Composition of Technical Ethephon [(2-Chloroethyl)phosphonic Acid] and Some Analogues Relative to Their Reactivity and Biological Activity

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The phosphorus-containing components in technical ethephon, $ClCH_2CH_2P(O)(OH)_2$, were assigned by comparisons with standards from synthesis involving ^{31}P NMR and GC/CI-MS (after derivatization with diazomethane). They were $ClCH_2CH_2P(O)(OH)_2$ (90%), $(HO)_2P(O)CH_2CH_2P(O)(OH)_2$ (3%), $ClCH_2CH_2P(O)(OH)OCH_2CH_2Cl$, $ClCH_2CH_2OCH_2CH_2P(O)(OH)_2$, and $H_2C{=}CHP(O)(OH)_2$ (each 1.5–1.8%), and $HOCH_2CH_2P(O)(OH)_2$, $HP(O)(OH)_2$, and $HOP(O)(OH)_2$ (each 0.3–0.6%), consistent with the synthetic route starting with tris(2-chloroethyl) phosphite and involving Arbuzov rearrangement and various hydrolysis and dehydrochlorination reactions. Urinary products of technical ethephon in rats are the parent compound, $HOP(O)(OH)_2$, and unmetabolized $(HO)_2P(O)CH_2CH_2P(O)(OH)_2$. Ethephon and its 2-bromoethyl analogue are more potent than its 2-fluoroethyl and thioic acid analogues and the impurities present in the technical grade material as plant growth regulators (tomato epinasty assay), in vitro inhibitors of plasma cholinesterase, and phosphorylating agents. The biological activity of technical ethephon appears to be associated with the reactions of its principal component, particularly ethylene liberation, and possibly phosphorylating activity.

INTRODUCTION

Ethephon (1) [(2-chloroethyl)phosphonic acid] is a major plant growth regulator (PGR) used to promote flower induction, fruit coloration, and fruit abscission, probably by stimulating the production of ethylene (Moore, 1979; Weed Science Society of America, 1989). It also has an unexpected biological activity for a phosphonic acid since in rats and mice it inhibits plasma cholinesterase (ChE) in a concentration-dependent manner in vitro at 20–100 μM and in vivo at 10–1500 mg/kg without obvious acute toxic effects and with little or no inhibition of acetylcholinesterase (Hennighausen et al., 1977; Hennighausen and Tiefenbach, 1978). The PGR activity and toxicological properties of technical ethephon could in theory be due to direct action of the principal ingredient, its decomposition product(s), or impurities. Ethephon is prepared by rearranging tris(2-chloroethyl) phosphite to bis(2-chloroethyl) 2-chloroethylphosphonate, which is in turn hydrolyzed to obtain 1 (Sittig, 1980). Technical grade ethephon is normally formulated as a concentrated aqueous solution that is highly acidic. It decomposes slowly below pH 3 but rapidly in the more alkaline ranges to produce ethylene and phosphate and chloride ions (Cooke and Randall, 1968; Edgerton and Blanpied, 1968). Under basic conditions it reacts with alcohols or phenols to form monoester alkyl or aryl phosphates, respectively, reflecting its phosphorylating activity (Maynard and Swan, 1963a,b).

This study considers the composition of technical ethephon and some analogues relative to their metabolism and their reactivity as ethylene generators (on the basis of PGR activity), inhibitors of plasma ChE, and alkylating and phosphorylating agents.

MATERIALS AND METHODS

Spectroscopy. ^1H and ^{31}P nuclear magnetic resonance (NMR) spectra, the latter with and without ^1H decoupling, were recorded at 300 and 121.5 MHz, respectively, for solutions in water containing 10% D_2O as the locking solvent (or in other solvent as specified) in 5-mm NMR tubes with a Bruker WM-300 spectrometer. Chemical shifts are referenced to internal sodium 3-(trimethylsilyl)propanesulfonate or tetramethylsilane for ^1H spectra in water or organic solvent, respectively, and to external trimethyl phosphate in water or organic solvent for ^{31}P spectra in water or the appropriate organic solvent, respectively. Chemical shifts are quoted on the δ scale and expressed as number of protons, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constant. Quantitative measurements utilized an inverted gate delayed acquisition pulse sequence (with a 10-s delay between pulses to minimize the nuclear Overhauser effects that would otherwise alter the precision of integration) and hexamethylphosphoramide (HMPA) as the internal standard.

Mass spectrometry (MS) was carried out with a Hewlett-Packard 5985B system operated in the chemical ionization (CI) mode with methane as reagent gas at 0.7 Torr. Pure compounds were analyzed via a direct insertion probe and mixtures by gas chromatography (GC) with a Hewlett-Packard 5840A gas chromatograph, fitted with an SPB5 fused silica capillary column (Supelco Inc., Bellefonte, PA) and using a splitless (1 min) injection at 220 °C. The temperature program used was 40 °C for 2 min and 20 °C/min to 300 °C. All CI-MS spectra of methyl esters gave ions at $[M + 1]^+$, $[M + 29]^+$, and $[M + 41]^+$.

Synthesis of Ethephon Components (Table I). Technical grade ethephon (product PREP-6, code S80866702GA, lot A71063) consisting of 1 (55.4%) and inert ingredients (44.6%, primarily water) was provided by Union Carbide Agricultural Products Co., Inc. (Research Triangle Park, NC). Pure 1 was prepared from the technical material by distilling off the water at reduced pressure, refluxing the brown residue with excess $SOCl_2$ for 12 h, distilling the resulting (2-chloroethyl)phosphonodichloridate (Rochlitz and Vilcsek, 1962) (62 °C/0.4 mmHg), and then hydrolyzing the dichloridate with ice. Removal of the water at 35 °C/0.01 mmHg and recrystallization from chloroform gave pure 1 as white plates: mp 76–77 °C; ^1H NMR (D_2O) δ 3.78 (2 H, dt, J_{P-H} = 13.6 Hz), 2.32 (2 H, dt, J_{P-H} = 17.9 Hz). Ethane-1,2-diphosphonic acid (2), mp 219–220 °C, was prepared from the corresponding tetrachloridate (mp 197–199 °C from acetone), which in turn had been made by cleaving the tetraisopropyl ester, described later, with PCl_5 (4 equiv) and distillation (bp 145 °C/0.5 mmHg). Hydrolysis of the tetrachloridate to obtain 2 involved

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addition of water which was then evaporated and trituration of the residue with acetone: ^1H NMR (D_2O) δ 1.99 (asymmetric quintet). Vinylphosphonic acid (**3**) was obtained in quantitative yield as an oil by dehydrochlorination of (2-chloroethyl)phosphonodichloridate with equimolar triethylamine in ether at room temperature followed by hydrolysis and solvent evaporation as above: ^1H NMR (acetone-d_6) δ 6.14 (m). 2-Chloroethyl (2-chloroethyl)phosphonic acid (**4**) was obtained ~80% pure by heating an equimolar mixture of ethephon and 2-chloroethanol at 100 °C for 45 h followed by vacuum stripping of the reaction mixture at 25 °C/0.1 mmHg for 2 h: ^1H NMR (D_2O) δ 4.21 (2 H, m), 3.79 (4 H, m), 2.34 (2 H, dt, J_{P-H} = 17.9 Hz). A mixture of [2-(chloroethoxy)ethyl]phosphonic acid (**5**) and (2-bromoethyl)phosphonic acid (**15**) (in a ratio of 3:2, respectively) was obtained by heating diisopropyl (2-bromoethyl)phosphonate (**11** described below) and 2-chloroethanol (2 equiv) at 100 °C for 6 days followed by treatment with PCl_5, removal of the resulting $POCl_3$ by distillation, and hydrolysis of the residue with ice: ^1H NMR for **5** (D_2O) δ 4.20 (4 H, m), 3.73 (2 H, m), 2.35 (2 H, dt, J_{P-H} = 17.8 Hz). (2-Hydroxyethyl)phosphonic acid (**6**) (mp 42 °C) was obtained in >95% purity by refluxing a solution of **15** (0.8 g, described below) in 25 mL of water adjusted to pH 2.5 for 21 days. The bulk of the water was removed at 60 °C/25 mmHg and then the remaining traces at 60 °C/0.1 mmHg over 5 h: ^1H NMR for **6** (D_2O) δ 3.84 (2 H, dt, J_{P-H} = 12.7 Hz), 2.09 (2 H, dt, J_{P-H} = 18.4 Hz).

Synthesis and Source of Related Compounds (Table II). Diisopropyl (2-haloethyl)phosphonates were prepared by Arbuzov reaction on heating under an N_2 atmosphere triisopropyl phosphite with 1-bromo-2-fluoroethane (0.72 equiv) (100 °C, 48 h) to obtain **9** (48%), with 1-bromo-2-chloroethane (4 equiv) (115 °C, 24 h) to obtain **10** (66%), or with 1,2-dibromoethane (4 equiv) (110 °C, 24 h) to obtain **11** (66%). The pure products were obtained by distillation to remove diisopropyl phosphite, triisopropyl phosphate, and tetraisopropyl ethane-1,2-diphosphonate. In contrast to earlier results (Saunders et al., 1948), attempts to prepare dimethyl or diethyl (2-fluoroethyl)phosphonate via 1-bromo-2-fluoroethane and sodium dimethyl or diethyl phosphite, respectively, in tetrahydrofuran or ether at −60 °C or room temperature surprisingly resulted in quantitative yield of tetramethyl or tetraethyl ethane-1,2-diphosphonate. Isopropyl (2-chloro- and 2-bromoethyl)phosphonochloridates (bp 66–68 °C/0.5 mmHg and 73–75 °C/0.5 mmHg, respectively), obtained by refluxing **10** and **11** in $SOCl_2$ (8 molar excess) for 14 h followed by distillation (70–80%), were converted to isopropyl (2-haloethyl)phosphonic acids **12** and **13** on mixing with ice, removing the water at reduced pressure, redissolving in chloroform, drying with Na_2SO_4, and evaporating the solvent to dryness. (2-Fluoro- and 2-bromoethyl)phosphonodichloridates (bp 54 °C/1.2 mmHg and 62 °C/0.1 mmHg, respectively) were obtained when **9** and **11** were reacted with PCl_5 (equimolar for **9** and 1.2 molar equiv for **11**) and distillation (90–95%); any excess PCl_5 in the preparation of the (fluoroethyl)phosphonodichloridate leads to a mixture of the 2-chloroethyl and 2-fluoroethyl compounds which is difficult to fractionate. (2-Haloethyl)phosphonic acids **14** and **15** were obtained by reacting their corresponding dichloridates with ice. (2-Chloroethyl)phosphonothioic acid (**16**) was prepared from (2-chloroethyl)phosphonodichloridothionate [obtained by refluxing (2-chloroethyl)phosphonodichloridate and P_4S_{10} (10% excess) in dry benzene (95%) with purification from traces of starting material on washing the benzene solution at 5 °C with water and then distilling (bp 59 °C/1 mmHg)]. Pure **16** was obtained by hydrolysis of the dichloridate in the presence of tetrahydrofuran or acetone, removal of the solvents, redissolving in chloroform, and drying (Na_2SO_4). Hydrogen phosphinate **17** was prepared by hydrolysis of (2-chloroethyl)dichlorophosphonite with ice under N_2, removal of the water under reduced pressure, redissolving in chloroform, drying (Na_2SO_4), and solvent evaporation. The dichlorophosphinite in turn had been prepared by refluxing (2-chloroethyl)dichloridophosphonothionate with triphenylphosphine (1.1 equiv) in dry benzene for 2 h under N_2. After the precipitated triphenylphosphine sulfide was removed by filtration, the product was distilled at 59 °C/20 mmHg.

Analysis of Technical Ethephon. Phosphorus-containing components in technical grade ethephon were assigned and quantitated by ^{31}P NMR, both before and after removal of the water, by direct and "in admixture" comparisons with synthetic standards. The composition was verified by GC/CI-MS analysis of technical ethephon from which the water had been removed and the residue treated with diazomethane in ether, again by direct comparisons with synthetic standards that were also methylated in the same manner.

Animal Studies. Male albino rats (180–220 g, Simonsen Laboratories, Gilroy, CA) were treated orally or intraperitoneally (ip) with pure or technical ethephon or with **14** or **16**. Prior to administration, aqueous solutions of acidic materials were adjusted to pH 7 with NaOH solution. The animals were placed in metabolism cages, and the urine was collected after 24 and 48 h. Phosphorus-containing urinary products were quantitated by ^{31}P NMR relative to HMPA as the internal standard following filtration of the urine samples (sterilizing filter unit, 0.22 μm, Millex-GV, Millipore Products Division, Bedford, MA). Male albino mice (18–22 g, Simonsen Laboratories) were also treated ip with ethephon (as an aqueous solution adjusted to pH 7) at 2500 mg/kg and sacrificed after 7 h. The livers were removed immediately, homogenized in a minimum volume of water, and deproteinated with an equal volume of 8% sodium sulfosalicylate, and the supernatant was analyzed by ^{31}P NMR spectroscopy.

Mice as above were used for 24-h LD_{50} determinations of compounds administered ip as aqueous solutions at pH 7. To determine the effects of **1** on glutathione (GSH) levels in the liver, neutralized **1** was administered ip to mice at 2000 mg/kg and the animals were sacrificed at 2 or 4 h after treatment. The livers were immediately removed, homogenized, and deproteinated with sodium sulfosalicylate, and GSH levels were determined by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) procedure (Mitchell et al., 1973).

ChE inhibition was assayed in vitro by incubation of plasma samples (from blood drawn by heart puncture) with the test compounds. Thus, mouse plasma (40 μL) was added to tubes containing the organophosphorus compound (10 μM) in phosphate buffer (100 mM, pH 8.0, 3 mL) and the mixture incubated at 25 °C for 15 or 90 min. Residual enzyme activity was determined by addition of acetylthiocholine and DTNB by the method of Ellman et al. (1961).

Plant Studies. Ethephon and 13 analogues were assayed with Pearson tomato plants (V.F.N. 8) over a 7-day period for symptoms of epinasty characterized as downward bending of the stems, leaves, and petioles caused by a more rapid growth in the tissues of the upper surfaces of those plant parts. Aqueous solutions (containing 1 drop of Tween 20 to 8 mL of water) of the compounds at concentrations ranging from 3 to 3000 ppm were sprayed on individual plants. For each of the more active compounds, four or five plants were used at each concentration, and, for the less active ones two or three plants were used. Control plants were sprayed with the water/detergent solution. After the spray had evaporated, the plants were exposed, outside, to morning shade and afternoon sun. Intercomparisons of treated plants were made visually within each series and photographically between experiments.

RESULTS

Composition of Technical Ethephon (Table I; Figure 1). Analysis of the phosphorus-containing components of technical ethephon served to assign eight compounds by ^{31}P NMR and GC/CI-MS comparisons with authentic standards or their methylated derivatives, respectively, and tentatively identify three additional compounds by GC/CI-MS of their methyl esters. Quantitation by ^{31}P NMR established 90% **1**, 3% **2**, 1.5–1.8% each **3–5**, approximately equal amounts (0.6%) of **6** and **7**, and 0.3% **8**. The ratio of phosphorus-containing components was unaffected by removal of the water. GC/CI-MS analysis revealed three additional components, which together comprised <0.5% of the mixture. They were tentatively identified as their methyl esters as $H_2C{=}CHP(O)(OEt)OMe$, $ClCH_2CH_2P(O)(OEt)OMe$, and $ClCH_2CH_2O(MeO)P(O)CH_2CH_2P(O)(OMe)_2$.

Reactivity of (2-Chloroethyl)phosphonic Acid and Related Compounds. The reactivity of **1** and related

Table I. Composition of Technical Ethephon and Biological Activity of Its Components

		^{31}P NMR[a]		GC/CI-MS[a,b] Me esters		plasma ChE inhib,[c] %		
no.	structure[b]	δ[d]	mol %[e]	RT min	$[M+1]^+$	15 min	90 min	tomato epinasty, ppm
1	$ClCH_2CH_2P(O)(OH)_2$[f]	25.20	90.1	8.03	173	88 ± 4 (n = 15)	48 ± 7 (n = 14)	10
2	$(HO)_2P(O)CH_2CH_2P(O)(OH)_2$[f]	28.46	3.1	11.51	247	103 ± 6 (n = 3)	101 ± 1 (n = 3)	>3000
3	$H_2C{=}CHP(O)(OH)_2$[f]	16.35	1.8	5.83	137	106 ± 1 (n = 2)	102 ± 2 (n = 2)	3000
4	$ClCH_2CH_2P(O)(OH)OCH_2CH_2Cl$	25.70	1.5	10.08	221			
5	$ClCH_2CH_2OCH_2CH_2P(O)(OH)_2$	26.10	1.5	10.25	217			
6	$HOCH_2CH_2P(O)(OH)_2$[f]	27.34	0.6	ND[g]	ND[g]	100 ± 6 (n = 3)	96 ± 6 (n = 3)	3000
7	$HP(O)(OH)_2$	4.57	0.6	4.17	111	101 ± 3 (n = 3)	96 ± 4 (n = 3)	
8	$HOP(O)(OH)_2$	0.30	0.3	5.67	141			

[a] See Figure 1. [b] Three minor components of the additional 0.5% are tentatively identified by GC/CI-MS (after methylation with diazomethane) as follows (structure, RT min, CI-MS $[M+1]^+$) (see Figure 1): (a) $H_2C{=}CHP(O)(OEt)OMe$ 7.16, 151; (b) $ClCH_2CH_2P(O)(OEt)OMe$ 8.39, 187; (c) $ClCH_2CH_2O(MeO)P(O)CH_2CH_2P(O)(OMe)_2$ 12.98, 295. An additional compound (10.59, 179) is not identified. [c] Candidate inhibitor a 10 μM. n = number of replicates. The mouse ip LD_{50} values of aqueous solutions of **1**, **2**, **3**, and **6** adjusted to pH 7 are >1500 mg/kg. [d] Relative to trimethyl phosphate in H_2O/D_2O 9:1. [e] Molar percentage based on ^{31}P NMR quantitation of technical ethephon analyzed directly. [f] Kosolapoff (1950). [g] ND, not detected.

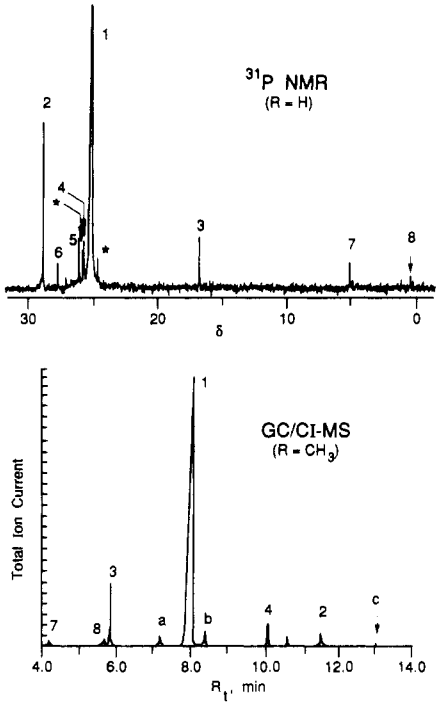

Figure 1. Analysis of technical ethepon by ^{31}P NMR as a solution in H_2O/D_2O 9:1 and by GC/CI-MS following methylation with diazomethane. Asterisks in the NMR spectrum indicate ^{13}C satellites of the signal of **1**. Identification of peaks (R = H for ^{31}P NMR and Me for GC/CI-MS): **1** $(ClCH_2CH_2P(O)(OR)_2$; **2** $(RO)_2P(O)CH_2CH_2P(O)(OR)_2$; **3** $(H_2C{=}CHP(O)(OR)_2$; **4** $ClCH_2CH_2P(O)(OR)OCH_2CH_2Cl$; **5** $ClCH_2CH_2OCH_2CH_2P(O)(OR)_2$; **6** $HOCH_2CH_2P(O)(OR)_2$; **7** $HP(O)(OR)_2$; **8** $ROP(O)(OR)_2$.

compounds was examined in aqueous solution at pH 7.4 or 13.8 at 25 °C with analysis by ^{31}P NMR. Compound **1** decomposes at pH 7.4 to give **8** in 98% yield with a half-life of 24 h. In addition, minor amounts of **3** and **6** (each ~1%) are formed, presumably by base-initiated elimination and substitution reactions, respectively. At pH 13.8 and 25 °C, the half-life of **1** is also approximately 24 h, but the final product mixture consists of **8** (91%), **6** (8%), and **3** (1%). (2-Bromoethyl)phosphonic acid (**15**) reacts almost 5 times faster than **1** at pH 7.4, both in its decomposition (to **8**) and in the dehydrohalogenation (to form **3**) and halogen substitution (to give **6**) reactions. (2-Fluoroethyl)phosphonic acid (**14**) and other compounds in Tables I and II are much less reactive as phosphorylating agents at pH 7.4.

No reaction is observed between **1** and various thiols, e.g., with GSH or *N*-acetylcysteine at pH 7.4 or with 4-nitrobenzenethiol in the presence of triethylamine in protic (methanol) or aprotic (acetone, acetonitrile, or chloroform) solvents, within 4 days at 25 °C, as determined by ^{31}P NMR. Consistent with these observations, **1** does not affect GSH levels in mouse liver 2 or 4 h after ip administration at 2000 mg/kg. In contrast, the (2-bromoethyl)phosphonates **11** and **15** are more reactive than **1** to substitution; i.e., the bromo substituent is readily displaced by equimolar 4-nitrobenzenethiol in acetone containing 4 equiv of triethylamine within 3 h at 25 °C, yielding the corresponding thioethers identified by ^{1}H and ^{31}P NMR and GC/CI-MS (after methylation of the phosphonic acid) (Table II).

Toxicity to Mice. Phosphonothioic acid **16** with an ip LD_{50} of 42 mg/kg (Table II) is more toxic to mice than the other compounds tested (Table I). Death occurred within 3 min of administration of **16** with poisoning signs similar to those obtained with NaSH or thioacetic acid.

Metabolism. ^{31}P NMR analysis of the 0–24-h urine of rats treated orally with technical ethephon at 500 mg/kg indicated only three phosphorus-containing products: **1** (58% of the administered dose); a minor product (~3% of the administered dose) at lower field not detected on administration of pure **1** and identified by comparison with the standard as **2**; and **8** (endogenous and possibly also as a decomposition product of **1**) (Figure 2). In urine samples collected at 48 h after treatment, only **8** was observed. A similar result was obtained for rats following ip administration of **1** at 500 mg/kg, where parent **1** and **8** were also the only phosphorus-containing compounds detected in the 0–24-h urine. On ip administration of **1** to mice at 2500 mg/kg, again only parent **1** and **8** were detected in the livers 7 h after treatment.

(2-Fluoroethyl)phosphonic acid (**14**) is stable in aqueous solution at physiological pH. Consistent with this stability, and in contrast to **1**, it is almost quantitatively excreted (>98% of the administered dose) in the 0–24-h urine with no detectable phosphorus-containing metabolites (Figure 2).

(2-Chloroethyl)phosphonothioic acid (**16**) is metabolized and excreted almost quantitatively in the urine within 24 h following oral administration to rats. In contrast to **1** and **14**, no **16** is detected by ^{31}P NMR analysis (Figure 2). ^{31}P chemical shifts (δ ^{31}P 18.9–19.3) indicate that the metabolites are phosphonates rather than phosphonothiolates; i.e., for the latter the resonances would be at 60–70 ppm. Moreover, the line broadening observed for these phosphonate metabolites ($w/2$ = 31.2 Hz; compared with $w/2$ = 6.2 Hz for HMPA, which is itself broadened from the nitrogen quadrupole effect) suggests that the phosphorus is bound to a macromolecule, presumably a protein.

Table II. Physical Properties, NMR Data, and Plant Growth Regulator Activity of Ethephon Analogues

no.	structure	bp/mmHg mp, °C	δ^a ^{31}P (solvent)	δ^b ^{1}H (solvent)	tomato epinasty,[c] ppm
di- and monoisopropyl phosphonates					
9	$FCH_2CH_2P(O)(OiPr)_2$	47/0.2	21.11 (d)[d] ($CDCl_3$)	4.73 (2 H, m), 4.59 (2 H, m), 2.22 (2 H, m), 1.32 (12 H, d), ($CDCl_3$)	
10	$ClCH_2CH_2P(O)(OiPr)_2$	83–3/0.6	21.04 ($CDCl_3$)	4.70 (2 H, m), 3.69 (2 H, m), 2.24 (2 H, m), 1.33 (12, H, d) ($CDCl_3$)	>3000
11	$BrCH_2CH_2P(O)(OiPr)_2$[e]	96/0.6	21.18 ($CDCl_3$)	4.70 (2 H, m), 3.41 (2 H, m), 2.35 (2 H, m), 1.32 (12 H, d) ($CDCl_3$)	>3000
12	$ClCH_2CH_2P(O)(OiPr)OH$	oil	32.46 ($CDCl_3$)	4.98 (1 H, m), 3.79 (2 H, m), 2.68 (2 H, m), 1.40 (6 H, d), ($CDCl_3$)	30–100
13	$BrCH_2CH_2P(O)(OiPr)OH$	oil	32.48 ($CDCl_3$)	4.96 (1 H, m), 3.58 (2 H, m), 2.75 (2 H, m), 1.41 (6 H, d) ($CDCl_3$)	30–100
phosphonic and phosphinic acids					
14	$FCH_2CH_2P(O)(OH)_2$[f,g]	44	25.28 (d)[d] (D_2O)	4.74 (2 H, ddt, J_{F-H} = 46.5 Hz, J_{P-H} = 17.9 Hz) (D_2O)	1000
15	$BrCH_2CH_2P(O)(OH)_2$[f,h]	84–86	24.64 (D_2O)	3.60 (2 H, dt, J_{P-H} = 12.2) 2.43 (2 H, dt, J_{P-H} = 18.2 Hz) (D_2O)	10
16	$ClCH_2CH_2P(O)OH(SH)$[i]	oil	67.43 ($CDCl_3$)	3.82 (2 H, dt, J_{P-H} = 12.2 Hz), 2.66 (2 H, dt, J_{P-H} = 16.3 Hz) ($CDCl_3$)	30
17	$ClCH_2CH_2P(O)(OH)H$[j]	oil	28.50 ($CDCl_3$) (J_{H-P} = 565 Hz)	12.40 (1 H, s), 7.25 (1 H, dt, J_{P-H} = 565 Hz, J_{H-H} = 2.0 Hz), 3.79 (2 H, dt, J_{P-H} = 15.4 Hz), 2.33 (2 H, ddt, J_{P-H} = 20.9 Hz, J_{H-H} = 2.0 Hz)	300

[a] Relative to trimethyl phosphate. [b] Relative to sodium 3-(trimethylsilyl)propanesulfonate in H_2D/D_2O 9:1 or tetramethylsilane in organic solvent. [c] Activity of related compounds: $ClCH_2CH_2C(O)OH$ and $ClCH_2CH_2S(O_2)OH$ > 3000. [d] J_{F-P} = 45 Hz for **9** and 26.6 Hz for **14**. [e] 2-(4-Nitrobenzenethiol) derivative ^{31}P NMR (acetone-d_6) δ 28.19; ^{1}H NMR (acetone-d_6) δ 8.20 (2 H, d), 7.53 (2 H, d), 4.69 (2 H, m), 3.61 (2 H, m), 2.42 (2 H, m), 1.21 (12 H, d); $[M + 1]^+$ 348. [f] Kosolapoff (1950). [g] Dichloridate ^{31}P NMR ($CDCl_3$) δ 39.53 (d, J_{F-P} = 24.8 Hz); ^{1}H NMR ($CDCl_3$) δ 4.89 (2 H, ddt, J_{F-H} = 46.2 Hz, J_{P-H} = 25.4 Hz), 3.10 (2 H, ddt, J_{F-H} = 20.3 Hz, J_{P-H} = 15.6 Hz). [h] 2-(4-Nitrobenzenethiol) derivative ^{31}P NMR (acetone) δ 21.67; ^{1}H NMR (acetone-d_6) δ 8.21 (2 H, d), 7.50 (2 H, d), 3.69 (2 H, m), 3.35 (2 H, m); $[M + 1]^+$ 264. [i] δ ^{31}P 78.92 (H_2O/D_2O 9:1, pH 1.5), 21.09 (H_2O/D_2O 9:1, pH 13). Mouse LD_{50} 42 mg/kg. Dichloridothionate ^{31}P NMR ($CDCl_3$) δ 75.66; ^{1}H NMR ($CDCl_3$) δ 3.94 (2 H, m), 3.30 (2 H, m). [j] (2-Chloroethyl)dichlorophosphine (59 °C/22 mmHg) ^{31}P NMR ($CDCl_3$) δ 182.1; ^{1}H NMR ($CDCl_3$) 3.92 (2 H, dt, J_{P-H} = 2.9 Hz), 2.82 (2 H, dt, J_{P-H} = 6.8 Hz).

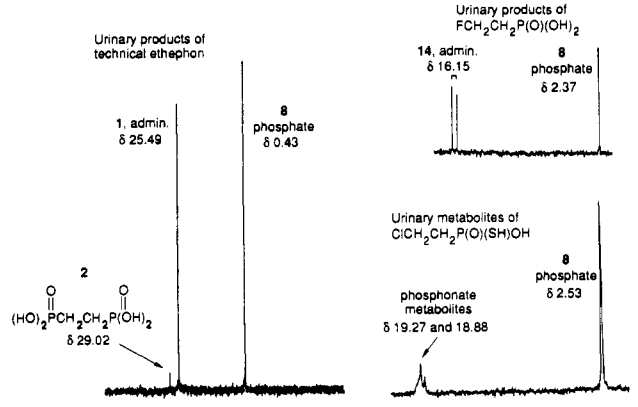

Figure 2. ^{31}P NMR spectra of the 0–24-h urine of rats treated orally with technical ethephon (500 mg/kg) and its fluoroethyl analogue **14** (250 mg/kg) and thio analogue **16** (70 mg/kg). Identification of peaks (δ): technical ethephon treatment, **1** 25.49, **2** 29.02, **8** (endogenous and originating from **1**) 0.43; (2-fluoroethyl)phosphonic acid (**14**) treatment, **14** 16.15, **8** (endogenous) 2.37; (2-chloroethyl)phosphonothioic acid (**16**) treatment, **8** (endogenous) 2.53, phosphonate metabolites 19.27 and 18.88.

It appears that the degradation of **16** occurs in the rat and not in the urine. When **16** is added to rat urine, it decomposes to **1**, with a half-life of approximately 2 h, yet neither **16** nor **1** is detected in the urine of treated rats. The conversion of **16** to **1** in urine is probably enzymatic since it occurs >100-fold faster than in water alone at the same pH.

Effect of Ethephon and Impurities and Related Compounds on Plasma ChE Activity. In in vitro assays, (2-haloethyl)phosphonic acids **1** (Table I) and **15** (Table III) at 10 μM undergo time-dependent reaction with mouse plasma ChE reaching inhibition levels of 52 and 87%, respectively, by 90 min. None of the other compounds tested is active as an inhibitor.

Table III. Inhibitory Activity of Ethephon Analogues for Mouse Plasma Cholinesterase Activity

no.	structure	plasma ChE activity,[a] % 15 min	90 min
15	$BrCH_2CH_2P(O)(OH)_2$	32 ± 1 (n = 3)	13 ± 1 (n = 3)
16	$ClCH_2CH_2P(O)(OH)SH$	83 ± 5 (n = 5)	83 ± 10 (n = 5)
	$(EtO)_2P(O)SH$	93 ± 7 (n = 6)	86 ± 12 (n = 6)

[a] Candidate inhibitor at 10 μM. n = number of replicates.

Effect of Ethephon and Impurities and Related Compounds on Tomato Plants (Tables I and II). (2-Chloroethyl)phosphonic acid (**1**) is ≥300-fold more potent than ethephon impurities **2**, **3**, and **6** in producing epinasty of the leaves. (2-Bromoethyl)phosphonic acid (**15**) is equal in potency to **1**, whereas (2-fluoroethyl)phosphonic acid (**14**) is 100-fold less active. The phosphonothioic acid (**16**) and hydrogen phosphinate (**17**) analogues are 3- and 30-fold less potent than **1**. Monoisopropyl esters **12** and **13** and particularly diisopropyl esters **10** and **11** are much less active than the corresponding phosphonic acids **1** and **15**, respectively. Other inactive compounds are 2-chloropropionic and (2-chloroethyl)sulfonic acids.

DISCUSSION

The observed composition of technical ethephon can be rationalized on the basis of its three-step synthesis (Sittig, 1980): reaction of PCl_3 and ethylene oxide to obtain tris(2-chloroethyl) phosphite (A); intramolecular Arbuzov rearrangement of A to bis(2-chloroethyl) 2-chloroethylphosphonate (B); hydrolysis of B to **1** (Figure 3). The impurities presumably result in the most part from side reactions in the formation of A and B. Thus, **2**, the most abundant impurity, results from reaction of phosphite A with diester B and hydrolysis of the resulting tetraester C. Impurity **3** originates from dehydrochlorination of **1** and **4** by partial hydrolysis of B. Impurity **5** may result

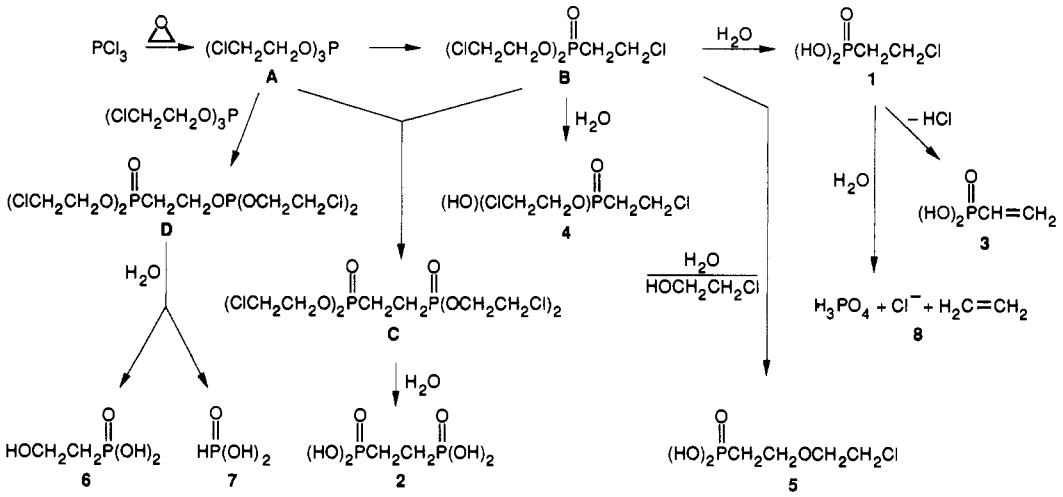

Figure 3. Synthesis of ethephon and proposed reaction pathways to impurities. Some of the products may also be formed by reactions other than those shown.

from condensation of B with 2-chloroethanol followed by hydrolysis. Impurities **6** and **7** could originate from hydrolysis of the mixed phosphite–phosphonate (D), which itself forms from intermolecular Arbuzov reaction between two molecules of starting material A; the occurrence of **6** and **7** in essentially equimolar amounts in technical ethephon is consistent with this pathway. Finally, **8** could originate from the slow decomposition of **1** in the presence of water. Although not shown in Figure 3, the minor impurity $ClCH_2CH_2O(HO)P(O)CH_2CH_2P(O)(OH)_2$ probably represents a partial hydrolysis product of C. The other two minor components, $ClCH_2CH_2P(O)(OEt)OH$ and $H_2C{=}CHP(O)(OEt)OH$, may originate from the involvement of ethanol, presumably present as an impurity in 2-chloroethanol or in ethylene oxide. It should be noted that the percentage composition of samples of ethephon may vary with their age and storage conditions.

Rats treated with technical ethephon excrete **1**, **2**, and phosphate, on the basis of ^{31}P NMR analysis of the urine. The phosphate represents both endogenous compound and that produced by decomposition of **1**. In relation to **1** and other components of technical ethephon, impurity **2** appears preferentially concentrated in the urine, suggesting that it is excreted without extensive metabolism or decomposition. Compound **2** may be a useful marker in urinary monitoring of possible exposure to technical ethephon. Other minor impurities in technical ethephon are not observed in the urine; either ^{31}P NMR spectroscopy is insufficiently sensitive as an analytical method for their detection or they decompose to phosphate. (Fluoroethyl)phosphonic acid (**14**) is much more stable than **1** to both chemical and metabolic degradation.

Plasma ChE is of similar sensitivity to technical ethephon and pure **1** (Hennighausen et al., 1977; Hennighausen and Tiefenbach, 1978), suggesting that **1** per se is the actual inhibitor, an observation supported by the present study which indicates that **1** is a much more effective inhibitor than ethephon impurities **2**, **3**, **6**, and **7**. Neither **1** nor impurity **2**, **3**, or **6** shows any signs of acute toxicity to mice when neutralized and administered ip at 1500 mg/kg.

(Chloroethyl)phosphonic acid (**1**) and its bromoethyl analogue (**15**) react readily in aqueous solution when the pH exceeds pK_1. Further increases in pH up to the pK_2 of these phosphonic acids do not markedly change the rate of decomposition but instead increase the proportions of hydroxy-substituted and dehydrohalogenation products (Segall, unpublished results). The chemical reactivity of analogues of **1** as phosphorylating agents at physiological pH parallels their potency as ChE inhibitors, i.e., **15** ≫ **1** ≫ **2**, **3**, **6**, **7**, or **16**. This parallel relationship suggests the possibility that the esteratic site of the enzyme is phosphorylated, perhaps at the serine residue forming an aged phosphoenzyme directly that, as such, is resistant to reactivation. If direct phosphorylation proves to be the case, it is an unusual example of esterase inhibition since relative to the normally accepted requirement for organophosphorus inhibitors, these phosphonic acids are dionizable.

Alkylation of S-nucleophiles is observed with **15** but not **1** and in the latter case does not appear to have toxicological significance. In addition, **1** does not affect the GSH levels of mouse liver in vivo. Consistent with this result, there is no evidence of any phosphorus-containing S-conjugates in the urine of ethephon-treated mice or rats.

Some features of the action of phosphonothioic acid **16** are worthy of note. It is the most toxic compound examined for mice with poisoning signs somewhat similar to those of H_2S intoxication. The parent compound **16** is not observed in the urine, but instead there are several phosphonate metabolites or degradation products, some of which appear to be bound to macromolecules. Possible oxidation to a reactive phosphono(thioperoxoic) acid with phosphorylating and sulfurylating properties (Segall et al., 1990) may be relevant to the presence of a protein adduct in the urine.

The PGR activity of ethephon is presumably due to its ability to release ethylene; three impurities that are not likely to liberate ethylene are much less active as PGRs. The relative potency of ethephon analogues for inducing epinasty in tomatoes is consistent with this proposal. Thus, **1** and **15** have the highest PGR activity and rate of ethylene liberation at physiological pH. Their monoisopropyl esters **12** and **13** are presumably metabolized to **1** and **15**, respectively, consistent with the reported activity of compound **4** (Cooke and Randall, 1968). The moderately active phosphonothioic acid **16** and hydrogen phosphinate **17** presumably undergo in vivo oxidation to **1** and then subsequent release of ethylene. Interestingly, this would mean conversion of **16** to **1** on application to tomato plants but not on administration to rats.

In summary, the biological activity of technical ethephon appears to be due to **1** itself rather than to impurities. The PGR activity is initiated by ethylene release on degradation of **1** and the plasma ChE inhibition is possibly due to its action as a phosphorylating agent.

ABBREVIATIONS USED

ChE, cholinesterase; CI, chemical ionization; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GC, gas chromatography; GSH, glutathione; HMPA, hexamethylphosphoramide; ip, intraperitoneal; MS, mass spectrometry; NMR, nuclear magnetic resonance; PGR, plant growth regulator.

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