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Studies of Pesticide Residues on Alfalfa Using C^{14}-Labeled Endosulfan

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Studies of Pesticide Residues on Alfalfa Using C^{14}-Labeled Endosulfan

GEORGE W. WARE[1]

FOREWORD

This bulletin combines several studies of the disappearance and degradation of endosulfan on alfalfa in Ohio. Since none of the investigations has been published, it seemed appropriate to combine and make these available to residue chemists, insect toxicologists, and allied scientists.

Residue information reported here includes data collected from 1958 through 1965. It covers several analytical methods including bioassay, colorimetry, paper chromatography, autoradiography, microcoulometric gas chromatography, electron capture gas chromatography, liquid scintillation spectrometry of C^{14}-labeled endosulfan, and counting in planchets using a windowless scaler.

INTRODUCTION

The organochlorine insecticide, endosulfan (Thiodan[2]), 6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepin-3-oxide, is a sulfur-containing toxicant developed by Farbwerke Hoechst AG, Germany. It is known chemically in the United Kingdom (British Standards Institute) as 1, 2, 3, 4, 7, 7-hexachlorobicyclo [2, 2, 1] -2-hepten 5, 6-bis (hydroxymethylen)-sulfite. The technical material (m.p. 80-90° C.) contains 90% endosulfan as a mixture of two stereoisomers: a high melting point isomer (HMP) (m.p. 208-210° C.) and a low melting point isomer (LMP) (m.p. 108-110° C). The remaining 10% consists of endosulfan alcohol and endosulfan ether (21). (See Fig. 1.)

Endosulfan is manufactured in the United States by the Niagara Chemical Division, Food Machinery and Chemical Corporation, and is used to control a variety of arthropod pests. Of the four synthesis components of technical endosulfan, only the HMP and LMP isomers appear to have appreciable insecticidal activity (10).

Barnes and Ware (1) applied gas chromatography, paper chromatography, and autoradiogram techniques to the study of C^{14}-labeled endosulfan penetration and metabolism in house flies. They identified

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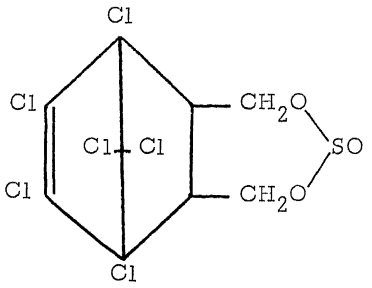
[2]Registered Trade Name.

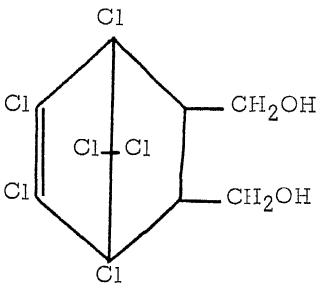
endosulfan sulfate as an oxidized metabolite of endosulfan (Fig. 1) and were able to synthesize it in dilute H_2SO_4 and $KMnO_4$.

Cassil and Drummond (7) also reported endosulfan sulfate formation on leafy plants such as spinach, celery, and alfalfa and on tree foliage. Endosulfan sulfate was reported on silage but not on growing coastal Bermuda grass, trash, or soil (4). It has been synthesized from the LMP and HMP isomers with calcium permanganate in chloroform and acetic acid at 5° C. (11).

Deema and Ware (9) studied the metabolism of endosulfan and its related products in the mouse. They found the chief metabolite to be the sulfate which was stored in fat and excreted in feces. A secondary metabolite found in urine and feces appeared to be endosulfan alcohol.



Endosulfan



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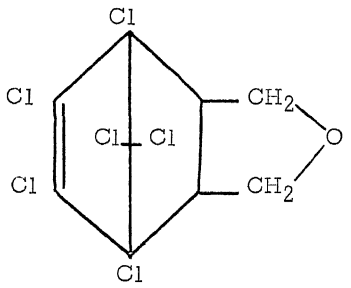
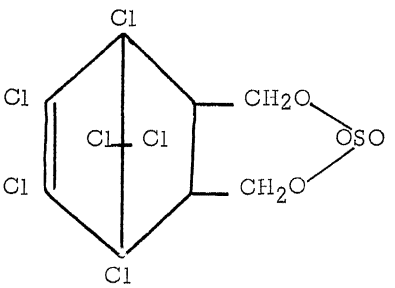


Endosulfan Ether



Endosulfan Sulfate

Fig. 1—Structures of endosulfan and related derivatives.

Lindquist and Dahm (17) investigated some of the chemical properties of endosulfan, using column and paper chromatography, melting point determinations, ultraviolet and infrared spectometry, and LD_{50} determinations on house flies and male white rats. They found a 6.7 μg./g. LD_{50} to susceptible house flies, while the oral LD_{50} to male white rats was between 40 and 50 mg./kg. of body weight.

Cassil (5), Graham (13), and Graham et al. (14) have described colorimetric methods of determining endosulfan residues, using the evolution of sulfur dioxide into an absorbing solution and addition of fuchsin to develop a color. In another method, described by Maitlen et al. (18), pyridine-methanolic sodium hydroxide was added to endosulfan extracts and the color measured photometrically. Paper and gas chromatography have been used to determine endosulfan residues in bean plants by Terranova and Ware (22).

As an experimental insecticide for control of the meadow spittlebug, *Philaenus spumarius* (L.), on alfalfa and red clover, endosulfan at ¼ lb. per acre has proved equal to BHC and lindane (24). However, one difficulty arising from endosulfan application on forage has been the determination of residues on harvested crops. The method developed by Cassil (5) and revised by Graham (13), which is sensitive in the range of 5-50 μg of toxicant, is well suited for the determination of endosulfan residues on plants and in soils but has been successful in only a few residue laboratories.

Several investigations of endosulfan residues and degradation on alfalfa are described in this report. These investigations were carried out between 1958 and 1965 and involve various analytical methods which are discussed and compared in terms of sensitivity and dependability.

1958 ENDOSULFAN RESIDUES ON ALFALFA

On April 25, 1958, duplicate plots of mixed alfalfa and red clover, 13 x 75 feet, were treated with endosulfan at the Ohio Agricultural Research and Development Center, Wooster. Emulsion sprays and 5% Attaclay granules were applied at three rates: 0.25, 0.5, and 1.0 lb. per acre. The sprays were made from emulsifiable concentrate containing 2 lb. per gallon and applied with a jeep-mounted, boom-type weed sprayer at 40 p.s.i. and 10 gal. per acre. The granules (24/48 mesh) were applied with a modified wheelbarrow-mounted Oliver grass seed drill. At the 0.25 lb. per acre rate, the original granules were diluted with an equal amount of 30/60 mesh Attaclay granules and applied at 10 lb. per acre. The 0.5 lb. treatment was made with the 5% granules at 10 lb. per acre while the 1.0 lb. rate was made with 20 lb. per acre.

The crop at time of treatment was between 5 and 7 inches tall. Six 1-lb. samples and a check were collected by hand from each of the six treatments at 0, 1, 3, 5, 7, 14, 21, 28, 35, and 42-day intervals for chemical and biological assay.

The samples were frozen until extracted as follows. A 500-gram alfalfa sample was cut on a hand-operated bacon slicer and then divided roughly into halves. These were placed in gallon jars with 400 ml. of redistilled hexane (Skellysolve B), minced on the Omnimixer for 5 minutes each, and combined in a large-mouth gallon jar. The mixer and gallon jars were rinsed with 200 ml. of hexane, bringing the total to 1000 ml.

The gallon jar was rolled on a rolling mill at 35 r.p.m. for 1 hour. The sample was filtered through Whatman No. 1 disc filters until 800 ml. were collected. The extract was then evaporated to 400 ml. with each ml. representing 1 gram of alfalfa. The extract was halved and placed in 2 half-pint prescription bottles which were held in the deep freeze.

The analytical method used was that described by Cassil (5) in which evolution of sulfur dioxide into an absorbing solution was measured colorimetrically by the addition of fuchsin dye, producing a violet color read at 570 mμ.

Biological assays were also conducted on these extracts, using second instar larvae of the mosquito, *Aedes aegypti,* following the methods described by Burchfield and Hartzell (3) and modified by France, Treece, and Ware (12). The minimum detectable level for the bioassay was 1.0 p.p.m. The results of chemical and biological assays are shown in Tables 1 and 2.

1958 ENDOSULFAN RESIDUES ON ALFALFA ANALYZED BY NIAGARA CHEMICAL DIVISION

In May 1961, the duplicate extracts from the 1958 endosulfan applications, which had been frozen since extraction, were sent by air express to Dr. C. C. Cassil at the Richmond, California, laboratory of the Niagara Chemical Division. Here they were analyzed by microcoulometric gas chromatography.

Analytical data from the 1958 applications are presented in Table 3, with all values corrected by the approximate column efficiency and recovery. The values of the untreated check samples were not subtracted due to the presence of a high control reading on the 5-day check sample. Details of recoveries of endosulfan from fortified check samples are presented in Table 4.

The values obtained in the spray versus granular study indicate a much higher initial deposit and more rapid dissipation of residues from the spray-treated plots (6).

TABLE 1.—Endosulfan Residues on First Cutting Alfalfa Measured by Chemical Assay, Expressed as Average of Three Assays, p.p.m. Based on Wet Weight of Samples, 1958.

Rate and Formulation	Days After Application 0	1	3	5	7	14	21	28
¼ lb. Spray	4.41	1.47	0.54	0.13	0.10	0.01	—	—
½ lb. Spray	24.54	7.09	1.59	0.80	0.33	0.03	0.00	0.00
1.0 lb. Spray	52.50	26.46	7.00	5.50	1.28	0.33	0.02	0.00
¼ lb. Granules	0.20	0.07	0.07	0.02	0.00	0.00	0.02	—
½ lb. Granules	0.16	0.14	0.08	0.02	0.07	0.00	0.00	0.00
1.0 lb. Granules	0.44	0.31	0.21	0.09	0.04	0.02	0.01	0.01

TABLE 2.—Endosulfan Residues on First Cutting Alfalfa Measured by Biological Assay Using the Mosquito Larvae, Aedes aegypti, **Expressed as Average of Three Assays, p.p.m. Based on Wet Weight of Samples, 1958.**

Rate and Formulation	Days After Application 0	1	3	5	7	14
¼ lb. Spray	6.18	2.83	2.06	0.00	0.00	0.00
½ lb. Spray	13.70	9.70	3.30	1.40	0.00	0.00
1 lb. Spray	43.20	21.19	9.07	5.53	4.03	0.00
¼ lb. Granules	0.00*	——	——	——	——	——
½ lb. Granules	0.00*	——	——	——	——	——
1 lb. Granules	0.00*	——	——	——	——	——

*Below detectable level.

TABLE 3.—1958 Endosulfan Residues on Alfalfa Analyzed by Microcoulometric Gas Chromatography, 1961.*

Rate and Formulation	Days After Application 0	5	14	21	28
Control	nil	1.84	0.07	nil	——
¼ lb. Spray	4.4	0.47	0.10	——	——
½ lb. Spray	20	1.84	——	0.07	——
1 lb. Spray	46	7.8	0.44	0.13	——
¼ lb. Granules	0.39	0.14	0.08	0.06	——
½ lb. Granules	0.53	0.26	0.12	0.06	0.03
1 lb. Granules	1.19	0.22	0.08	0.08	——

*Analyses by Dr. C. C. Cassil.

TABLE 4.—Recovery of Endosulfan from Fortified Alfalfa Extracts by Microcoulometric Gas Chromatography, 1961.*

Control Sample (Days)	Endosulfan Added (p.p.m.)	Endosulfan Found (p.p.m.)**	Percent Recovery**
7	1.0	0.94	94
14	0.5	0.46	92
21	0.1	0.098	98
28	0.05	0.056	112
		Average	99

*Analyses by Dr. C. C. Cassil.
**Corrected for column efficiency.

1959 ENDOSULFAN RESIDUES ON ALFALFA

Aerial applications of sprays and granular materials were made on April 22 and 23, 1959, at Don Scott Field, to duplicate plots 80 x 400 feet in size. The crop was mixed alfalfa and red clover 6 inches tall. Applications were made with a Piper Cub equipped for crop dusting and spraying.

The following sprays were made from 2-lb. emulsifiable concentrate: 0.25 lb. in 2 gal. per acre; 0.5 lb. in 2 gal. per acre; 0.5 lb. in 4 gal. per acre. All granular formulations were 24/48 mesh and supplied for this experiment by the Niagara Chemical Division, Food Machinery and Chemical Corporation, Middleport, N. Y. These were: 0.25 lb.

TABLE 5.—Endosulfan Residues on Mixed Alfalfa-Red Clover from Aerial Application of Emulsion Sprays and Granular Formulations, Expressed as Average of Three Chemical Assays, p.p.m. Based on Wet Weight of Samples, 1959.

Rate and Formulation	Days After Application 0	4	7	15
¼ lb. Spray 2 gal.	25.33	24.00	1.15	0.05
½ lb. Spray 2 gal.	11.67	11.80	0.77	0.05
½ lb. Spray 4 gal.	49.67	13 53	0.97	0.08
¼ lb. 15 lb. Attaclay Granules	0.57	10.08	0.50	0.04
½ lb. 15 lb. Attaclay Granules	1.90	10.50	0.31	0 10
½ lb. 15 lb. Volclay Granules	2.20	10.58	0.06	——

in 15 lb. Attaclay granules per acre; 0.5 lb. in 15 lb. Attaclay granules per acre; and 0.5 lb. in 15 lb. Volclay granules per acre. An untreated plot served as the control.

The sprays were applied between 7:00 and 8:00 p.m. with a 4-5 m.p.h. northwest wind and a temperature of 45° F. The granules were applied the following morning between 7:00 and 7:30 a.m. with a 4-8 m.p.h. north wind and 35° F.

Three 500-gram samples were collected by hand from each treatment at 0, 4, 7, 15, 22, and 32 days for chemical analysis. The samples were frozen until they were extracted as described in the 1958 study. The analytical method was that described by Cassil (5) and modified by Graham (13). The results appear in Table 5. The residues for 22 and 32 days were less than the minimum detectable level and are not presented.

1961 C^{14}-ENDOSULFAN RESIDUES ON ALFALFA

In April 1961, C^{14}-tagged endosulfan was applied to 6-inch high alfalfa. The labeled material furnished by the Niagara Chemical Division weighed 1.75 grams and yielded 1.01 millicuries of C^{14} activity, a specific activity of 0.577 microcurie per milligram.

Paper chromatographic separation of the constituents, followed by liquid scintillation of 0.5 cm. strip segments, indicated the following composition: LMP isomer, 55.3%; HMP isomer, 30.6%; endosulfan ether, 13.4%; and endosulfan alcohol, 0.7%. Gas chromatographic analysis of the same material by the Niagara Chemical Division indicated 58.3%, 35.6%, 6.0%, and less than 1.0%, respectively. A multiple chromatogram utilizing all four constituents alone and together with C^{14}-labeled toxicant is shown in Fig. 2.

Experimental plots were located at The Ohio State University Swine Evaluation Station (Fig. 3) on 2nd year alfalfa. The soil types for this general locality are Miami and Crosby. The four treated plots were contained in an area 260 feet long by 80 feet wide. The area was surrounded by 5-foot high hog wire fencing attached to steel fence posts spaced 20 feet apart, as specified by the Atomic Energy Commission.

The spray equipment was designed and prepared by the Department of Agricultural Engineering, U.S.D.A. and Ohio Agricultural Research and Development Center. It consisted of a self-contained, air pressure spray apparatus mounted on a tractor with axles extended to 6 feet 4 inches. An "L" steel frame attached to the rear of the tractor placed the nozzles 6 feet behind the driver (see cover photo). Four T-Jet fan nozzles spaced 18 inches apart on the ½-inch I.D. steel pipe boom were mounted on this frame.

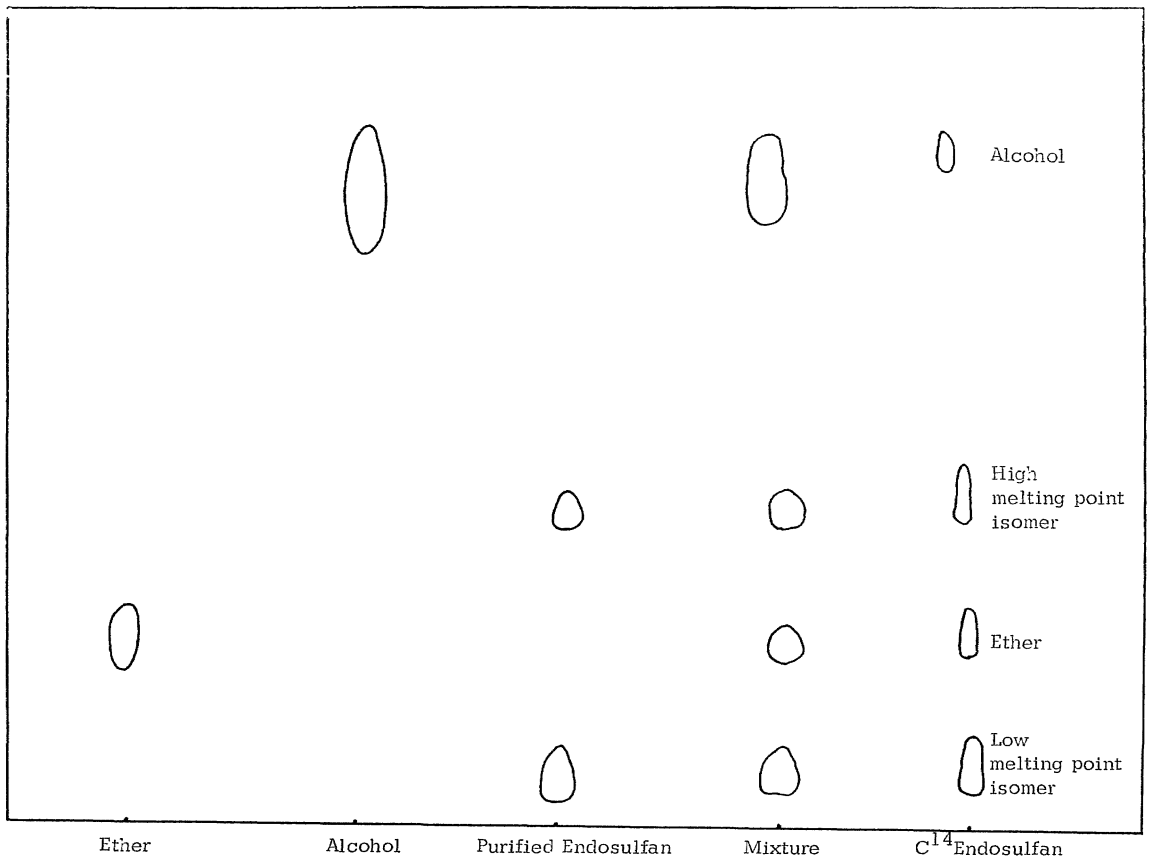


Fig. 2.—Multiple paper chromatogram of normal and C^{14}-labeled endosulfan.

Plastic hoses connected the pressure chamber, which held a 1-qt. insecticide reservoir, to the spray boom and to the compressed air supply tank near the tractor engine. A cut-off valve in the fluid line between the reservoir pressure chamber and the spray boom was controlled by the driver through an extension arm.

Two framed, polyethylene shields mounted on the steel frame midway between the driver's seat and the boom protected the driver from

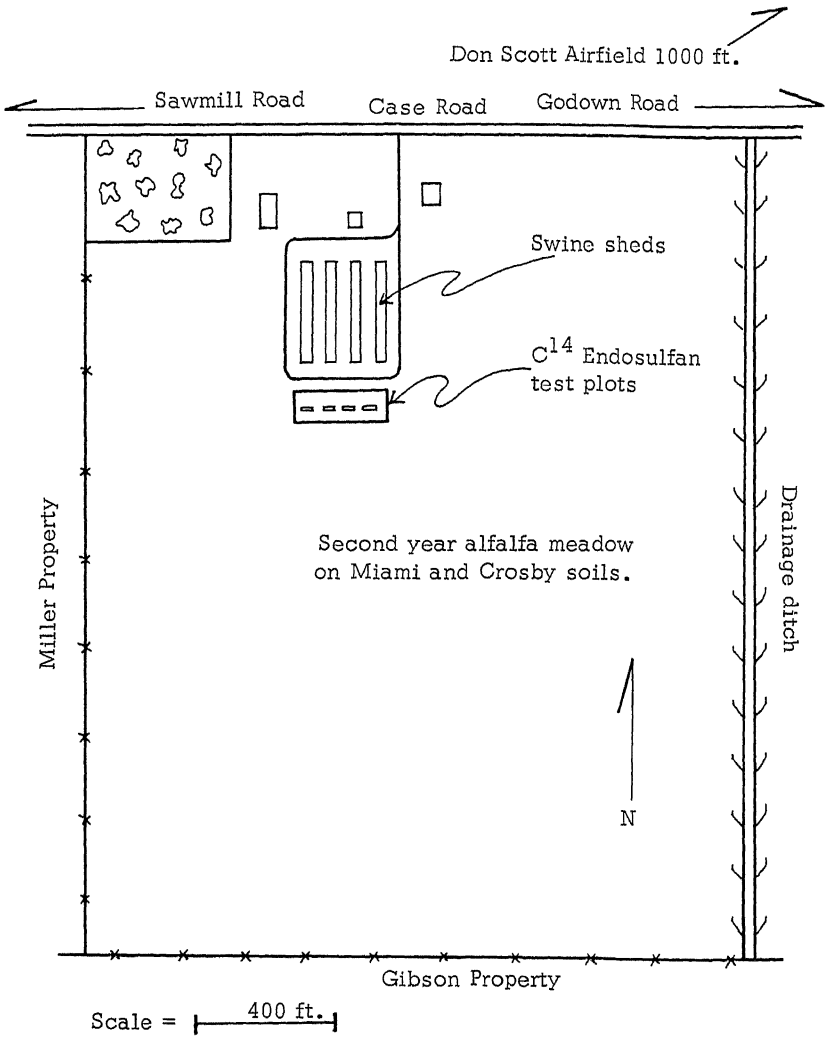


Figure 3.—Scale drawing of The Ohio State University Swine Evaluation Station where C^{14}-labeled endosulfan test plots were located.

forward spray drift and shielded the spray pattern from wind during application. The apparatus was calibrated to discharge 10 gal. per acre at 2 m.p.h. and 30 p.s.i.

Prior to applying the tagged compound, a practice run was conducted April 25 on a set of plots arranged in tandem. Four 6 x 25-foot plots, with untreated lengths between, were sprayed with the experimental spray rig under the described conditions. The spray rig exhausted air on the last 2 feet of the fourth plot, indicating accurate calibration of equipment.

In this practice run, 1.5615 grams of unlabeled endosulfan were dissolved in 4.75 ml. of emulsifying liquid and brought to 522 ml. in water. Under these conditions, the plots were treated with 0.25 lb. of endosulfan, as 2.0 lb. E.C. in 10 gal. of spray per acre.

For the actual application of C^{14}-tagged endosulfan, all of the above conditions were repeated at 5:00 p.m. April 26, with the exception of slightly reduced tractor speed and an 8 m.p.h. west wind. The application was made upwind. Because of the large protective windshields, no noticeable effect was observed in the spray pattern. This was borne out later when drift planchets were checked for radioactivity.

On this occasion the spray rig exhausted air 10 feet from the end of the fourth plot. This would increase slightly the rate of application from the calculated 0.25 lb. to 0.278 lb. per acre. After the first sampling 1 hour following application, all other sampling was compensated for by collecting stems only from the treated portion of the plot.

Samples were taken 0, 7, 14, 21, 28, 35, 42, and 47 days after application. Each sample consisted of two five-stem subsamples, A and B, picked from opposite sides of a plot. The stems were picked individually 2-inches from the ground.

The 50 stem samples were folded over, placed in 0.05 gauge polyethylene poultry freezer bags, and sealed with masking tape. Each sample was marked with date of harvest, plot number, and subsample. Sample 14-3-B, for example, indicated 14 days after application, plot 3, and side B. The samples were returned to the laboratory as soon after collection as possible and frozen until extracted.

On each sampling date, several 200-stem control samples were collected from surrounding alfalfa. A kilogram sample of untreated alfalfa was collected on each sampling date and dried for 1 week in a greenhouse cubicle for dry weight determinations.

All alfalfa within the protective fence, including the test plots, was mowed on August 2, left to dry, and burned on August 9.

Soil samples were then collected from each plot and in the natural runoff area. Gross activity of all samples was measured with a win-

dowless, gas-flow counter and found to be 1.7 times background or less. Since the radioactivity in the soil samples was less than 2 times background, the fence was removed and the alfalfa crop returned to normal cultural practices.

Extraction of the alfalfa samples was conducted during the 2 weeks following the 47-day sampling. To avoid contamination from samples containing higher residues, controls were extracted first, followed by day 47, 42, 35, etc., and ending with day 0.

For extraction, a frozen sample was weighed to the nearest gram and then cut with scissors or a bacon slicer, depending on stem thickness. The chopped material was then placed in one or two 1-gallon jars with 2 ml. redistilled hexane for each gram of sample. The jars were attached to a high speed mixer, minced for 5 minutes each, capped with aluminum foil and screw caps, and then rolled for 1 hour on a rolling mill at 20 r.p.m.

After rolling, the alfalfa-hexane mixture was filtered through Whatman No. 1 filter discs into a liter-graduated cylinder. The volume was recorded and the extracts were transferred to one or more pint prescription bottles, marked with sample number and volume, and held in the deep freeze.

Cleanup was accomplished in a deep freeze. After adding 4 grams of Attaclay per 100 ml. to each extract, the mixture was shaken for a few moments and allowed to stand for 30 minutes. The clear extracts were then filtered into pint prescription bottles. Masking tapes from the original bottles were transferred to the new containers, which were returned to the deep freeze.

Originally it was planned to determine the radioactivity of dried plant extracts on planchets with a gas-flow counter and automatic sample changer. Numerous attempts were made to measure the activity of the extracts but there was little reproducibility among individual samples prepared from the same batch of extract. Some of the difficulties encountered were contamination of sample holders, buildup of activity within the counting chamber, and variation of count rate of a given sample during repeated measurements.

Since initial measurements with a Packard Tri-Carb Liquid Scintillation Spectrometer Model 314X proved successful, it was decided to make all measurements with this equipment.

A liquid phosphor, used for counting non-aqueous test solutions (15), was prepared as follows: 15.12 grams PPO (2,5 diphenyloxazole, primary scintillator) (4 g./liter); 0.378 gram POPOP (1, 4 bis 2 (phenyloxazolyl)-benzene, secondary scintillator) (0.1 g./liter); 3.78 liters toluene.

TABLE 6.—C^{14}-Tagged Endosulfan Residues on Alfalfa Determined by Liquid Scintillation, Expressed as the Average of Eight Subsamples, 1961.

Sampling Day	Endosulfan (p.p.m.) (Wet Weight)*	Endosulfan (p.p.m.) (Dry Weight)
0	13.10	80.75
7	1.19	5.58
14	0.080	0.458
21	0.011	0.059
28	0.011	0.051
35	0.005	0.020
42	0.003	0.013
47	0.002**	0.007**

*Corrected for recovery.
**Average of 2 subsamples.

One-ml. samples of the radioactive alfalfa extracts were pipetted into 20-ml. screw cap counting vials and 20 ml. of liquid phosphor were added. Each vial was sealed with its screw cap and the sample, plot, and vial numbers were written on it. Background vials containing only the liquid phosphor were also prepared and counted with the plant extracts. Up to 100 samples could be prepared and placed in the automatic sample changer. A C^{14} Tri-Carb standard was counted with the other samples to determine counter efficiency, which was 55% during these measurements.

The results shown in Table 6 indicate that endosulfan or its degradation products are present on alfalfa 47 days after application. The 95% confidence limit values were obtained from nomographs presented by Jarrett (16).

Table 7 indicates an average recovery of 75.7% of the C^{14}-endosulfan from 0, 7, 14, and 21-day control samples. In each case the

TABLE 7.—Recovery of C^{14}-Endosulfan from Fortified Control Samples at Different Days. Figures Represent Average of Three Samples Measured by Liquid Scintillation of Plant Extracts Carried Through the Clean-up Procedures.

Control Sample Day	Gross Counts per Minute	Net Counts per Minute	Percent Recovery
0	2956	2938	77.22
7	2846	2828	74.35
14	2627	2608	73.78
21	2970	2952	77.61
		Average	75.74

sample was fortified with 1.0 ml. of C^{14}-tagged endosulfan in acetone added to the stripping solvent in the jar immediately before mincing. The samples were rolled for 1 hour, filtered, and carried through the clean-up procedure. In one instance in which some of the pigmented extract was counted without clean-up, the recovery was 100%.

In correcting the data for recovery losses, as seen in Table 6, the individual recovery percentage was used for its respective sampling date through 21 days. Beyond that, the average recovery of 75.74% was applied (see Table 7).

It was suggested by Bowman (2) that waxes, lipids, and hexane may interfere with the counting rate by quenching or absorbing the emitted photons. The results of several tests conducted to determine this are presented in Table 8.

As indicated by the 98% recovery obtained in the fortified extracts, there is no appreciable loss of activity in alfalfa extracts due to quench-

TABLE 8.—Results of Study to Determine the Quenching Effect of Plant Waxes on Counting Rates in Normal Extracts and 10X Concentrated Plant Extracts Containing C^{14}-Endosulfan. Figures Represent Average of Three Samples Measured by Liquid Scintillation.

Sample No.	Sample Description	Av. Gross Counts/Min.	Av. Net Counts/Min.	Percent of Fortified Counts	Average Recovery
1					
2	Fortified normal	1896.33	1878.40	98.32	
3	14-day extract				
4	Fortified normal				
5	21-day extract	1887.98	1870.05	97.90	98.07
6					
7	Fortified normal				
8	35-day extract	1890.35	1872.42	98.01	
9					
10					
11	Fortified hexane	1928.02	1910.09	100.00	
12	Standard				
13	Fortified 10X conc.				
14	14-day extract	1921.84	1903.91	99.63	
15					
16	Fortified 10X conc				
17	21-day extract	1895.14	1877.21	98 27	98.65
18					
19	Fortified 10X conc.				
20	35-day extract	1891.55	1873.62	98.06	
21					

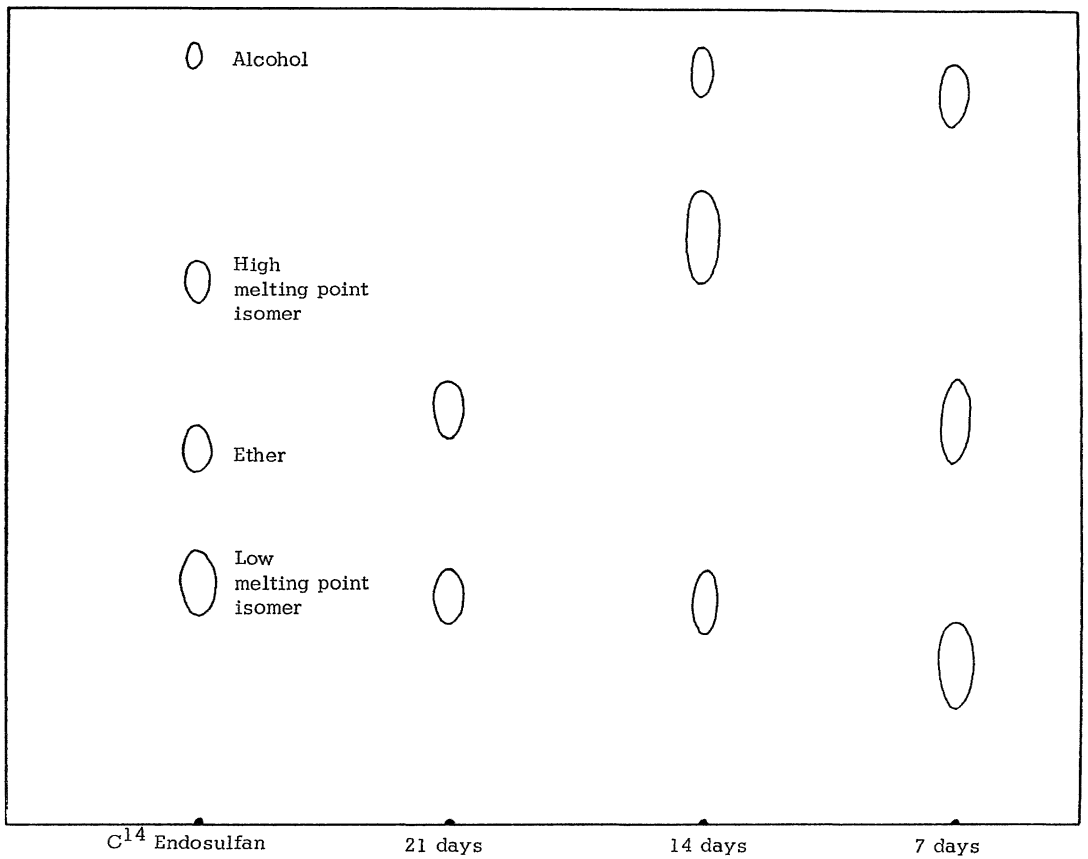


Fig. 4.—Reconstructed paper chromatogram of 7, 14, and 21-day C^{14}-endosulfan-treated alfalfa extracts with the radioactive standard.

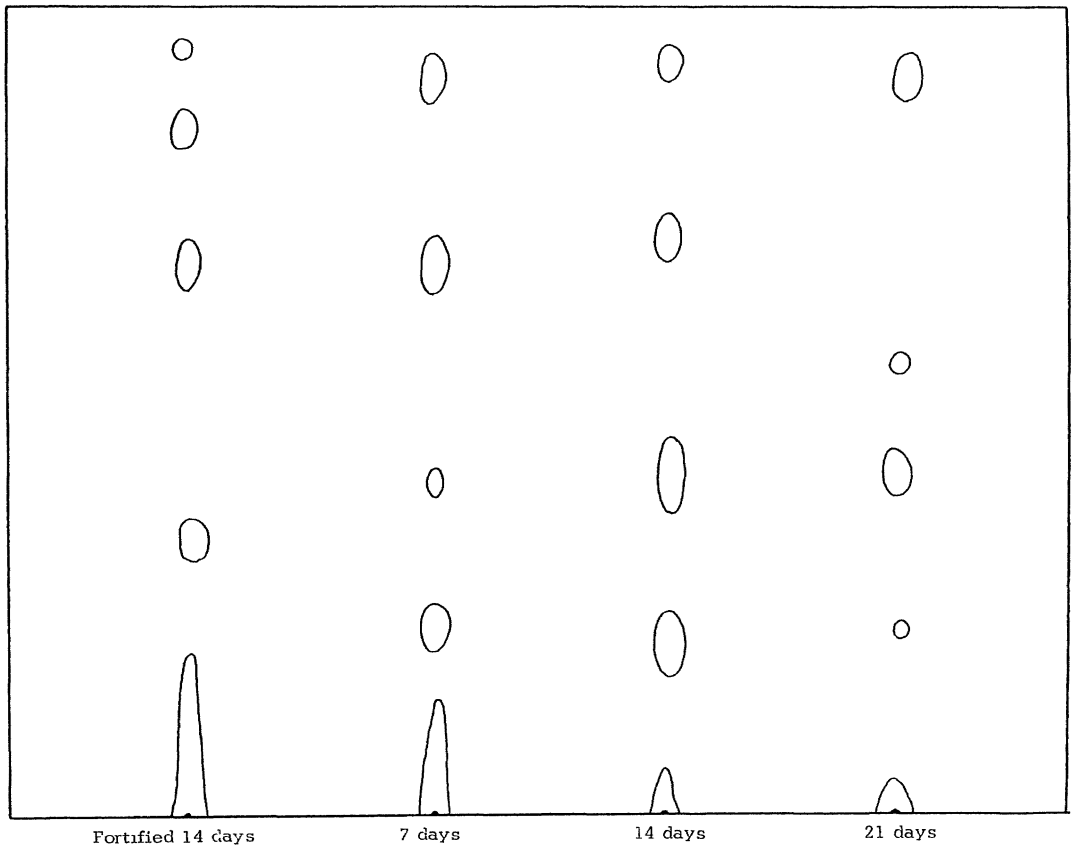


Fig. 5.—Reconstructed paper chromatogram of 7, 14, and 21-day C^{14}-endosulfan-treated alfalfa extracts with radioactive endosulfan-fortified 14-day extract.

ing, either in normal extracts or in those concentrated 10 times. It is believed that raw hexane extracts of alfalfa, diluted as they were with the phospor (20:1), could have been counted satisfactorily without clean-up. However, with extracts concentrated 10 times, the pigment should be given serious consideration as a quenching agent.

A modification of the chromatographic separation of chlorinated insecticides developed by Mitchell (19, 20) was applied to the 7, 14, and 21-day sample extracts with the exception that no chromogenic agents were applied to the papers after development. The two multiple chromatograms presented in Figures 4 and 5 were prepared as follows.

Sheets 9½ x 10 inches were cut from Whatman No. 1 chromotographic sheets and sprayed on both sides with a 5% solution of U.S.P. medium heavy mineral oil in reagent grade anhydrous diethyl ether. Figure 4 represents the chromatogram which received 20 μl of highly concentrated (100X) alfalfa extract and Figure 5 represents 50 μl of the same extract at the points of application. Both were developed in hard rubber photographic tanks for 6 and 6.5 hours respectively, using 65% methyl cellosolve in water as the mobile solvent.

After being dried in the hood for 1 hour, the chromatograms were cut into strips and each strip was cut into ¼-inch lengths. The individual strips in Figure 4 were 3 cm. wide and those in Figure 5 were 4 cm. wide. The ¼-inch lengths were placed diagonally in scintillation vials, with one length to a vial containing 0.5 ml. of phosphor.

The caps were marked and the vials were placed in the automatic scintillation spectrometer. The sample changer temperature was held at 30° F., with time and total counts set for 30 minutes or 10,000 counts for each vial. Figure 5 was repeated, using 50 minutes or 10,000 counts for each vial. This is a modification of the methods reported by Wang (25) and Davidson (8). No increase in count rate was found by filling the vials with phosphor.

These chromatograms show spots at the positions indicated. Further work was conducted with the extracts in which the interfering waxes and lipids were removed and autoradiograms were made. One of these is illustrated in Figure 6.

The data from these liquid scintillation analyses of radioactive materials found in alfalfa extracts are presented in Table 6 and illustrated in Figure 7. The points on both wet and dry weight curves in Figure 7 follow a pattern with the exception of the 21-day results.

These same extracts, uncleaned, were analyzed by microcoulometric gas chromatography and reported by Cassil (6). The slope of these data is included in Figure 7. Results obtained by the two methods of analyses appear to agree, especially at the origin and end of the

microcoulometric gas chromatography at 28 days. Beyond that time, the liquid scintillation method should be more reliable.

There was a significant count rate above background at 35, 42, and 47 days after application. These counts were translated into p.p.m. and, although quite low, appear to follow a uniform pattern of degradation.

A close examination of the slopes from this study and that of Bowman (2) shows a break in the straight-line degradation function in the 15-20 day vicinity. This break suggests the conversion of endosulfan to the cyclic sulfate, which has residual life somewhat greater than that of either isomer.

Supporting this theory is the work conducted on the periodical counting of etched glass planchets sprayed in the field with the alfalfa

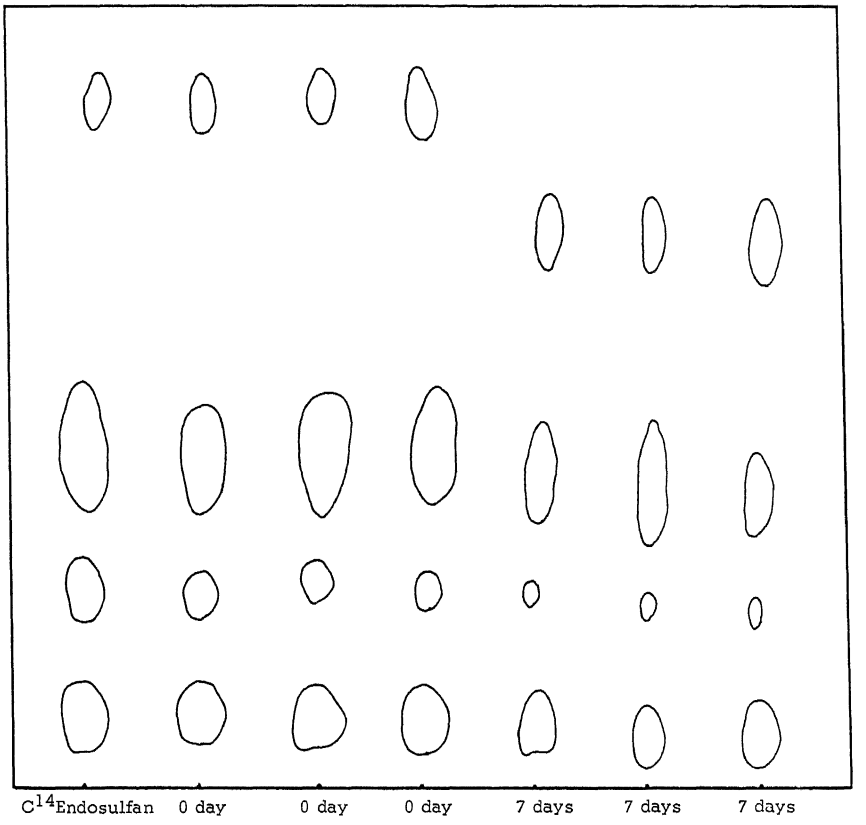


Fig. 6.—Autoradiogram of C^{14}-endosulfan-treated alfalfa extracts at 0 and 7 days after field application of 0.25 lb. per acre.

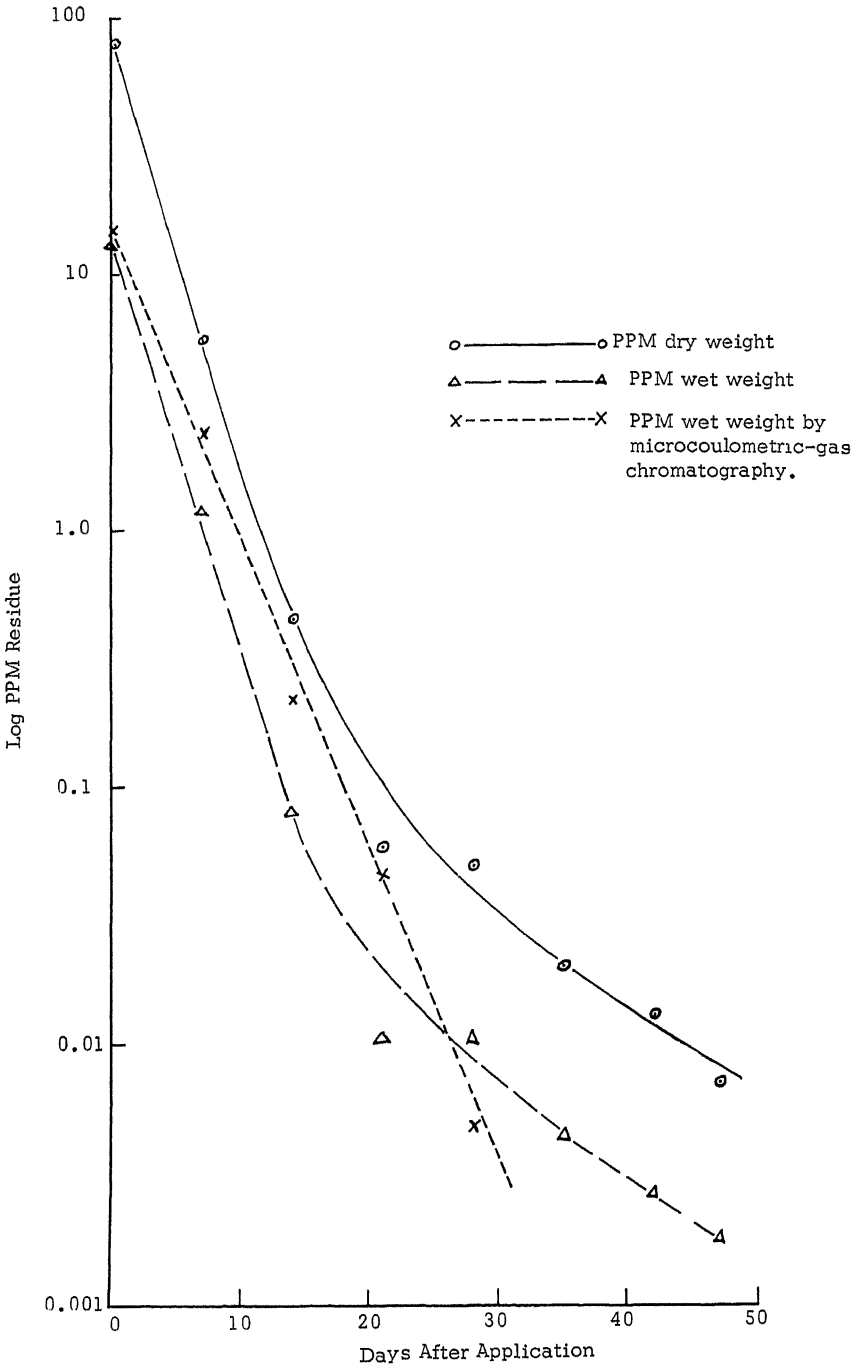


Fig. 7.—Degradation curves of C^{14}-endosulfan applied to 6-inch alfalfa, measured by liquid scintillation and microcoulometric gas chromatography.

plots. The data from several of these planchets, plotted individually, indicate two functions of activity loss. One which contributes greatly to the original steepness of the slope appears to have an approximate half-life of 3.5 days. Another component shows a half-life of 43 days. A representative curve of this disappearance is illustrated in Figure 8.

Since this study was completed, additional work indicates that the cyclic sulfate is not produced on glass plates. Thus the secondary slope is probably that of the high melting point isomer, which has a considerably longer residual life on all surfaces and is not converted to the cyclic sulfate as readily as the low melting isomer.

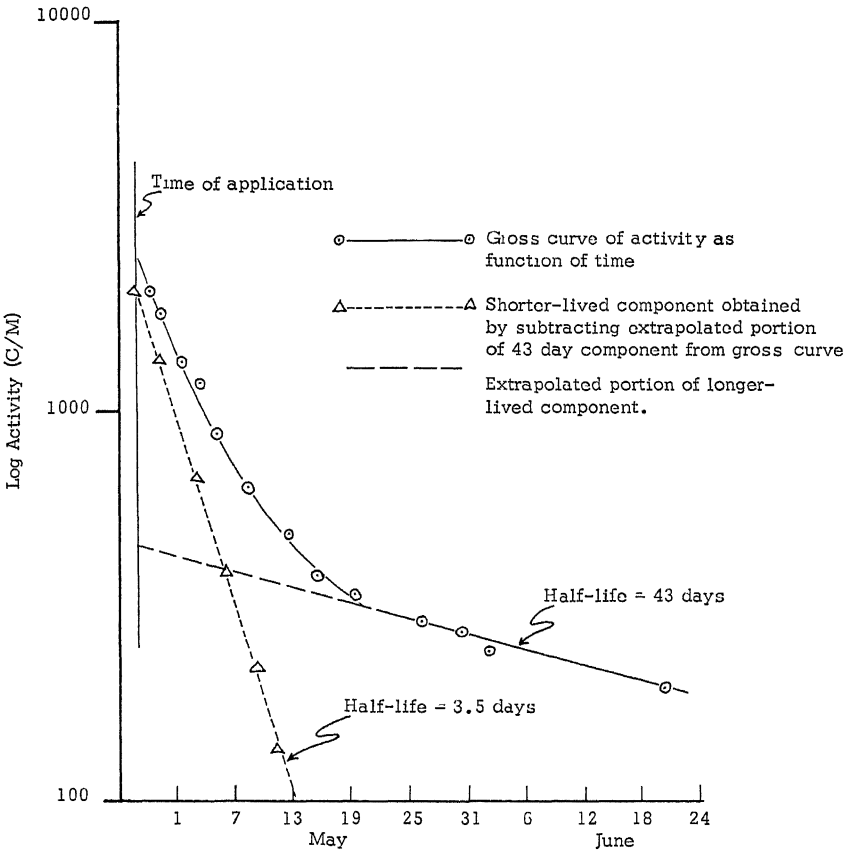


Fig. 8.—Radioactivity curve of glass planchet sprayed in field with C^{14}-endosulfan test plots and subsequently retained in reactor building counting room.

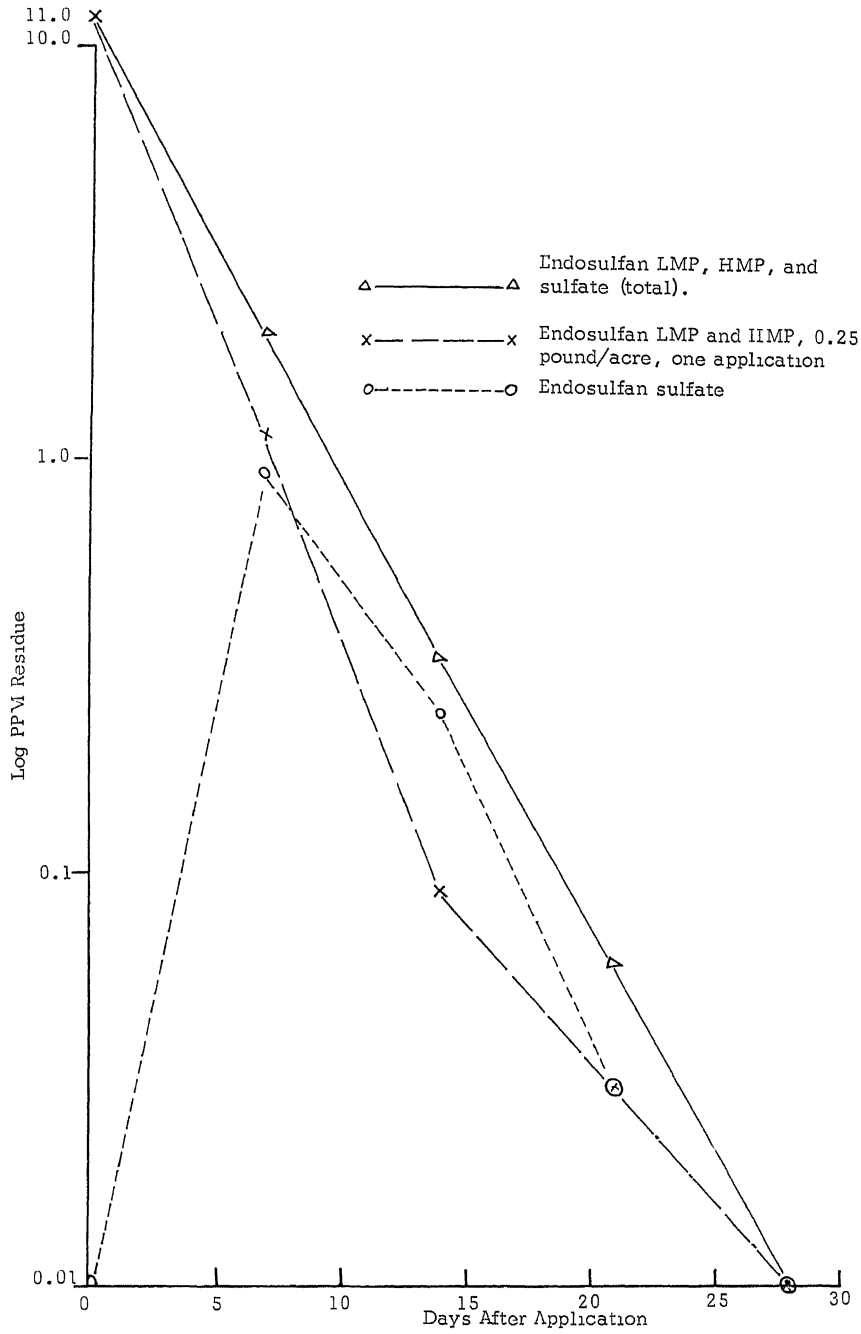


Fig. 9.—Degradation of 0.25 lb. per acre endosulfan on alfalfa and its conversion to endosulfan sulfate, measured by microcoulometric gas chromatography.

1961 C^{14}-ENDOSULFAN RESIDUES ANALYZED BY NIAGARA CHEMICAL DIVISION

One hundred-ml. aliquots of the hexane extracts from the previous study, taken before clean-up, were shipped in 4-ounce prescription bottles to the Niagara Chemical Division, Richmond, California, in July 1961. These were analyzed by Dr. C. C. Cassil, using microcoulometric gas chromatography as in the 1958 studies. The results (Table 9 and Fig. 7) indicate that the residues dissipated from an average value of 14.6 p.p.m. at 0 days to 0.04 p.p.m. at 21 days. The replicates at a given time are in good agreement and exhibit a dissipation rate of 80 to 90% per 7-day interval (Table 9). The values for the 35, 42, and 47-day intervals were less than 0.01 p.p.m., the minimum detectable level.

1963 ENDOSULFAN RESIDUES ON ALFALFA

On April 25, 1963, triplicate 50 x 50-foot plots of 2-year-old alfalfa were treated with endosulfan emulsions at 0.25 and 1.0 lb. per acre made from 25E containing 2 lb./gal. The sprays were applied with the experimental Jeep-mounted, boom-type weed sprayer at the rate of 12.5 gal./acre at 34 p.s.i. and a ground speed of 1.9 m.p.h. The applications were made between 1:30 and 2:30 p.m. on Ohio State University fields located at the Antenna Laboratory. For the 0.25 lb. treatment, 5.3 oz. of concentrate were added to 4.2 gal. of water; 21.3 oz. were used with 4.2 gal. of water for the 1.0 lb. rate.

All samples were hand collected, beginning with control and followed by the 0.25 lb. and 1.0 lb. applications. Approximately 10 lb. of alfalfa were collected from three plots of the same treatment, combined in a large paper grocery bag, returned to the laboratory, and mix-

TABLE 9.—C^{14}-Endosulfan Residues on Alfalfa Measured by Microcoulometric Gas Chromatography, Expressed as Average of Eight Subsamples, 1961.*

Sample Day	Endosulfan (p.p.m.)	
	Uncorrected	Corrected
0	12.59	14.46
7	2.12	2.43
14	0.188	0.213
21	0.039	0.046
28	0.020	0.005
35**	0.04	nil
42**	0.03	nil
47**	0.02	nil

*Analyses by Dr. C. C. Cassil.
**Composite sample constituting one analysis.

ed thoroughly. From this, six 250-gram samples were placed in polyethylene poultry freezer bags, taped, marked with date of collection and treatment, and frozen. Samples were collected 0, 7, 14, 21, 28, 36, and 42 days after application.

A frozen 250-gram sample was cut into small pieces and mixed thoroughly. A 100-gram subsample was macerated in the Waring blendor for 10 minutes with 300 ml. of redistilled Skellysolve B. The extract was filtered through glass wool and a 250-ml. aliquot was collected. This was evaporated to 200 ml. in a graduated cylinder with a filtered air stream and gentle heating with a hair dryer. It was then halved and each half was carried through a different clean-up.

One half was cleaned on a polyethylene-coated alumina column after drying over Celite 545, as described by Thornburg (23), and returned to 100 ml. final volume in benzene for analysis by gas chromatography.

The second 100-ml. portion of the hexane extract was evaporated to dryness, returned to 100-ml. volume with benzene, and then diluted

TABLE 10.—Residues of Endosulfan and Its Known Components on Alfalfa Treated at 0.25 and 1.0 Lb./Acre Spray in 1963 and Measured by Electron Capture Gas Chromatography in 1965.

Sample Day and Treatment		Endosulfan (p.p.m.)			
		Ether	Low Melting Isomer	High Melting Isomer	Sulfate
0	0.25 P	trace	3.72	1.52	0.00
0	0.25	0.0024	4.07	1.68	0.00
0	1.0 P	0.30	16.35	9.00	0.00
0	1.0	0.36	19.50	9.24	0.00
7	0.25 P	trace	0.18	0.216	0.228
7	0.25	trace	0.134	0.156	0.151
7	1.0 P	0.0072	1.68	2.40	0.547
14	0.25 P	0.00	0.0090	0.0156	0.049
14	0.25	0.00	0.0118	0.0168	0.072
21	0.25 P	0.00	0.0014	0.0108	0.0085
21	0.25	0.00	0.0010	0.0011	0.0040
21	1.0 P	0.00	0.0067	0.0372	0.054
21	1.0	0.00	0.0079	0.0139	0.046
28	0.25 P	0.00	0.0043	0.0094	0.019
28	1.0 P	0.00	trace	0.043	trace
36	0.25 P	0.00	0.00	0.003	trace
36	1.0 P	0.00	0.00	0.009	0.008
42	0.25 P	0.00	0.00	0.00	0.00
42	1.0 P	0.00	0.00	0.00	0.00

P = Samples cleaned on polyethylene-coated alumina column.

or concentrated, depending on the results of preliminary gas chromatographic analysis. An aliquot from each sample was cleaned with Nuchar-Attaclay as described by Thornburg (23), filtered, and dried over sodium sulfate before injection into the electron capture gas chromatograph.

Each sample was injected three times for analysis, using 2 to 5 μl per injection. The average peak heights of the three injections were quantitated, using standard curves which were run daily and checked several times daily for reliability. Recovery studies of the several endosulfan components after this clean-up method indicated 100% for the ether, 87% for the low melting point isomer, 85% for the high melting point isomer, and 93% for the sulfate.

For these analyses, a Barber-Coleman Model 15 gas chromatograph with a tritium source electron capture detector was used. The column was u-shaped, Pyrex, 3 feet x 1/4 inch ID, packed with 1.5% SE 52 silicone rubber on 70/80 mesh Anakrom ABS. The column, detector, and injection block temperatures were 215°, 220°, and 230° C., respectively. The peaks were eluted with prepurified grade nitrogen at 100 ml./minute and passed through a molecular sieve. The results are presented in Table 10.

1963 ENDOSULFAN RESIDUES ANALYZED BY NIAGARA CHEMICAL DIVISION

In May 1964, three 250-gram frozen green alfalfa samples from each sampling date and treatment of 1963 were packed in dry ice and sent to the Niagara Chemical Division, Middleport, N. Y. These were held at 0° C. until extraction.

Samples were extracted by maceration in a Waring blendor with 500 ml. of benzene and 250 ml. of isopropanol for 3 minutes. Five hundred ml. of 2% aqueous NaCl were added and the benzene layer was decanted. The benzene extract was dried over sodium sulfate, filtered, and cleaned up according to the procedure described by Thornburg (23). Due to the high level of extraneous material present in the forage extracts, the clean-up was repeated. A known volume of the cleaned extract was concentrated to 2 ml. in a Kuderna-Danish evaporator and an aliquot injected into the microcoulometric gas chromatograph.

The stationary phase used for these analyses was 15.5% SE-30 silicone gum rubber on Chromosorb W. Operating conditions were block temperature, 260° C., and column, 240° C. The results are shown in Figures 9 and 10, which are the averages of the subsample data shown in Table 11.

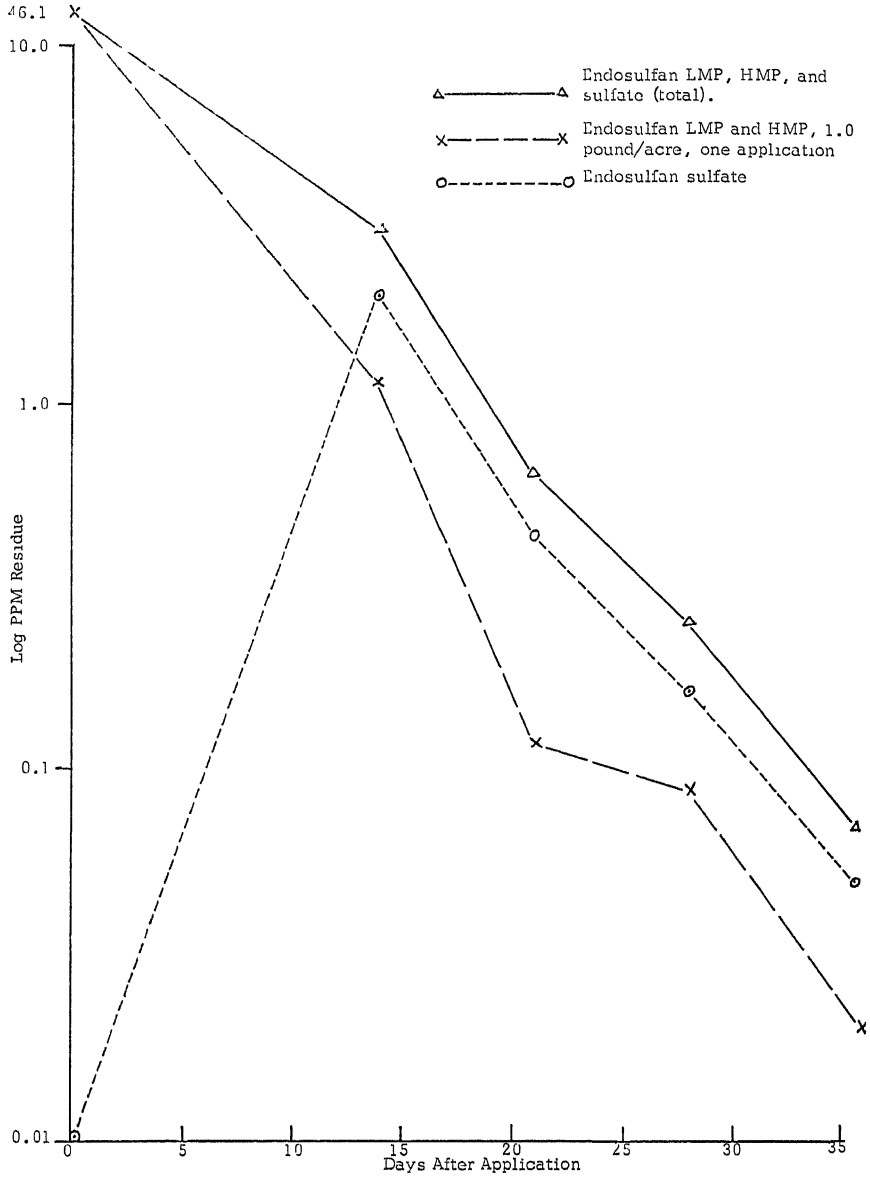


Fig. 10.—Degradation of 1.0 lb. per acre endosulfan on alfalfa and its conversion to endosulfan sulfate, measured by microcoulometric gas chromatography.

1965 ENDOSULFAN RESIDUES ON ALFALFA

On July 26, 1965, a single 10 x 80-foot plot of 2nd-year alfalfa at the Ohio Agricultural Research and Development Center was treated with 0.25 lb. endosulfan per acre at 20 gal. emulsion per acre under 50 p.s.i. When treated, the alfalfa was 8 to 12 inches high after the third

TABLE 11.—Results of Microcoulometric Gas Chromatography of Alfalfa Extracts from 1963 Ohio Applications.

Sample Day and Treatment (lb./acre)	Endosulfan (p.p.m.)	
	LMP and HMP*	Sulfate*
0 day check	0.00	0.00
0 day 0.25	11.49	0.00
0 day 0.25	12.41	0.00
0 day 0.25	9.07	0.00
0 day 1.0	39.80	0.00
0 day 1.0	52.20	0.00
0 day 1.0	46.40	0.00
7 day 0.25	1.15	0.85
7 day 0.25	1.17	0.97
7 day 0.25	1.04	0.89
14 day 0.25	0.11	0.25
14 day 0.25	0.09	0.32
14 day 0.25	0.08	0.14
14 day 1.0	1.28	2.02
14 day 1.0	1.29	2.20
14 day 1.0	1.08	1.88
21 day 0.25	0.02	0.02
21 day 0.25	0.03	0.01
21 day 0.25	0.04	0.04
21 day 1.0	0.12	0.40
21 day 1.0	0.16	0.56
21 day 1.0	0.08	0.38
28 day check	0.00	0.00
28 day 0.25	0.00	0.00
28 day 0.25	0.00	0.00
28 day 0.25	0.00	0.00
28 day 1.0	0.05	0.06
28 day 1.0	0.13	0.27
36 day check	0.00	0.00
36 day 0.25	0.00	0.00
36 day 0.25	0.00	0.00
36 day 0.25	0.00	0.00
36 day 1.0	0.01	0.02
36 day 1.0	0.03	0.09
36 day 1.0	0.03	0.04

*Corrected for recovery values and column efficiency.

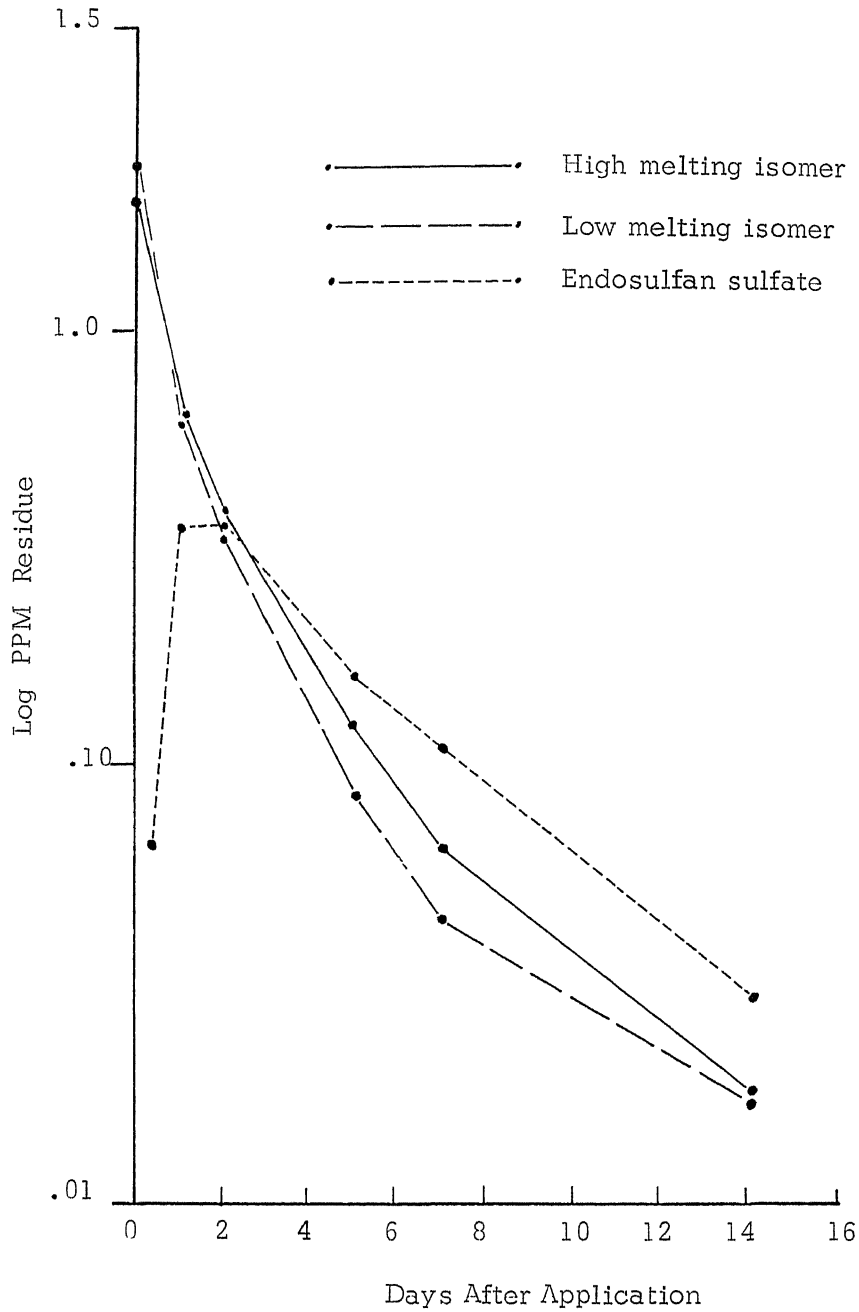


Fig. 11.—Degradation of 0.25 lb. per acre endosulfan applied to 12-inch high, third-cutting alfalfa and its conversion to endosulfan sulfate, measured by electron capture gas chromatography.

TABLE 12.—Results (p.p.m.) of Electron Capture Gas Chromatography of Alfalfa Extracts from July 1965 Application of Endosulfan at 0.25 Lb. Emulsifiable Concentrate per Acre, Columbus, Ohio.

Days	Endosulfan-Ether	Endosulfan-Diol	Low Melting	High Melting	Sulfate	Total
Control	0.00	0.00	0.043	0.031	0.00	0.074
0	0.006	trace	2.32	2.040	0.066	4.432
1	trace	trace	0.606	0.625	0.351	1.582
2	trace	0.00	0.335	0.381	0.351	1.067
5	0.00	0.00	0.087	0.123	0.157	0.367
7	0.00	0.00	0.044	0.064	0.107	0.215
14	0.00	0.00	0.017	0.018	0.030	0.065
21	0.00	0.00	0.00	0.00	trace	trace
28	0.00	0.00	0.00	0.00	trace	trace

cutting. Three-pound samples were collected immediately before and after application (as control and 0 days) and at 1, 2, 5, 7, 14, 21, and 28 days. The samples were frozen until extraction.

The frozen samples were passed through a Hobart salad chopper until finely cut and well mixed. A 100-g. subsample was extracted and cleaned up as follows. The subsample was put in a Waring blendor with 100 ml. of isopropanol and minced for 2 minutes. Then 200 ml. of reagent-grade benzene were added and the mixture blended for 5 minutes. The extract was filtered through glass wool into a separatory funnel, where the benzene was separated from the isopropanol by addition of 100 ml. of saturated NaCl solution and 500 ml. of distilled water. The aqueous portion was removed and the benzene measured. Two ml. of benzene represented 1 gram of alfalfa.

The benzene extract was cleaned by the Nuchar-Attaclay method, dried over sodium sulfate, and condensed or diluted as necessary for electron capture gas chromatography of 2 to 5 μl quantities of the extract. The results are presented in Table 12 and Figure 11.

SUMMARY AND CONCLUSIONS

Endosulfan applied to Ohio alfalfa in late April disappears at a predictable, logarithmic rate, as do most other organochlorine insecticides. This cyclodiene insecticide resembles aldrin and heptachlor in that it undergoes oxidation to form a primary, insecticidal metabolite, endosulfan sulfate, which endures approximately as long as the parent compound. Field temperatures apparently influence the rate of sulfate production. It was found on alfalfa samples 1 hour after application in July but not on those treated in April.

Autoradiograms of C^{14}-endosulfan extracts of alfalfa indicate that the sulfate is the only prominent product formed, although endosulfan alcohol would be expected. This was confirmed by electron capture and microcoulometric gas chromatography.

According to several studies in Ohio, 0.25 lb. per acre applications in late April should leave between 10 and 15 p.p.m. as an initial deposit. This deteriorates and is diluted by growth to 10 p.p.b. by the 28th day and to 1.8 p.p.b. by the 47th day, This residue is approximately equal to a 7.0 p.p.b. residue on dried hay.

Of the analytical methods used in these studies, the two most sensitive were the radioisotope labeled material measured by liquid scintillation and autoradiography, followed by electron capture gas chromatography. The better extraction and clean-up methods were those used by the Niagara Chemical Division in 1963 and Ohio in the 1965 studies.

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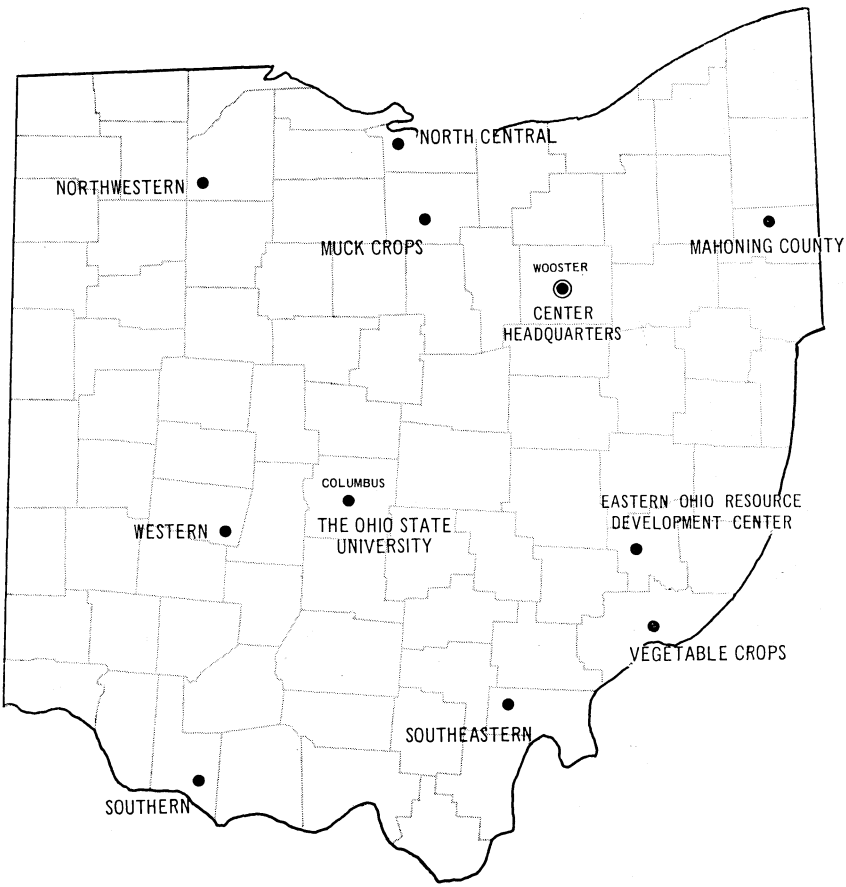

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North Central Branch, Vickery, Erie County: 335 acres

Northwestern Branch, Hoytville, Wood County: 247 acres

Southeastern Branch, Carpenter, Meigs County: 330 acres

Southern Branch, Ripley, Brown County: 275 acres

Vegetable Crops Branch, Marietta, Washington County: 20 acres

Western Branch, South Charleston, Clark County: 428 acres