The berry group showed a sixfold decrease in residues over a 7-day period, indicating a half life of 2 days. Similar curves are shown for apples, green beans, and the lettuce group.

Another pattern demonstrated by the analytical program is the rapid rate at which Sevin degrades in soil. Tests involving Sevin at three different concentrations were conducted in Norfolk sandy loam soil at the Union Carbide Agricultural Research Station at Clayton, N. C. The chemical was tilled into the top 6 inches of soil, which was then sampled periodically for analysis. The results are shown graphically in Figure 2. The half life of Sevin is demonstrated to be approximately 8 days at all concentrations, and in every case the residue appeared to be completely degraded within 40 days.

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Received for review August 18, 1964. Accepted December 21, 1964. Division of Agricultural and Food Chemistry, Symposium on Carbamate Insecticides, 148th Meeting, ACS, Chicago, Ill., September 1964.

END OF SYMPOSIUM

HERBICIDE DEGRADATION

Microbial Degradation of Simazine

D. D. KAUFMAN, P. C. KEARNEY, and T. J. SHEETS

Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

Soil microorganisms effective in the degradation of the herbicide simazine were isolated by the enrichment method. Bioassays of simazine culture solutions of *Aspergillus fumigatus* Fres. indicated that this organism degraded simazine to nonphytotoxic or at least less phytotoxic compounds. This organism utilized simetone as a sole source of both carbon and nitrogen. Decreases in radioactivity in culture solutions containing chain-labeled simazine were greater than those observed from solutions containing ring-labeled simazine. C^{14} from chain-labeled simazine was found largely in lipids, proteins, and $C^{14}O_2$, whereas little C^{14} from ring-labeled simazine was incorporated and no $C^{14}O_2$ evolved. Chromatographic analysis of both ring- and chain-labeled simazine culture solutions indicated that simazine was transformed into several compounds not previously reported. Data obtained indicate that degradation of simazine by *A. fumigatus* occurs by a dealkylation, deamination, or both, of the side chains, and that hydroxysimazine is not an intermediate in this mechanism.

MICROBIAL degradation is an important factor in the behavior of pesticides applied to soil. The decomposition of *s*-triazine herbicides by soil microorganisms has been studied by several investigators (*1–4, 9, 10, 15, 20–23*). Until recently, few investigators have been able to demonstrate clearly that soil microorganisms can utilize 2 - chloro - 4,6 - bis(ethylamino) - *s* - triazine (simazine) as a source of carbon, nitrogen, or both. Guillemat *et al.* (*10*) and Waeffler (*25*) concluded that the carbon of the simazine molecule was unavailable to soil fungi. Guillemat *et al.* (*10*) also concluded that the nitrogen of simazine could be utilized in the presence of an adequate carbon source, whereas Waeffler (*25*) concluded that simazine nitrogen could be utilized by soil fungi, but not as a sole source of nitrogen. Burnside (*3*) isolated five microorganisms which were able to subsist for 3 months in media containing simazine as the sole source of nitrogen and nearly sole source of carbon. Bioassay of the growth medium, however, revealed no significant decrease in toxicity during a 30-day incubation period. Kaufman, Kearney, and Sheets (*15*) reported the isolation of several organisms capable of degrading simazine. Although one organism, *Aspergillus fumigatus* Fres., utilized simazine as a sole source of carbon, degradation was more rapid in the presence of a supplemental carbon source. The purpose of this study was to investigate further the degradation of simazine by soil microorganisms.

Materials and Methods

A population of soil microorganisms effective in the degradation of simazine was developed by an enrichment technique.

This technique consisted of placing 10 grams of a Hagerstown silty clay loam in a 500-ml. flask containing 250 ml. of distilled water. Sufficient simazine was added (1.25 grams of wettable powder formulation per 1000 ml.; Simazine 80W) to establish an active ingredient concentration of 1000 p.p.m. A duplicate set of flasks which contained no simazine was used as a blank. Four replications were used. The flasks were maintained on a reciprocating shaker at 24° C. Simazine degradation was determined by measuring chloride ion liberation by the method of Iwasaki, Utsumi, and Ozawa (*13*) at 3-day intervals. A soil dilution–plate method (*14*) was used for the final isolation of effective organisms from enriched culture solutions. A 5-ml. sample of solution from simazine-treated flasks served as the starting point for dilution series. The plating medium consisted of 0.2 gram of K_2HPO_4, 0.3 gram of NH_4NO_3, 0.2 gram of $CaSO_4$, 0.2 gram of $MgSO_4$.-

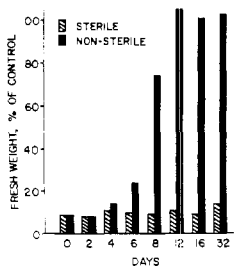

Figure 1. Effect of additions of sterile and nonsterile culture solutions to soil on fresh weights of oats

$7H_2O$, 1 mg. of $FeSO_4.7H_2O$, and 20 grams of agar in 1000 ml. of distilled H_2O. Simazine (5 mg. per liter) was supplied as the sole source of carbon. Simazine, sterilized by filtration, was added to the concentrated mineral salts medium after autoclaving.

An oat bioassay method was used to determine whether the isolated organisms reduced simazine to nonphytotoxic or less phytotoxic compounds. Aliquots from culture solutions in which effective organisms had been growing for 0, 2, 4, 6, 8, 12, 16, and 32 days were added to Hagerstown silty clay loam in plastic pots. The chemical and physical characteristics of this soil are: pH 5.5, 12.5 meq. per 100 grams cation exchange capacity, 29.1% moisture content at field capacity, 4.3% organic matter, 7% sand, 63% silt, and 30% clay. Culture solutions contained 0.2 gram per liter of sucrose and 5 mg. per liter of simazine in addition to the mineral salts. The addition of 100 ml. of culture solution to 300 grams per pot of soil was equivalent to an application rate of 3.2 pounds per acre of simazine on day 0. Inoculated simazine solutions were compared with sterile simazine solutions at each planting date. Four replications were used for each experimental variable. Oat seedlings were harvested after a 3-week growing period, and the fresh weight was expressed as per cent of the sterile control.

Because of its greater solubility [3200 p.p.m. (*3*)], 2,4-bis(ethylamino)-6-methoxy-*s*-triazine (simetone) was used to determine whether or not the soil fungus *Aspergillus fumigatus* Fres. could use an *s*-triazine compound as a sole source of carbon, nitrogen, or both. Five media were prepared using the salts listed previously, with the exception of NH_4NO_3. The media combinations used were: simetone and salts; simetone, sucrose, and salts; simetone, $NaNO_3$, and salts; simetone, sucrose, $NaNO_3$, and salts; sucrose, $NaNO_3$, and salts; sucrose and salts; and salts. Concentrations of simetone, sucrose, and $NaNO_3$ were 1, 0.1, and 2 grams per liter, respectively. The media were sterilized by filtration and dispensed in 100-ml. aliquots into sterile 250-ml. flasks. One milliliter of a spore suspension was placed in each flask. Duplicate flasks without the spore suspension served as controls. Five replications were used. All cultures were maintained on the reciprocating shaker at 24° C. for 7 days. Cells were harvested on preweighed, fritted glass filters oven-dried at 60° C. for 24 hours, and weighed. The cell weights obtained were corrected with weights obtained from sterile control flasks and recorded as milligrams of oven-dried cells.

The degradation of both ring- and chain-labeled simazine (specific activity 5.06 and 4.97 μc. per mg., respectively) was compared in a closed system; the basal medium was amended with 0.1 gram per liter of sucrose and 5 p.p.m. of labeled simazine. CO_2-free air was circulated through the system and $C^{14}O_2$ evolved from each culture was trapped in 0.5*N* NaOH. Two 1-ml. samples were removed from each CO_2 collection flask at 24-hour intervals and plated, and the amount of activity (counts per minute) was determined. All counts were corrected for self-absorption and background and the data expressed as per cent C^{14} evolved as $C^{14}O_2$. Residual radioactivity of the culture solution was measured at 48-hour intervals, and a 2-ml. sample was concentrated and chromatographed descendingly on Whatman No. 1 filter paper with isoamyl alcohol saturated with 0.1*N* HCl. Chromatograms prepared in this manner were first scanned with a 4-pi strip scanner; the per cent distribution of C^{14} appearing on each chromatogram was determined with a hand counter, and then exposed to "no-screen" x-ray film for 2 weeks.

At the conclusion of the incubation period, the cells were harvested by filtration, washed twice with 0.85% NaCl, and then fractionated according to the procedures of Roberts *et al.* (*23*). Two 1-ml. portions of each fraction were then plated and dried, and residual C^{14} was determined.

Results

Degradation of simazine in enrichment solutions progressed slowly under conditions used in this investigation. Degradation began almost immediately and continued as a slow process, only 3 to 4% being degraded over a period of 30 days. Periodic isolations of microorganisms from the enrichment solutions on a solid medium containing simazine as a sole carbon source, however, repeatedly produced characteristic populations of soil microorganisms. Microbial populations of simazine-containing solutions differed from those which did not contain simazine. These organisms, predominantly fungi, were effective in degrading simazine under pure culture conditions. Organisms which utilized simazine as a sole or supplemental source of carbon were *Aspergillus flavipes* (Banier and A. Sartory) Thom and Church, *A. fumigatus* Fres., *A. ustus* (Banier) Thom and Church, *Fusarium moniliforme* Sheldon, *F. oxysporum* Schlect., *Penicillium purpurogenum* Stoll, *P.* sp., *Rhizopus stolonifer* (Ehr. ex Fr.) Vuill, *Stachybotrys* sp., *Trichoderma viride* (Pers. ex Fr.), three species of *Streptomyces*, and four bacterial isolates believed to belong in the genus *Arthrobacter*. In comparative studies, *A. fumigatus* was most effective in the degradation of simazine.

Bioassays of culture solutions revealed that *A. fumigatus* could degrade simazine (Figure 1), detoxication being almost complete in 12 days. Similar results were obtained with other effective organisms. Occasionally some stimulation of the assay plant was observed after detoxication was completed. Whether the stimulation was due to simazine degradation products or to other metabolic products produced by the organism was not determined.

Simetone was utilized by *A. fumigatus* as a sole source of nitrogen, carbon, or both (Table I). Growth of *A. fumigatus* was significantly greater, however, when sucrose was supplied as a supplemental source of carbon, or when mineral nitrogen was supplied as supplemental nitrogen source. No growth was observed in media containing only the basal salts and sucrose with no nitrogen source, or basal salts and nitrogen with no carbon source.

Studies comparing the metabolism of ring- or chain-labeled (C^{14}) simazine in the enclosed incubation system indicated that $C^{14}O_2$ was evolved only from culture solutions containing chain-labeled simazine (Figure 2). No $C^{14}O_2$ was evolved from culture solutions containing ring-labeled simazine. Planchet counts indicated decreases in C^{14} remaining in external culture solutions (Figure 3). The loss of activity in culture solutions containing chain-labeled simazine was greater than that observed in solutions containing ring-labeled simazine. Of the C^{14} which could be accounted for at

Table I. Oven-Dry Mycelial Weights of *A. fumigatus* Produced in Media Containing Simetone as a Sole or Supplementary Source of Carbon, Nitrogen, or Both

Carbon Source	Nitrogen Source	Oven-Dried Cells, Mg.
Simetone	Simetone	1.8
Simetone + sucrose	Simetone	2.8
Simetone	$NaNO_3$	2.4
Simetone + sucrose	$NaNO_3$ + simetone	10.2
Sucrose	$NaNO_3$	10.8

Table II. Distribution of C^{14} among External Solution, Cellular Components, and $C^{14}O_2$ on Day 11

Fraction	% of Total C^{14}	
	Ring-labeled	Chain-labeled
Total activity remaining in culture solution	99.4	54.2
Total activity in cellular components	0.6	6.5
Total activity evolved as $C^{14}O_2$	0	39.3
Total activity	100.0	100.0

the conclusion of the experiment (Table II), virtually all of the C^{14} from ring-labeled simazine remained in the external culture solution, with a small fraction appearing as cellular components and none appearing as $C^{14}O_2$. Approximately half of the original activity present in chain-labeled simazine remained in the external culture solution, with 39.3% having been evolved as $C^{14}O_2$ and 6.5% remaining as cellular components.

Cell fractionation data also indicated differences in the distribution of C^{14} from the different forms of labeled simazine (Table III). The incorporation of C^{14} from chain-labeled simazine was 8 to 9 times that from ring-labeled simazine. The major portion of C^{14} from chain-labeled simazine appeared in lipid, nucleic acid, and residual protein fractions, with only small amounts appearing as transient intermediates. Only a small amount of C^{14} from ring-labeled solutions appeared as cellular components.

Chromatograms for both ring- and chain-labeled simazine culture solutions revealed a major shift in peaks of radioactivity. Examination of the distribution of C^{14} appearing on each chromatogram revealed that simazine had been largely converted to one or more unidentified products (Figures 4 and 5). A broad peak of radioactivity (unknown I, R_f 0.81) which was distinguishable from simazine (R_f 0.91) was observed on chromatograms from both ring- and chain-labeled simazine culture solutions. A second peak of activity (unknown II, R_f 0.22) occurred only on chromatograms prepared from ring-labeled simazine culture solutions. Radioautographs of these chromatograms revealed that trace amounts of several additional compounds were also present in both ring- and chain-labeled culture solutions. The C^{14}-simazine used did not contain any of these substances in measurable amounts.

An attempt was made to identify unknowns I and II by cochromatography with several suspected intermediates. Unknown I was eluted from the chromatograms with a mixture of butanol, acetic acid, and water (4:1:5) and rechromatographed descendingly on Whatman No. 1 filter paper with isoamyl alcohol saturated with 0.1*N* HCl. A portion of the eluate was cochromatographed with authentic 2-hydroxy-4,6-bis(ethylamino)-*s*-triazine (hydroxysimazine). Solutions of simazine and hydroxysimazine were also chromatographed in this system. R_f values of simazine, hydroxysimazine, and unknown I were found to be 0.91, 0.62, and 0.81, respectively, in this solvent system.

Unknown II was eluted from chromatograms with distilled water and cochromatographed with 2,4,6-trihydroxy-*s*-triazine (cyanuric acid) in butanol, acetic acid, and water (4:1:5). In this system, cyanuric acid had an R_f value of 0.32, whereas the R_f value of unknown II was 0.60. Unknown II was also cochromatographed with 2-chloro-4,6-bis(amino)-*s*-triazine in a thin-layer chromatography system with silica gel as an adsorbent and benzene–acetic acid (1:1) as the solvent. The R_f value of unknown II did not compare with that of 2-chloro-4,6-bis(amino)-*s*-triazine. *A. fumigatus* was unable to utilize either cyanuric acid or 2,4-bis-(hydroxy)-6-amino-*s*-triazine (ammelide) when these compounds were supplied as a sole source of carbon in the basal salts medium.

The rate of chloride ion liberation was determined in mass cultures of *A. fumigatus*. In these studies only small amounts (2 to 3%) of the chloride introduced with simazine were detected in the ionic form. Chloride ion was first detected on day 6 of the incubation period and increased slowly to a maximum of 3% at the conclusion of the incubation period (12 days).

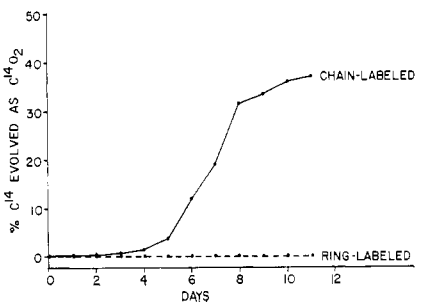

Figure 2. $C^{14}O_2$ evolution from ring- and chain-labeled simazine

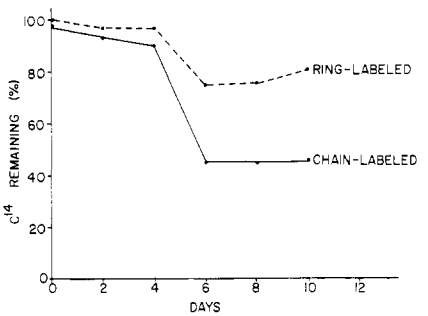

Figure 3. Disappearance of C^{14} from culture solutions of *A. fumigatus*

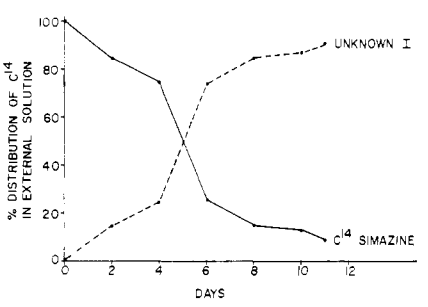

Figure 4. Distribution of C^{14} on chromatographs prepared from culture solutions containing chain-labeled simazine

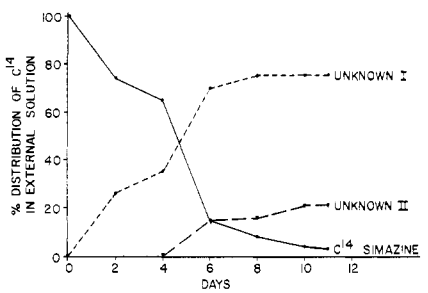

Figure 5. Distribution of C^{14} on chromatographs prepared from culture solutions containing ring-labeled simazine

Discussion

The results of this investigation further substantiate that *s*-triazine herbicides can be degraded by soil microorganisms. Growth studies with simetone, the 2-methoxy analog of simazine, confirmed previous reports (*15*) that *A. fumigatus* can utilize an *s*-triazine as a sole source of carbon, nitrogen, or both. Further evidence that *A. fumigatus* can utilize the carbon of simazine was obtained in studies employing C^{14} chain-labeled simazine.

The distribution of C^{14} from chain-labeled simazine is similar to what one might expect if the organism had been fed a labeled 2-carbon moiety such as acetate or ethanol (*23*).

Soil fungi appear to be major participants in the degradation of *s*-triazine herbicides (*2*, *3*, *10*, *15*). In contrast, degradation of many other pesticides appears to occur largely by bacterial

Table III. Distribution of C^{14} in Cellular Components

	Ring-Labeled		Chain-Labeled	
Fraction	C./2 min.	% of total	C./2 min.	% of total
Transient intermediates	230	34.1	443	7.4
Lipids	216	32.1	2733	45.6
Nucleic acids	160	23.7	1228	20.5
Residual proteins	68	10.1	1588	26.5
Total	674	100.0	5992	100.0

metabolism. Several workers (*1*, *15*, *22*), however, have reported that a few bacteria can degrade *s*-triazine compounds.

Bioassays of simazine culture solutions demonstrated that the soil fungus, *A. fumigatus*, reduced simazine to a nonphytotoxic or at least a less phytotoxic compound. Similar results were obtained with other effective isolates. Differences between these results and those obtained by Burnside (*2*) and Burnside, Schmidt, and Behrens (*3*) are best explained by comparison of the culture media employed. The culture media assayed in this investigation contained additional carbon and nitrogen sources, whereas media assayed by Burnside and coworkers (*2*, *3*) contained no additional nitrogen and very little additional carbon. Degradation of simazine occurs more rapidly in the presence of supplemental carbon sources (*9*, *10*, *15*).

Until recently, the nature of the metabolic products involved in degradation of simazine by soil microorganisms was unknown. A degradation scheme was proposed by Gysin and Knusli (*11*), who suggested that 2-chloro-4,6-bis(ethylamino)-*s*-triazine (simazine) is first transformed to 2-hydroxy-4,6-bis(ethylamino)-*s*-triazine (hydroxysimazine), followed by cleavage of the triazine ring and subsequent formation of a biguanide-type compound and CO_2. Recent investigations (*5*, *8*, *12*, *18*, *24*) with C^{14} ring-labeled simazine clearly demonstrated that hydroxysimazine is an early major degradation product in higher plants. The conversion of simazine to hydroxysimazine is a nonenzymatic reaction (*5*, *12*, *24*); corn and certain other plants contain a benzoxazine derivative which catalyzes the conversion of simazine to hydroxysimazine. Presumably, hydroxysimazine is further degraded in corn plants when the carbon in the 2-position of the ring is removed in the form of CO_2.

The results of this and earlier investigations (*15*, *16*) provide insight into a different mechanism involved in the degradation of *s*-triazine herbicides by soil microorganisms. It was demonstrated earlier (*15*) that the loss of C^{14} in *A. fumigatus* culture solutions containing chain-labeled simazine was greater than that observed in solutions containing ring-labeled simazine. Similar observations were made in this investigation. In addition, $C^{14}O_2$ evolved from cultures growing in solutions containing chain-labeled simazine, but none evolved from cultures growing in solutions containing ring-labeled simazine. Three possibilities exist, therefore: degradation of simazine by *A. fumigatus* involves merely a dealkylation or deamination of the side chains and ring cleavage does not occur; both dealkylation or deamination and ring cleavage occur with complete incorporation of ring constituents and no evolution of $C^{14}O_2$ from ring-labeled simazine; either dealkylation or deamination and incorporation of intact ring occur. Ring cleavage appears unlikely, however, since virtually no C^{14} was observed in the cellular components of cells growing in presence of ring-labeled simazine. Whether or not the 0.06% of ring C^{14} present in cellular components could be attributed to contamination, impurities in the parent compound, or incorporation of the intact ring was not determined. Data obtained in this investigation and others (*15*, *16*) support the hypothesis for dealkylation or deamination without ring cleavage.

Attempts to identify the intermediates by cochromatography with known *s*-triazine compounds were unsuccessful. Unknown I was not comparable with hydroxysimazine. This is not surprising, however, since very little chloride ion was detected in culture solutions. If hydroxysimazine were involved as an intermediate, substantial amounts of chloride ion should have been present, since nearly all of the simazine was degraded. Although chlorine has no known essentiality in the nutrition of fungi (*7*), chlorinated metabolites are not uncommon (*6*). Kearney, Kaufman, and Sheets (*16*), working with the same unknown, also observed that it was not comparable chromatographically with hydroxysimazine, the dealkylated simazine analog 2-chloro-4-amino-6-ethylamino-*s*-triazine, or the deaminated dehalogenated analog 2,4-bis(hydroxy)-6-ethylamino-*s*-triazine. They concluded, however, that their results did not necessarily preclude the existence of hydroxy- or amino-substituted *s*-triazines as intermediates in the metabolism of simazine.

A C^{14}-labeled form of unknown II was present only on chromatograms prepared from culture solutions containing ring-labeled simazine. Absence of a C^{14}-labeled form of this compound from chromatograms of culture solutions containing chain-labeled simazine indicates that both chain portions of the original molecule are absent. Cochromatography indicated that unknown II was not comparable with either cyanuric acid or 2-chloro-4,6-bis(amino)-*s*-triazine. 2,4-Bis(hydroxy)-6-amino-*s*-triazine (ammelide) was excluded as a possibility, since the known R_f values of this compound in these solvent systems were considerably lower than those obtained for unknown II. In short-term experiments with mass cultures of *A. fumigatus*, Kearney *et al.* (*16*) detected small amounts of ammelide in culture solutions containing ring-labeled simazine. Failure to detect substantial amounts of ammelide in culture solutions examined in this investigation may have been due to differences in methods of study.

Since ring cleavage evidently does not occur and *A. fumigatus* is unable to utilize either ammelide or cyanuric acid as a sole source of carbon, one of these compounds conceivably may constitute one of the end products of simazine degradation by *A. fumigatus*. Degradation of simazine by this organism therefore would merely involve dealkylation, deamination, or both, in addition to an eventual dehalogenation of the molecule. Until recently, little was known concerning the metabolism of triazines except through research on derivatives possessing antimalarial properties (*26*). 2,4-Diamino-*s*-triazine (formoguanamine) is converted in man to ammelide (*17*). C^{14} ring-labeled 2,4,6-triethyleneimino-*s*-triazine (triethylenemelamine), injected into mice, was excreted within 24 hours (*19*). No radioactivity occurred in respired air, indicating that the ring was not destroyed. The principal metabolite excreted was cyanuric acid. Side chain degradation of *s*-triazine compounds is, therefore, not uncommon. Whether or not simazine degradation by *A. fumigatus* involves dealkylation, deamination, or both is not known at this time.

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Received for review August 20, 1964. Accepted January 4, 1965.

INSECTICIDE RESIDUES

Colorimetric Analytical Method for Bidrin Residues in Alfalfa, Celery, Lemon Peel, Lettuce, Orange Peel, Potatoes, String Beans, and Tomatoes

R. T. MURPHY, L. K. GASTON, and F. A. GUNTHER

University of California Citrus Research Center and Agricultural Experiment Station, Riverside, Calif.

A procedure is presented for the determination of microgram quantities of Bidrin in crop samples. Cleanup is accomplished with an acid reflux followed by distillation of interfering materials. The Bidrin is then hydrolyzed with alkali and the resulting dimethylamine distilled and collected. The amine is determined colorimetrically as dimethyl dithiocarbamate following the addition of cupric ion and carbon disulfide. Residues as low as 0.20 p.p.m. can be accurately determined from samples as large as 125 grams. An average of 12 samples per day can be analyzed by this procedure.

THE candidate insecticide 3-(dimethoxyphosphinyloxy) - *N,N*-dimethyl-*cis*-crotonamide, commonly called Bidrin, has been shown by various workers to be effective against certain insect pests of several crops. However, before registration of a pesticide at the state level and by the Federal Government is possible, an acceptable method of residue analysis must be submitted along with data showing persistence of the compound on and in the crops involved in the petition presented. The method should have good degrees of specificity and reliability, and also sufficient sensitivity to determine adequately the persistence characteristics of the compound and any *in situ* metabolites of pharmacological significance. The present method meets the requirements for specificity for the parent compound, reliability, and sensitivity to 0.2 p.p.m. or less in the presence of the benzene-extractable substances from alfalfa, celery, lemon peel, lettuce, orange peel, potato peel, potato pulp, string beans, and tomatoes.

Cleanup consists of refluxing the benzene extractables with acid and steam distillation of interfering materials; the nondistilled Bidrin is hydrolyzed by alkali to yield dimethylamine, which is in turn steam-distilled and determined colorimetrically by the highly specific Stanley, Baum, and Gove (*3*) procedure. Dimethylamine reacts with carbon disulfide and ammonia to form the benzene-soluble, amber-colored cupric dimethyldithiocarbamate in the presence of cupric ion. This reaction is specific for dialkylamines and therefore other pesticides with a monoalkylamine group give no interference. Other dialkylamine-containing pesticides interfere only if such compounds are hydrolyzed to steam-volatile amines by the described conditions.

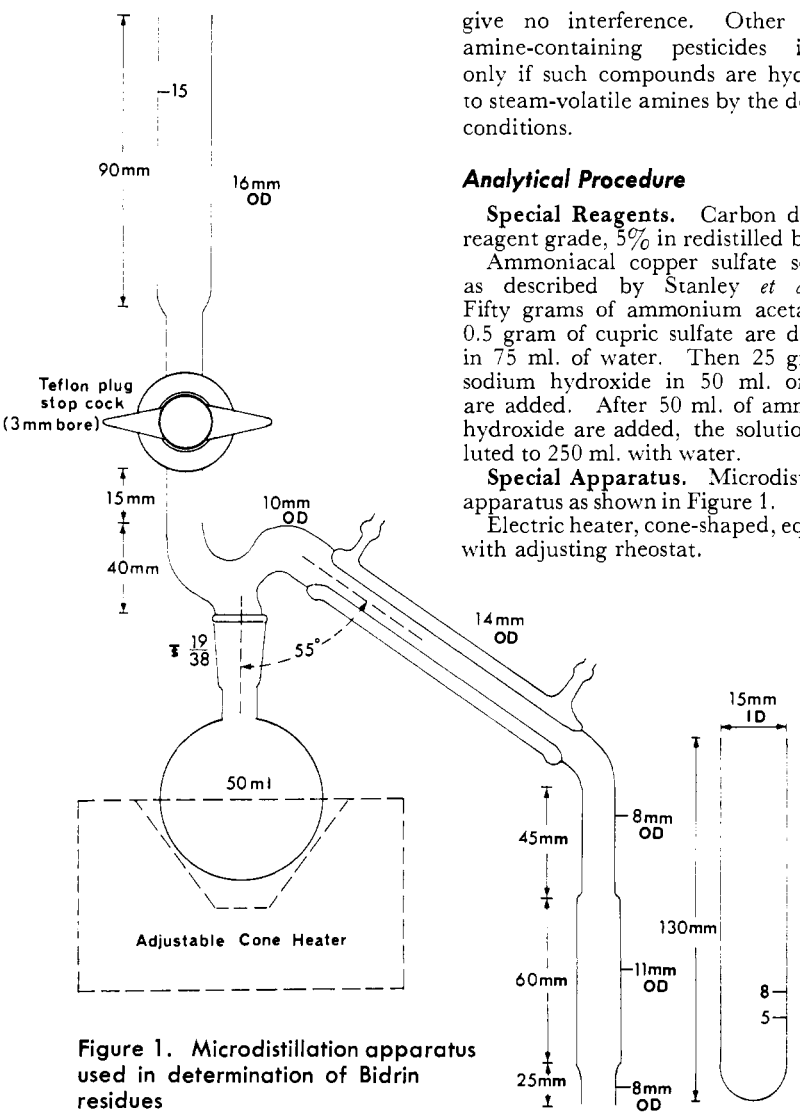

Figure 1. Microdistillation apparatus used in determination of Bidrin residues

Analytical Procedure

Special Reagents. Carbon disulfide, reagent grade, 5% in redistilled benzene.

Ammoniacal copper sulfate solution, as described by Stanley *et al.* (*3*). Fifty grams of ammonium acetate and 0.5 gram of cupric sulfate are dissolved in 75 ml. of water. Then 25 grams of sodium hydroxide in 50 ml. of water are added. After 50 ml. of ammonium hydroxide are added, the solution is diluted to 250 ml. with water.

Special Apparatus. Microdistillation apparatus as shown in Figure 1.

Electric heater, cone-shaped, equipped with adjusting rheostat.