Effect of Impurities on the Mammalian Toxicity of Technical Malathion and Acephate

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A number of impurities present in technical malathion and acephate were isolated by column and thin-layer chromatography and the structures of 11 impurities in malathion and seven impurities in acephate were determined by nuclear magnetic resonance, infrared and mass spectroscopy, and by gas and thin-layer chromatography. Several of the impurities potentiated rat toxicity of purified malathion and highest potentiating activity was observed with *O,S,S*-trimethyl phosphorodithioate and the *S*-methyl isomeride of malathion. Potentiating effects were significantly smaller in the mouse compared with the rat. 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 which were noticeably more toxic to mice. In contrast, a significant reduction in mouse toxicity was observed after storage of technical acephate under the same conditions.

Enhancement of the toxicity of insecticides and other biologically active compounds by synergists or potentiators is a well-known phenomenon. In the case of insecticides, synergists have been used for many years to increase the effectiveness of these materials in controlling pest insects. While synergism of insecticides by adjuvants is in principle a useful occurrence, the same phenomenon in warm-blooded animals is obviously a matter of considerable concern. Numerous examples are known where enhancement of toxicity has been observed when a combination of chemicals is administered to a mammal. Perhaps the first example of this is the potentiation of the mammalian toxicity of malathion by EPN which was observed over 20 years ago and subsequently reported by Frawley et al. (1957). Although the above example cites a case of potentiation between two insecticides, potentiation also is caused by minor impurities present in technical organophosphorus insecticides. An early example of this was discovered by Casida and Sanderson (1963) who reported the potentiation of dimethoate by a polar impurity, possibly the *O*-demethylated product of dimethoate. More recently Pellegrini and Santi (1972) have described the potentiating activity of a variety of simple trimethyl phosphorothioate and phosphorodithioate esters on the mammalian toxicity of such safe organophosphorus insecticides as phenthoate and malathion. This report is concerned with the identification of the major impurities which are commonly present or may be developed upon storage in technical malathion and acephate, and the evaluation of the effect of the impurities on the mammalian toxicity of the purified insecticides.

MATERIALS AND METHODS

General. All melting points were obtained on a Fisher-Johns melting point apparatus and were uncorrected. Infrared (IR) spectra were taken on a Perkin-Elmer Model 700A or Beckman Model 4240 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were determined in a Varian T-60 spectrometer using tetramethylsilane as the internal standard.

Silica gel F-254 (EM Laboratories, Inc.) or QIF (Quantum Industries) plates of 0.25 mm thickness were used for analytical thin-layer chromatography (TLC). Preparative TLC was conducted by using silica gel QIF or GF (Analtech, Inc.) plates of 0.5 or 1.0 mm thickness. Several different solvent systems were used and these are indicated as appropriate in subsequent sections. Location of the compounds on TLC plates was accomplished by 2,6-dibromoquinone-4-chloroimide (DBQ) spray reagent (Menn et al., 1957), iodine vapor, or ultraviolet detection. Silicic acid (Mallinckrodt cc-7) was used for column chromatography.

Mass spectra of purified compounds were determined by direct insertion probe in a Finnigan Model 1015 mass spectrometer interfaced with a data acquisition and reduction system (System 150). Unless otherwise specified, the ionizing voltage was 70 eV. Gas chromatography (GC) was carried out in a Varian Aerograph Model 1400 gas chromatograph equipped with a glass, $^{1}/_{4}$ in. × 6 ft column and alkaline flame ionization detector. Packing material consisted of 5% OV-101 or 4% AN-600 on Gas-Chrom Q (80–100 mesh). The carrier gas (usually nitrogen) flow rate was 32 mL/min. The same instruments were used for GC–mass spectrometry utilizing a 4% AN-600 column and helium as the carrier gas (35 mL/min). Samples in ethyl acetate (1 mg/mL) were injected.

Chemicals. Technical malathion and acephate were obtained through the Environmental Protection Agency from the repository at the Battelle Memorial Institute, Columbus, Ohio. Samples of purified (99.3%) and technical (95%) malathion also were obtained from the American Cyanamid Co., Princeton, N.J. Purified acephate (99.3%) was obtained from the Chevron Chemical Co., Richmond, Calif. *O,O*-Dimethyl phosphoramidothioate, *O,S*-dimethyl phosphoramidothioate (methamidophos), sodio *O,S*-dimethyl phosphorothioate, *O,O*-dimethyl *N*-acetylphosphoramidothioate, sodio *O*-methyl and sodio *S*-methyl *N*-acetylphosphoramidothioate were available from previous studies (Quistad et al., 1970; Kao and Fukuto, 1977; Larson, 1975).

O,O,S-Trimethyl phosphorodithioate (Norman et al., 1952) was prepared by heating at reflux for 3 h a mixture of sodio *O,O*-dimethyl phosphorodithioate and excess dimethyl sulfate in methanol. The product, a colorless liquid, was distilled at 80–82 °C (7.0 mm), n^{25}_{D} 1.5263. *O,O,S*-Trimethyl phosphorothioate (Hilgetag et al., 1960) was similarly prepared from the potassium salt of *O,O*-dimethyl phosphorothioic acid and dimethyl sulfate. The product distilled at 42–44 °C (0.04 mm), n^{25}_{D} 1.4558.

O,S,S-Trimethyl phosphorodithioate (Pellegrini and Santi, 1972) was prepared as follows. *O,O,S*-Trimethyl phosphorodithioate and a 10% mole excess of sodium ethyl

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xanthate in methanol was heated at reflux for 3 h. The mixture was concentrated to dryness and the residual solid, sodio *O,S*-dimethyl phosphorodithioate, was methylated with dimethyl sulfate. The crude product was purified by column chromatography, using silicic acid and progressive elution with benzene and benzene–ethyl acetate (1:4 and 1:1). The product was recovered in the 1:1 benzene–ethyl acetate fraction.

O,S-Dimethyl *S*-(1,2-dicarboethoxy)ethyl phosphorodithioate was prepared from malathion. A mixture of malathion and 10% excess of sodium ethyl xanthate in methanol was heated at reflux for 2 h. Concentration of the mixture gave sodio *O*-methyl *S*-(1,2-dicarboethoxy)ethyl phosphorodithioate which was purified by silicic acid column chromatography, eluting progressively with hexane, ethyl acetate, and ethyl acetate–methanol (4:1). The intermediate was recovered in the ethyl acetate–methanol fraction. The sodium salt was treated as usual with dimethyl sulfate, yielding the final product which was purified by column chromatography, eluting progressively with hexane, benzene, and benzene–ethyl acetate (9:1 and 7:3). The product was recovered in the 7:3 benzene–ethyl acetate fraction.

Malathion monoacid was prepared as previously described (March et al., 1956) and separated into the α and β isomers according to Welling et al. (1970). Diethyl mercaptosuccinate and tetraethyl dithiosuccinate were prepared according to literature methods (Ailman, 1965).

The structures of all synthesized compounds were routinely verified by NMR.

Purification of Malathion and Acephate. High-purity malathion was obtained by repeated recrystallization from methanol. Technical malathion was dissolved in methanol at room temperature and chilled slowly with stirring in dry ice–methanol to -50 °C. The crystalline malathion was collected on a chilled Büchner funnel and recrystallized four more times. Purification of malathion also was achieved by column chromatography, using silicic acid and sequential elution with hexane–benzene and benzene–ethyl acetate solvents. Details of the procedure are given in the section describing the separation of malathion impurities. Purified acephate, mp 90 °C, was obtained by repeated recrystallization from ethyl acetate–ether (1:1) solvent.

Determination of Impurities. Impurities present in technical malathion and acephate were determined by TLC and GC. TLC analyses were carried out by the method of Pellegrini and Santi (1972). Identification of the impurity was achieved by cochromatography with authentic standards and quantification was achieved by visual estimation of the intensity of the color developed with DBQ reagent relative to known amounts of the standard. In the case of GC, quantification was accomplished by comparing the height of the peak with those of the standard.

Effect of Storage on Technical Malathion and Acephate. Samples (1.0–1.5 g) of technical malathion and acephate were sealed in glass ampules and placed in constant temperature cabinets at 20 and 40 °C. The ampules were opened at intervals of 3 and 6 months and examined for content and toxicity to mice.

Toxicological Evaluation. Rat and mouse oral LD_{50} determinations were made with 175–200-g female albino rats (Sprague-Dawley derived) and 22–27-g Swiss white mice obtained from Simonsen Laboratories, Gilroy, Calif. Solutions of the toxicants in corn oil, propylene glycol, or water were administered orally at 1.0 mL/200-g rat and 0.10 mL/mouse. The animals were fasted for 6 h before

Table I. Toxicity of Purified and Technical Malathion to Rats, Mice, and Houseflies

Malathion	LD_{50} rat, mg/kg	LD_{50} mouse, mg/kg	LD_{50} housefly, μg/g
Purified I[a]		3200	29.0
Purified II[b]	9500	3000	
Purified III[c]	12500	3600	
Technical I[d]	1500	1850	29.0
Technical II[e]		1850	

[a] Purified by column chromatography. [b] Obtained from the American Cyanamid Company (99.3% pure). [c] Purified by recrystallization. [d] Obtained from the EPA Battelle Repository. [e] Obtained from the American Cyanamid Company (95% pure).

treatment and kept under observation for 2 days. LD_{50} values were based on 24-h mortality using 16 to 20 rats (four at each dose) and 20 to 30 mice (five at each dose). LD_{50} values were determined by probit analysis. Housefly toxicity was determined as previously described (March and Metcalf, 1949).

RESULTS

Toxicity of Purified and Technical Malathion. As shown in Table I, technical malathion (~95% pure) varied in acute rat oral LD_{50} from about 1400 to 1600 mg/kg, while the purified materials gave values of about 9500 (99.3% pure) and 12 500 (recrystallized) mg/kg. In the case of mice, technical malathion varied in acute LD_{50} from 1750 to 2050 mg/kg, while purified materials gave values of 3000 (99.3% pure) and 3600 (recrystallized) mg/kg. No toxicity difference was observed between the purified and technical malathion following topical application to houseflies. These results indicated that impurities present in technical malathion potentiated the toxicity of malathion to rats and mice.

Isolation and Identification of Impurities in Technical Malathion. A sample of technical malathion (95% pure) was initially examined qualitatively by TLC and GC. At least four impurities less polar than malathion (referred to as nonpolar impurities or NPI) were detected by TLC using benzene as the developing solvent, and at least eight impurities more polar than malathion (referred to as polar impurities or PI) were detected using benzene–ethyl acetate (9:1 and 1:1) as solvent. At least 11 impurities were observed by GC.

In order to obtain sufficient amounts of impurities for structure determination, 11 g of technical malathion was subjected to column chromatography with 350 g of silicic acid. Stepwise elution with 50 mL of hexane, 800 mL of hexane–benzene (2:1), 500 mL of hexane–benzene (1:1), 1000 mL of benzene, 1500 mL of benzene–ethyl acetate (9:1), 1000 mL of ethyl acetate, and 500 mL of ethyl acetate–methanol (7:3) separated the impurities in three fractions, i.e., NPI fraction (460 mg) consisting mainly of four nonpolar impurities A–D, malathion fraction (9.821 g) consisting mainly of malathion and small amounts of impurities A–D and polar impurities E–F, and PI fraction (327 mg) consisting mainly of ten polar impurities E–N. The NPI fraction eluted with benzene and benzene–ethyl acetate, malathion fraction with benzene–ethyl acetate, and PI fraction with the last three solvent systems. The entire procedure was repeated three times using a total of 33 g of technical malathion. Figure 1 shows the various impurities detected by TLC. The impurities were designated in alphabetical order according to their R_f values.

The NPI fraction consisted of two major components A and B. Impurities C and D were present in trace amounts and no attempt was made to identify them. The

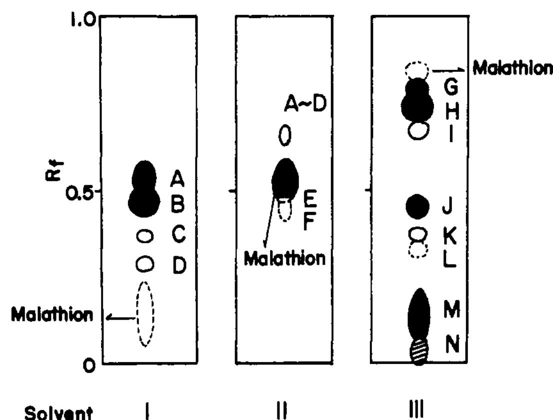

Figure 1. Silica gel thin-layer chromatograms of nonpolar impurities fraction (left), malathion fraction (center), and polar impurities fraction (right). Solvent systems: I, benzene; II, benzene–ethyl acetate (9:1); III, benzene–ethyl acetate (7:3).

NPI fraction (250 mg) was subjected to preparative TLC using a total of seven QIF silica gel plates and two developments with hexane–benzene (7:3). The zone containing A (R_f 0.55–0.65) and B (R_f 0.31–0.41) were collected and extracted with ethyl acetate. Removal of the solvent gave 97 mg of A and 49 mg of B. TLC purification of A was repeated, this time using 1:1 hexane–benzene as the developing solvent. The material at R_f 0.60–0.68 was isolated, giving a light-yellow oil which gave identical R_f values by TLC and retention time by GC as a synthesized sample of *O,O,S*-trimethyl phosphorodithioate. NMR spectrum of A (δ, 60 MHz, $CDCl_3$) showed a doublet at 2.26 (3 H, SCH_3, J = 16 Hz) and a doublet at 3.73 (6 H, OCH_3, J = 16 Hz). Mass spectrum gave the following major peaks: m/e 172 [M^+] (52.4%), 141 (5.6%), 126 (16.4%), 125 (57.1%), 109 (18.0%), 93 (100%), 79 (42.8%), and 63 (57.1%). Both NMR and mass spectra were in agreement with the synthesized sample of *O,O,S*-trimethyl phosphorodithioate, thus confirming the structure of A.

Repeated TLC of B, again using 1:1 hexane–benzene solvent, gave a light-yellow oil (R_f 0.50–0.55, two migrations) which still contained a trace of another component. Two-dimensional development showed that B decomposed to a small extent on the silica gel plates. NMR spectrum of the oil, however, showed only a doublet at 3.87 (J = 17 Hz), indicating the presence of the $POCH_3$ moiety. Infrared spectrum gave relevant absorptions which supported the presence of $POCH_3$ and P=S moieties. Impurity B was subjected to GC–mass spectrometry using a 6-ft column packed with 4% AN-600 on Gas-Chrom Q. Conditions for optimum isolation of B were found with the temperature programmed at 4 °C/min over a range of 130–185 °C. The ionizing voltage was set at 17 eV in order to detect the molecular ion. Under these conditions, B gave the following major peaks: m/e 282 [M^+] (4.3%), 188 (18.1%), 157 (2.3%), 156 (23.7%), 125 (29.1%), 93 (100%), 79 (10.0%), and 63 (4.3%). The mass spectrum, therefore, ruled out *O,O,O*-trimethyl phosphorothioate and bis(dimethoxyphosphinothioyl) disulfide and confirmed bis(dimethoxyphosphinothioyl) sulfide as the structure of B.

The PI fraction (945 mg) was subjected to column chromatography using 45 g of silicic acid and stepwise elution with solvents consisting of 50 mL of hexane, 130 mL of hexane–benzene (1:1), 200 mL of benzene, 300 mL of benzene–ethyl acetate (19:1), and 200 mL each of 9:1, 4:1, 1:1 benzene–ethyl acetate, 130 mL of ethyl acetate, and 200 mL of ethyl acetate–methanol (7:3). After the first 500 mL, a total of 56 fractions (20 mL each) were collected and TLC or GC analysis showed the presence of at least 12 components. Fractions no. 21–25 (188 mg), containing mostly G and a small amount of H, were combined, and 100 mg was subjected to TLC using hexane–ethyl acetate (19:1) as solvent (two migrations). Extraction of the R_f zone 0.49–0.58 gave G, a yellow oil whose mass spectra showed a molecular ion at m/e 410. NMR spectrum showed two triplets at 1.27 and 1.32 (6 H, CH_3, J = 7 Hz), multiplet at 2.47–3.27 (2 H, CH_2), multiplet at 3.60–3.90 (1 H, CH), and two quartets at 4.12 and 4.20 (4 H, OCH_2, J = 7 Hz). Infrared spectrum showed the following absorptions (thin film, cm^{-1}): 2975, 2930, 2900, 2865, 1730, 1465, 1445, 1405, 1390, 1370, 1345, 1320, 1300, 1255, 1230, 1200, 1155, 1090, 1025, 975, 935, 885, 850, and 785. The spectroscopic data provided evidence that G was tetraethyl dithiosuccinate. Both isolated and synthesized tetraethyl dithiosuccinate gave identical TLC properties, NMR, IR, and mass spectra. Based on the recovery by the separation procedure, the amount of G in technical malathion was estimated to be >0.3%.

Fractions no. 26–29 consisted of H (151 mg) contaminated by a small amount of G. Analytical TLC using hexane–ethanol (19:1) separated H into two components (two migrations), H-1 (R_f 0.49) and H-2 (R_f 0.43). Preparative TLC of 100 mg of H gave 21 mg of H-1 and 48 mg of H-2. H-1 gave a faint orange color to DBQ reagent while H-2 gave a red color. Neither compound responded to the alkaline flame ionization detector, indicating the absence of phosphorus. NMR spectra showed no proton absorptions for $POCH_3$ or $PSCH_3$ but revealed the presence of the diethyl succinyl moiety. In fact, the NMR spectrum of H-1 was virtually identical with that of G. The mass spectra of H-1 gave a molecular ion peak at m/e 378 [M^+]. Based on these data, the structure of H-1 was concluded to be tetraethyl thiosuccinate. The NMR spectrum of H-2 was similar to that of G and H-1 but had distinct differences. The peaks attributable to protons in the succinic acid moiety were shifted slightly downfield, and the splitting pattern was less complex. The structure was not discernible from the mass spectrum and H-2, therefore, remains an unknown although it is probably similar in nature to G and H-1. Based on recovery in the separation procedure, the amount of H-1 and H-2 in technical malathion was ≥0.5%.

Fractions no. 42–45, containing compounds J, K, L, and M (50 mg), were combined and subjected to preparative TLC using benzene–ethyl acetate (7:3) solvent. The zones at R_f 0.56–0.69 (J), 0.28–0.56 (K and L), and 0.04–0.28 (M) were extracted with ethyl acetate and each fraction was further purified by preparative TLC. The NMR spectrum of J showed two triplets at 1.25 and 1.28 (6 H, CH_3, J = 7 Hz), doublet at 2.40 (3 H, SCH_3, J = 17 Hz), multiplet at 3.07 (2 H, CH_2, J = 8 Hz), doublet at 3.88 (3 H, OCH_3, J = 14 Hz), and two quartets at 4.17 and 4.25 (4 H, OCH_2, J = 8 Hz). The NMR data suggested that J was *O,S*-dimethyl *S*-(1,2-dicarboethoxy)ethyl phosphorodithioate and, therefore, this compound was synthesized and compared with J. The NMR and IR spectra, TLC and GC properties of J and the synthesized material were identical, and J was concluded to be the *S*-methyl isomeride of malathion. The amount of J in technical malathion was approximately 0.2% as estimated by TLC.

The R_f 0.28–0.56 zone obtained by TLC of fractions no. 42–45 contained impurities J, K, and L. L gave a yellow color on TLC plates upon treatment with DBQ reagent and its TLC movement corresponded to that of *O,S,S*-trimethyl phosphorodithioate after development with a variety of solvent systems. L was separated from J and K by preparative TLC using benzene–ethyl acetate (7:3)

and analyzed by GC. The retention time of L was identical with that of *O,S,S*-trimethyl phosphorodithioate, thus confirming its structure. Based on GC, the amount of L in technical malathion was estimated to be 0.003%.

The NMR spectrum of M gave a triplet at 1.27 (3 H, CH_3, J = 7 Hz), a multiplet at 2.90–3.13 (2 H, CH_2), doublet at 3.80 (6 H, OCH_3, J = 16 Hz), quartet at 4.23 (2 H, OCH_2, J = 7 Hz), and a broad singlet at 6.5–7.0 (1 H, OH). M gave a red color on TLC plates with DBQ reagent. The results suggested that M must be α- or β-malathion monoacid, i.e., either *O,O*-dimethyl *S*-(1-carboxy-2-carboethoxy)ethyl phosphorodithioate or the isomeric *S*-(1-carboethoxy-2-carboxy)ethyl analogue. The NMR, IR, and TLC properties of M were compared with those of synthesized α- and β-malathion monoacid, and the results conclusively showed that M was the β-monoacid. The amount of M in technical malathion was estimated to be 0.6–0.8% based on estimation of color development on TLC plates.

PI fractions no. 54–56 were subjected to TLC analysis using three different solvent systems: benzene–ethyl acetate (1:1), benzene–ethyl acetate–acetic acid (50:50:3), and hexane–ether–acetic acid (40:60:3). Each chromatogram showed two major spots with DBQ reagent, one corresponding to β-malathion monoacid, R_f 0.19–0.33, 0.56, and 0.26, respectively. The other spot representing impurity N cochromatographed with synthesized α-malathion monoacid with R_f values of 0–0.18, 0.52, and 0.20, respectively. The amount of N or α-malathion monacid in technical malathion was 0.1–0.15% as estimated by TLC.

PI fraction no. 17, consisting of malathion and impurity F (99 mg total), was subjected to preparative TLC using hexane–ether (3:1) as the developing solvent (two migrations). Extraction of the zone at R_f 0.39–0.47 gave a viscous oil (20 mg) which gave a red color with DBQ and responded to alkaline flame ionization detection in GC. The NMR spectrum of F showed two triplets at 1.25–1.27 (3 H, CH_3, J = 7 Hz), a multiplet of 2.85 (2 H, CH_2, J = 7 Hz), a singlet at 3.65 (3 H, OCH_3), a doublet at 3.90 (6 H, $POCH_3$, J = 14 Hz), and a multiplet at 4.0–4.2 (2 H, OCH_2). The NMR data suggested a structure for F similar to malathion except for the presence of only one carboethoxy moiety, the other carboethoxy being replaced by a carbomethoxy group. GC–mass spectrometry at an ionizing voltage of 17 eV gave the following major peaks: m/e 159 (100%), 158 (32.0%), 127 (12.0%), 125 (8.0%), 113 (40.0%), and 93 (12.0%). Under the operating conditions, a peak for the molecular ion at m/e 316 could not be obtained. However, the fragmentation peaks at 159 (100%) and 158 (32%) confirmed the presence of the methyl ethyl succinate and dimethyl phosphorodithioate moieties, respectively. Malathion has been shown to fragment in a similar manner, giving diethyl succinate (m/e 173) as the base peak and a peak at m/e 158 (Damico, 1969). Impurity F, therefore, is a mixture of *O,O*-dimethyl *S*-(1-carbomethoxy-2-carboethoxy)ethyl phosphorodithioate or the corresponding 1-carboethoxy-2-carbomethoxy analogue.

Pellegrini and Santi (1972) reported the presence of a small amount (0.1%) of *O,O,S*-trimethyl phosphorothioate in technical malathion. This compound, however, was not detected in the PI fraction where it was expected. Therefore, a sample of technical malathion (101 mg) was subjected directly to preparative TLC using two silica gel plates and benzene–ethyl acetate (7:3) as the solvent. The R_f zone 0.33–0.50, the place where synthesized *O,O,S*-trimethyl phosphorothioate was localized, was extracted and subjected to GC analysis. A peak (impurity O) of retention time identical with *O,O,S*-trimethyl phosphorothioate was detected. The amount of impurity O or *O,O,S*-trimethyl phosphorothioate in technical malathion was 0.04% as estimated by GC.

The malathion fraction contained, besides malathion, impurities E and F. The R_f value of E was very close to that of malathion and consistently migrated with malathion with a variety of eluting solvents. In contrast to malathion, E gave an immediate yellow color after spraying with DBQ (at this time malathion gave no color) and, after heating at 120 °C for 5 min, the color turned brown. These observations, along with TLC comparison using a variety of solvent systems, indicated that E was identical with a synthesized sample of diethyl α-mercaptosuccinate.

The structures of the various impurities identified in technical malathion, along with amounts detected and TLC and GC characteristics, are presented in Table II.

Toxicological Evaluation of Malathion Impurities. Of the impurities identified in technical malathion, eight were examined for their toxicological effects when added to purified malathion. Impurity B or bis(dimethoxy)phosphinothioyl) sulfide was not examined since this material, synthesized by an independent method, itself contained a number of impurities. Table III contains toxicological data for the effect of the various impurities on the toxicity of purified malathion to the rat and mouse. Included also are data for the toxicity of the impurity itself to each animal (indicated as 100% impurity in the table). It is evident from the data that *O,O,S*-trimethyl phosphorodithioate (A), *S*-methyl isomeride of malathion (J), *O,S,S*-trimethyl phosphorodithioate (L), *O,O,S*-trimethyl phosphorothioate (O), and malathion monoacid all increase the toxicity of purified malathion to mice and rats. Based on the log dose–probit response (LD–p) lines for toxicity determination and the amount of each impurity added to malathion, it can be concluded that the increase in toxicity is attributable to potentiating and not to additive effects. The potentiation of phenthoate and malathion by A, L, and O was previously reported by Pellegrini and Santi (1972).

Potentiating effects were decidedly greater in the rat compared with the mouse. This becomes apparent when the toxicity ratios (relative to the toxicity of purified malathion) are compared. For example, impurity L added to purified malathion at 1.0% increased mouse toxicity 1.4-fold while 0.05% L added to malathion increased rat toxicity about fourfold. Of the various impurities examined, L showed the highest potentiating activity to rats, followed by the *S*-methyl isomeride of malathion (J) and A. In light of the relatively large amounts of A and J in technical malathion and their potentiating activity, these two impurities undoubtedly contribute to the higher toxicity of technical malathion. Although L showed the highest potentiating activity, it was present to only a minor extent in technical malathion (0.003%). It is noteworthy that A, J, L, and O, impurities which potentiated rat toxicity to a higher degree than mouse toxicity, were all intrinsically more toxic to the rat than to the mouse.

Impurities E and G, i.e., diethyl mercaptosuccinate and the corresponding disulfide, are nonphosphorus containing impurities and showed no potentiating activity. In fact, both compounds appeared to antagonize malathion toxicity in mice since 5% E or G added to purified malathion reduced mouse toxicity by 32 and 28%, respectively.

Toxicity of Purified and Technical Acephate. As shown in Table IV, technical acephate varied in acute oral LD_{50} from 1580 to 2100 mg/kg for rats and 480 to 520 mg/kg for mice, while purified (recrystallized) acephate

Table II. Structures and TLC and GC Properties of Impurities Present in Technical Malathion

Impurity	Structure	Amount present, %	Color DBQ	TLC R_f[a] I	II	III	IV	Rel. retention times, s 5% OV-101[b]	4% AN-600[c]	4% AN-600[d]
A	$(CH_3O)_2P(S)SCH_3$	1.1	Red	0.68	0.86		0.55	15		33
B	$(CH_3O)_2P(S)SP(S)(OCH_3)_2$	~0.5	Red	0.67	0.86		0.46	84		
E	$HSCH(COOEt)CH_2COOEt$	Tr	Brown	0.47	0.82		0.0			
F	$(CH_3O)_2P(S)SCH(COOEt)CH_2COOCH_3$ and $(CH_3O)_2P(S)SCH(COOCH_3)CH_2COOEt$	>0.1	Red	0.45	0.81			147		
G	$[SCH(COOEt)CH_2COOEt]_2$	>0.3	Orange	0.43	0.80					
H-1	$S[CH(COOEt)CH_2COOEt]_2$	>0.5[e]	Light orange	0.37	0.79					
J	$(CH_3O)(CH_3S)P(O)SCH(COOEt)CH_2COOEt$	0.2	Yellow to brown	0.12	0.56			216		
L	$(CH_3S)_2P(O)OCH_3$	0.003	Yellow	0.07	0.40				54	100
M	$(CH_3O)_2P(S)SCH(COOEt)CH_2COOH$	0.6–0.8	Red	0.05	0.17	0.50				
N	$(CH_3O)_2P(S)SCH(COOH)CH_2COOEt$	0.1–0.15	Red	0.05	0.05	0.45				
O	$(CH_3O)_2P(O)SCH_3$	0.04	Yellow	0.07	0.31				30	48

[a] Solvent systems: I, benzene-ethyl acetate (9:1); II, benzene-ethyl acetate (1:1); III, benzene-ethyl acetate-acetic acid (50:50:3); IV, hexane-benzene (1:1). [b] Column temperature, 190 °C; port temperature, 220 °C. [c] Column temperature, 170 °C; port temperature, 200 °C. [d] Column temperature, 150 °C; port temperature, 185 °C. [e] Includes H-2.

Table III. Effect of Malathion Impurities on the Toxicity of Purified Malathion to Mice and Rats

Impurity	Mouse toxicity: % added impurity	LD_{50}, mg/kg	Ratio[a]	Rat toxicity: % added impurity	LD_{50}, mg/kg	Ratio[a]
A	5.0	2530	1.27	0.5	6900	1.8
	100[b]	1850		1.0	3200	3.9
				5.0	2450	5.0
				100	660	
E	5.0	4230	0.76	2.0	>8000	
G	5.0	4100	0.78	2.0	>8000	
J	0.5	2350	1.37	0.05	4400	2.9
	2.0	1150	2.78	0.1	3100	4.0
	100	330		0.5	1975	6.3
				1.0	1920	6.7
				2.0	1250	10.0
				100	120	
L	1.0	2230	1.42	0.05	3100	4.2
	2.0	2060	1.57	0.2	2100	5.9
	100	400		0.5	1775	7.2
				100	110	
M, N	5.0[c]	2720	1.18	2.0[d]	8900	1.4
O	1.0	3080	1.04	0.2	4100	3.0
	100	530		1.0	2900	4.4
				100	260	

[a] LD_{50} of recrystallized malathion (rat, 12 500 mg/kg; mouse, 3200 mg/kg) divided by the LD_{50} of recrystallized malathion plus impurity. [b] The value 100% denotes that impurity was used alone. [c] Malathion β-monoacid was used. [d] An approximately 1:1 mixture of M and N was used.

Table IV. Toxicity of Purified and Technical Acephate to Rats, Mice, and Houseflies

Acephate	LD_{50} rat, mg/kg	LD_{50} mouse, mg/kg	LD_{50} housefly, μg/g
Purified (recrystallized)	1480	425	1.65
Technical	1800	500	1.68
Purified + 5% conc. imp.[a]		520	1.30

[a] The concentrated impurities remaining in the mother liquor after recrystallization of acephate were added to purified acephate.

gave values of 1350 to 1650 for rats and 395 to 450 mg/kg for mice. Further, addition of the concentrated impurities (5% by weight), obtained from the mother liquor after recrystallization of technical acephate, to purified acephate decreased the toxicity of the pure material by about 22%. No toxicity difference was observed between the purified and technical acephate following topical application to houseflies. These results suggest that impurities present in technical acephate antagonize mammalian toxicity of acephate.

Isolation and Identification of Impurities in Technical Acephate. Analytical silica gel TLC showed that technical acephate contains a number of impurities but in very small amounts. Therefore, the technical material was recrystallized and the mother liquor was used for isolation of the impurities. Technical acephate (20 g) was dissolved in 1000 mL of ethyl acetate–ether mixture (1:1) by stirring at room temperature for 3 h. At no time was heat applied to the mixture. After removal of insoluble materials, the solution was cooled slowly to -78°C. The solid acephate was collected, the filtrate was concentrated to about 5 mL and chilled again to -78 °C. The mixture was filtered and the solvent was removed from the filtrate to give the concentrated impurities fraction (CI, 868 mg) which was subjected to TLC analysis. At least 11 impurities (I-XI) were detected by DBQ reagent. Seven of

Table V. Structures and TLC and GC Properties of Impurities Present in Technical Acephate

Impurity	Structure[a]	Amount present, %	Color DBQ	TLC R_f[b]				Rel. retention times, s	
				A	B	C	D	4% AN-600[c]	4% AN-600[d]
IV	$(CH_3O)_2P(S)NH_2$	0.07	Red	0.65	0.90	1.0		42	27
V	$(CH_3O)(CH_3S)P(S)NH_2$	Tr	Red	0.55	0.88				60
VI	$(CH_3O)_2P(S)NHCOCH_3$	0.44	Red	0.48	0.84	1.0		150	78
VII	$(CH_3O)_2P(O)SCH_3$	0.20	Yellow	0.31	0.79	0.80	0.62	39	27
VIII	$^+M^-SP(O)(OCH_3)NHCOCH_3$	Tr	Red	0	0.53	0.59			
X	$^+M^-OP(O)(SCH_3)OCH_3$	2.0	Yellow	0	0.22	0.54	0.12		
XI	$^+M^-OP(O)(SCH_3)NHCOCH_3$	Tr	Yellow	0	0.18	0.47	0.05		

[a] M^+ is an unknown cation. [b] Solvent systems: A, benzene–ethyl acetate (1:1); B, acetonitrile–methanol–H_2O (16:3:1); C, ethyl acetate–methanol (1:1); D, diethyl ether–isopropanol (2:3). [c] Column temperature, 160 °C; port temperature, 205 °C. [d] Column temperature, 185 °C; port temperature, 215 °C.

these were identified as follows.

The CI fraction (850 mg) was subjected to column chromatography using 40 g of silicic acid and stepwise elution with the following solvents: 30 mL of hexane; 120 mL of hexane–benzene (1:1); 120 mL of benzene; 100 mL each of 9:1, 4:1, 7:3, and 3:2 benzene–ethyl acetate; 150 mL of benzene–ethyl acetate (1:1); 250 mL of ethyl acetate; and 200 mL of ethyl acetate–methanol (1:1). A total of 47 fractions, approximately 25 mL each and a final fraction of 150 mL were collected. Impurities I, II, and III were extremely minor, and insufficient amounts were recovered for structure analysis. Fraction no. 16 contained IV as the sole impurity. Mass spectral analysis of IV gave the following major peaks: *m/e* 141 [M^+] (27.5%), 111 (20%), 110 (14%), 94 (10%), and 78 (100%). The mass spectral data and TLC properties were identical with that of an authentic sample of *O,O*-dimethyl phosphoramidothioate and, therefore, IV was assigned this structure. The amount of IV in technical acephate was 0.5–0.8%, as estimated by GC.

Fractions no. 17–21 contained IV and V. TLC, using benzene–ethyl acetate (3:2) as the solvent, gave V. Mass spectral analysis gave the following major peaks: *m/e* 158 [M^+] (25.5%), 111 (21.5%), 110 (52.0%), 79 (64.5%), 63 (18.0%), and 47 (35.5%). The molecular ion and fragmentation pattern suggest that V is *O,S*-dimethyl phosphoramidodithioate.

The impurities (38 mg) in fractions no. 24–28, consisting mainly of VI, were subjected to preparative TLC using benzene–ethyl acetate (1:1) as the solvent. The zone at R_f 0.55–0.64, which gave a red color with DBQ, was extracted, yielding a viscous oil (VI). The mass spectrum of this material gave the following major peaks: *m/e* 183 [M^+] (12%), 142 (89%), 141 (21%), 125 (11%), 112 (37%), 111 (35%), 93 (25%), 79 (49%), 78 (100%), 63 (33%), 47 (38%), 43 (73%), and 42 (33%). These data suggested that VI was *O,O*-dimethyl *N*-acetylphosphoramidothioate which was independently synthesized and found to give an identical mass spectrum. VI also showed TLC and GC properties identical with the synthesized material. The amount of VI in technical acephate was 0.46%, as estimated by GC.

Impurity VII, localized in fractions no. 34–38 (30 mg), was purified further by TLC using benzene–ethyl acetate (7:3). The zone at R_f 0.35–0.50 which gave a yellow color with DBQ was extracted, giving 14 mg of an oil. The mass spectrum of VII gave the following major peaks: *m/e* 156 [M^+] (21%), 141 (0.5%), 125 (9%), 110 (81%), 109 (59%), 80 (29.5%), 79 (100%), 49 (100%), and 45 (50%). TLC and GC properties of VII were identical with *O,O,S*-trimethyl phosphorothioate, an impurity which also was present and identified in technical malathion. The mass spectrum of synthesized *O,O,S*-trimethyl phosphorothioate was the same as that of VII, thus confirming its structure. The amount of VII in technical acephate was 0.20%, as estimated by GC.

Fraction no. 47 contained the largest amount of impurities (640 mg) and consisted of acephate and impurities VIII–XI. This fraction was resubjected to column chromatography using 48 g of silicic acid and stepwise elution with 30 mL of benzene, 70 mL of ethyl acetate, 300 mL of ethyl acetate–methanol (4:1), and 300 mL of ethyl acetate–methanol (3:2). After the first 100 mL, a total of 20 fractions (30 mL each) were collected. Concentration of subfractions no. 14–15 yielded X as colorless crystals, mp >360 °C, which gave a single yellow spot on TLC plates with DBQ. The NMR spectrum of X (D_2O) showed only two doublet signals of equal intensities at 2.20 (SCH_3, J = 14 Hz) and 3.67 (OCH_3, J = 12 Hz). The NMR and IR spectra, and TLC properties of X (cf. Table V) were identical with those of an authentic sample of sodio *O,S*-dimethyl phosphorothioate. X, therefore, is *O,S*-dimethyl phosphorothioic acid or a corresponding salt. The amount of X in technical acephate was about 2%, as estimated visually on a TLC plate after color development with DBQ.

Impurities VIII and XI were localized in subfractions 8–11 and 16–18, respectively, and each was further purified by preparative TLC using ethyl acetate–methanol (1:1). In this system, the R_f of VIII was 0.59 and the R_f of XI was 0.47. Because of their highly polar nature, neither compound was amenable to mass spectral analysis. By analytical TLC using different solvent systems (cf. Table V), VIII cochromatographed with sodio *O*-methyl *N*-acetylphosphoramidothioate and XI with sodio *S*-methyl *N*-acetylphosphoramidothioate. VIII and XI were, therefore, identified as salts of *O*-methyl and *S*-methyl *N*-acetylphosphoramidothioic acid, respectively. They were present in trace amounts in technical acephate. Impurity IX was present in a very minute amount and attempt was not made to identify it.

The structures of the various impurities identified in technical acephate, along with amounts detected, and TLC and GC characteristics, are presented in Table V.

Toxicological Evaluation of Acephate Impurities. Table VI contains toxicological data for the effect of impurities IV, VI, VII, and X on the toxicity of purified acephate to mice. The toxicity of each impurity alone to mice also is included. As indicated in the table, IV and X had virtually no effect on the mouse toxicity of acephate. *O,O,S*-Trimethyl phosphorothioate (VII) caused a slight degree of potentiation, e.g., 1% VII added to purified acephate changed the LD_{50} from 570 to 460 mg/kg. In contrast, *O,O*-dimethyl *N*-acetylphosphoramidothioate

Table VI. Effect of Acephate Impurities on the Toxicity of Purified Acephate to Mice

Impurity	% added impurity	Mouse LD_{50}, mg/kg	Ratio[a]
IV	5.0	570	1.0
	100[b]	430	
VI	1.0	720	0.79
	5.0	800	0.71
	100	1150	
VII	0.5	540	1.05
	1.0	460	1.25
	100	530	
X	5.0	570	1.0
	100	>3600	

[a] LD_{50} of recrystallized (570 mg/kg) divided by the LD_{50} of recrystallized acephate plus impurity. [b] The value 100% denotes that impurity was used alone.

Table VII. Effect of Storage on the Toxicity of Technical Malathion and Acephate to Mice and Houseflies

		Malathion LD_{50}		Acephate LD_{50}	
Storage period, months	Temp, °C	Mouse, mg/kg	House-fly, μg/g	Mouse, mg/kg	House-fly, μg/g
0		2400	33.0	500	1.25
3	20	2300		640	
6	20	2350		720	
3	40	2000	32.0	790	1.25
6	40	1780	32.0	820	1.10

(VI) added to purified acephate caused a significant decrease in mouse toxicity. As indicated in the table, VI was one of the major impurities present in technical acephate and it probably contributed to the lower mouse toxicity of the technical material compared with purified acephate.

Considerable variation in the susceptibility of white mice to both technical and purified acephate was noted. For example, the toxicity of purified acephate to mice in Table IV is given as 425 mg/kg, while a value of 570 was used to calculate toxicity ratios in Table VI. The data presented in Table IV were obtained from a single batch of mice purchased from Simonsen Laboratories, while data in Table VI were obtained from mice purchased 6 months later from the same supplier. Owing to variation in mice sensitivity, care was taken to obtain toxicological data in each table with the same batch of mice. Therefore, it is reasonable to compare toxicity data within each table, but data between tables should not be compared.

Effect of Storage on the Toxicity of Technical Malathion and Acephate. The effect of storage of technical malathion and acephate on toxicity was examined at 20 and 40 °C for a period of 6 months. Table VII provides data which show the change in toxicity of these two materials to mice and houseflies during the 6-month storage period. Little change in malathion mouse toxicity was observed after storage at 20 °C, but a notable increase in toxicity was apparent after storage at 40 °C. GC analysis of technical malathion stored at 40 °C for 6 months showed a slight decrease in malathion content, from 95 to 94%, but this small difference was within experimental error. These results showed, however, that a minute change in malathion content and, therefore, the composition of technical malathion had a strong effect on its toxicity to mice. No change in toxicity to houseflies was observed.

In contrast to technical malathion, mouse toxicity of technical acephate decreased with storage. For example, after 6 months storage, technical acephate was about two-thirds as toxic to mice as the original material. Toxicity to houseflies, however, was virtually unchanged, indicating that the decrease in mouse toxicity was not attributable to acephate decomposition.

Table VIII. Effect of Storage on the Change in Amounts of Some of the Impurities in Technical Malathion and Acephate

	Amount of impurity, %				
		20 °C		40 °C	
Impurity[a]	0 month	3 months	6 months	3 months	6 months
In malathion					
A	1.1	0.92	0.88	0.82	0.92
J	0.2	0.2	0.2	0.35	0.45
In acephate					
IV	0.073	0.052	0.04	0.12	0.01
VI	0.44	0.38	0.33	0.30	0.27
VII	0.20	0.20	0.19	0.22	0.20
Methamidophos	0.10	0.25	0.25	0.26	0.36

[a] Analyzed by GC except for J which was analyzed by TLC.

Table VIII gives data obtained by GC or TLC analysis of some of the major impurities present in malathion and acephate during the storage period. Impurities A and J were monitored in malathion and impurities IV, VI, VII, and methamidophos (*O,S*-dimethyl phosphoramidothioate) were monitored in acephate. The results show that the amount of A decreased slightly during the storage period at both temperatures, while J increased about twofold after 6 months storage at 40 °C. In light of the strong potentiating activity of J, the higher concentration of J is in agreement with the increased toxicity of malathion to mice.

Changes in content of some of the impurities present in technical acephate also were noted during the storage period. *O,O*-Dimethyl phosphoramidothioate (IV) and the corresponding *N*-acetyl derivative (VI) both decreased slightly, while *O,O,S*-trimethyl phosphorothioate remained unchanged. An increase in methamidophos content was observed. It should be mentioned that methamidophos was not isolated by the procedures described earlier utilizing column and thin-layer chromatography. Evidently, the chromatographic and solubility properties of methamidophos are very similar to those of acephate and the detection of methamidophos was masked by the large amounts of acephate present. Methamidophos, however, was readily separable from acephate and other constituents by GC.

It is difficult to explain the decrease in mouse toxicity of technical acephate following storage since the content of VI, the impurity which showed antagonizing activity, decreased with time. Methamidophos, in contrast, increased with time, but this material with an LD_{50} to mice of 14 mg/kg (Kao and Fukuto, 1977) is substantially more toxic to mice than acephate. At the present time, it is not possible to account for the decrease in mouse toxicity of acephate after storage and further work is required. It is possible that one or more of the unknown impurities also contribute to antagonism of acephate.

DISCUSSION

Malathion and acephate contain acyl moieties which are vulnerable to hydrolytic degradation in biological systems. The mammalian safety of malathion has been attributed to the susceptibility of the carboethoxy groups to hydrolytic detoxication to the corresponding carboxylic acid derivative. This reaction, mediated by the carboxylesterases, evidently takes place in mammals at a rate faster than the activation reaction leading to malaoxon, the metabolite responsible for inhibition of the cholinesterase

enzymes (Dauterman, 1971). On the other hand, the low mammalian toxicity of acephate and related *N*-acylphosphoramidothioates has been explained on the basis of their ability to avoid hydrolytic activation to the corresponding phosphoramidothioate, i.e., to the more effective anticholinesterase methamidophos (Kao and Fukuto, 1977; Larson, 1975). If the assumption is made that impurities in malathion and acephate are able to interfere with esterase cleavage of the *O*-acyl or *N*-acyl linkages, respectively, then the impurities would be expected to have a potentiating effect on malathion but an antagonizing effect on acephate. The toxicological data were, in general, consistent with this prediction, indicating that esterases are involved in the potentiation of antagonism of malathion and acephate. Work is currently in progress on the mode of action of the toxicological effects produced by the impurities.

Although there were a number of notable exceptions, on the whole the various impurities identified in technical malathion and acephate were those which might normally be expected in these materials. The formation of impurities, of course, would depend on the industrial method of synthesis. The trimethyl phosphorothioate and phosphorodithioate impurities (A, L, and O) were previously reported to be present in phenthoate and malathion by Pellegrini and Santi (1972) and, therefore, were expected. The presence of L and O, however, is not readily explainable, but these were found in only minor amounts. The relatively large amount of bis(dimethoxyphosphinothioyl) sulfide (B) in technical malathion was somewhat of a surprise since this compound is not attainable through conventional synthetic methods. It may have been formed as a side product in the synthesis of the starting *O*,*O*-dimethyl phosphorodithioic acid or by the reaction between this acid and malathion. The latter reaction also would explain the formation of diethyl mercaptosuccinate and its coupled products (E, G, H-1). The presence of the mixed carbomethoxy and carboethoxy esters (F) also was surprising. Evidently methanol was present in the reaction mixture for the preparation of malathion, and ester interchange occurred to a small extent. Malathion is prepared on an industrial scale by the addition of *O*,*O*-dimethyl phosphorodithioic acid to diethyl maleate (Cassady, 1951), and methanol might have been carried forward from the synthesis of the acid.

Acephate is prepared industrially by demethylation and remethylation of *O*,*O*-dimethyl *N*-acetylphosphoramidothioate (VI) (Magee, 1974). The detection of *O*,*S*-dimethyl phosphoramidodithioate was, therefore, completely unexpected, and a logical explanation of its presence is not available since this compound contains two sulfur atoms. The presence of *O*,*O*,*S*-trimethyl phosphorothioate (VII) also is not obvious. It possibly may be formed by reaction between two molecules of VI (or its precursor *O*,*O*-dimethyl phosphoroamidothioate) by S-methylation and subsequent attack by the acyl oxygen on the phosphorus atom via a four-center rearrangement.

Storage, particularly at higher temperatures, has a significant effect on the toxicity of technical malathion and acephate to mice, increased toxicity being observed with malathion and decreased toxicity with acephate. Small changes in the composition of the technical materials evidently cause the toxicity differences. Needless to say, malathion should not be stored for prolonged periods under conditions where it is subjected to consistently high temperatures.

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Received for review December 20, 1976. Accepted March 14, 1977. This investigation was supported from Federal Funds from the Environmental Protection Agency under Contract No. 68-01-1920 and Grant R804345-01. The contents do not necessarily reflect the views and policies of the Environmental Protection Agency, nor does mention of trade names or commerical products constitute endorsement or recommendation for use.

COMMUNICATIONS

2-Ethyl-3-methylbutyric Acid, a New Volatile Fatty Acid Found in Rum

2-Ethyl-3-methylbutyric acid was isolated from rum and identified by coupled GC–MS and IR spectrometry. An NMR spectrum for synthesized 2-ethyl-3-methylbutyric acid is also presented. So far the acid has not been found in any other alcoholic beverage than rum.

Acetic acid forms the major part of the volatile acids in alcoholic beverages, contributing 50–95% in whiskies, about 75% in cognacs and other brandies, and about 80% in rums (Nykänen et al., 1968). These beverages also