Determination of Phorate and Five of Its Metabolites in Corn

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Residues of the insecticide phorate, {*O,O*-diethyl *S*-[(ethylthio)methyl] phosphorodithioate}, and five of its metabolites were determined in corn treated with the insecticide in granular form. The plant was extracted in a Soxhlet apparatus for 8 hours with 10% methanol in chloroform, and the extract was separated into three fractions by liquid chromatography on silica gel. Aliquots of the concentrated fractions were injected into a gas chromatograph equipped with a flame photometric detector sensitive to phosphorus. No interference from the crop extracts was encountered. Recoveries of the compounds from fortified samples were 96% or more, except for the oxygen analog of phorate; its recovery was only 62%. Sensitivity was 0.004 p.p.m. or better. Residues in various parts of corn plants treated with 1 pound of phorate per acre were determined at seven intervals after application. The toxicities of the six compounds to the European corn borer, *Ostrinia nubialis* (Hübner), were determined. Partition and chromatographic data are also given.

Phorate (Thimet), *O,O*-diethyl *S*-[(ethylthio)methyl] phosphorodithioate, a product of the American Cyanamid Co., Princeton, N. J., is a systemic insecticide registered for use on a variety of crops and ornamental plants. A 10% granular formulation, Thimet 10-G, is recommended for use on field corn or on corn grown for seed.

As part of an investigation of insecticide residues on field-grown corn, a gas chromatographic method was devised to determine residues of phorate and five of its metabolites, which are formed by oxidation of the sulfide in the side chain to a sulfoxide or sulfone and formation of a P=O (O-analog) from the P=S group of the parent compound. The formulas of these compounds are shown in Figure 1. Different parts of the plant were analyzed at six intervals after application of the insecticide and at harvest. The relative toxicities of phorate and the five metabolites to the European corn borer, *Ostrinia nubialis* (Hübner), were also determined by a laboratory screening procedure.

Many methods have been advanced for the determination or detection of phorate and its metabolites. They include the use of colorimetry (Giang and Schechter, 1960; Manuel, 1968; Waldron *et al.*, 1963; Winnett and Katz, 1965), cholinesterase inhibition (Bowman and Casida, 1957; Curry, 1961), paper chromatography (Jacquinot, 1964), thin-layer chromatography (Blinn, 1963, 1964; Getz, 1962; Ragab, 1967a, 1967b), and gas chromatography with a variety of detectors (Bache and Lisk, 1966; Egan *et al.*, 1964; Nelson, 1967; Ruzicka *et al.*, 1967;

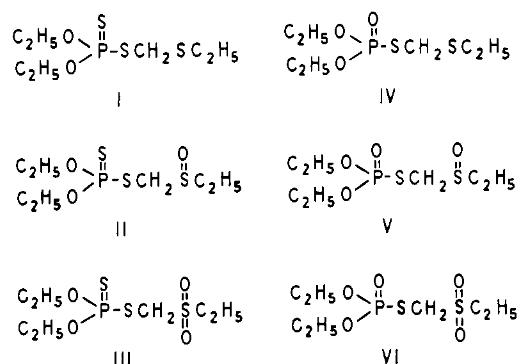

Figure 1. Phorate (I) and five of its metabolites

I, P=S,S; II, P=S,SO; III, P=S,SO_2; IV, P=O,S; V, P=O,SO; VI, P=O,SO_2

Sans, 1967). The determination of phorate has also been discussed in several reviews of analytical methodology for organophosphorus pesticides (Abbott and Egan, 1967; Chilwell and Hartley, 1961; Sutherland *et al.*, 1964). Some of the methods did not determine the metabolites, and some attempted an estimate of the metabolites by oxidizing them to the sulfone. However, none of the procedures could be used to determine phorate and its five metabolites individually and quantitatively. The wide differences in the toxicities of the compounds to the European corn borer make the determination of the individual compounds desirable.

EXPERIMENTAL

Apparatus. An F & M Scientific Corp. (Avondale, Pa.) Model 700 gas chromatograph was equipped with the flame-photometric detector of Brody and Chaney (1966) (MicroTek Instruments, Inc., Baton Rouge, La.) and the 526-mμ interference filter, which detects phosphorus.

Soxhlet extraction apparatus (Fisher Scientific Co., No. 9-556B).

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Chromatographic column (Kontes No. K-42000), 12-mm. I.D. glass.

Reagents and Solvents. Phorate and its five metabolites were analytical grade chemicals supplied by the American Cyanamid Co.

The silica gel for the chromatography of the extract was used as received from the J. T. Baker Chemical Co. (No. 3405). It lost 3.5% of its weight after overnight heating at 110° C.

Sodium sulfate was the anhydrous reagent grade chemical. Acetone, acetonitrile, benzene, chloroform, hexane, and absolute methanol were C.P. grade solvents. Except for the methanol, all were distilled before use.

Sample Preparation and Extraction. Samples of various parts of corn treated with a granular formulation of phorate (10-G) at the rate of 1 pound of active insecticide per acre (1.12 kg. per hectare of Thimet) were taken at 1, 3, 7, 14, 21, and 28 days after treatment and at harvest. The samples, which had been kept frozen while awaiting analysis, were finely chopped (ca. 20-mesh) in a Hobart cutter and well mixed. Twenty grams of each sample were transferred to a Soxhlet extraction apparatus containing a plug of glass wool to prevent insoluble plant material from siphoning over during solvent exchanges. Each sample was extracted under nitrogen for 8 hours with 150 ml. of chloroform-methanol, 9 to 1 by volume, at the rate of six solvent exchanges per hour. The extract was allowed to cool and then percolated through a plug of sodium sulfate 25 mm. in diameter and 30 mm. thick. After the extraction flask and plug were washed with another 10 ml. of chloroform, the percolate was evaporated to dryness on a 50° C. water bath under water pump vacuum (ca. 35 mm. of Hg). (Chloroform, methanol, and water must be removed to avoid difficulty in the ensuing liquid chromatography.) The residue was taken up in 10 ml. of benzene for liquid chromatography.

Liquid Chromatography. The silica gel column was prepared by adding successively to the glass chromatographic column a plug of glass wool, 2 grams of sodium sulfate, 4 grams of silica gel, and 2 grams of sodium sulfate. The column was washed with 20 ml. of benzene, and the filtrate was discarded. When the benzene extract was added to the column, collection of the eluate was begun. While the extract percolated into the adsorbent, the vessel that held the extract was rinsed with 5 ml. of 1% acetone in benzene, and these rinsings were used to wash the extract into the adsorbent. Forty-five milliliters more of the same solvent were added to the column. The eluate (fraction A) contained phorate (I) and its sulfone (III).

The receivers were changed, and 50 ml. of 10% acetone in benzene were allowed to percolate through the column. The eluate (fraction B) contained the sulfide of the O-analog (IV), the sulfoxide of phorate (II), and the sulfone of the O-analog (VI).

The sulfoxide of the O-analog (V) remaining on the column was eluted with 50 ml. of acetone (fraction C).

Each fraction was evaporated to near dryness under water pump vacuum by using a 50° C. water bath. The residue was transferred to a calibrated tube with benzene and its volume adjusted to 2 ml. for gas chromatographic analysis. Five microliters of the solution were equivalent to 50 mg. of plant material.

Gas Chromatographic Analysis. The following conditions were used:

Column. Glass, 90 cm. × 4-mm. I.D. (6-mm. O.D.).
Packing. DC 200, 10% (w./w.) on 80- to 100-mesh Gas-Chrom Q (Applied Science Laboratories, State College, Pa.).
Carrier Gas. Nitrogen at 160 ml. per minute.
Other Gases. Oxygen at 40 ml. per minute; hydrogen at 200 ml. per minute.
Temperatures. Column 180° C. for fractions A and B, and 200° C. for fraction C.; injection port 225° C.; detector (external) 200° C.

The column was conditioned overnight at 230° C. and then conditioned further with the gas chromatograph operating as described by injecting 250-ng. amounts of the appropriate insecticides in extract equivalent to 50 mg. of plant until several successive injections of 5-ng. amounts of insecticide in plant extract (0.1 p.p.m.) produced a constant response. Difficulty of conditioning the column was as follows: sulfide < sulfone < sulfoxide and P=S < P=O.

Five microliters of each fraction (or a dilution thereof if too concentrated) were injected for analysis. Response to the compounds in the extracts and the standards was measured in terms of peak height.

Toxicity of Compounds to Corn Borers. Several dilutions of each compound (31.25 to 500 p.p.m.) in acetone were prepared, and 0.2 ml. of each solution was distributed evenly on the surface of 2 ml. of artificial diet contained in plastic jelly cups (18 mm. deep × 26-mm. bottom diameter × 34-mm. top diameter). After the solvent evaporated, five 7-day-old larvae from a laboratory-reared strain of the European corn borer were introduced into each cup and confined there with a paper-board cap lined with Saran. Cups and larvae were held at 27° C. and 70% relative humidty. Mortality was recorded after 72 hours.

Each treatment was replicated eight times, and the experiment was designed as a randomized block. The data were analyzed for sources of variance by use of the *F*-test, and treatment means were separated with Duncan's new multiple-range test (Duncan, 1955).

RESULTS AND DISCUSSION

The choice of extraction procedure for the compounds was not based on recoveries from fortified samples but on maximum recoveries obtained by exhaustive extraction of field specimens. An 8-hour Soxhlet extraction period was used; it was the minimum time required to remove more than 99% of the residue recoverable from the field samples by the extraction method. The need for exhaustive or adequate extraction to recover pesticides from crops has been cited by Mumma *et al.* (1966) and by others (Archer and Crosby, 1967; Bertuzzi *et al.* 1967; Burke and Porter, 1967; Root, 1967; Wheeler and Frear, 1966; Wheeler *et al.*, 1967). The mixed solvent, 10% methanol in chloroform, was used for extraction because in a previous study it gave maximum recoveries of six phosphorus insecticides and nine of their metabolites from field-treated crops without removing excessive quantities of co-extractives (Bowman *et al.*, 1968b). Extractions were performed in a nitrogen atmosphere to eliminate possible oxidation of residues.

Gas chromatograms of fractions A, B, and C obtained from the liquid chromatography of the standards on silica gel are shown in Figure 2. Retention times of the six compounds and minimum detectable concentrations (based on twice noise level) in a sample equivalent to 50 mg. of plant material are given in Table I. The data show that the gas chromatographic analyses are rapid, and the sensitivity is very high (at least 0.004 p.p.m.). Response (peak height) was linear with concentration to at least the 250-ng. level. Nothing in the corn extracts interfered with the analysis of the six compounds. In the chromatography of fraction C, column temperature was maintained at 200° C. rather than at 180° C. to speed the analysis and increase the response to the P=O,SO metabolite. This effect may be observed in Figure 2, which compares the P=O,SO peaks obtained (dotted-line peak in *A* at 180° C. and solid-line peak in *C* at 200° C.) when the two temperatures were used.

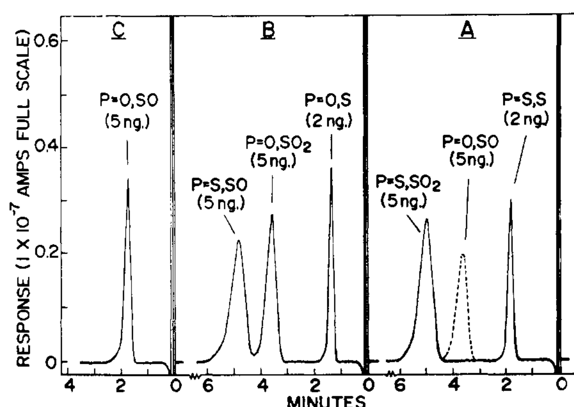

Figure 2. Gas chromatograms of phorate and five of its metabolites in fractions A, B, and C obtained by liquid chromatography on silica gel (solid lines only)

Column temperature was 180° C. for fractions A and B and 200° C. for fraction C. The dotted line peak in chromatogram *A* and the peak in chromatogram *C* show chromatograms of 5 ng. of the same compound (P=O,SO) at 180° and 200° C., respectively (P=O,SO is not found in fraction A)

Table I. Retention Times of Phorate and Five of Its Metabolites and Minimum Detectable Levels in 50-Mg. Equivalents of Corn per Analysis

Compound	Retention Time, Min.	Minimum Detectable Level, P.P.M.
P=S,S(I)	1.90	0.002
P=S,SO(II)	4.90	0.004
P=S,SO_2(III)	5.00	0.004
P=O,S(IV)	1.40	0.002
P=O,SO(V)	1.75[a]	0.004
P=O,SO_2(VI)	3.65	0.004

[a] This retention time was obtained with the chromatographic column at 200° C. At a column temperature of 180° C., the retention time for this compound is 3.65 minutes.

When untreated corn leaves were fortified with phorate and the five metabolites at the 0.05 p.p.m. level just prior to extraction, percentage recoveries were: P=S,S 96; P=S,SO 96; P=S,SO_2 100; P=O,S 62; P=O,SO 98; P=O,SO_2 98. Recoveries were therefore 96 to 100% except for the 62% value for the P=O,S metabolite. (The low recovery of this compound could arise from its instability. Samples fortified with P=O,S produced no other observable product. In the absence of crop, the recovery of P=O,S was 96 to 100%.) These data parallel those obtained in a method for fenthion, {*O,O*-dimethyl *O*-[4-(methylthio)-*m*-tolyl] phosphorothioate}, and its metabolites (Bowman and Beroza, 1968a) which likewise gave good recoveries (above 90%) for five of the compounds and a low recovery (75%) for its P=O,S metabolite.

Additional data that may be useful in analyzing crops for residues of phorate and its metabolites are given in Table II and Figure 3. Table II gives the *p*-values (fraction of compound partitioning into the nonpolar phase of an equivolume two-phase system) of the compounds in seven solvent systems. These values, which remain constant regardless of chromatographic column employed,

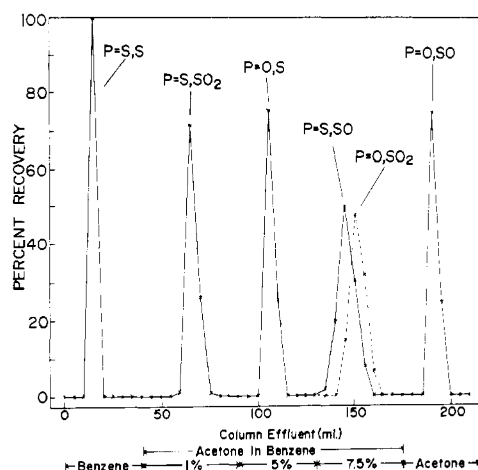

Figure 3. Liquid chromatography of phorate and five of its metabolites on 4 grams of silica gel

Table II. *p*-Values of Phorate and Metabolites

Compound	Hexane-Water	Hexane-20% Aq. Acetonitrile	Hexane-40% Aq. Acetonitrile	Hexane-60% Aq. Acetonitrile	Hexane-80% Aq. Acetonitrile	Hexane-Acetonitrile	Benzene-Water[a]
P=S,S	1.00	1.00	1.00	0.81	0.54	0.21[b]	...
P=S,SO	0.54	0.35	0.10	0.02	0.01	0.00	...
P=S,SO_2	0.98	0.79	0.30	0.05	0.02	0.01	...
P=O,S	0.89	0.73	0.36	0.11	0.05	0.02	...
P=O.SO	0.00	0.00	0.00	...	...	...	0.21
P=O,SO_2	0.01	0.00	0.00	...	...	...	0.78

[a] This solvent system was used for P=O,SO and P=O,SO_2, which were not distinguished in the other systems.
[b] This value is lower than the 0.26 previously reported for another sample of phorate which probably contained impurities.

may be useful in confirming the identity of residues at the nanogram level (Beroza and Bowman, 1965). When micrograms rather than nanograms of residues are available in a sample, infrared, ultraviolet, or mass spectrometry may be used to confirm the identity of residues. In these instances, the liquid chromatogram of the six compounds, each in 100-μg. amount, on 4 grams of silica gel (Figure 3) could be helpful in devising a means of collecting enough material to make such confirmations. However, the P=S,SO and P=O,SO_2 metabolites were not separated by this procedure. The residues of phorate and its metabolites in various parts of the corn plant at seven intervals after treatment of the plants with 1 pound of active phorate per acre are shown in Table III. It is important that the insecticide find its way into the whorl to be effective against the European corn borer. The high residues in the whorl at the 1- and 3-day intervals indicate that this result was achieved with the phorate granules. The rapid growth of the plant appears to move the residues out of the whorl and on to the leaves. This movement of the granules can actually be observed on the plant. At silage time, no residue remains in the whorl, and the leaves have the greatest amount of residue. The ease with which phorate was oxidized to its sulfoxide is also noteworthy. One day after the insecticide was applied, the residue of phorate sulfoxide (P=S,SO) was more than three times that of phorate. In general, phorate itself was essentially gone in 14 days, while very low levels of its sulfoxide and sulfone (0.1 p.p.m. or less) persisted to the 28-day interval. At harvest time the plant was essentially free of insecticide (less than 0.01 p.p.m.).

The results of the toxicity tests with phorate and the five metabolites are given in Table IV. Phorate appeared to be the most toxic of the six compounds, and its sulfoxide (P=S,SO) and sulfone (P=S,SO_2) were next in toxicity, in that order. The effective toxicites of the three O-analogs were low compared to those of the three thionophosphates. Residues of the O-analogs apparently have little insecticidal value. Inasmuch as the O-analogs of P=S insecticides are usually more toxic than their thionophosphate precursors, their low activity could be a consequence of the instability of the compounds. Another indication that the O-analogs might not be stable is the very low level of their residues; only two of these residue values in Table III exceeded 0.1 p.p.m.

The relative toxicities of the compounds were determined to examine the relationship between the residues of the compounds and the degree of insect control obtained. These data plus the residues of the six compounds on the different plant parts at the seven intervals after application of phorate provided a good idea of the location and persistence of the residue on the plant.

Table III. Residues of Phorate and Five Metabolites on Corn Treated with 1 Lb. of Phorate per Acre for First-Generation European Corn Borer Control

Days after Treatment	Plant Parts[a]	Dry Matter, %	Mean Residues[b] (P.P.M., Wet Basis)				
			P=S,S	P=S,SO	P=S,SO_2	P=O,SO	P=O,SO_2
1	L	23.7	0.36	2.95	1.38	0.092	0.021
	STA	9.1	1.89	3.76	0.12	<0.004	<0.004
	W	14.6	8.41	28.0	0.49	0.098	0.014
	A	14.0	1.71	5.14	0.83	0.074	0.011
3	L	21.6	0.32	4.17	2.27	0.30	0.091
	STA	8.3	0.66	1.21	0.11	<0.004	<0.004
	W	13.2	4.39	21.3	1.12	0.11	0.017
	A	11.3	1.30	3.94	0.42	0.055	0.012
7	L	23.3	0.084	0.44	0.50	0.037	0.050
	STA	10.8	0.003	0.16	0.024	<0.004	<0.004
	W	17.2	0.016	0.28	0.062	<0.004	<0.004
	A	13.0	0.003	0.11	0.11	<0.004	<0.004
14	L	23.4	0.023	0.49	0.26	0.063	0.037
	STA	12.4	0.003	0.022	0.022	<0.004	<0.004
	W	25.9	0.007	0.12	0.049	<0.004	<0.004
	A	15.1	0.020	0.40	0.11	<0.004	<0.017
21	L	23.2	<0.002	0.086	0.11	<0.004	0.006
	STA	15.5	<0.002	0.018	0.009	<0.004	<0.004
	W	25.4	0.020	0.10	0.17	<0.004	<0.004
	A	16.5	0.022	0.050	0.023	<0.004	<0.004
28 (silage time)	L	23.6	<0.002	0.066	0.044	<0.004	<0.004
	STA	15.7	<0.002	0.011	0.004	<0.004	<0.004
	E	14.2	<0.002	<0.004	<0.004	<0.004	<0.004
	A	15.8	<0.002	0.018	0.014	<0.004	<0.004
83 (harvest)	STL	38.3	<0.002	0.009	<0.004	<0.004	<0.004
	K	69.5	<0.002	<0.004	<0.004	<0.004	<0.004
	C	54.1	<0.002	<0.004	<0.004	<0.004	<0.004
	H	50.3	<0.002	<0.004	<0.004	<0.004	<0.004

[a] L = leaves, STA = stalk and axils, STL = stalk and leaves, W = whorl, A = aliquot of entire plants, E = ear, K = kernels, C = cobs, H = husk of ear.
[b] Two or three replicates. No phoratoxon (P=O,S) was found except in three of the six 1- and 3-day whorl samples, and they contained 0.07 p.p.m.

Table IV. Relative Toxicity of Phorate and Five of Its Metabolites to 7-Day-old European Corn Borer Larvae in Laboratory Tests

Compound	Concentration in Acetone, P.P.M.	Mean No. Larvae Surviving per Cup[a]	Percentage Mortality
P=S,S	500	0.13 f	97
	250	0.13 f	97
	125	1.63 e	67
	62.5	3.75 cd	25
	31.25	4.63 ab	7
P=S,SO	500	1.25 e	75
	250	3.38 d	32
	125	4.25 abc	15
	62.5	4.88 a	2
	31.25	5.00 a	0
P=S,SO_2	500	4.00 bc	20
	250	4.63 ab	7
	125	4.88 a	2
	62.5	4.88 a	2
	31.25	4.88 a	2
P=O,S	500	5.00 a	0
	250	5.00 a	0
	125	4.88 a	2
	62.5	4.88 a	2
	31.25	5.00 a	0
P=O,SO	500	4.75 ab	5
	250	5.00 a	0
	125	5.00 a	0
	62.5	5.00 a	0
	31.25	5.00 a	0
P=O,SO_2	500	4.88 a	2
	250	4.75 ab	5
	125	4.88 a	2
	62.5	4.88 a	2
	31.25	4.88 a	2
Untreated	...	4.88 a	2
	...	4.75 ab	5
	...	5.00 a	0

[a] Each value is the mean of eight replicates. Means followed by the same letter are not significantly different at the 0.01 level of probability (Duncan, 1955).

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