Metabolism of *S*-[(2-Methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl)methyl]-*O,O*-dimethyl phosphorodithioate (Supracide) in Alfalfa

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Metabolic studies of alfalfa treated with C^{14}*S*-[(2-Methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl)-methyl]*O,O*-dimethyl phosphorodithioate (Supracide) are described. Though the cholinesterase-inhibiting oxygen analog is present, it is not the major route of metabolism. It decreases steadily after treatment. Water-soluble C^{14} residues are not cholinesterase-inhibiting. One of these, based on thin-layer chromatographic R_f values, appears to be a labile conjugate which, upon standing, hydrolyzes to the desmethyl compound. The majority of the water-soluble radioactivity is polar, and based on R_f's, the same pattern is found whether the ring- or methoxy-labeled pesticide is used. Fragmentation of methoxy- or ring-labeled parent compound is suggested by $C^{14}O_2$ release to the atmosphere or incorporation into the carbon pool of a plant.

Supracide, a new Geigy insecticide, *S*-[(2-Methoxy-5-oxo-Δ^2-1,3-4-thiadiazolin-4-yl) methyl]*O,O*-dimethyl phosphorodithioate, is presently being evaluated on several crops. Since this compound, designated GS-13005, effectively controls alfalfa weevil, alfalfa was selected as the crop for this metabolic study. Some work has been done with radioactive GS-13005 on other plants. Esser and Müller (1966), using beans, recovered 30% of the applied 5-carbonyl-C^{14} GS-13005 (Figure 1) as $C^{14}O_2$ after 14 days. Bull (1968), using the above labeled compound and P^{32} GS-13005 on cotton and alfalfa, reported the presence of several aqueous soluble metabolites, some of these being hydrolytic products.

Since many metabolic studies have shown the oxygen analog of thiophosphates to be not only a major cholinesterase inhibitor, but also the primary metabolite in plants (Heath, 1961; O'Brien, 1960), special emphasis was placed on the oxygen analog of GS-13005 (Figure 1). Any metabolite that is a strong cholinesterase inhibitor is important and must be detected. GS-13005 contains a heterocyclic ring which was believed to be important in this metabolic study; consequently, all the radioactive compounds were either labeled in the 5 position of the ring or in the methoxy group attached to the ring (Figure 1).

Methods for the preparation of radioactive compounds used in this study are presented. The synthesis of these compounds is based on the fundamental laboratory synthesis of GS-13005 by Rüfenacht (1968).

SYNTHESIS OF C^{14}-LABELED COMPOUNDS

2-Methoxy-Δ^2-1,3,4-thiadiazolin-5-C^{14}-5-one (RH). Thiocarbazinic acid-*O*-methylester (120 mmoles) was dissolved in 40 ml. of acid (1-to-3 concd. HCl-H_2O)

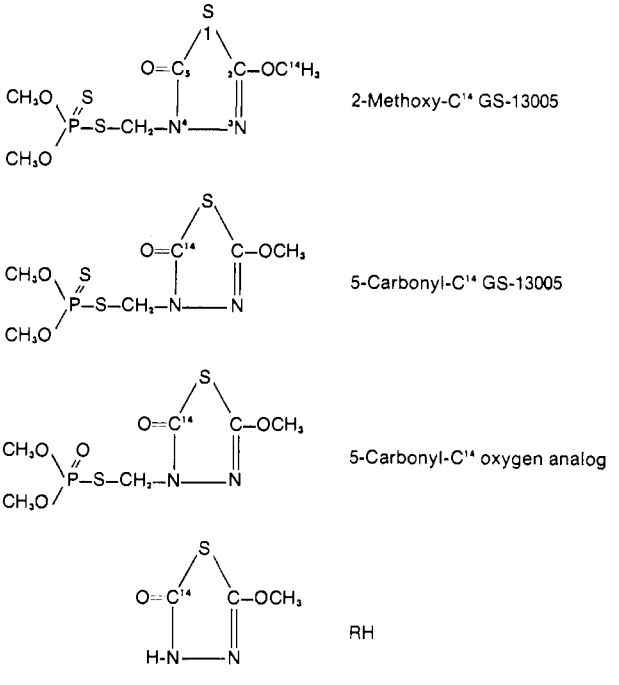

Figure 1. Chemical structures of GS-13005 and related compounds

R–the thiadiazole structure

and the solution cooled to 8° to 10° C. With stirring, phosgene-C^{14} (100 mmoles in 30 ml. of benzene) was added dropwise over a period of 75 minutes. Afterward the reaction mixture was kept at 10° C. for an hour, and then at room temperature for 20 hours. Stirring was maintained at all times. Nitrogen was bubbled through the organic layer to remove the benzene. To the resulting slurry, 40 ml. of the 1-to-3 acid solution was added, and the mixture extracted five times with 100-ml. portions of chloroform. The hazy extract was filtered and concentrated to dryness; white

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crystals (8.7 grams) were obtained, m.p. 111-14° C. This compound has been designated RH (Figure 1).

***S*-[(2-Methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl-5-C^{14})-methyl]*O,O*-Dimethyl Phosphorodithioate.** The potassium salt of *O,O*-dimethyl phosphorodithioic acid (65 mmoles) was added over a period of 15 minutes to 80% sulfuric acid (208 mmoles). Compound RH (65 mmoles) and paraformaldehyde (65 mmoles) were added, each over a period of 15 minutes. The reaction was water-cooled, and stirring was continuous for 3 hours.

The two-layer reaction system was transferred and extracted three times with 60 ml. of water. The lower oily layer was neutralized with 6% $NaHCO_3$ solution. The system was seeded with nonradioactive GS-13005, and the mixture stirred overnight in a refrigerator. The product was removed by filtration, washed until free of sulfate, and desiccated over P_2O_5. Purification was by preparative thin-layer chromatography using GF silica gel; 9.6 grams of pure material was obtained, m.p. 39.5–40.0° C., specific activity 6.67 μc. per mg., purity determined by thin-layer chromatography and subsequent radioautography. This compound has been designated 5-carbonyl-C^{14} GS-13005 (Figure 1).

***S*-[(2-Methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl-5-C^{14})-methyl]*O,O*-Dimethyl Phosphorothioate.** The same general procedure as that used for the 5-carbonyl-C^{14} GS-13005 was followed in preparing its oxygen analog, except that ammonium *O,O*-dimethyl phosphorothioate was used, and that the reaction was carried out using smaller amounts. The product was purified by preparative thin-layer chromatography using GF silica gel. The zone containing the oxygen analog was immediately scraped from the wet plate, and then the oxygen analog was extracted with acetone. If the plate was allowed to dry, evidence of breakdown into several unknown compounds was found by thin-layer chromatography. A colorless oil was obtained; specific activity was 2.25 μc. per mg.; purity was determined by thin-layer chromatography and subsequent radioautography. This compound has been designated 5-carbonyl C^{14} oxygen analog (Figure 1).

Methyl-C^{14}-xanthic Acid (I). Methanol-C^{14} (100 mmoles) and CS_2 (100 mmoles) in 15 ml. of toluene were added over a period of 20 minutes to a stirred and water-cooled mixture of KOH (100 mmoles) and 15 ml. of toluene. The slurry was stirred overnight. After filtration and drying, the yellow salt (12.4 grams) had a purity of 96.2%, as indicated by $HClO_4$ titration.

***O*-Methyl-C^{14} Thiocarbazate (II).** Hydrazine hydrate (81.6 mmoles) was added to a stirred and ice-bath-cooled solution of compound I (81.6 mmoles and 5.5 ml. of water). Stirring was continued for 4 hours at room temperature. The yellow slurry was extracted six times with 50-ml. portions of chloroform and the extract was dried over $MgSO_4$. The latter was removed by filtration. After concentration and drying, a white solid product (5.6 grams) was obtained, m.p. 72–4° C. Titration with $HClO_4$ indicated a purity of 99.5%.

2-Methoxy-C^{14}-Δ^2-1,3,4-thiadiazolin-5-one (III). Compound III was prepared using compound II.

The same general procedure as that used to prepare compound RH was followed. A white crystalline material (4.7 grams) was obtained, m.p. 111–14° C.

4-(Hydroxymethyl)-2-methoxy-C^{14}-Δ^2-1,3,4-thiadiazolin-5-one (IV). Formaldehyde (32.4 mmoles, 36.8%) was added to compound III. The mixture was heated to 120° to 125° C. and then refluxed for 1 hour. On cooling to room temperature, the green mixture was treated with 2.7 ml. of 5% Na_2CO_3. The solution was extracted with three 20-ml. portions of chloroform. The extract was filtered and then taken to dryness. A white crystalline product was dried over P_2O_5, m.p. 86.5–7.5° C. (5.05 grams).

4-(Chloromethyl)-2-methoxy-C^{14}-Δ^2-1,3,4-thiadiazolin-5-one (V). Thionyl chloride (42.4 mmoles) was added to 5 ml. of chloroform and cooled to −10° C. To this solution, compound IV (31.2 mmoles) in 30 ml. of chloroform was added over a 25-minute period. The mixture was stirred at −10° C. for 30 minutes. The temperature of the bath was slowly increased to 70° C., and the reaction mixture refluxed for 2 hours and then stirred at room temperature overnight. The solvent was removed using vacuum, and an oily product (5.5 grams) was recovered.

***S*-[(2-Methoxy-C^{14}-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl)-methyl]*O,O*-dimethyl phosphorodithioate.** The potassium salt of *O,O*-dimethyl phosphorodithioic acid was slurried in 10 ml. of acetone and warmed to 30° to 35° C. Compound V (30.5 mmoles), dissolved in 15 ml. of acetone, was added to the slurry over a 10-minute period. The slurry was cooled to room temperature and stirred overnight. The acetone was removed under nitrogen, and then 25 ml. of water was added. The mixture was extracted five times with 50-ml. portions of ether. The extracts, after drying over $MgSO_4$, were stripped of solvent. The crude product was purified in the same manner as the 5-carbonyl-C^{14} GS-13005; the yield of pure material was 4.7 grams and the specific activity was 2.63 μc. per mg. This compound has been designated 2-methoxy-C^{14} GS-13005 (Figure 1).

TREATMENT OF ALFALFA

The 2-methoxy-C^{14} GS-13005 was injected into alfalfa grown in pots in a greenhouse during the winter. The 5-carbonyl-C^{14} GS-13005 and the 5-carbonyl C^{14} oxygen analog were injected into alfalfa grown in open outdoor hotbeds during the early summer. Each sample consisted of three stalks which at harvest weighed from 3 to 6 grams, with a mean of about 4 grams. Ethanol was used as the injecting solvent. All samples were extracted and analyzed immediately after harvest.

In another experiment designed to simulate application under field conditions, a 90-square-foot plot of alfalfa was sprayed at the Geigy Research Farm, New Paltz, N. Y. A liquid concentrate containing 1 gram of 5-carbonyl-C^{14} GS-13005 was diluted with water and applied using a hand boom sprayer, this application being equivalent to 1 pound per acre. Analysis of the radioactive material prior to formulation indicated that it contained 0.05 to 0.10% of the oxygen analog.

METHODS

The alfalfa acetone extracts were partitioned between chloroform and water to obtain a rudimentary separation of nonpolar and polar metabolites. The number of metabolites was determined by taking aliquots from each fraction and separating them by thin-layer chroma-

tography. Additional procedures were developed for the field samples which are very similar to those used for residue analyses. A flyhead homogenate (Mattson *et al.*, 1969) was used to detect cholinesterase-inhibiting metabolites on thin-layer chromatograms.

Procedure 1: Extraction and Partition of Alfalfa. Alfalfa was cut into sections and macerated for 10 minutes with 30 ml. of acetone and water (9 to 1) in an Omni-Mixer. After settling, the liquid extract was decanted into a fritted glass funnel containing a 2-mm. pad of Hyflo Super-Cel, vacuum being used to aid filtration. The residue and pad were extracted and filtered again. The acetone was removed from the combined filtrates using a rotary evaporator.

The concentrate was transferred to a separatory funnel and diluted to 20 ml. with water. Twenty milliliters of chloroform was added, and the mixture shaken gently. After separation, each layer was washed with 2 ml. of solvent, the chloroform with water, and vice versa. The washings were added to the appropriate fractions.

The aqueous fractions were centrifuged at least 1 hour. The lower chloroform layer was transferred to the main chloroform layer. The aqueous fraction was filtered as described earlier, and again centrifuged. With a Pasteur pipet (9-inch), the aqueous layer was transferred without disturbing the chloroform layer, which should be clear and about 0.3 ml. If not clear, small amounts of water and chloroform were added, shaken, and allowed to separate. To prevent contamination of the aqueous layer, it was transferred before the chloroform layer.

The combined aqueous portions and the combined chloroform portions were each diluted to 25 ml. From each fraction, an aliquot was added to 20.0 ml. of a scintillation solution, Bray's solution for an aqueous aliquot (0.50 ml.) and a toluene solution (5 grams of PPO and 0.3 gram of POPOP diluted to 1 liter with toluene) for a chloroform aliquot (0.25 ml.). A Packard Tri-Carb 3365 was used for all counting, and the disintegrations per minute (d.p.m.) for a sample were determined by internal standardization.

When alfalfa was extracted immediately after injection, over 90% of the radioactivity was recovered in the chloroform fraction. Extracts of healthy, rapidly growing plants did not form emulsions which were difficult to break, while weak slow-growing plants, or plants which had been stored frozen, were frequently troublesome. These emulsions were broken by prolonged centrifuging. All chloroform had to be separated from the aqueous layer to prevent carryover of nonpolar radioactivity.

Procedure 2: Two-Dimensional Thin-Layer Chromatography. For the chloroform fractions, 250 μl. from each was spotted directly onto one corner of a 250-micron layer of silica gel G. Initial development was with benzene–chloroform–ethyl acetate (85:15:5). The plate was dried for 15 minutes. A small amount of the oxygen analog was then spotted below the original spot. The plate was turned 90° and developed with chloroform-acetone (9 to 1). When dry, the plate was either sprayed with flyhead homogenate (Procedure 5) and then exposed to x-ray film for two weeks, or the radioactivity in the GS-13005 and oxygen analog zone areas was determined by scintillation counting after the zones were scraped. The colored plant material was used as a guide in locating the zones. All scrapings were added to vials containing 20 ml. of toluene scintillation solution. The scrapings from oxygen analog zones also required the addition of 0.54 gram of Cab-O-Sil (Cabot, M-5). Radioactive standards of C^{14} GS-13005 and the C^{14} oxygen analog carried through the separating, scraping, and counting procedures resulted in recoveries of 97 and 93%, respectively. Without addition of Cab-O-Sil, the recoveries for the C^{14} oxygen analog were only about 80%.

For the aqueous fractions, a 5.00-ml. aliquot was concentrated to 1.00 ml. using a rotary evaporator; then a 25-μl. aliquot of the concentrate was spotted on a thin-layer chromatographic plate (250-micron, silica gel G). The oxygen analog was always spotted as a reference standard.

Two separate chromatograms were developed for each aqueous fraction of the many extracts prepared. One plate was developed in a nonpolar system of chloroform-acetone (9 to 1) and the other plate was developed in a polar system of acetonitrile-water-ammonia (40:9:1). The plates were allowed to dry, sprayed with flyhead homogenate, evaluated for cholinesterase-inhibiting zones, and then exposed to x-ray film for two weeks. No difference was found in the radioautograms of samples or of standards that had been, or had not been sprayed with flyhead homogenate.

The average R_f values for GS-13005, its oxygen analog, and RH using these three developing systems are given in Table I. The two-dimensional development as outlined was used only for alfalfa extracted as outlined in Procedure 1.

Procedure 3: Methanol Extracts of Field Alfalfa. Twenty grams of alfalfa was blended with 80 ml. of Nanograde methanol for 5 minutes in a blender. The liquid extract was filtered; the filtrate was immersed in an acetone–dry ice bath, and filtered again. Although only small amounts of plant material were removed, the thin-layer chromatographic characteristics of the extract were improved. Total radioactivity in the filtered extract was determined using the toluene scintillation solution.

Aliquots of the methanol extracts were spotted on a 250-micron layer of silica gel. Polar and nonpolar systems B and C, given in Table I, were used on different plates for one-dimensional development. By use of the flyhead homogenate (Procedure 5), the amount of the oxygen analog was estimated by visual comparison with zones representing known amounts of this analog. The dried chromatograms were then exposed to x-ray film for two weeks.

Table I. Thin-Layer Chromatographic R_f Values of GS-13005, Its Oxygen Analog, and RH in Various Systems

Compound	Average R_f[a]		
	A	B	C
GS-13005	0.50	0.68	0.85
Oxygen analog	0.05	0.38	0.75
RH	0.09	0.38	0.60

[a] A. Benzene–chloroform–ethyl acetate (85:15:5).
B. Chloroform-acetone (9 to 1).
C. Acetonitrile-water-ammonia (40:9:1).

Table II. Residues (P.P.M.) in Fractions of Alfalfa Extracts from Greenhouse Alfalfa Injected with 2-Methoxy-C^{14} GS-13005 and Hotbed Alfalfa Injected with 5-Carbonyl-C^{14} GS-13005

Days Post Injection	Chloroform-Soluble				Water-Soluble (Equivalent to GS-13005)
	GS-13005		Oxygen Analog		
	GLC[a]	TLC[b]	TLC[b]	CHE[c]	
			Greenhouse		
3 1/2	175	214	0.8	0.8	25
6 1/2	92	99	0.9	1.0	34
10	96	93	0.4	0.4	30
16	46	46	0.5	0.4	34
23	20	24	0.4	0.4	36
			Hotbed		
3 1/2	6.9	7.1	0.4		39
	23.5	27.7	0.7		38
6 1/2	1.4	1.4	0.1		28
	1.8	2.1	0.2		42
19 1/2	<0.5	0.4	0.2		12
	<0.5	0.2	0.2		15

[a] Nonradioactive method.
[b] Radioactive method.
[c] Hestrin method.

Aliquots were also injected directly into a gas chromatograph equipped with an electron capture detector. Column operating conditions were the same as those given in Procedure 4.

To determine the residue level of GS-13005, corrections were necessary for the decreased detector response caused by large amounts of plant material.

Procedure 4: Acetone Extracts of Field Alfalfa. One hundred grams of alfalfa was blended with 400 ml. of acetone for 5 minutes in a blender. The extract was filtered using vacuum. The residue level of the oxygen analog was determined in aliquots of the filtered extract as described for methanol extracts (Procedure 3)—i.e., only one-dimensional development was done.

An aliquot of the acetone extract representing 50 grams of alfalfa was mixed with 200 ml. of benzene and 100 ml. of water. The radioactivity was determined in each layer after the mixture had separated. Part of the benzene fraction, equivalent to 48 grams of alfalfa, was partitioned and column chromatographed using a column of Florisil. The column eluate was analyzed for GS-13005 by gas chromatography. This procedure was also used to determine GS-13005 in milk, as reported by Cassidy *et al.* (1969).

Procedure 5: Flyhead Homogenate Spray. Flyhead cholinesterase is extremely sensitive to the oxygen analog of GS-13005. As little as 100 pg. of the analog can be detected on thin-layer chromatograms. Moreover, this enzyme is inhibited wherever compounds having antiesterase activity occur on developed thin-layer chromatograms. Because of the extreme sensitivity of this method, overloading of chromatograms with plant extracts must be avoided. Extracts of alfalfa that were known not to have been treated with cholinesterase-inhibiting pesticides have produced zones of inhibition when excessively large aliquots were chromatographed. These zones are thought to be naturally occurring cholinesterase-inhibiting compounds.

The procedure used in this study has been reported in detail by Mattson *et al.* (1969).

RESULTS AND DISCUSSION

In this study of the metabolism of C^{14} in alfalfa, using both injected and sprayed plants, acetone extracts were separated into chloroform and aqueous fractions (Procedure 1). The organic fraction contained only one significant radioactive substance besides the parent compound, the oxygen analog. This material was also the only cholinesterase-inhibiting substance present. The aqueous fraction contained radioactive substances, one of which was labile, breaking down soon after extraction, and which was not detected when alfalfa was injected with the radioactive oxygen analog. The labile aqueous-soluble substance, about 20% of the radioactivity, appears to be a conjugate of the desmethyl compound of GS-13005. It is not a cholinesterase inhibitor, and thus does not constitute a residue problem.

Table I presents the R_f values of GS-13005, the oxygen analog, and RH. Table II presents the results of analyses of 2-methoxy C^{14} and 5-carbonyl-C^{14} GS-13005 injected into alfalfa. Tables III and IV present the results of analyses of 5-carbonyl C^{14} GS-13005 sprayed on field-growing alfalfa. Table V shows the R_f values of the major zones of radioactivity found in the aqueous fractions of alfalfa injected with C^{14} GS-13005. These R_f values were determined using eight different polar systems, following initial development with a nonpolar system. The results of the organic soluble fractions are discussed separately from those of the aqueous fractions.

Chloroform-Soluble C^{14} Residues. Two-dimensional chromatograms of all chloroform fractions showed the same pattern when sprayed with flyhead homogenate. Neither the labeling position nor the alfalfa being injected or sprayed made any difference. Three radio-

Table III. Total Radioactivity (P.P.M.)[a] in Fractions of Field Alfalfa Sprayed with 1 Pound per Acre 5-Carbonyl-C^{14} GS-13005 and Extracted by Various Solvents

Harvest, Days	Procedure 1 Acetone-Water		Procedure 3 Methanol	Procedure 4 Acetone	
	Chloroform	Water	Not Fractionated[b]	Benzene	Water
0	42.5	1.5	46	44	1.8
3	3.9	3.8	7.8	5.0	3.2
6	2.0	3.9	5.0	1.9	3.5
10	1.7	5.0	4.6	1.6	3.9
18	1.1	4.5	3.0	0.7	3.7
22	0.7	3.5	2.0	0.5	2.7

[a] P.p.m. equivalent to GS-13005.
[b] Slight ppt. in counting vials.

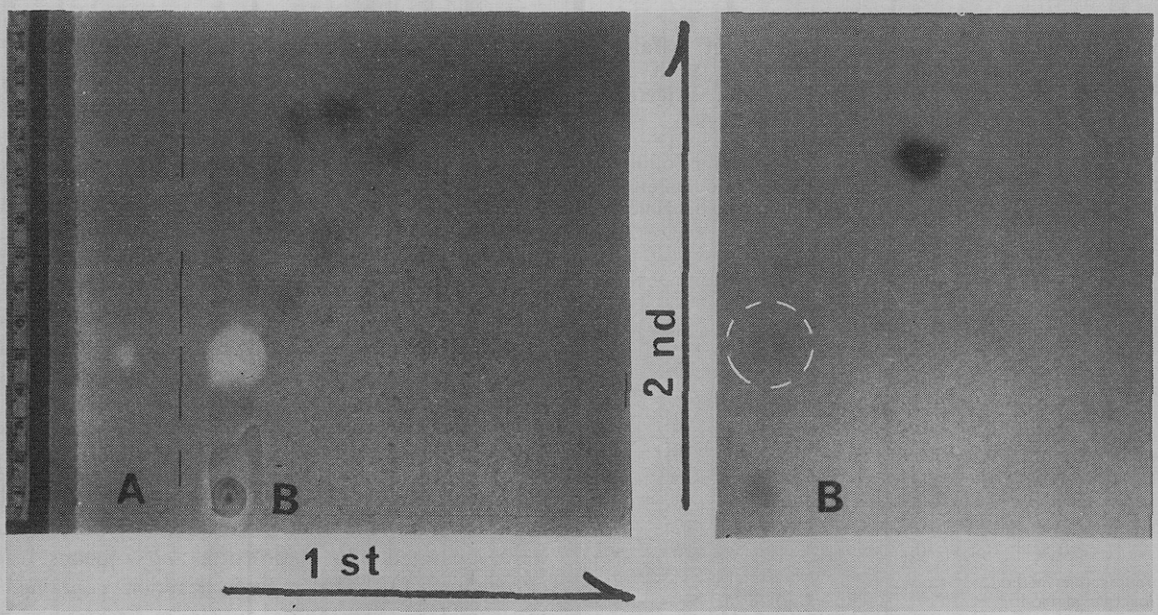

Figure 2. Typical chromatogram for chloroform fraction of alfalfa treated with C^{14} GS-13005

A. Cholinesterase inhibition of 100 pg. of oxygen analog. B (left). Cholinesterase inhibition of chloroform aliquot. B (right). Radioautogram of B (left) (zone encircled in white). 1st system benzene–chloroform–ethyl acetate (85:15:5). 2nd system chloroform-acetone (9 to 1)

active zones were found by radioautograms: GS-13005, the oxygen analog, and polar material that remained at the origin. For alfalfa injected with the C^{14}-oxygen analog, no zone for GS-13005 was found. The relatively small amount of polar radioactivity remaining at the origin was caused by incomplete separation of the chloroform and water phases.

The only zone of cholinesterase inhibition found on the two-dimensional chromatograms corresponded to the oxygen analog. A typical chromatogram is shown in Figure 2. This zone of inhibition could be detected even when trace amounts of the oxygen analog were present and when no corresponding zone could be detected on radioautograms. Because of the extreme sensitivity of the flyhead homogenate, no other cholinesterase-inhibiting metabolite could be present without it being detected by this procedure. To further substantiate this conclusion, the cholinesterase inhibition of chloroform extracts prepared from alfalfa injected with methyl-C^{14} GS-13005 were determined by the Hestrin (1949) method. The ChI_{50} value of 0.001 mg. per 2 ml. was determined for a pure oxygen analog standard using flyhead homogenate. This value was used to calculate the part-per-million values of the oxygen analog, assuming that all of the inhibition in the chloroform was due to this compound. The resulting values (Table II) show excellent agreement with those determined by radioactivity (Procedure 2)—*i.e.*, by scraping the zones from thin-layer chromatograms and determining the radioactivity in the silica gel. These results, obtained by two techniques, substantiate the conclusion that the oxygen analog is the only cholinesterase-inhibiting metabolite in the chloroform fraction.

The rate of metabolism is greatly influenced by growing conditions. This fact is quite evident by comparing the data for greenhouse alfalfa injected with 2-methyl-C^{14} GS-13005 and that for hotbed alfalfa injected with 5 carbonyl-C^{14} GS-13005 (Table II). The dissipation of residues is much more rapid under ideal hotbed growing conditions.

The most important information is that obtained from alfalfa sprayed with 5-carbonyl C^{14} GS-13005 at the rate of 1 pound per acre. The extracted radioactivity and the partitioning of this radioactivity between chloroform and water, and benzene and water (Table III), show that extracting efficiencies using procedures 1, 3, and 4 are not significantly different. The water-soluble radioactivity peaked on the tenth day, and then decreased

Table IV. Radioactive Residues (P.P.M.) in Field Alfalfa Sprayed with 1 Pound Per Acre 5-Carbonyl-C^{14} GS-13005

	GS-13005			Oxygen Analog	
				Procedure 1,2, acetone-water extract	
Harvest, Days	Procedure 1,2, acetone-water extract TLC-radioassay	Procedure 3, methanol extract electron capture	Procedure 4, acetone extract microcoulometric	TLC-radioassay	Fly head spray
0	32	34	33	0.4	0.3
3	3.3	3.3	3.2	0.2	0.2
6	1.3	1.5	1.5	0.2	0.1
10	0.9	0.7	1.2	0.2	0.04
18	0.4	<0.5	0.4	0.1	0.02
22	0.2	<0.5	0.3	<0.1	0.01

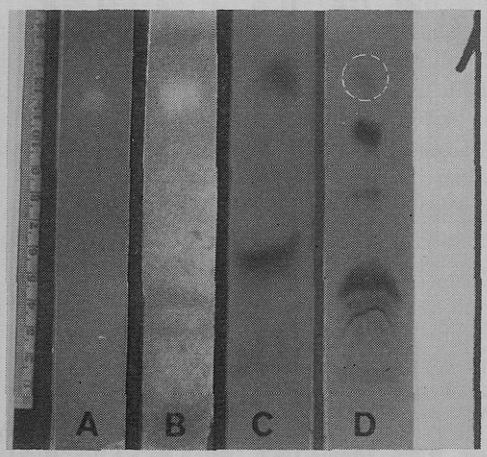

Figure 3. Typical chromatogram for aqueous fraction of alfalfa treated with C^{14} GS-13005

A. Cholinesterase inhibition of 100 pg. of oxygen analog
B. Cholinesterase inhibition of an aqueous aliquot
C. Radioautogram of B
D. Radioautogram of same fraction after standing 3 days (top zone outlined in white)
System, acetonitrile-water-ammonia (40:9:1)

slowly. The radioactivity in the organic fraction, as was the case for the injection work (Table II), declined steadily.

Analyses for GS-13005 and the oxygen analog in field samples were done by several different methods. The dissipation of these two compounds (Table IV) indicates that no unusual accumulation occurs under normal field practice. The zero day value of 0.3 p.p.m. for the oxygen analog is less than 1% of the initial level of GS-13005. The dissipation curve for the oxygen analog based on these values does not show an increase after application, as does the typical initial metabolic curve for the oxygen analog of many organothiophosphates—e.g., data presented by O'Brien (1960). The residue half-life (RL_{50}) (Gunther and Blinn, 1958) for the oxygen analog was 4.2 days during this 22-day field study. This short half-life indicates that whether the analog is or is not a metabolite, its transient nature will not result in any build-up in alfalfa.

The oxygen analog values determined by radioassay (Procedure 2) for the 10- to 22-day samples are too high, since more radioactivity was streaked into the zone of the oxygen analog as the ratio of the oxygen analog to polar material decreased. When the relative ratios of the solvents in the developing system of benzene–chloroform–ethyl acetate were changed from 85:15:5 to 4:4:2, more radioactivity was removed from the oxygen analog zone. The values determined by cholinesterase inhibition are more specific for the oxygen analog than the values by radioassay.

Table V. R_f Values of Major Radioactive Zones Found in the Aqueous Fractions by Two-Dimensional Development Using Chloroform-Acetone (9 to 1) Initially and Then Various Polar Developing Systems

Polar Developing System	Solvent Ratio	Upper Zone	Lower Zone
CH_3CN–H_2O	40 to 10	0.60	0.25
CH_3CN–H_2O	40 to 20	0.75	0.40
CH_3CN–H_2O	10 to 40	0.85 one zone	
CH_3CN–H_2O–NH_3	40:9:1	0.75	0.20
CH_3CN–H_2O–NH_3	40:5:1	0.65	0.20
CH_3CN–NH_3	50 to 1	0.35	0.15
H_2O–NH_3	50 to 1	0.75 one zone	
$(CH_3)_2CHOH$–H_2O–NH_3	20:2:1	0.65	0.25

Aqueous-Soluble C^{14} Substances. The polar metabolites found in all aqueous fractions after partitioning (procedures 1 and 4) constituted about 10 to 30% of the radioactivity injected into alfalfa, and only 3 to 11% of that deposited by spraying. Aliquots of all aqueous fractions (Procedure 2) were chromatographed using two systems. For every sample, the radioactivity on thin-layer chromatograms remained at the origin using chloroform-acetone (9 to 1), but separated into discrete zones using acetonitrile-water-ammonia (40:-9:1). All radioautograms of alfalfa treated with C^{14} GS-13005 had the same pattern.

To determine if the aqueous soluble polar metabolites were the same whether 2-methoxy-C^{14} or 5-carbonyl-C^{14} GS-13005 was injected, aliquots were chromatographed two-dimensionally, initially with chloroform-acetone (9 to 1), and then with a very polar system. Eight different polar systems were used. The radioautograms showed that for six systems two major zone areas of radioactivity (Table V) were detected and for two, the most polar systems, only one zone was detected. Since the 9-to-1 system would remove GS-13005, its oxygen analog, and RH from the origin, the zones on radioautograms do not represent these compounds. In all systems, no difference was found in the chromatograms of the 2-methoxy-C^{14}- and the 5-carbonyl-C^{14}-treated samples. Since the chromatographic patterns are the same, cleavage of the methoxy group from the thiadiazole ring is not a primary metabolic step.

The more polar radioactive zone that remained closer to the origin contained about 80% of the aqueous soluble radioactivity and most of the plant material, while the zone nearer the solvent front contained about 20% of the radioactivity. When the aqueous fractions of alfalfa injected with the oxygen analog were chromatographed, the resulting radioautograms showed no upper zone. The absence of such a zone is further evidence that the oxygen analog is not the primary route of metabolism of GS-13005 in alfalfa; moreover, this absence indicated that this upper zone represents a primary metabolite for GS-13005.

Additional work showed the material in the upper zone to be very labile. When an aqueous fraction is allowed to stand for a couple of days, and then an aliquot is chromatographed, the upper zone becomes a doublet (upper part of doublet encircled in white), as shown in Figure 3. Eluting and rechromatographing radioactivity in these two zones resulted in further breakdown, whereas GS-13005 and its oxygen analog are relatively stable under identical treatment. Thus, attempts to isolate enough of these substances for identification by either mass spectrometry or infrared were unsuccessful.

That the lower zone of the doublet represented the desmethyl derivative of GS-13005 {*S*-[(2-methoxy-5-oxo-Δ^2-1,3,4,-thiadiazolin-4-yl)methyl]*O*-methyl, *O*-hydrogen phosphorodithioate} was considered, but attempts to prepare it as a standard by chemical synthesis were unsuccessful. Bull (1968) claims to have prepared this compound. His thin-layer and paper chromatography of plant extracts are the same as those in this study.

Based on R_f's, the lower zone of the doublet does seem to be the desmethyl GS-13005. The presence of this compound would explain the data for alfalfa injected with the 5-carbonyl-C^{14} GS-13005 oxygen analog—i.e., the desmethyl GS-13005 cannot be formed from the oxygen analog. It is thought that a labile conjugate of desmethyl GS-13005 exists in alfalfa which is readily hydrolyzed.

Based on the data of Bull (1968), Esser and Müller (1966), and that in this study, there is a strong possibility that a plant can cleave the RH moiety, releasing $C^{14}O_2$. Though the thiadiazole ring is stable even to acid, a plant seems to be able to break this ring. The radioactive zones found in the lower part of chromatograms for aqueous fractions, as shown in Figure 3, were always present in considerable plant material. Regardless of the C^{14} position and dependent on growing conditions, varying amounts of evolved $C^{14}O_2$ would be detected analytically.

Traces of the oxygen analog, up to 0.01 p.p.m., were found on thin-layer chromatograms of the aqueous fraction whenever difficulty was encountered in separating the chloroform and water layers. The zones of cholinesterase inhibition caused by these traces of oxygen analog did not coincide with either zone of the upper doublet when the acetonitrile-water-ammonia system (40:9:1) was used. A typical chromatogram is shown in Figure 3. When the chloroform-acetone system (9 to 1) was used, the zone showing cholinesterase inhibition always corresponded to that of the standard of the oxygen analog. No zone exhibiting anticholinesterase activity was found on chromatograms of aqueous fractions other than that for the oxygen analog.

LITERATURE CITED

Bull, D. L., J. AGR. FOOD CHEM. **16,** 610 (1968).
Cassidy, J. E., Murphy, R. T., Mattson, A. M., Kahrs, R. A., J. AGR. FOOD CHEM. **17,** 571 (1969).
Esser, H. O., Müller, P. W., *Experienta* **22,** 36 (1966).
Gunther, F. A., Blinn, R., "Analysis of Insecticides and Acaricides," p. 138, Interscience, New York, 1958.
Heath, D. F., "Organophosphorus Poisons," p. 172, Pergamon, New York, 1961.
Hestrin, Shlomo, *J. Biol. Chem.* **180,** 249 (1949).
Mattson, A. M., Kahrs, R. A., Murphy, R. T., J. AGR. FOOD CHEM. **17,** 564 (1969).
O'Brien, R. D., "Toxic Phosphorus Esters," Chap. 8, Academic Press, New York, 1960.
Rüfenacht, Kurt, *Helv. Chim. Acta* **51,** 518 (1968).

Received for review April 29, 1968. Accepted December 9, 1968.