XXIV, and XXV). R_3 may also be an unsaturated group such as methallyl (XIX) or methylpropenyl (XX).

Activity of methylcarbamate insecticides has been correlated with molecular weight (*1*). This relationship does not hold true for methylcarbamate crabgrass herbicides, because 2,4-di-*tert*-butyl-*o*-tolyl methylcarbamate (XXVI), an isomer of Azak, is inactive.

Acknowledgment

The authors thank T. K. Dickson for conducting many of the tests on these compounds.

Literature Cited

(1) Audus, L. J., "Physiology and Biochemistry of Herbicides," p. 27, Academic Press, New York, 1964.
(2) Gysin, H., Knüsli, E. (to J. R. Geigy A.G.), U. S. Patent **2,776,197** (Jan. 1, 1957).
(3) Haubein, A. H. (to Hercules Powder Co.), *Ibid.*, **3,140,167** (July 7, 1964).
(4) Kilsheimer, J. R., Moorefield, H. H., "Structural Requirements for Insecticidal Activity in the Phenyl Carbamate Series," Division of Agricultural and Food Chemistry, 139th Meeting, ACS, St. Louis, Mo., March 25, 1961.
(5) Metcalf, R. L., "Organic Insecticides," p. 317, Interscience, New York, 1955.

Received for review March 27, 1965. Accepted June 25, 1965. Division of Agricultural and Food Chemistry, 149th Meeting, ACS, Detroit, Mich., April 1965.

METABOLISM BY PLANTS

Translocation and Metabolism of 2,6-Dichloro-4-nitroaniline by Lettuce and Tomato

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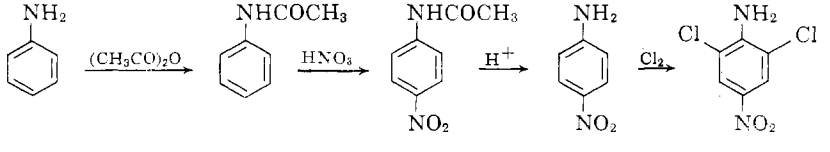
Lettuce plants were treated with soil and nutrient applications of C^{14}-labeled 2,6-dichloro-4-nitroaniline (DCNA). After 50 days, labeled carbohydrates and unchanged DCNA were recovered from the plants grown in soil. The amino acid, chlorophyll, and uronic acid portions of the plants did not contain radioactivity, whereas a carbohydrate fraction was labeled. The possible transient metabolites, 2-chloro-4-nitroaniline, 2,6-dichloro-*p*-phenylenediamine, and *p*-nitroaniline, were not detected. DCNA was shown to be translocated by tomato seedlings.

THE FUNGICIDE, 2,6-dichloro-4-nitroaniline, has been used for the control of *Botrytis* and *Rhizopus* fungal diseases on plants and perishable fruits and vegetables under a temporary tolerance registration. The first commercial application of this material was in the United Kindgom, where it was used successfully to control *Botrytis* infections in greenhouse lettuce (*1*). A spectrophotometric method for the determination of DCNA was described by Roburn (*10*), who indicated that neither DCNA nor DPPD (2,6 - dichloro - *p* - phenylenediamine), a possible metabolite, was absorbed by the roots or leaves of plants. In spite of this report, the possible uptake, translocation, and metabolism of DCNA by lettuce was investigated.

Materials and Methods

Preparation of DCNA-C^{14} and DPPD-C^{14}. Uniformly labeled 2,6-dichloro-4-nitroaniline was prepared by the following sequence of reactions:



A yield of 30.7% (333.2 mg.) of uniformly labeled 2,6-dichloro-4-nitroaniline [m.p. 191–92° C. (0.97 mc./mmole)] was obtained from 5.1 mc. (0.147 gram) of uniformly labeled aniline sulfate. The radiochemical purity of the preparation was assessed by paper chromatography using a benzene-formamide system. The material to be separated was placed as a spot on Whatman No. 1 paper impregnated with formamide. The chromatogram was developed with benzene saturated with formamide as the solvent using the descending technique. The labeled DCNA gave only one radioactive peak at R_f 0.8. Good separations of 2-chloro-4-nitroaniline (R_f 0.4), *p*-nitroaniline (R_f 0.15), 2,6-dichloro-*p*-phenylenediamine (DPPD) (R_f 0.7), and DCNA were obtained with this system.

Uniformly C^{14}-labeled 2,6-dichloro-*p*-phenylenediamine was prepared from uniformly C^{14}-labeled DCNA by reduction with zinc and hydrochloric acid under reflux. Purification by transfer to water as the hydrochloride, neutralization, and extraction into ether gave, on removal of the solvent, DPPD-C^{14} (m.p. 119–120° C.) containing about 3% of labeled impurities when paper chromatographed using the benzene-formamide system. The DPPD-C^{14} was stored in benzene solution under N_2 in the dark at 5° C.

Radiocounting Equipment and Methods. All samples were counted using a Packard Tri-Carb liquid scintillation spectrometer, Model 314 EX-2, with a 200-sample counting chamber at 25° F. One channel was optimized for counting samples in toluene scintillator solution, 15 ml. per sample (18.75 grams of PPO (2,5-diphenyloxazole) plus 0.375 gram of POPOP {*p*-bis[2-(5-phenyloxazolyl)] benzene} in 3750 ml. of toluene). The second channel was optimized for counting Hyamine hydroxide Schöniger combustion (*7*) samples using Diotol (*5*) scintillator solution, 15 ml. per sample (73 grams of recrystallized naphthalene, 4.6 grams of PPO, and 0.88 gram of POPOP in a mixture of 350 ml. of toluene, 350 ml. of dioxane, and 210 ml. of methanol). Aqueous alkaline solutions (0.5-ml. aliquots) were counted in 15 ml. of Diotol plus 0.6 gram of Cab-O-Sil M5, a thixotropic dry silica–water absorbent and suspending agent. All samples were corrected for quenching by recounting following the addition of an aliquot of C^{14}-labeled toluene. Paper chromatograms were counted using a Vanguard 880 chromatogram scanner or by cutting the paper strip into $^1/_2$-inch × $^5/_8$-inch sections and

counting each section separately in toluene scintillator solution in the Tri-Carb. Thin-layer chromatograms were counted by scraping off 0.5- × 2.0-cm. sections of the absorbent, suspending each section in Diotol plus Cab-O-Sil M5, and counting in the Tri-Carb.

Experimental Procedures

Recovery of DCNA-C^{14} and DPPD-C^{14} from Lettuce. To a Bibb lettuce plant (49.27 grams) in a Waring Blendor was added 1.0 ml. of a solution of DCNA-C^{14} in benzene (76.8 μg./ml.) containing 7.92×10^5 d.p.m. The plant was macerated with two 50-ml. portions of benzene, giving 76 ml. of benzene which was recovered by centrifugation. Triplicate 0.5- and 1.0-ml. samples of this extract were counted and recounted after adding toluene C^{14} standard. A total of 7.57×10^5 d.p.m. were found in the benzene extract. This represented a 95.5% recovery of the radioactivity added. The DCNA-C^{14} in the extract was identified by paper chromatography.

An aqueous fraction (16 ml.) was separated from the lettuce residues during the centrifugation. Samples of this were counted and found to contain negligible radioactivity.

In a similar experiment, 49.35 grams of lettuce were macerated with two 50-ml. portions of benzene and 1.0 ml. of the DPPD-C^{14} standard solution (2.05×10^6 d.p.m.). Centrifugation, followed by re-extraction of the residue and combination of the extracts, gave 77.5 ml. of benzene solution which was counted. In the benzene solution, there was 2.86×10^5 d.p.m.—i.e., 14% recovery. The aqueous phase (15.5 ml.) contained 2.92×10^4 d.p.m., equivalent to 1.4% of the activity added. Distillation of the benzene extract under reduced pressure at 25° C. gave a residue which was dissolved in 2.5 ml. of benzene. Chromatography of 20 μl. of this solution did not give any definite radioactive peaks. In order to determine whether the DPPD-C^{14} was unstable under the conditions of a simple benzene-water partition, 0.1 ml. of the standard DPPD-C^{14} benzene solution (2.05×10^5 d.p.m.) in 100 ml. of benzene was added to 100 ml. of water. The phases were separated and aliquots of each were counted. The partition ratio under these conditions was found to be 245 to 1 benzene to water, the total recovered being 81%. The benzene solution was evaporated to dryness at room temperature, and 0.5 ml. of benzene was added. Paper chromatography of 5 μl. of this solution using the benzene-formamide system gave an intense radioactive zone at R_f 0.65 (DPPD) and a much smaller peak at R_f 0.78. This experiment indicated that even if DPPD was formed, under these conditions of extraction it would not be detected.

Uptake of DCNA-C^{14} by Tomato and Lettuce Seedlings from Nutrient Solution

Preliminary experiments using unlabeled DCNA were performed to discover whether the chemical would penetrate the roots and translocate under laboratory conditions. The roots of 25 tomato seedlings (variety Rutgers, 6 to 8 inches) were placed in Hoagland's nutrient solution containing 10 p.p.m. of DCNA. Another 25 seedlings were grown in Hoagland's nutrient solution as controls. At various time intervals, up to 10 days after beginning the treatment, the stems and leaves were removed from selected plants and analyzed for DCNA by the Kilgore procedure (*6*). The results are shown in Figure 1. In a similar experiment, tomato seedlings were grown for 40 hours in a 10-p.p.m. DCNA Hoagland's nutrient solution and then transferred to nutrient solution without the DCNA. The stems and leaves were analyzed for DCNA at various time intervals. The results are shown in Figure 2. A similar experiment using Bibb lettuce seedlings growing in 10-p.p.m. DCNA in Hoagland's nutrient solution gave concentrations of 0.39 p.p.m. after 40 hours, 2.73 p.p.m. after 4 days, and 0.034 p.p.m. of DCNA after 7 days in the leaf tissue. About 7 days after starting treatment, the lettuce showed severe phytotoxic symptoms consisting of marginal browning and necrosis of the leaves.

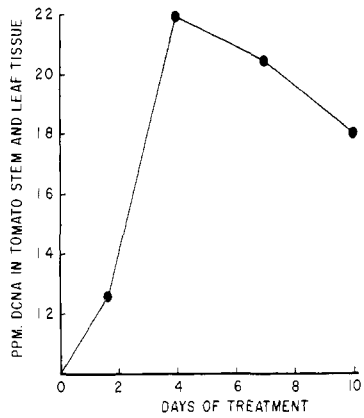


Figure 1. Concentration of DCNA in upper stem and leaf tissue of tomato seedlings growing in 10-p.p.m. DCNA nutrient solution

Average of duplicate determinations

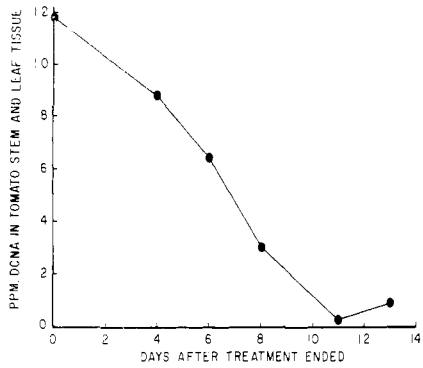


Figure 2. Disappearance of DCNA from upper stem and leaf tissue of tomato seedlings after growing for 40 hours in 10-p.p.m. DCNA nutrient solution

Table I. Distribution and Recovery of Radioactivity from Tomato Plants Grown in DCNA-C^{14} Nutrient Solution

	D.P.M. (× 10^6)	% of Total Radioactivity Supplied
Benzene extract	20.22	31.3
Water extract	0.33	0.5
80% Methanol extract	0.27	0.4
Residue (by combustion)	2.24	3.5
Recovered nutrient	37.27	57.8
	60.33	93.5

Hoagland's nutrient solution containing 6.06 p.p.m. of DCNA-C^{14} (64.5×10^6 d.p.m./liter) was prepared and 24 tomato seedlings (variety Rutgers, 6- to 8-inch stems) were grown in the solution for 4 days in the laboratory under 700-foot-candle artificial light. The plants were extracted by maceration and centrifugation successively with benzene, water, and 80% MeOH. Aliquots of the extracts and the recovered nutrient were counted. The remaining white fibrous residue was counted using the Schöniger combustion method. The results are shown in Table I.

Paper chromatography of the benzene fraction using the benzene-formamide system and a similar system using *n*-butyl acetate saturated with 50% aqueous formamide as the developing solvent (R_f of DCNA-C^{14} was 0.9) indicated that the only radioactive component was DCNA-C^{14}.

Paper chromatography using the upper phase of a mixture of 667 ml. of Skellysolve C, 333 ml. of benzene, 800 ml. of methanol, and 200 ml. of water, after equilibration of the spotted paper for 12 hours at 37° C., also showed only DCNA-C^{14} in the benzene extract. Thin-layer chromatography on Silica Gel G plates and development with ethyl acetate (DCNA-C^{14} R_f was 0.75) or with benzene-chloroform, 8 to 2 (DCNA-C^{14} R_f was 0.5), again showed the presence of only DCNA-C^{14}.

Hydrolysis of a portion of the insoluble residue (16 grams) with 100 ml. of refluxing 2*N* H_2SO_4 for 30 minutes, cooling, neutralization with ammonia, and extraction with ethyl acetate gave about 35% of the radioactivity in the extract. Paper chromatography using the benzene-formamide system indicated that about one third of the extract activity was present as DCNA-C^{14}. Hence, of the DCNA-C^{14} radioactivity absorbed by the plants, 87.7% was benzene extractable DCNA-C^{14}, 1.0% was DCNA-C^{14} not extractable with benzene, and 2.6% was water or aqueous methanol extractable. Only 8.7% was completely insoluble.

It was evident from these experiments that the DCNA was translocated to the leaves and stems of tomatoes and rapidly degraded. A similar situation was observed with the Bibb lettuce seedlings. To confirm these latter observations, two-week-old Bibb lettuce seedlings

Table II. Counting Data from Benzene Extracts and Dry Residues from DCNA-C^{14} Soil-Treated Lettuce

	Benzene Extract		Residue	
Replicate No.	D.p.m.	DCNA-C^{14}, p.p.m.	D.p.m.	Equivalent p.p.m. DCNA-C^{14}
1	1.87×10^5	0.423	1.03×10^6	2.66
2	1.68×10^5	0.248	1.13×10^6	3.09
3	1.64×10^5	0.288	1.00×10^6	2.74

were grown for one week in Hoagland's nutrient solution in order to allow the plants to recover from transplanting shock. The roots of each plant were then placed separately in 300 ml. of Hoagland's nutrient containing 1.47 mg. of DCNA-C^{14} (4.9 p.p.m.). The plants were grown under artificial light (700 foot-candles) and one plant was harvested at each time interval.

Leaf tissue of the plants was macerated with 50 ml. of benzene and centrifuged and the benzene decanted. The fibrous residue was again macerated with 50 ml. of benzene and centrifuged and the benzene solution decanted. The residue was dried at 40° C. overnight under reduced pressure. The combined benzene extracts were sampled and counted. The remaining solution was evaporated to dryness under reduced pressure at 35° C. and the dark green residue was dissolved in 1 ml. of benzene. Paper chromatography of this solution using the benzene-formamide system identified the radioactivity as DCNA-C^{14}. The dried fibrous residue was counted using the Schöniger combustion technique.

After the seedlings had grown for 2 days in the DCNA-C^{14} solution, the remaining plants were placed in ordinary Hoagland's nutrient solution and selected plants were extracted as above at various time intervals. The results are shown graphically in Figures 3 and 4. In order to be able to compare the radioactivity in each fraction of each plant, the data for Figures 3 and 4 were calculated on the basis of d.p.m. per milligram of leaf tissue.

The apparent reduction in the concentration of DCNA-C^{14} between 6 and 12 hours after the start of the experiment was unexpected. It was, however, reproducible during two subsequent experiments. Such a break in the curve indicates that a different mechanism operates in the latter stages of the uptake time and a sequence comprising active uptake, partial poisoning of the tissue, followed by mechanical transport in the transpiration stream, could be operating.

Uptake of DCNA-C^{14} from Soil by Bibb Lettuce

Five four-week-old Bibb lettuce plants, grown in 3-inch pots, were treated with DCNA-C^{14} by mixing 4.082 mg. of DCNA-C^{14} with 25 grams of dry potting soil and spreading the mixture $^1/_4$ inch deep on the top of the soil. The rate was equivalent to four times the recommended usage. Fifty days after treatment, the plants were harvested. Two plants showed phytotoxic and/or nutritional deficiency symptoms and were used in a preliminary experiment to check the techniques. The plants were watered from the top once a day. Soluble fertilizer was applied once per week. Each plant was extracted separately with 50-ml. portions of benzene as previously described and the fibrous residue was again dried at 40° C. under reduced pressure. The radioactivity in the extract was determined by counting aliquots in the Packard Tri-Carb spectrometer and the radioactivity in the residue was determined using the Schöniger combustion method. The results are shown in Table II. Paper chromatography of the benzene extracts indicated most of the radioactivity was present as unchanged DCNA-C^{14}. However, there were also radioactive zones at the origins of the chromatograms indicating the presence of polar metabolites. The calculated parts per million of DCNA-C^{14} in Table II were corrected for the presence of these latter labeled substances. Approximately 4% of the radioactivity applied to the soil was recovered in the plant tissue.

Ethanol Extraction of Residues from DCNA-C^{14} Soil-Treated Lettuce

In order to determine the nature of the highly polar labeled product(s) observed in the chromatograms from the benzene extracts from the soil treatment experiments, the fibrous residue from replicate 2 was extracted three times, each with 60 ml. of boiling 70% aqueous ethanol for 15 minutes. After filtration, the extracts were combined and aliquots were counted. A total of 1.17×10^5 d.p.m. was found in the extract—i.e., about 9.1% of the total activity in the plant as calculated from the benzene extract plus the combustion data. The extract was evaporated to dryness at 30° C. under reduced pressure and 2.5 ml. of methanol were added. The residue, which was completely soluble in the methanol, was paper chromatographed using the benzene-formamide system and a similar system in which the developing solvent was formamide saturated with benzene. The benzene-formamide system gave a radioactive zone at the origin—i.e., no DCNA-C^{14} was detected. The paper chromatogram developed in formamide saturated with benzene had one radioactive peak at R_f 0.5. Similar chromatograms which were sprayed with silver nitrate–ethanolic NaOH reagent produced a brown spot at R_f 0.5 matching the R_f of the radioactive zone. Ninhydrin reagent produced two pink spots (amino acids) at R_f 0.35 and 0.4.

A 1.0-ml. portion of the methanol solution was diluted with 50 ml. of de-ionized

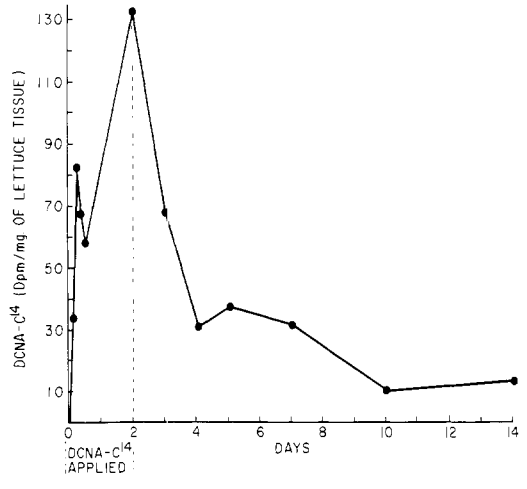


Figure 3. Uptake of DCNA-C^{14} from nutrient solution by lettuce during 2 days and its subsequent disappearance

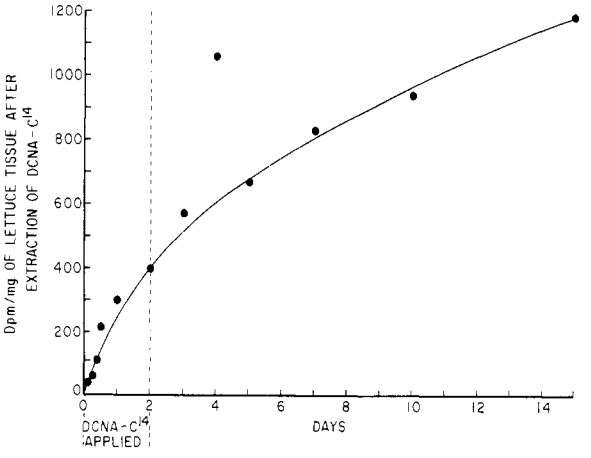


Figure 4. Appearance of non-DCNA-C^{14} radioactivity in lettuce plants from 2-day root soak in DCNA-C^{14} nutrient solution

Table III. R_f's of Spots Obtained by Thin-Layer Chromatography of Nonamino Acid Fractions from 70% Ethanol Extract of DCNA-C^{14} Soil-Treated Lettuce

Color Reagent			
$AgNO_3$–NaOH	Naphtho-resorcinol		Radio-activity
0.15	0.15	Blue	
0.27	0.25	Blue	
0.37	0.35	Brown	
0.49	0.45	Brown	
0.58	0.55	Brown	0.58
0.72	0.78	Brown	0.75
0.82	0.86	Red-brown	
0.94	0.92	Red-brown	

water and poured onto 300 grams of Dowex 50-2X (H^+) resin in a short column. The Dowex 50-2X was first conditioned by stirring with 500 ml. of 6*N* HCl for 10 minutes, followed by washing and filtration with de-ionized water until the washings were neutral. The column containing the extract was eluted with 200 ml. of de-ionized water followed by 600 ml. of 6*N* HCl. Aliquots of the eluates were counted. There was a total of 3.98×10^4 d.p.m. in the water eluate and 7.2×10^3 d.p.m. in the acid eluate (100% recovery). These data indicate that 85% of the activity in the 70% ethanol extract was in the nonamino acid, with 15% being recovered in the 6*N* HCl amino acid eluate. The latter figure was probably high, since only 200 ml. of water were used to elute the 300 grams of resin.

Each aqueous fraction was evaporated to dryness, were the residues were dissolved in 2.5 ml. of methanol, and 10 μl. were chromatographed on thin-layer plates of Silica Gel G using the Fahmy system (*3*). The amino acid fraction gave only one unresolved spot with a positive ninhydrin reaction. On repeated thin-layer chromatography, the level of radioactivity was too low to allow the determination of the positions of the radioactive zones.

The nonamino acids, however, gave two radioactive zones which coincided with two spots showing positive silver nitrate colors. This plate did not give any ninhydrin-positive spots. Repeated thin-layer chromatography of the nonamino acids (three spots), spraying one spot and associated chromatogram with the silver nitrate reagent, one spot with naphthoresorcinol (*11*), and counting the third spot produced the results shown in Table III.

Discussion

In contrast to the supposition that DCNA was not absorbed by the roots of lettuce plants (*10*), the present study indicated that DCNA was readily taken up and translocated by both tomato and lettuce, producing appreciable concentrations in the leaf tissues. DCNA was rapidly degraded to polar metabolites and the labeled carbon from DCNA-C^{14} appeared in carbohydrate plant constituents presumably by re-entry of fragments into the metabolic pool. Transient metabolites, such as related compounds with chlorine removed or the reduction product, 2,6-dichloro-*p*-phenylenediamine, were not detected, leading to the hypothesis that the primary degradation products are formed by ring opening. Figures 3 and 4 demonstrate the relationship between the disappearance of the absorbed DCNA-C^{14} and the appearance of benzene-insoluble C^{14}-labeled products. Paper chromatograms of the chlorophyll-containing benzene extracts from DCNA-C^{14}–treated lettuce and tomatoes showed the presence of only DCNA-C^{14} and the polar re-entry products, indicating that the labeled carbon was not incorporated into the chlorophyll. Similarly, a carbohydrate–uronic acid fraction obtained by aqueous ethanol extraction and ion-exchange separation showed that the labeled carbon was present in the naphthoresorcinol-positive carbohydrate material. No attempt was made to collect respired CO_2 from the DCNA-C^{14}–treated lettuce but further work on this aspect of the problem may be enlightening. A proportion of the incorporated radioactivity remained in the tissue even after extraction with benzene and aqueous methanol or ethanol. This tissue was comprised chiefly of cellulose and lignins (*2*) but on refluxing with sulfuric acid, little additional DCNA-C^{14} was produced. In the case of barban, 4-chloro-2-butynyl *N*-(3-chlorophenyl) carbamate, a water-soluble 3-chloroaniline–containing metabolite was formed in plants which produced 3-chloroaniline when hydrolyzed (*8*, *9*). 2,6-Dichloro-4-nitroaniline was not conjugated in this manner.

Literature Cited

(1) Clark, N. G., Hams, A. F., *J. Sci. Food Agr.* **12,** 751 (1961).
(2) Edelman, J., Ginsburg, V., Hassid, W. Z., *J. Biol. Chem.* **213,** 846 (1955).
(3) Fahmy, A. R., *Helv. Chim. Acta* **44,** 2022 (1961).
(4) Heftman, E., "Chromatography," pp. 365, 505, Reinhold, New York, 1961.
(5) Hergerg, R. J., *Anal. Chem.* **32,** 42 (1960).
(6) Kilgore, W. W., Cheng, K. W., Ogawa, J. M., J. AGR. FOOD CHEM. **10,** 399 (1962).
(7) Lisk, D. J., *Pesticide Revs.* **1,** 152 (1962).
(8) Riden, J. R., Jr., Hopkins, T. R., J. AGR. FOOD CHEM. **10,** 455 (1962).
(9) Riden, J. R., Jr., Hopkins, T. R., Dubrovin, K. P., Abstracts Division of Agricultural and Food Chemistry, 144th Meeting, ACS, Los Angeles, April 1963.
(10) Roburn, J., *J. Sci. Food Agr.* **12,** 767 (1961).
(11) Smith, I., "Chromatographic Techniques," p. 168, Heinemann, London 1958.
(12) Trevelyan, W. E., *Nature* **166,** 444 (1950).

Received for review April 29, 1965. Accepted July 4, 1965. Second in a series on translocation and metabolism of pesticides. Paper I, "Translocation and Persistence of Tritium-Labeled Cycloheximide in Eastern White Pine Seedlings," by A. J. Lemin and R. C. Thomas, was published in J. AGR. FOOD CHEM. **9,** *254 (1961).*