Demonstration of Lindane and a Lindane Metabolite in Plants by Paper Chromatography

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A paper chromatographic method used to detect lindane and oil-soluble chlorinated metabolites of lindane in various species of crop plants indicated the presence of a metabolite in lindane-treated carrot plants. Results of experiments to characterize this substance, as yet unidentified, are presented.

Little is known of the plant metabolism of hydrocarbon compounds containing chlorine. This investigation was limited to a consideration of the metabolism of one such compound, lindane, the gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane. An objective was to demonstrate the presence of a metabolite or metabolites of lindane in lindane-treated plants. Translocation (*10*) and metabolism (*2, 6*) of the insecticide by plants have been studied and it is known that its application results in the development of off-odors and off-flavors in the edible portions of some crop plants (*1, 3, 11*).

"Reversed phase" paper chromatography has been used for the analysis of pesticide compounds (*13*). Plant waxes, oils, and pigments, however, usually interfere with the chromatographic analysis of plant extracts and, therefore, rigorous cleanup procedures are necessary (*9*). Because there is a possibility that chemical changes of metabolites occur during drastic cleanup procedures, paper chromatography was adapted for the qualitative analysis of lindane and similar compounds in the presence of appreciable quantities of oil and plant pigments. In some cases a supplementary procedure was used to clean up an extract partially before chromatography. Only oil-soluble compounds are considered in this report. Emphasis was placed on the study of the lindane-treated carrot plant, because it was known to accumulate lindane.

Material and Methods

Plant-Growth Conditions. Various plant species were grown in the greenhouse. Treated plants were those grown in soil containing lindane (99% gamma isomer). Lindane was incorporated in a weighed quantity of soil by spraying the soil (spread out in a 1-inch layer) with a known volume of an acetone solution of lindane. Acetone alone was sprayed on soil in which control plants were grown. The spraying procedure in each case was interrupted at least 5 times and the soil was thoroughly mixed. All other growth conditions were the same for treated and control plants.

Preparation and Extraction of Plant Tissue. Plant material was usually prepared for extraction within 1 hour after harvest. First the plant material was thoroughly washed with tap and distilled water to remove adhering soil particles. In addition, the fibrous root samples were freed of all visible soil or composted material with forceps. The plant material was rinsed several times with methylene chloride and again with distilled water, then quickly blotted dry and weighed.

The plant material was extracted twice by blending with freshly distilled methylene chloride (Fisher certified) in a Waring Blendor. In recovery experiments an average of 90% of standard lindane added to control-plant tissue was recovered. These initial extracts were filtered through dry cotton and then stored at 3° C. The use of methylene chloride as a solvent did not interfere with subsequent chromatographic analyses.

Cleanup of Plant Extracts. In some special cases plant extracts were partially freed of various plant extractives by a well-known method for obtaining chlorophyll in the colloidal state (*21*). The initial methylene chloride extract was carefully evaporated to dryness. Fifteen milliliters of acetone were used to transfer the residue to a 50-ml. separatory funnel. This acetone solution-suspension then was added dropwise (about 1 drop per second) to 600 ml. of distilled water in a 1-liter Erlenmeyer flask. During the addition of the acetone the water was stirred vigorously by a rotating borosilicate glass-enclosed magnetic stirring bar. The resulting aqueous fraction was transferred to a 1-liter separatory funnel after filtration through a 5-cm. layer of glass wool in a glass Gooch crucible held at the top of the separatory funnel. (This glass wool filter retains any chlorophyll and carotene which may have precipitated.) The flask, filter, and stirrer bar were rinsed with three successive 10-ml. portions of distilled water and the rinse water was used to wash the precipitated substances. The aqueous fraction was then shaken for 2 to 3 minutes each time with four successive 100-ml. portions of petroleum ether (ACS, boiling point 37° to 43° C.). In some extractions it was difficult to effect a substantial separation of the water and petroleum ether phases, because of emulsification. Patient manipulation of the separatory funnel, however, invariably resulted in satisfactory separations.

After completion of the extraction procedure, the combined petroleum ether fraction was washed by being shaken briefly with three successive 100-ml. portions of distilled water. The washed petroleum ether fraction was filtered through a 1-inch plug of dried cotton into a 1-liter Erlenmeyer flask. This fraction, clear and only lightly colored, was stored at 3° C. An important consideration relevant to the nature of this cleanup procedure was the avoidance of drastic treatment (heat, acid, and alkali) which might have affected the presence or nature of metabolites.

Paper Chromatography Method. The chromatographic procedure finally adopted was a modification of a reversed phase method described by Mitchell (*13, 15*). Whatman No. 1 paper was used in his method. Of Mitchell's two solvent systems, aqueous and nonaqueous, only the latter could be modified to permit the chromatography of chlorinated pesticides in the presence of appreciable quantities of plant oils and pigments. *n*-Hexane–acetonitrile (*12*)

Table I. R_f Values of Compounds in N,N-Dimethylformamide–n-Hexane System on Whatman No. 17 Paper[a]

Compound	Purity of Compound	R_f
Aldrin	Purified	0.82
α-BHC	Purified	0.54
γ-BHC (lindane)	99%	0.33
Chlordan	99%	0.68, (0.49)[c]
p-Dichlorobenzene	Technical	0.87
2,4-Dichlorophenoxyacetic acid	Purified	0.00
p,p'-DDT	Purified	0.61
p,p'-DDT (dehydrochlorinated)	Purified	0.69
Dieldrin	Purified	0.65
Endrin	95%	0.63, 0.20
Heptachlor	Purified	0.76
Isodrin	97%	0.70
Methoxychlor	Technical	0.25, 0.14, 0.02
p-Nitrochlorobenzene[b]	Purified	0.22
Toxaphene	Technical	0.55
1,3,5-Trichlorobenzene	Purified	0.65, (0.93)[c]

[a] Each compound with 0.015 ml. of mineral oil at origin spot. All R_f values based on measurement to centers of final spots. R_f value for major constituent given first.
[b] Gives brownish spot with $AgNO_3$ indicator reagent.
[c] Present but not clearly defined.

Table II. Lindane in Treated-Plant[a] Tissues

Age of Plants at Harvest, Days	Plant Tissue, Analyzed	No. of Different Tissue Extracts, Analyzed[b]	Fresh Weight of Tissue, Grams	Treatment of Extract[c]	Av. of Lindane Estimations, P.P.M.
	Carrot, *Daucus carota*, var. Imperator (10 P.P.M.)				
100–120	Fibrous roots	4	1	C and D	25
	Edible portion	4	40	C	3
	Stem and leaf	1	57	C	0.25
	Carrot, *Daucus carota*, var. Imperator (5 P.P.M.)				
53	Fibrous roots	2	7	D	25
120	Fibrous roots	2	2	D	17
	Edible portion	2	40	C	0.25
	Snap bean, *Phaseolus vulgaris*, var. Topcrop (5 P.P.M.)				
50	Edible beans	1	50	C	0
60	Fibrous roots	1	3	D	3
	Tomato, *Lycopersicon esculentum*, var. Rutgers (5 P.P.M.)				
90	Fibrous roots	1	16	C	1.5
	Sweet potato, *Ipomoea batatas*, var. Porto Rico (5 P.P.M.)				
120	Fibrous and edible roots	2	40	C	−0.5
	Wheat, *Triticum aestivum*, var. Atlas 66 (5 P.P.M.)				
70	Fibrous roots	1	24	C	1
	Potato, *Solanum tuberosum*, var. Katahdin (5 P.P.M.)				
30	Fibrous roots	1	10	D	5
120	Fibrous roots	2	2	D	2.5
	Potato	1	40	C	0.5
	Potato peel	1	0.7	D	0.0
	Corn, *Zea mays*, var. Golden Bantam (1 P.P.M.)				
70	Roots	1	20	C	0

[a] Plants grown in soil containing lindane. Soil concentration of lindane given in parentheses.
[b] At least two aliquots of each tissue extract chromatographed.
[c] D. Initial extract of tissue applied directly to spot line of chromatographic paper.
C. Extract cleaned up before chromatography.

and *n*-hexane–*N,N*-dimethylformamide (7) have been used to separate chlorinated insecticides from plant extractives. The paper chromatography method presented employed the latter solvent pair and effected such a cleanup during chromatography.

The essential feature of the modification was the use of thick chromatography paper, Whatman No. 17, which contained a considerable quantity of *N,N*-dimethylformamide (DMF) as the immobile phase. Samples of two slightly thicker papers (obtained through the courtesy of the Eaton-Dikeman Co., Mount Holly Springs, Pa.), E & D filter paper grades 652 and 901, each 0.05 inch thick, were also found satisfactory. All papers used for chromatography were thoroughly washed in distilled water and air-dried before use, to remove interfering chloride ions.

The size of the chromatographic sheet was 8.5 × 9.5 inches. A pencil line drawn 0.5 inch from the lower edge across the width of the paper marked off a strip by which the paper was conveniently handled by the fingers. A parallel line 2 inches from this line was the spot line. The solutions to be chromatographed were spotted at 1-inch intervals along this line. Care was taken to keep the diameter of the spots as small as possible.

After the extracts and appropriate standard solutions were spotted, the chromatographic sheet was dipped in a shallow pan, only once in a solution consisting of 50 ml. of *N,N*-dimethylformamide and of 50 ml. of diethyl ether. The paper was quickly submerged in the solution so that it was thoroughly and uniformly wetted from the top margin just to the spots. After removal from this solution the paper was hung for several minutes in a well-ventilated room to permit the ether to volatilize. At this stage there was an average of 0.22 gram of *N,N*-dimethylformamide per square inch on the paper. The paper was attached to a glass rod and the handling strip was cut off, then it was placed in a chromatographic chamber and the mobile solvent, *n*-hexane, allowed to ascend the paper. The paper was removed from the chromatographic chamber when the *n*-hexane front had ascended to about 15 cm. This occurred within a conveniently short time, 30 to 40 minutes, at 15° to 20° C.

After the front was marked, the paper was hung in the room until the odor of *n*-hexane was not noticeable. The paper (still damp because of the *N,N*-dimethylformamide) was then sprayed on both sides with Mitchell's silver nitrate reagent (*14*). After 5 minutes at room temperature, the paper was irradiated with ultraviolet light to develop the chromatogram as described by Mitchell.

The sensitivity of the chromatographic method described is considerably less than in chromatography utilizing Whatman No. 1 paper. For example, 5 to 10 γ of standard lindane chromatographed on thick paper will result in a just discernible spot after reduction of the silver nitrate indicator. As much as 3 mg. of lindane have been chromatographed on Whatman No. 17 paper without overloading the paper.

Representative R_f values obtained for the different compounds chromatographed on Whatman No. 17 paper, by the method described, are presented in Table I.

Paper Chromatography of Plant Extracts. Many of the compounds (Table I) were added to plant extracts and chromatographed. It is hoped that the chromatographic method may prove useful, at least as a supplementary method, in the detection of possible metabolites of the different pesticides, containing chlorine, applied to plants.

(Whether the cleanup procedure was applicable to extracts containing any pesticide except lindane was not determined.) Because the methods were used primarily to study lindane-treated plants, the following discussion refers to analysis of extracts from such plants only.

Spots representing various quantities of standard lindane were always included when plant extracts were chromatographed. The spotted quantity of pure lindane varied from 10 to 300 γ, the amount depending on the quantity of lindane in the treated plant extracts.

Preferably, the lindane standard was added to a control plant extract before spotting; otherwise, oil was added to lindane standards at the time of spotting. Mineral oil (Nujol), Wesson oil, or olive oil can be used. Oil was most easily applied by dissolving a calculated quantity in the standard solution, so that 0.15 ml. of oil was spotted with the desired quantity of lindane.

Aliquots of the two kinds of plant extracts (initial methylene chloride, final petroleum ether) were carefully evaporated (the last 5 to 10 ml. at room temperature) to final volumes of about 0.2 ml. contained in tapered centrifuge tubes. In each case, the total final volume was applied by repeated spotting to the spot line of the chromatographic paper.

Initial methylene chloride extracts representing up to 10 grams of wheat root tissue or 3 grams of fibrous root tissue of carrot were applied directly to the spot line of the chromatographic paper and successfully chromatographed. Other examples of this "tolerance" of natural plant constituents (oil, pigments) by the chromatographic analysis described are given in Table II.

Though all methods described were considered qualitative, recovery experiments indicated that no great loss of lindane occurred when extracts were cleaned before chromatography. In these recovery experiments, 100 γ of lindane was added to methylene chloride extracts of 50-gram samples of control, edible carrot or spinach leaf tissue. The extract was then cleaned up. The final 0.2-ml. volumes of these extracts are referred to as test extracts. Checks consisted of the addition of 100 γ of lindane to the final 0.2-ml. volumes of similarly cleaned extracts to which no lindane had been added prior to cleanup. Checks were spotted on the chromatography paper beside the spots representing the test extracts. Visual comparison of the final areas and of color intensities of the chromatographed lindane spots in six separate recovery experiments indicated that 75 to 100% of the lindane added prior to tissue extraction had been recovered and chromatographed. In addition, chromatographic analysis of *D* and *C* extracts of fibrous roots of carrot (Table II) did not indicate a loss greatly exceeding that found in the recovery experiments.

The effect of the cleanup procedure on substances other than lindane is not known. For this reason the cleanup procedure was considered only as an exploratory step in the investigation of such substances. Its use facilitated the chromatographic demonstration of metabolite substances in extracts which were too colored to permit successful direct chromatography. The direct and much simpler procedure of applying the initial extract to the chromatographic paper was used whenever possible.

Experimentation and Results

At different growth stages, treated and control plants were harvested and specific tissues were analyzed for lindane and lindane metabolites. The tops of the treated plants did not exhibit any acute toxicity symptoms during the period of plant growth. Many of the treated plants, however, did exhibit swelling of the root tips. Except for this typical symptom of root growth inhibition, the treated plants appeared only slightly less vigorous than the controls.

Presence of Lindane. A summary of data from experiments pertaining to the presence of lindane in various treated plant tissues is presented in Table II, which includes only data obtained from the analysis of treated plants. Without exception, an equal quantity of tissue from control plants was analyzed in the same manner and at the same time. Lindane was never found in any control extract.

The substance identified as lindane on the chromatograms of treated carrot extracts was demonstrated to be actually lindane. The methods used were comparison of R_f values in different solvent systems and the determination of *m*-dinitrobenzene by the chromatographic method of Sternburg and Kearns (*20*) following dechlorination and nitration of substances in the treated carrot extract.

Presence of Other Substances. Two other classes of substances, not shown in Table II, usually present in plant extracts, reacted with the silver nitrate indicator reagent. These extraneous reducing substances were present in apparently equal quantities in both the treated and the control extracts of a given plant tissue. These substances, sometimes visibly colored prior to application of the indicator spray, were present near the initial spot line (R_f about 0.17) or near the mobile solvent front line (R_f + 0.9).

In addition to lindane and the extraneous substances just noted, chromatographic analysis of extracts of tissue from treated carrot plants indicated the presence of a silver nitrate reacting substance not present in the extracts of control carrot plant tissue. This substance, furthermore, did not appear on the chromatograms of any of the other extracts, treated or control, of plants listed in Table II. Extracts of fibrous root, edible root, and stem-leaf tissue from treated carrot plants were shown to contain this unknown substance in various quantities. A summary of these data is presented in Table III.

Table III. Concentration of Unknown Substance in Treated Carrot Tissues

Lindane in Soil, P.P.M.	Tissue Analyzed	Relative Concn. of Unknown Substance[a]
	Fibrous roots	0.1–1.0
10	Edible portion	0.1
	Stem and leaves	0.1
	Fibrous roots	0.1
5	Edible portion	0.01
	Stem and leaves	0.0

[a] Highest concentration is represented by unity. If color reaction of chromatographed unknown substance is arbitrarily considered to equal that of lindane, highest concentration of unknown found would approximate 5 p.p.m.

Lindane and Unknown Substance in Tissue and Soil. Though the concentration of the unknown substance was low and somewhat variable, the highest concentration usually was found in the fibrous roots of the carrot (Table III). Similarly, the highest concentrations of lindane were also found in this root tissue, the main function of which was the uptake of water and nutrients from the soil. Such data clearly indicate the importance of analyzing this type of root tissue in metabolite studies involving soil-applied pesticides.

In preliminary experiments, various tissue regions of the treated edible carrot were extracted and analyzed separately. Results indicated that in the outer layers of the carrot there was a considerably higher concentration of lindane and of the unknown substance than in the core region. Of unknown significance at present is the finding that the species (carrot) which had "accumulated" the greatest quantity of lindane was the only species in which the unknown substance was detected.

Soil from around the roots of treated and control carrot plants was also analyzed for lindane and other substances. The spot representing the unknown substance found in the treated carrot tissue, however, was not detected on the chromatograms of the treated soil samples. If the unknown substance found in carrot tissue was present in the soil around the roots of such carrots, its concentration was only a very small percentage of that found in the fibrous roots.

Characterization of Unknown Substance Found in Treated Carrot. Various attempts to identify the un-

known substance in treated carrot yielded ambiguous results. Analyses of eluates of different R_f zones on chromatograms of control and treated carrot extracts indicated that the unknown substance contained organic chloride. The substance as chromatographed did not react with either bromophenol blue or ninhydrin spray indicator solutions. To date, the substance is best described by its behavior on the Whatman No. 17 paper chromatogram. As lindane always accompanied the unknown substance in treated carrot extracts, a ratio of the R_f values (R_f lindane to R_f unknown substance), in addition to the R_f values, was used as a measurement. The migration distances were measured to the top of the spots in contrast to the measurements for the R_f values given in Table I.

In plant extracts in 24 trials, the average R_f and ratio values found were lindane R_f 0.40 (0.30 to 0.52), unknown R_f 0.60 (0.43 to 0.77), and a ratio of R_f values of 0.66 (0.59 to 0.78).

Because 1,2,4-trichlorobenzene (TCB) and γ-pentachlorocyclohexene (PCCH) have been demonstrated to be lindane metabolites in houseflies (*5, 20*), the possibility that the unknown substance was one of these compounds was investigated. The R_f value for 1,2,4-trichlorobenzene chromatographed with control carrot extract was 0.77. The ratio R_f lindane to R_f 1,2,4-trichlorobenzene was 0.45. In view of these data it was considered that the unknown substance was not 1,2,4-trichlorobenzene. The value of the R_f of standard pentachlorocyclohexene, however, appeared similar enough to that of the unknown to warrant further tests. In 32 trials the R_f value of pentachlorocyclohexene chromatographed with control plant extract was 0.67 (0.47 to 0.79) and the R_f lindane to R_f pentachlorocyclohexene ratio was 0.61 (0.54 to 0.66).

The ease with which lindane can be separated from pentachlorocyclohexene is due to the type of reversed phase chromatography employed. These compounds are not easily separated by a chromatographic system in which oil is the immobile phase.

In view of the questionable reliability of using R_f data alone, an attempt was made to identify the unknown as pentachlorocyclohexene by the identification of 1-chloro-2,4-dinitrobenzene as the nitrated derivative (*20*) of the unknown substance after dechlorination by the method of Schechter and Hornstein (*19*). Attempts to do this with the unknown eluted from Whatman No. 17 paper chromatograms were unsuccessful. An attempt was then made to demonstrate the presence of pentachlorocyclohexene in treated carrot tissue extracts. This involved the dechlorination and nitration of substances in the initial plant extracts without prior chromatographic separation on Whatman No. 17 paper. The nitration products were then chromatographed (*20*). A positive test for 1-chloro-2,4-dinitrobenzene was obtained in these tests. Though the evidence is not conclusive, it indicates that the unknown substance may be pentachlorocyclohexene or a closely related compound. It is expected that greater quantities of plant material containing a higher concentration of the unknown substance will facilitate identification.

Chromatographic analysis involving the nitrated derivatives of substances originally present as chlorine-containing compounds in plant tissue extracts was not as satisfactory as that reported for such compounds from insect material (*20*). An indicator method to demonstrate a small quantity of 1-chloro-2,4-dinitrobenzene in the presence of a large quantity of *m*-dinitrobenzene was developed by employing a reaction suggested by Schechter, based on a colorimetric method (*18*). After migration of the nitrated derivatives, the chromatographic paper was first sprayed with pyridine, then heated for 5 minutes at 105°C. before being sprayed with 1% solution of sodium hydroxide in methanol. 1-Chloro-2,4-dinitrobenzene reacts and gives a purplish color. The nitrated derivative of lindane, *m*-dinitrobenzene, does not react under these conditions.

Discussion

Production of oil-soluble metabolites of lindane in houseflies appears to be variable (*5, 20*). Further study may indicate that varietal and environmental factors affect such metabolism in plants. Though pentachlorocyclohexene has a particularly disagreeable musty odor, correlation of off-flavor or off-odor with presence of pentachlorocyclohexene in plants has not been attempted.

The potato plant, noted for the development of off-flavors and off-odors, contained only a relatively low concentration of lindane. Moreover, no other substances were detected in potato tissue extracts by the methods used. In view of the difference in oil-soluble chlorinated compounds between carrots and potatoes, it would be desirable to know the nature and concentration of water-soluble metabolites of lindane in plants. Such compounds have been shown to be present in lindane-treated houseflies (*4*). Recent studies of natural "bitter principles" in carrots (*8*) and cucurbits (*16, 17*) may indicate that another source of off-flavors and off-odors may exist. It is not impossible that natural plant substances, affected only indirectly by the presence of lindane within the plant, may contribute to or be responsible for the production of off-flavors and off-odors.

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