Fate of C^{14}-Labeled Chloroneb in Plants and Soils

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Uptake of C^{14}-labeled chloroneb (1,4-dichloro-2,5-dimethoxybenzene) into cotton and bean plants was demonstrated by autoradiography and by quantitative C^{14} analyses. Chloroneb was primarily concentrated in the roots and lower stem portions of the plants, where the need for protection against soil fungi is greatest. Metabolism studies with extracts of the treated plants showed that over 95% of the C^{14}-residue in the treated plants was intact chloroneb and its principle metabolite, 2,5-dichloro-4-methoxyphenol, in about a 1:1 ratio. Small amounts of 2,5-dichloroquinone and 2,5-dichlorohydroquinone were also detected. Radiochemical residue analyses of beans and cotton seed from plants grown with recommended treatments of C^{14}-ring-labeled chloroneb showed total residues that were less than the established federal tolerances. The half-life of C^{14}-ring-labeled chloroneb in soil has been determined to be approximately 3 to 6 months when incorporated into the soil 2 to 3 in. below the surface at a rate of 2 lb active per acre. About 90% of the residual activity recovered from the treated soil was intact chloroneb.

Chloroneb (Demosan, E.I. du Pont de Nemours & Co., 1,4-dichloro-2,5-dimethoxybenzene) has been shown to be an effective aid in reducing seedling diseases of cotton, beans, and soybeans, when used in conjunction with a standard seed protective fungicide. Chloroneb enters the plants through the roots and is primarily concentrated in the roots and lower stem portions, where the need for protection against soil fungi is greatest (Fielding and Rhodes, 1967; Kirk *et al.*, 1969; Sinclair and Darrag, 1966). Chloroneb is metabolized to 2,5-dichloro-4-methoxyphenol in rats and dogs (Rhodes and Pease, 1971), in cows (Rhodes and Pease, 1971; Gutenmann and Lisk, 1969) and by *Rhizoctonia solani* (Hock and Sisler, 1969).

This paper reports the synthesis of C^{14}-ring-labeled chloroneb, the results of disappearance and metabolism studies in soil, and uptake, distribution, and metabolism studies in beans and cotton. Chloroneb was synthesized with the C^{14}-label in the ring so that the C^{14}-label would be retained by all metabolites in which the aromatic ring had not been degraded.

EXPERIMENTAL

Synthesis of C^{14}-Ring-Labeled Chloroneb. Chloroneb was synthesized by a two step reaction from 2,3,5,6-C^{14}-labeled hydroquinone, with an overall yield of 48.7%. The C^{14}-labeled hydroquinone was obtained from Volk Radiochemical, Irvine, Calif.

Water and 1 *N* sodium hydroxide were deoxygenated by bubbling nitrogen through the liquids for a period of 1 hr. Dimethyl sulfate and sulfuryl chloride were purified by fractional distillation shortly before use.

C^{14}-labeled hydroquinone (121 mg, 1.25 mCi), unlabeled hydroquinone (979 mg, recrystallized from water) and 22 ml of 1 *N* deoxygenated sodium hydroxide were placed in a 250-ml Erlenmeyer flask under a continuous purge of nitrogen. Dimethyl sulfate (2.4 ml) was added and the mixture stirred for 2 hr. Solids, first curdy, then granular, separated. The reaction mixture was extracted four times with a total of 50 ml of chloroform. The chloroform extracts were combined and 2.5 g of magnesium sulfate was added. The drying agent was removed by filtration and washed thoroughly with chloroform. A boiling stone, three drops of ethanol, and 3.24 ml of sulfuryl chloride were added to the chloroform extract, and the resulting solution was heated for 2 hr in a water bath at 40° C. The chloroform was removed by drawing a stream of air over the solution. The resulting oily crystals were recrystallized twice from 25-ml portions of methanol to yield 759 mg of white crystals (A).

Methylation of a small sample of unlabeled hydroquinone in the aqueous mother liquor scavenged any partially methylated C^{14}-hydroquinone. This was chlorinated and recrystallized as described above to give Sample B (1085 mg). A sample of unlabeled chloroneb recrystallized from the methanol recrystallization mother liquors gave Sample C (500 mg).

The radiochemical purity of each sample was determined by thin-layer chromatography, radioautography, and liquid scintillation counting. The radiochemical purities of the samples were 96.7, 96.4, and 95.6%, respectively, for A, B, and C, and the specific activities were 0.0927, 0.0378, and 0.0095 mCi/mmol. The total radiochemical yield was 48.7%.

Exposure of C^{14}-Ring-Labeled Chloroneb to Field Conditions. Soil Treatment and Sampling. During the summer of the year, four 12-in. sections of 4-in. diameter stainless steel tubing were driven into the ground (Keyport silt loam) on a test site in Delaware to isolate undisturbed columns of soil. About 0.5 in. of each cylinder was left protruding above the ground surface to protect against run-off. The upper 3 in. of soil was removed from each cylinder and 1.854 mg (0.839 μCi) of the labeled chloroneb, in 1 ml of acetone, was incorporated in the 2–3 in. layer of soil from each cylinder. The treated soil was replaced in the cylinder and covered with the 0–2 in. layer of untreated soil. The level of treatment corresponds to an application rate of about 2 lb active/acre.

One cylinder each was dug up after 1, 3, 6, and 12 month exposures to field conditions. The soil in each cylinder was removed for analysis in the following increments as measured from the surface: 0–1, 1–3, 3–5, 5–8, and 8–12 in. All increments were wet ballmilled for 2 hr, spread on large trays to dry, then passed through a 10 mesh screen.

Analysis and Results. Duplicate 2-g aliquots of each soil increment were analyzed for total C^{14} by the wet combustion method described by Smith *et al.* (1964). Results of these analyses are given in Table I.

Aliquots of the 1–3 in. layers of soil from the 6 month exposure sample were extracted and analyzed for chloroneb and possible metabolites. Fifty grams of soil, 100 ml of 2 *N* HCl,

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Table I. Percent of Original C^{14}-Activity in Field Soil Fractions

Soil Depth (in.)	Exposure Time (Months) 1	3	6	12
0–1	6.00	6.05	4.15	6.43
1–3[a]	59.90	43.38	46.12	32.53
3–5	2.47	2.10	1.65	0.86
5–8	<0.01	<0.01	<0.01	<0.01
8–12	<0.01	<0.01	<0.01	<0.01
Weeds[b]	3.59	...	...	...
Total	71.96	51.53	51.92	39.82

[a] Treatment increment; treatment level equivalent to 2 lb C^{14}-labeled chloroneb/acre. [b] Weeds, which were growing in the 1-month cylinder, were analyzed for C^{14} content.

and 250 ml of *n*-butanol were heated under reflux for 36 hr. The solution was filtered to remove the soil and the volume of the solution was reduced to *ca.* 125 ml in a hood. The resulting solution was extracted for 72 hr with diethyl ether in a continuous extractor. The ethereal phase was reduced to *ca.* 2 ml and quantitatively transferred to a 5-ml volumetric flask and made to volume with acetone.

Duplicate 1-g samples of the extracted soil were analyzed for total unextracted C^{14}-residues by the combustion method cited above, and aliquots of the ethereal extract and aqueous fraction were counted in a liquid scintillation spectrometer to determine the total C^{14} in the extracts. The results showed that about 70% of the total C^{14} was extracted into the ether, 11% remained in the aqueous phase, and 19% remained on the soil.

One milliliter of the extract was applied to a thin-layer plate of kieselgel (600 μ thickness) as a streak, and a chloroneb reference spot was applied next to the streak. The plate was developed to 15 cm in chloroform and placed next to X-ray film. The resulting radioautogram (Figure 1) showed 2-C^{14}-labeled compounds at R_f values of 0.81 and 0.00. The R_f values for chloroneb and possible metabolites under these conditions are listed in Table VI. The areas of adsorbent containing the C^{14}-materials were removed from the plate and eluted with 50 ml of acetone. Aliquots of the eluted compounds were counted and the results showed that 90% of the C^{14} extracted from soil was chloroneb, and 10% was an unknown compound that remained at the origin of the chromatogram. The structural assignment and analyses of chloroneb were confirmed by gas chromatographic analysis, according to the procedure of Pease (1967).

Uptake and Distribution of C^{14}-Ring-Labeled Chloroneb by Cotton and Bean Plants. GREENHOUSE STUDIES. Stoneville 213 reginned cotton seed, treated with Ceresan L, and Wade snapbeans treated with Delsan AD were used in these studies. C^{14}-Labeled chloroneb, formulated as a 75% wettable powder, was applied as a seed overcoat at rates of 6.75 oz and 3 oz active per 100 lb, respectively, and the seeds (three seeds per pot) planted in 5-in. pots of soil in the greenhouse. Similar plantings were made for additional studies where the C^{14}-labeled-chloroneb was thoroughly mixed in a 2 in. by 2 in. band to simulate field in-furrow treatment at a rate equivalent to 2 lb active per 12,000 row ft. Three plants from each treatment were harvested every week until the plants reached maturity.

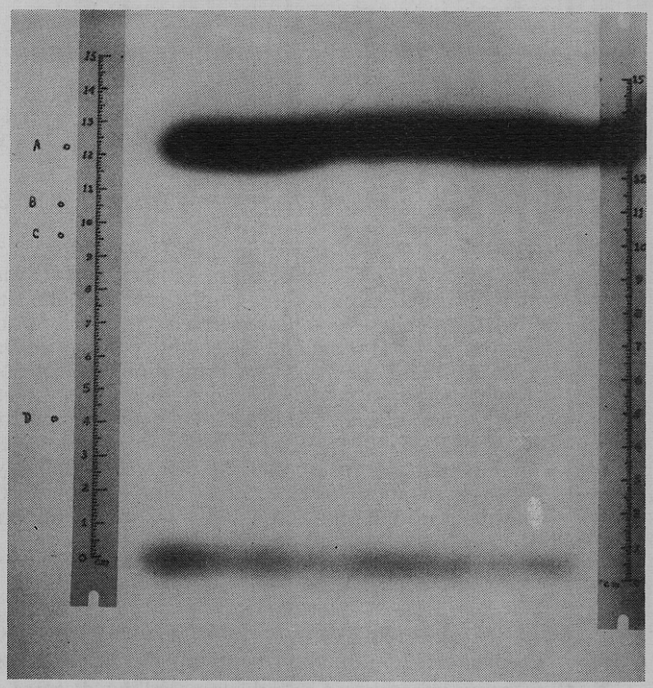

Figure 1. Tlc radioautogram of soil extract. A—Chloroneb; B—2,5-dichloroquinone; C—2,5-dichloro-4-methoxyphenol; D—2,5-dichlorohydroquinone

One plant from each harvest was prepared for autoradiography by conventional methods and placed next to Ansco high speed X-ray film (nonscreen) for 2 weeks. The film was removed from the plant and developed. Typical radioautograms of the plants are shown by Fielding and Rhodes (1967).

A second plant from each harvest was thoroughly washed with tap water and cut into sections for quantitative analyses.

Table II. C^{14} Equivalent to ppm of Chloroneb in Cotton Plants Grown in a Greenhouse With C^{14}-Ring-Labeled Chloroneb Treatments

Weeks After Planting	Seed Treatment Upper[a] Portion	Lower Portion	Seed	Total Plant	In-Furrow Treatment Upper Portion	Lower Portion	Seed	Total Plant
1	...	152.4	...	152.4	...	397	...	397
2	0.38	7.7	...	6.8	36.3	309	...	241
4	0.30	5.2	...	2.7	18.7	235	...	51.7
8	0.11	1.2	...	0.67	3.26	45.8	...	7.0
16	0.01	0.77	<0.01	0.18	...	...	...	...
18	0.01	1.01	<0.01	0.21	...	...	...	...
19	<0.01	0.77	<0.01	0.12	...	...	...	...
20	...	...	<0.01	...	...	...	...	...
22	...	...	...	...	...	...	0.04	...

[a] Upper portion defined as everything above cotyledon leaves.

Table III. C^{14} Equivalent to ppm of Chloroneb in Bean Plants Grown in a Greenhouse With C^{14}-Ring-Labeled Chloroneb Treatments

Weeks After Planting	Seed Treatment				In-Furrow Treatment			
	Upper[a] Portion	Lower Portion	Bean	Total Plant	Upper Portion	Lower Portion	Bean	Total Plant
1	...	127	...	127	...	290	...	290
2	35.5	49.5	...	40.3	35.6	217	...	131
3	3.20	44.0	...	18.1	12.8	165	...	67.5
4	0.50	29.6	...	2.70	4.10	123	0.48	17.5
5	0.60	43.6	0.04	2.60	2.37	121	1.50	18.0
9	0.13	12.9	0.03	0.81	...	...	...	...

[a] Upper portion defined as everything above cotyledon leaves.

Duplicate samples of each plant part were analyzed for total C^{14} by combustion techniques according to the procedure of Smith *et al.* (1964). The results of these analyses are listed in Tables II and III.

Field Studies. Snapbeans and cotton were treated with radiolabeled chloroneb under actual field conditions to determine the total residue in the beans and cotton seed from plants grown with recommended treatments. Three rows of snapbeans were grown, each with a different C^{14}-ring-labeled chloroneb treatment. One row was treated with a seed overcoat at a rate of 2.6 oz active per 100 lb, the second with an in-furrow treatment at the rate of 2 lb active per 12,000 row ft, and the third with an in-furrow treatment at the rate of 2 lb active/acre. Cotton was grown with an in-furrow treatment of 2 lb active per 12,000 row ft. The plants were grown to maturity and the beans, cotton seeds, and plants were harvested and analyzed for total C^{14}. The results of these analyses are given in Table IV.

Snapbeans, grown at several locations with recommended chloroneb treatments, were analyzed for chloroneb and the metabolite by the procedure of Pease (1967) to compare the results of radiochemical and conventional analyses. The results of these conventional analyses, given in Table V, compared very well with the special radiochemical analyses of beans grown with comparable treatments of C^{14}-labeled chloroneb.

Metabolism Studies. Wade snapbean plants were grown in a greenhouse with an in-furrow treatment of C^{14}-ring-labeled chloroneb at a rate of 1 lb active per 12,000 row ft. The plants were harvested 12 days after planting and the average weight per plant was 4 g.

Ninety grams of plant tissue was digested in 5 *N* phosphoric acid according to the procedure of Pease (1967). Chloroneb and 2,5-dichloro-4-methoxyphenol were distilled from the aqueous solution and continuously extracted into hexane. The volume of the hexane extract was reduced to about 5 ml in a hood and made to 5.0 ml with hexane. The total C^{14} extracted into the hexane was determined by counting an aliquot of the extract in a liquid scintillation spectrometer. The remainder of the solution was applied to a thin-layer plate of kieselgel (250 μ thickness) next to a reference spot of chloroneb. The tlc plate was developed to 10 cm in chloroform and placed next to X-ray film for 2 weeks.

The residual phosphoric acid was adjusted to pH 7 with 50% sodium hydroxide and extracted three times with an equal volume of ethyl acetate. The volume of the ethyl acetate solution was reduced to *ca.* 4 ml under a hood, and the resulting solution was quantitatively transferred to a 5-ml volumetric flask and made to volume with ethyl acetate. The total C^{14} extracted into the ethyl acetate was determined and the remainder of the solution chromatographed on a tlc plate as described above.

The radioautograms showed the presence of five C^{14}-labeled compounds in the extracts. The area of adsorbent containing each compound was removed from the plate and the sorbate was eluted with 50 ml of acetone. The volume of

Table IV. C^{14} Equivalent to ppm of Chloroneb in Beans and Cotton at Harvest Time from Plants Grown in the Field with C^{14}-Ring-Labeled Chloroneb Treatments

Crop	Upper[a] Portion	Lower Portion	Bean	Cotton Seed	Total Plant
Cotton[b]	0.056	1.14	...	0.030	0.230
Bean[c]	0.050	3.32	0.003	...	0.320
Bean[d]	0.061	13.66	0.009	...	0.833
Bean[b]	0.207	36.53	0.078	...	2.14

[a] Upper portion defined as everything above cotyledon leaves. [b] Treatment: In-furrow at rate of 2 lb active per 12,000 ft of row. [c] Treatment: Seed overcoat at rate of 2.6 oz active per 100 lb. [d] Treatment: In-furrow at rate of 2 lb active per acre.

Table V. Residue Analyses of Chloroneb in Snapbeans

Treatment	Rate[a]	Location	Residue (ppm) Radiochemical	Residue (ppm) Chemical[b]
In-furrow	2.0	Newark, Del.	0.078	...
In-furrow	1.0	Hollister, Calif.	...	<0.04
In-furrow	1.7	Linden, Calif.	...	0.09
In-furrow	3.0	Linden, Calif.	...	0.14
Seed overcoat	2.6	Newark, Del.	0.003	...
Seed overcoat	4.0	Raleigh, N.C.	...	<0.04
Seed overcoat	6.0	Niles, Mich.	...	<0.04
Seed overcoat	6.0	Bowling Green, Ohio	...	<0.04
Seed overcoat	8.0	Fletcher, N.C.	...	<0.04

[a] In-furrow rate—lb chloroneb per 12,000 ft of row. Seed overcoat rate—oz chloroneb per 100 lb. [b] Chemical analysis—Sum of chloroneb plus metabolite concentrations.

Table VI. Percent of Chloroneb and Metabolites in Plants Extracts

Compound	Percent of Extract Bean	Percent of Extract Cotton	R_f Value[a]
Chloroneb	50.6	52.1	0.81
2,5-Dichloro-4-methoxyphenol	45.8	44.2	0.65
2,5-Dichlorohydroquinone	0.6	0.7	0.27
2,5-Dichloroquinone	0.9	0.9	0.71
Unknown	2.1	2.1	0.00
Total	100.0	100.0	

[a] Tlc separations done on 250 μ kieselgel plates developed in chloroform.

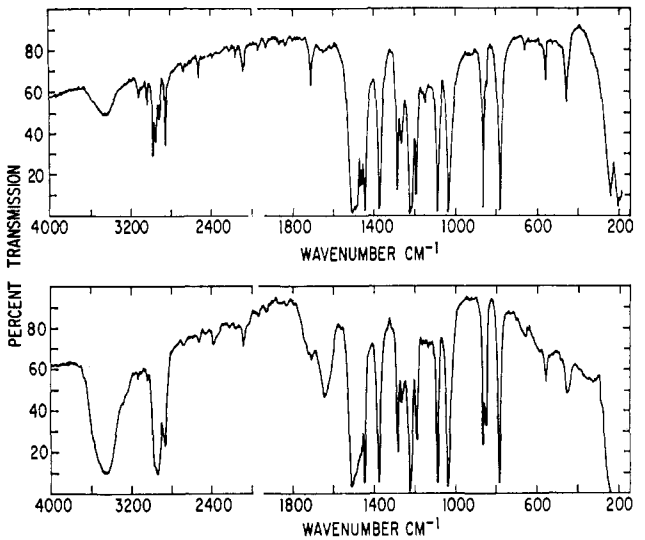

Figure 2. Infrared spectra ofchloroneb. Upper—chloroneb isolated from bean extract. Lower—chloroneb reference

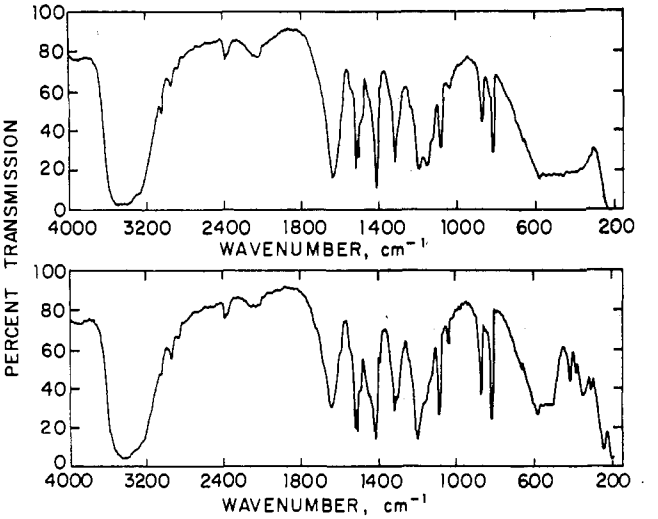

Figure 4. Infrared spectra of 2,5-dichlorohydroquinone. Upper—2,5-dichlorohydroquinone isolated from bean extract. Lower—2,5-dichlorohydroquinone reference

each solution was reduced to 5.0 ml and the C^{14} in each fraction was determined. Each C^{14}-labeled compound was purified by rechromatographing on tlc plates. Infrared and mass spectra were obtained for each compound. The identity of four of the compounds was confirmed by comparison of R_f values on tlc plates, infrared, and mass spectra with those for the appropriate reference compounds. The infrared spectra are shown in Figures 2–5, and the results of the analyses are given in Table VI. The four identified compounds are chloroneb, 2,5-dichloro-4-methoxyphenol, 2,5-dichlorohydroquinone, and 2,5-dichloroquinone.

The amount of C^{14} not extracted from the plants was determined by combustion analyses. The percent of total activity in each phase was 58.7% in the hexane extract, 22.8% in ethyl acetate, 3.4% in the aqueous solution, and 15.1% remained in the plant tissue. Two compounds, chloroneb and 2,5-dichloro-4-methoxyphenol, were found in the hexane. The ethyl acetate extract contained 2,5-dichlorohydroquinone, 2,5-dichloroquinone and the unidentified compound plus the chloroneb and 2,5-dichloro-4-methoxyphenol that were not removed in the hexane extract.

Cotton plants (100 g) grown in the greenhouse with a 0.92 lb active per 12,000 row ft treatment of C^{14}-ring-labeled chloroneb were extracted and analyzed by the procedure described for beans. The extraction removed 84.3% of the C^{14} from the plants. The results of these analyses and the R_f values for each compound are given in Table VI.

RESULTS AND DISCUSSION

Chloroneb was shown to have a half-life of approximately 3 to 6 months in Keyport silt loam in Delaware when the C^{14}-labeled compound was incorporated into field soil 2 to 3 in. below the surface. The concentration of the compound in soil remained constant during the winter months when the ground was frozen, but disappearance was observed again in the spring. It was also found that essentially all of the chloroneb remains in the treated layer of soil (1–3 in. layer). Rhodes *et al.* (1970) have shown that chloroneb is immobile

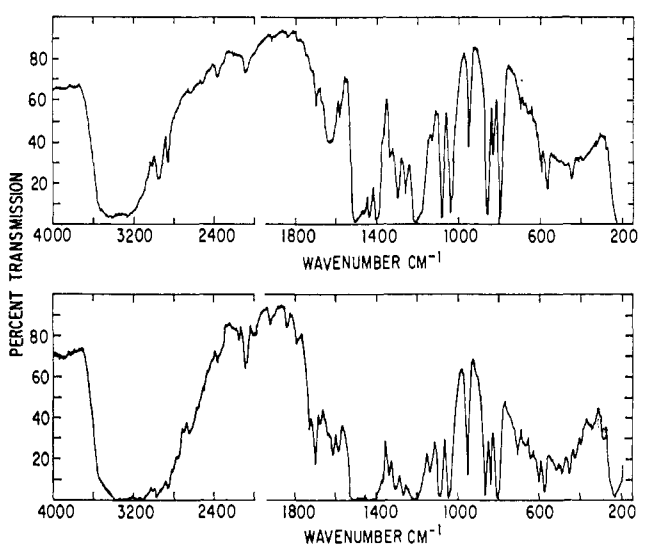

Figure 3. Infrared spectra of 2,5-dichloro-4-methoxyphenol. Upper—2,5-dichloro-4-methoxyphenol isolated from bean extract. Lower—2,5-dichloro-4-methoxyphenol standard

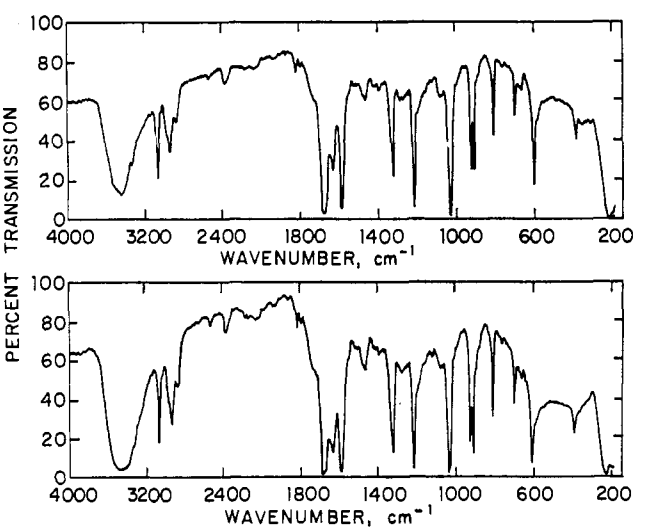

Figure 5. Infrared spectra of 2,5-dichloroquinone. Upper—2,5-dichloroquinone isolated from bean extract. Lower—2,5-dichloroquinone reference

in a variety of soil types. Analyses of the treated soils showed that about 90% of the residual activity recovered from the soil was chloroneb, and the remainder of the activity was unidentified polar compound(s). It has been shown by tlc-radioautography that the unknown is not 2,5-dichloro-4-methoxyphenol, 2,5-dichlorohydroquinone, or 2,5-dichloroquinone.

Chloroneb is taken up through the roots by cotton and bean plants, and is primarily concentrated in the roots and lower stem portions of the plant where the need for protection against soil fungi is greatest. The concentration of the compound in each plant part decreases with time. Quantitative radiochemical analyses of cotton seed and beans from plants grown in the field with recommended treatments of C^{14}-ring-labeled chloroneb show that only trace amounts (<0.1 ppm) of total residue are found in these plant parts under field conditions. Federal tolerances for chloroneb and its metabolite, 2,5-dichloro-4-methoxyphenol, calculated as chloroneb, are set at 0.1 ppm in beans and cotton seed.

It was observed that the residues in the upper plant parts were greater in plants grown in pots than in plants grown under normal field conditions. This difference illustrates that plants grown in the greenhouse where the roots are continuously in contact with the compound take up larger amounts of chemicals from soils than plants grown under normal field conditions where the roots grow through the treated zone.

Chloroneb and 2,5-dichloro-4-methoxyphenol accounted for greater than 95% of the C^{14} residue in cotton and bean plants (Table VI). Trace amounts of 2,5-dichlorohydroquinone, 2,5-dichloroquinone, and an unidentified polar metabolite were also detected in the plant extracts. Chloroneb and the three metabolites are all stable under the extraction conditions described above. When more rigorous hydrolysis conditions were used, a decrease in extraction efficiency and decomposition of 2,5-dichloro-4-methoxyphenol was observed.

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