridothioate.

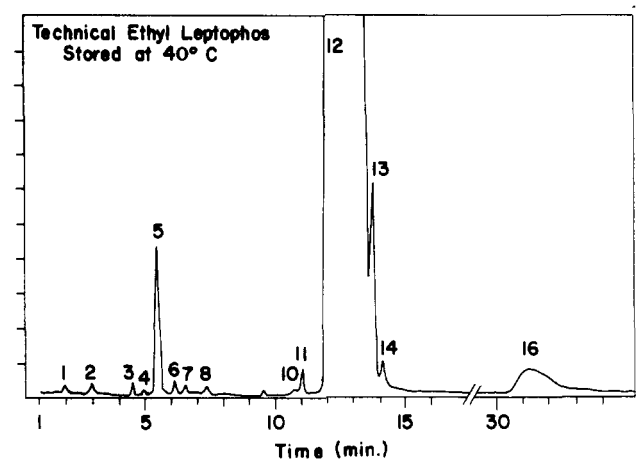


Figure 3. GC/CIMS chromatogram reconstructed from total ion current from m/e 90 to 650 for technical ethyl leptophos stored at 40 °C for 4.5 months. The column was programmed from 80 to 225 °C at 12 °C/min.

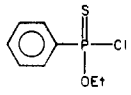
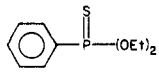
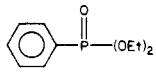
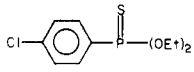
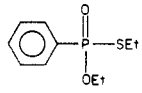
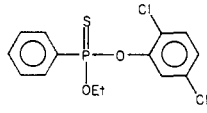
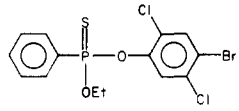
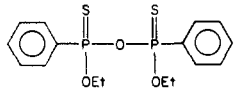
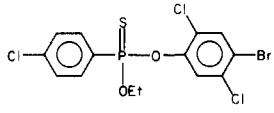
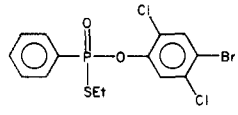
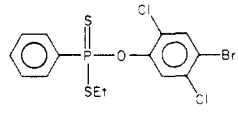
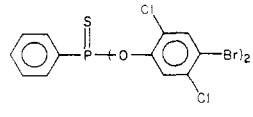
Table I shows the percent composition of technical ethyl leptophos before and after storage. The technical material was nearly 90% pure and was stable under the storage conditions of this experiment. Furthermore, all of the impurities measured were also stable except for *O*-ethyl phenylphosphonochloridothioate, peak 4.

Delayed Neurotoxicity of Technical vs. Purified Ethyl Leptophos. A comparison of the delayed neurotoxic potential of technical vs. purified ethyl leptophos as single oral doses to hens is shown in Table II. The MED was 1000–1250 mg/kg for purified ethyl leptophos while the MED for the technical material was at most 750 mg/kg. The onset of ataxia in hens treated with purified material was also 5–10 days later than for hens given the technical compound. A statistical evaluation of these data, and those shown in Tables III and IV, was not conducted because some stages of ataxia were not always observed in all of the hens under similar treatment. The variation in response time among hens that did develop the same degree of ataxia was generally 2–5 days.

Table III shows a comparison of the same two materials as in Table II except that the hens were given daily oral doses of 10 or 50 mg kg^{-1} day^{-1} for periods of 24 or 45 days. Hens given 50 mg kg^{-1} day^{-1} of either material developed severe ataxia, stage T_4, during both treatment periods whereas none of the hens given 10 mg kg^{-1} day^{-1} showed any sign of ataxia. At those dose levels there appears to be no difference in the delay period between technical and purified materials. Surprisingly, however, hens given either material for 24 days developed ataxia 7–15 days earlier than hens under similar treatment for 45 days.

In a previous paper (Hollingshaus et al., 1979), we reported ethyl leptophos was not delayed neurotoxic to hens at 1000 mg/kg. The first stage of ataxia is often difficult to positively identify either clinically or histopathologically (Abou-Donia and Graham, 1978a,b, 1979; Abou-Donia et al., 1979). In the present study, hens given 1000 mg/kg of purified ethyl leptophos, although slightly clumsy, were difficult to distinguish from control animals while hens given doses greater than 1000 mg/kg developed severe ataxia. It seems apparent that the highest dose examined in our previous study was just at threshold and positive

Table I. Chemical Composition of Technical Ethyl Leptophos before and after Storage at 40 °C for 4.5 Months As Determined by Gas Chromatography-Mass Spectroscopy

peak no.[a]	structure	% composition unstored	% composition stored
1	unknown (nonphosphorus)		
2	unknown (nonphosphorus)		
3	unknown (nonphosphorus)		
4		1.4	<0.1
5		1.5	1.7
6		0.1	0.1
7		<0.1	<0.1
8		<0.1	<0.1
10		<0.1	<0.1
12		89.5	89.5
12b		<0.1	<0.1
12c		2.1	2.1
13b		0.6	0.8
13c			
14	unknown (phosphorus)		
15	unknown (phosphorus)		
16		1.6	1.6
	total:	96.8-97.2	95.8-96.3

[a] See Figure 1.

ataxia was therefore not observed. It is also evident that some component of the technical material is enhancing its neurotoxicity.

The difference in neurotoxic potential between technical and purified ethyl leptophos prompted an analysis of the chemical composition of the technical material and its

Table II. Delayed Neurotoxic Activity of Technical vs. Purified Ethyl Leptophos as Single Oral Doses to Hens

compd	dose, mg/kg	mean days after treatment when ataxia was observed T_1[a]	T_2	T_3	T_4
technical (89% pure)	1500	14	16	19	21
	1250	14	16	18	20
	1000	14	20	30	–[b]
	750	?	23	26	30
purified (>99% pure)	1500	24	25	27	29
	1250	21	23	24	27
	1000	25	–	–	–
	750	–	–	–	–

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand. [b] This stage of ataxia was not observed.

Table III. Delayed Neurotoxicity of Technical vs. Purified Ethyl Leptophos Administered as Daily Oral Doses to Hens

compd	dose, mg/kg	no. days treated	mean days after treatment when ataxia was observed T_1[a]	T_2	T_3	T_4
technical (89% pure)	10	24	–[b]	–	–	–
	50	24	–	37	40	52
	10	45	–	–	–	–
	50	45	–	49	63	67
purified (>99% pure)	10	24	–	–	–	–
	50	24	–	36	38	49
	10	45	–	–	–	–
	50	45	–	43	52	64

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand. [b] This stage of ataxia was not observed.

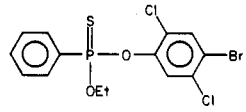
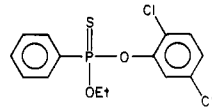
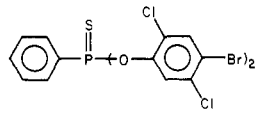
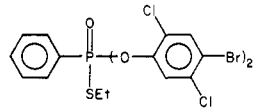
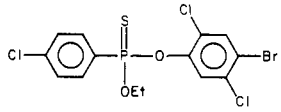
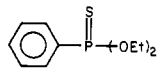
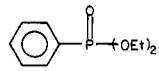
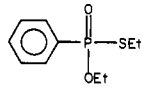
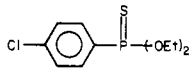
Table IV. Effects of Storage at 40 °C on the Delayed Neurotoxicity of Technical Ethyl Leptophos Administered Orally to Hens

compd	dose, mg/kg	no. treated	mean days after treatment when ataxia was observed T_1[a]	T_2	T_3	T_4
technical	1250	3	14	16	18	20
	1000	3	14	21	29	31
	750	3	–[b]	23	25	30
technical (stored)	1250	3	23	26	–	–
	1000	3	–	–	–	–
	750	3	–	–	–	–

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand. [b] This stage of ataxia was not observed.

stability during storage at 40 °C for 4.5 months. As shown in Table IV, the MED for technical material after storage was 1250 mg/kg compared to at most 750 mg/kg for nonstored material. The delay period was also ~10 days longer for the stored material than for the nonstored material. When these values are compared with those for technical and purified ethyl leptophos shown in Table II, the MED and delay period for technical material after storage are similar to those of purified ethyl leptophos. The reason for the change in delayed neurotoxic pattern is not readily evident in light of the apparent stability of

Table V. Delayed Neurotoxicity of Some Impurities in Technical Ethyl Leptophos Administered Orally to Hens

compd	no. hens treated	dose, mg/kg[a]	no. ataxic[b]
	3	1250	3
	3	1000	0
	3	1000	0
	3	200	0
	3	100[c]	0
	3	500	3
	3	250	3
	3	500[d]	0
	3	200	3

[a] Highest dose tested. [b] Showing any stage of ataxia. [c] This compound was tested both orally and intraperitoneally. [d] Required several doses of atropine to offset acute effects.

the technical material during storage.

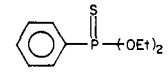
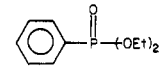
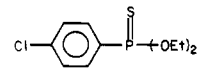
An analysis of the delayed neurotoxicity of organophosphorus impurities present in technical ethyl leptophos in excess of 0.1% is shown in Table V. While none of the aryl esters tested except ethyl leptophos caused any sign of ataxia at the doses examined, each of the *O,O*-diethyl esters caused severe ataxia. The *O,S*-diethyl phenylphosphonothioate, however, was not neurotoxic at 500 mg/kg.

A more extensive study of the diethyl esters is summarized in Table VI. The MED for *O,O*-diethyl phenylphosphonothioate was 150 mg/kg and was 100 mg/kg for its oxon analogue, *O,O*-diethyl phenylphosphonate. *O,O*-Diethyl (4-chlorophenyl)phosphonothioate was neurotoxic at 150 mg/kg. The delay period for each compound was generally 10–12 days for the first signs of ataxia but complete paralysis developed within 2–6 days.

This delay pattern was similar to that observed for hens given technical ethyl leptophos, shown in Table II. It now seems evident that the difference in delayed neurotoxicity between technical and purified ethyl leptophos is due to these diethyl phenylphosphonate impurities.

Johnson (1975) proposes that the specificity of a neurotoxic organophosphorus compound depends on its ability to react with neurotoxic esterase, but it is very likely to have detoxification and excretion pathways common to other nonneurotoxic organophosphates and possibly other

Table VI. Delayed Neurotoxicity of *O,O*-Diethyl Esters of Phenylphosphonothioate, Phenylphosphonate, and (4-Chlorophenyl)phosphonothioate

compd	dose, mg/kg	no. treated	mean days after treatment when ataxia was observed: T_1[a]	T_2	T_3	T_4
	500	3	–[b]	10	11	13
	300	3	–	12	13	15
	200	3	–	11	12	16
	150	3	–	–	10	14
	100	3	?	–	–	–
	250	3	–	–	10	13
	100	3	20	–	–	–
	200	3	11	12	15	18
	150	1	–	14	16	18
	125	1	–	–	–	–
	100	3	–	–	–	–

[a] T_1 = clumsy; T_2 = drunken; T_3 = sitting on hocks; T_4 = unable to stand. [b] This stage of ataxia was not observed.

compounds also. If those pathways are saturated or inhibited by another compound, the threshold dose of a neurotoxic compound is likely to be decreased. Such was reported to be the case with a series of alkyl esters of DDVP (Albert and Stearns, 1974). Lotti and Johnson (1978) have also reported that organophosphorus esters may have an affinity for both acetylcholinesterase and neurotoxic esterase, but that one may predominate over the other; whichever is greatest will dictate the physiological response. It is possible that the mixture of impurities present in technical ethyl leptophos is potentiating its delayed neurotoxicity, but additional studies are necessary to establish the mechanism involved. Since these diethyl phenylphosphonate impurities are probably present in other *O*-ethyl phenylphosphonothioate pesticides, e.g., EPN, cyanofenphos, and S-Seven (Abou-Donia, 1979), their effect on the delayed neurotoxic potential of such compounds should be investigated. The unexpectedly high neurotoxic activity observed for *O,O*-diethyl phenylphosphonothioate and phenylphosphonate is also of considerable interest since, in contrast to other neurotoxic organophosphorus esters, these compounds are not considered to be active inhibitors of esterases owing to their relatively inert nature. Further studies of these esters are continuing.

Supplementary Material Available: Fragment ions with relative intensities for each peak (8 pages). Ordering information is given on any current masthead page.

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Lotti, M.; Johnson, M. K. *Arch. Toxicol.* **1978**, *41*, 215.
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Effects of Dietary Hexachlorobenzene on in Vivo Biotransformation, Residue Deposition, and Elimination of Certain Xenobiotics by Rats

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The effect of hexachlorobenzene (HCB) pretreatment of male albino rats on the in vivo biotransformation, residue deposition, and elimination of radiocarbon-labeled aldrin, 1-naphthol, DDT, HCB, and mirex was investigated. In rats pretreated with 250 ppm of dietary HCB for 4 weeks, the percentage of ^{14}C associated with the more polar urinary metabolites was either increased (aldrin; mirex), decreased (HCB), or unchanged (DDT; 1-naphthol). There was no evidence of qualitative changes in biotransformation of any of the five ^{14}C-labeled test compounds that could be attributed to HCB pretreatment. Rats fed the HCB diet and subsequently treated with [^{14}C]aldrin retained less radiocarbon residues in adipose and kidney tissue than comparably treated control rats retained, whereas rats fed the HCB diet and subsequently treated with [^{14}C]DDT or [^{14}C]mirex retained more radiocarbon residues in their adipose tissue than control rats retained. There were no differences due to HCB pretreatment in tissue radiocarbon residues of rats treated with [^{14}C]HCB or [^{14}C]-1-naphthol. Excretion rates of radiocarbon in HCB-diet rats were enhanced after treatment with each of the radiocarbon-labeled compounds. Rats fed the HCB diet gained more body weight than controls during the 4-week pretreatment period but subsequently lost more weight than controls during the 2 weeks posttreatment.

Since 1945, hexachlorobenzene (HCB) has been used worldwide as a fungicidal seed treatment on wheat and other small grains. In addition to its uses in agriculture, HCB is used as an additive for certain military pyrotechnic compositions, as a porosity controller in the manufacture of electrodes, as a chemical intermediate in dye manufacture and organic synthesis, and as a wood preservative (Mumma and Lawless, 1975). The primary commercial use of HCB in 1974 was as a peptizing agent in the manufacture of nitroso- and styrene-type rubber automobile tires. HCB is produced as a byproduct of industrial chlorination processes (including the manufacture of carbon tetrachloride, perchloroethylene, and trichloroethylene) and the production of chlorine by the electrolysis of brine (Mumma and Lawless, 1975).

HCB is a relatively stable, widespread environmental contaminant (U.S. EPA, 1973; National Academy of Sciences, 1975), and its misuse has resulted in serious human health problems as well as a major incidence of livestock contamination (U.S. EPA, 1973). In the early 1960s, an outbreak of cutaneous porphyria affecting over 5000 persons in Turkey resulted from consumption of bread prepared from HCB-treated seed (Schmid, 1960). In late 1972, HCB residues resulted in the quarantine of over 20 000 cattle in Louisiana. Apparently HCB spilled or blown from open trucks hauling industrial waste from a perchloroethylene plant to a dump was the primary source of the HCB contamination (U.S. EPA, 1973; Himbry et al., 1975).

Because HCB is an environmental contaminant of significance and is a potent inducer of the hepatic mixed function oxidase enzyme systems, (Rajamanickam and Padmanaban, 1974; Stonard and Nenov, 1974; Turner and Green, 1974; Koss and Koransky, 1975; Mehendale et al., 1975; Stonard, 1975; Iverson, 1976), we need to obtain information regarding its interactions with various components of the environment. The current study was undertaken to evaluate certain aspects of the interactions of HCB with mammals: specifically, its effects on in vivo biotransformation, residue deposition, and excretion of certain other chemicals by the laboratory rat. The chemicals selected for this study, aldrin, DDT, HCB, mirex, and 1-naphthol, represent a range of polarity and lipid solubilities and have functional groups that are subject to various biotransformation pathways (Gunther et al., 1968; Menzie, 1969, 1974).

MATERIALS AND METHODS

Chemicals. Unlabeled HCB (>99% purity) was obtained from Chem Service, Inc., West Chester, PA. Uniformly labeled [^{14}C]HCB (99% radiochemical purity,

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