Metabolism of Lindane to Organic-Soluble Products by Houseflies

Walter T. Reed[1] and Andrew J. Forgash

Three strains of houseflies (susceptible, moderately resistant, and highly resistant) were used to evaluate the metabolism of lindane (1,2,3,4,5,6-hexachlorocyclohexane) to organic-soluble products as a mechanism of resistance. Metabolites of treated flies were identified, utilizing mass spectrometry and gas-liquid chromatography. Greater amounts of 1,2,4- and 1,2,3-trichlorobenzene, 1,2,4,5- and 1,2,3,4-tetrachlorobenzene, pentachlorobenzene, and iso-pentachlorocyclohexene were formed by lindane-treated highly-resistant flies than were formed by the more susceptible strains. The highly-resistant strain also produced more 1,2,4-trichlorobenzene and 1,2,4,5-tetrachlorobenzene from γ-pentachlorocyclohexene and more pentachlorobenzene from iso-pentachlorocyclohexene, although 1,2,4- and 1,2,3-trichlorobenzene and 1,2,4,5- and 1,2,3,4-tetrachlorobenzene were detected in equal amounts among the strains following iso-pentachlorocyclohexene application. Following application of individual chlorinated benzenes, no further metabolism was detected. Since all metabolites were found in relatively small amounts, the principal metabolic pathway of lindane was probably not by way of the chlorinated benzenes and, along with penetration differences, the metabolism of lindane to organic-soluble products was a minor resistance mechanism.

Several factors on different chromosomes contribute to lindane (1,2,3,4,5,6-hexachlorocyclohexane)-resistance in houseflies (Oppenoorth and Nasrat, 1966). While some of these factors are not concerned with the reduction of the amount of toxicant in the body, the rapid metabolism to nontoxic products may be an important factor in some resistant strains.

Oppenoorth (1954) first noted that while both resistant and susceptible strains of flies metabolized lindane, the resistant strains did so more quickly. Using ^{14}C-labeled lindane, Bradbury and Standen (1955) determined that, in addition to decreased penetration in resistant fly strains, lindane was metabolized to water-soluble metabolites of unknown structure, although degradation did not proceed to the CO_2 stage. Pentachlorocyclohexene (PCCH) was reported as a major metabolite of flies (Sternburg and Kearns, 1956); however, isotope dilution techniques indicated that γ-PCCH (the basic monodehydrochlorination product of lindane) was formed in relatively small amounts (Bridges, 1959). A second isomer of PCCH, whose formation appears to be limited to metabolic means, was tentatively identified by Reed and Forgash (1968), although it was also found in relatively small amounts.

Several chlorine-substituted benzenes have been reported as organic-soluble metabolites of lindane. Trichlorobenzene (TCB) was reported present in relatively small amounts (Bradbury and Standen, 1958), and materials having gas-liquid chromatographic (glc) retention times identical to those of 1,2,4- and 1,2,3-TCB were found by Reed and Forgash (1969). The latter group also tentatively reported 1,2,4,5-tetrachlorobenzene (TetCB) as a metabolite and noted the presence of two compounds having retention times identical to those of 1,2,3,4-tetrachlorobenzene and pentachlorobenzene (PCB) following lindane application. Following the application of γ-PCCH, both 1,2,4-TCB and 1,2,4,5-tetrachlorobenzene were present while treatment with the second isomer of PCCH yielded materials with the retention times of 1,2,4- and 1,2,3-TCB, both isomers of tetrachlorobenzene, and pentachlorobenzene.

Considerably less is known about the metabolism of lindane to water-soluble products. Autoradiographical techniques indicated the presence of at least 11 different metabolites (Bradbury, 1957). Also, the six possible isomers of dichlorothiophenol were detected, leading to the hypothesis that lindane metabolism takes place *via* conjugation with glutathione (Bradbury and Standen, 1959). This was supported by Ishida and Dahm (1965) who demonstrated that the same *in vitro* enzyme system metabolized lindane and γ-PCCH in the presence of glutathione. Also, Clark *et al.* (1966) isolated a compound that appeared to be S(2,4-dichlorophenyl) glutathione, following lindane application. They did not find, however, any S(pentachlorocyclohexyl) glutathione, although Sims and Grover (1965) reported that α-3,4,5,6-tetrachlorocyclohexene and γ-PCCH were conjugated with glutathione *in vitro* by a rat-liver enzyme that was probably glutathione S-aryltransferase.

It is apparent that lindane is metabolized to several different compounds. The relationship of this metabolism to resistance is much less clear. It is the objective of this work to evaluate the metabolism of lindane to organic-soluble metabolites as a mechanism of resistance.

MATERIALS AND METHODS

Housefly Strains. Three housefly strains were used for this work. (1) The WHO/IN strain, a standard reference strain supplied by the World Health Organization; this was considered to be a susceptible strain, and was used as a basis for evaluating the resistance of the other strains. (2) The Rutgers G strain. This was a moderately resistant strain obtained in New Jersey, which had been in contact with lindane and reared in the laboratory in the absence of insecticidal pressure (Forgash and Hansens, 1967). (3) The Rutgers RL strain. This was a highly resistant strain selected from the Rutgers G strain by topical application with micronized lindane (99+ % purity) until no significant mortality could be obtained. Resistance was maintained by submerging the pupae in micronized lindane, thereby requiring the emerging flies to crawl through it. Unless otherwise noted, females were utilized in all experimentation.

The resistance levels of these strains were determined by both topical and injection methods, using the procedure of Forgash (1967), with the exception that dimethylsulfoxide (DMSO) was used as the injection solvent.

The resistance was measured in terms of LD_{50}'s (lethal dose for 50% of the population) and LD_{80}'s. The LD values and

Department of Entomology and Economic Zoology, Rutgers University, New Brunswick, N.J. 08903
[1] Present address: Biological Sciences Research Center, Shell Development Co., P. O. Box 4248, Modesto, Calif. 95352

their 95% confidence limits were determined by probit analysis.

Standard Reference Chemicals. Several standards were used in the identification of metabolites. They consisted of: 1,2,4- and 1,2,3-trichlorobenzene (Hooker Electrochemical Co., Niagara Falls, N.Y.); 1,2,4,5-tetrachlorobenzene (Matheson, Coleman, and Bell, East Rutherford, N.J.); 1,2,3,4-tetrachlorobenzene and pentachlorobenzene (K and K Laboratories, Inc., Plainview, N.Y.).

Since γ-PCCH was not commercially available, its laboratory synthesis was necessary. This was accomplished using the procedure of Nakajima *et al.* (1949) with several modifications. Ten grams of lindane were dissolved in 1.5 liter of acetone. This was mixed with 2.5 liters of 1/50 N NaOH for 20 min; the reaction was quenched with 0.1 N HCl. The solution was submitted to steam distillation, the organic solvent was removed by vacuum evaporation, and the solution was extracted with diethyl ether. The ether was removed by vacuum evaporation and the residue extracted with hexane. The final purification was accomplished by passing the solution through a 3-cm glass column packed with 100 g of silicic acid in a hexane slurry. The fraction that eluted between 235 and 290 ml contained only γ-PCCH and hexane.

Verification of the identity of the material produced in this manner involved four analytical techniques: glc, infrared spectrometry, mass spectrometry, and elemental analysis. The glc was a Research Specialties Model 600 with a ^{90}Sr electron capture detector. The column used was a 3.2-mm × 2.43-meter aluminum, hair-pin type, packed with 15% QF-1 on Chromosorb-P; the column temperature was 185° C and the nitrogen carrier gas flow rate was 45 ml per min. The infrared spectrum was determined with a Perkin-Elmer Model 21 infrared spectrometer. The sample was contained between 2 NaCl plates; the spectrum was recorded from 2 to 15.5 μ. The elemental analysis consisted of quantitative determinations of C, H, and Cl (Schwarzkoph Microanalytical Laboratory, Woodside, N.Y.). The mass spectra were obtained on a CEC 21-104 mass spectrometer (Gollob Analytical Service, Inc., Berkeley Heights, N.J.).

Metabolic Studies. Sublethal and lethal doses of lindane were applied topically (in 1 μl of acetone) and by injection (in 1/3 μl of DMSO) to 20 3- to 5-day-old flies, lightly anesthetized with CO_2. The application was made with a Dutky-Fest micro-applicator equipped with a 100-μl, Hamilton No. 710 microliter syringe and a Yale B-D 30-gauge needle. The flies were incubated in glass petri dishes, 10 flies per dish, for 1, 2, and 4 hr.

All three strains were treated with identical doses, incubated for the same period of time, and processed simultaneously. This was done so that the error in the determination of the differences in metabolism between strains would be kept at a minimum.

At the end of the incubation periods, the flies were anesthetized with CO_2, rinsed rapidly with acetone, and homogenized in a size C Potter-Elvehjem tissue grinder containing 4 ml of acetone and 0.3 g of Na_2SO_4. Particulate matter was removed by rapid centrifugation. The acetone was removed from the samples by vacuum evaporation and the residue was redissolved in hexane. The petri dishes were rinsed with hexane (external fly rinses and petri rinses were omitted for injection tests). Polar impurities were removed from the samples by means of Florisil columns as described by Moats (1963) with several modifications. Four-gram portions of Florisil (Florisil Co., Tallahasee, Fla.) were heated to 350° C for 18 hr, cooled, and 0.2 ml of distilled water were added. After 24 hr of equilibration, a hexane slurry of Florisil was packed into a 1-cm glass column plugged with glass wool. Each sample was eluted through the column in hexane, followed by an additional 75 ml of hexane. Sample volumes were then adjusted for optimum glc response.

A Micro-Tek model MT-220 (Tracor, Inc., Austin, Texas) equipped with ^{63}Ni electron capture detection was used for glc analysis. The column was an aluminum, hair-pin type (6.39-mm, × 1.22-meter) packed with 15% F-50 on Gas-chrom-Q; the argon-methane flow rate was 80 ml per min at an oven temperature of 180° C. The inlet temperature was 270° C; the detection temperature was 235° C. For quantitative purposes, the peak area was calculated by multiplying the peak height times the peak width at 1/2 the peak height.

This analytical procedure was repeated using sublethal doses of γ-PCCH applied topically and by injection, and sublethal injected doses of the remainder of the suspected metabolites. Proper controls and untreated fly homogenates with lindane or its derivatives added were used to insure that metabolites were not formed artificially.

Identification of Metabolites. When possible, the tentative identification of metabolites was accomplished by the comparison of their glc retention times to those of standards. In three instances, however, mass spectrometry was utilized to aid in the analysis. In order to obtain adequate amounts of pure material, large numbers of resistant flies (RL strain) were used. Approximately 1000 flies (mixed sex) were confined to lindane-coated beakers for 6 hr, at which time they were anesthetized with CO_2, rinsed with acetone, and homogenized in a Waring Blendor containing 400 ml of acetone. The particulate material was removed by filtration, and the solvent removed from the filtrate by vacuum evaporation. The resulting viscous material was extracted with hexane. Polar impurities were removed by passing the hexane layer through two successive Florisil columns. The first column consisted of a glass tube (3.5-cm diameter) with a sintered glass filter packed with 18 g of dried Florisil in a hexane slurry. The sample was eluted through in hexane, followed by an additional 200 ml of hexane. The volume of the eluant was reduced to 5 ml by vacuum evaporation. The second Florisil column consisted of 50 g of dried Florisil with 2% water added packed in a hexane slurry into a tube (3-cm diameter). The metabolites were eluted with hexane between 130 and 250 ml.

The final purification of the metabolites was accomplished by the use of preparative glc. A 4.8-mm × 2.43-meter hair-pin type, aluminum column packed with 15% QF-1 on Chromosorb-P was used at 180° C. The inlet temperature was 280° C and the detector temperature was 235° C. A stream-splitter with a splitting ratio of approximately 200 to 1 was employed to allow collection of the sample without first passing it through the detector. The solution containing the metabolite was reduced to 50 μl by vacuum evaporation and was injected onto the column. The material having the retention time of the desired metabolite was collected at the exit port of the stream-splitter using a 20-cm, open-end melting-point capillary cooled by an ice cube. A small sample of the collected material was removed with a fine wire and rinsed with hexane into a test tube. If an aliquot of this solution produced a single peak of the desired retention time, the capillary was cut to a length of 10 cm and sealed. For mass spectrographic analysis, the capillary was inserted into a copper tube connected to the inlet. Upon evacuation of the copper tube, the glass tube was broken, thereby introducing the sample into the system.

Radioisotope Techniques. Uniformly labeled ^{14}C lindane (34 mc/mM, International Chemical and Nuclear Corp., City of Industry, Calif.) was used in an attempt to demonstrate that radioactive material not recovered as lindane or organic-

soluble metabolite was present in the water-soluble form. Fifteen nanograms of the labeled lindane were applied topically in acetone to 10 resistant flies. At the end of 4 hr incubation, the flies were homogenized in acetone and Na_2SO_4. The homogenate was centrifuged, and the supernatant decanted and combined with an acetone rinsing of the petri dishes. An aliquot was placed in a stainless steel planchet along with 0.1 ml of 1% Triton X-100, evaporated to dryness, and counted in a SC90 Shielded Manual Sample Changer equipped with a thin-window (1.49 mg per cm^2) G.M. radiation detector. The particulate residue remaining after centrifugation was extracted with methanol in a Soxhlet extractor for 18 hr. An aliquot of this was also evaporated to dryness and counted. A separate sample of the centrifuged residue was liquified in concentrated aqua regia, evaporated to dryness, and counted.

RESULTS AND DISCUSSION

Resistance of Fly Strains. The standard reference strain used for this work possessed some degree of resistance to lindane, as was evident in the existence of a more lindane-susceptible strain, the Wilson strain (Forgash and Hansens, 1967). The WHO/IN strain was considered to be more appropriate for use as a susceptible reference strain because of its universal availability and its controlled rearing conditions. By topical application of lindane, its LD_{50} was 0.088 μg per fly ($P\{0.080 \leq LD_{50} \leq 0.097\} = 0.95$); the LD_{80} was 0.169 μg per fly ($P\{0.145 \leq LD_{80} \leq 0.195\} = 0.95$). The injected LD_{50} was 0.032 μg per fly ($P\{0.029 \leq LD_{50} \leq 0.036\} = 0.95$) and the LD_{80} was 0.058 μg per fly ($P\{0.051 \leq LD_{80} \leq 0.067\} = 0.95$). The Rutgers G strain, the moderately-resistant strain, was approximately ten-fold resistant and possessed a topical LD_{50} of 0.849 μg per fly ($P\{0.782 \leq LD_{50} \leq 0.920\} = 0.95$) and an LD_{80} of 1.43 μg per fly ($P\{1.71 \leq LD_{80} \leq 2.48\} = 0.95$). Injected, the LD_{50} was 0.303 μg per fly ($P\{0.27 \leq LD_{50} \leq 0.336\} = 0.95$) and the LD_{80} was 0.510 μg per fly ($P\{0.433 \leq LD_{80} \leq 0.619\} = 0.95$). The highly-resistant Rutgers RL strain possessed an immeasurably high topical resistance level; no significant mortality could be obtained following the application of lindane equal to 1000 $\times$ the WHO/IN LD_{50}. By injection it was approximately fifty-fold as tolerant as the WHO/IN strain; it possessed an LD_{50} of 1.48 μg per fly ($P\{1.35 \leq LD_{50} \leq 1.61\} = 0.95$), and an LD_{80} of 2.66 μg per fly ($P\{2.29 \leq LD_{80} \leq 3.10\} = 0.95$).

Identification of Metabolites. To aid in their identification, mass spectra were obtained on three of the metabolites recovered by preparative glc from lindane-treated flies. The material possessing the retention time of γ-PCCH also produced a mass spectrum identical to that of γ-PCCH. Another metabolite yielded a mass spectrum similar to that of γ-PCCH, in that all of the peaks produced by the metabolite were at the same locations as those produced by γ-PCCH; however, some of the peak height ratios differed. This led to its tentative identification as an isomer of PCCH (Reed and Forgash, 1968), referred to as Iso-PCCH in this report. The third metabolite produced a mass spectrum indicative of tetrachlorobenzene and a glc retention time comparable of that for the 1,2,4,5-isomer. The remaining organic-soluble lindane metabolites, tentatively identified according to glc retention times, were 1,2,4-TCB, 1,2,3-TCB, 1,2,3,4-TetCB, and PCB (Reed and Forgash, 1969).

Lindane Metabolism. Two dosages of lindane were applied to the three strains of flies by injection and topical application. The first, 4 μg per fly, lethal to all strains by injection, was sublethal to the RL strain and lethal to the WHO/IN and G strains by topical application. The other dosage, 0.015 μg per fly, was sublethal to all strains following both topical application and injection. Following the application of the lethal doses, a moribund state was quickly produced, but no mortality occurred in 4 hr, the longest incubation period.

Upon examination of the data obtained following topical application (Table I) and injection (Table II) of 4 μg per fly, several trends were evident. Although trace amounts of γ-PCCH (0.1% or an extremely small amount of material whose quantitation was obscured by impurities) were present in the lindane solution prior to application, it was apparent that γ-PCCH was formed by the fly, as it appeared in both internal homogenates and fly rinses in amounts greater than those present in the insecticide solution prior to application. The γ-PCCH found in the external rinse was probably either formed in the cuticle and leached out by the rinsing solvent, or was formed by nonenzymic means on the outside of the fly. The second alternative is favored, as γ-PCCH is the first intermediate in the basic dehydrochlorination of lindane and is formed commonly in very small quantities under a variety of nonbiological conditions. Also, no other metabolites were found in the external rinse. Thus, there appeared to be no relationship between the presence of γ-PCCH found on the outside of the cuticle and resistance following lindane application.

There seemed to be no relationship between the amount of γ-PCCH formed inside the fly and resistance following either the injection or topical application of 4 μg of lindane per fly. If anything, there seemed to be a negative relationship, with susceptibles containing more then resistants in terms of percentage of the homogenate and in terms of the percent of the total applied (Table I). This could have been because the susceptibles formed more γ-PCCH or because the highly-resistant strain metabolized the γ-PCCH at a faster rate, the latter of which is supported by γ-PCCH injection data to be presented later.

The formation of Iso-PCCH appeared to follow a different pattern than that observed for γ-PCCH. Following topical application of 4 μg of lindane per fly, the percentage of Iso-PCCH based on total material in the homogenate increased as incubation periods lengthened, with the highly-resistant RL strain possessing more than the other strains. Based on the total material applied, however, there was no increase with resistance. Inasmuch as a smaller amount of lindane was present inside the resistant flies, less was available for degradation; this may explain the lack of increase. Injection tests tended to support this hypothesis, as the RL's produced more Iso-PCCH in terms of both the amounts found in the homogenate and the total applied. The G strain was less proficient at producing Iso-PCCH following both injection and topical application than the more susceptible WHO/IN strain. One reason could have been that the G strain flies generally appeared to be smaller and less vigorous than the WHO/IN's. When metabolites were present in unexpectedly low quantities (*e.g.*, the injection of lindane and 4-hr incubation in Table II) the flies seemed smaller and less vigorous than normal.

The production of 1,2,4,5-TetCB appeared to follow a pattern similar to that for Iso-PCCH. Following the topical application of lindane (4 μg per fly), RL's formed more 1,2,4,5-TetCB in terms of material in the homogenate than did the more susceptible strains, but no differences were noted between strains when the recovery was in terms of the applied dose. When lindane was injected, however, the RL strain produced more 1,2,4,5-TetCB by both methods of calculation. Other chlorine-substituted benzenes (1,2,4-TCB, 1,2,3-TCB, 1,2,3,4-TetCB, and PCB) were found only in the RL strain

Table I. The Recovery of Organic-Soluble Products Following the Topical Application of Lindane (4 μg per fly)

Incubation Time (Hours)	Strain	Sample[a]	% Recovery 1,2,4-TCB	1,2,3-TCB	1,2,4,5-TetCB	1,2,3,4-TetCB	γ-PCCH	PCB[b]	Iso-PCCH[b]	Lindane	Total[c]
	WHO/IN	H	0	0	0	0	3.0	0	0.3	96.7	
		HT	0	0	0	0	0.3	0	T[d]	10.3	
		UA	0	0	0	0	0.2	0	0	73.4	83.5
1	G	H	0	0	0	0	1.6	0	0.2	98.2	
		HT	0	0	0	0	0.2	0	T	9.0	
		UA	0	0	0	0	0.2	0	0	63.1	71.7
	RL	H	0	0	0	0	1.3	0	0.8	98.0	
		HT	0	0	0	0	0.1	0	T	4.4	
		UA	0	0	0	0	0.2	0	0	63.8	68.2
	WHO/IN	H	0	0	0.2	0	1.9	0	0.7	97.2	
		HT	0	0	T	0	0.3	0	0.1	13.7	
		UA	0	0	0	0	0.2	0	0	66.9	81.6
2	G	H	0	0	T	0	1.4	0	0.1	98.4	
		HT	0	0	T	0	0.4	0	T	29.4	
		UA	0	0	0	0	0.2	0	0	49.6	81.4
	RL	H	0	0	0.8	0	1.3	0.5	2.1	95.4	
		HT	0	0	T	0	0.1	T	0.1	5.7	
		UA	0	0	0	0	0.2	0	0	66.0	73.2
	WHO/IN	H	0	0	0.4	0	3.0	0	0.9	95.7	
		HT	0	0	0.1	0	0.7	0	0.2	22.0	
		UA	0	0	0	0	0.5	0	0	57.8	81.6
4	G	H	0	0	0.1	0	1.8	0	0.6	97.4	
		HT	0	0	T	0	0.5	0	0.2	29.0	
		UA	0	0	0	0	0.4	0	0	60.8	91.6
	RL	H	1.1	T	1.6	0.6	1.7	0.4	3.3	91.2	
		HT	0.1	T	0.1	T	0.1	T	0.2	6.4	
		UA	0	0	0	0	T	0	0	50.9	58.7

[a] H = Recovery based on total amount of all metabolites and parent compound in the homogenate. HT = Recovery based on amount of parent compound applied. UA = Amount unabsorbed, *i.e.*, the percent of applied dose recovered in the cuticle rinse. [b] Quantitation based upon γ-PCCH standard curve. [c] Represents the sum of all materials found in the petri rinse, external rinse, and homogenate. [d] T = Trace. This represents less than 0.1% or a small amount of material that was unquantitatable because of impurities.

Table II. The Recovery of Organic-Soluble Products Following the Injection of Lindane (4 μg per fly)

Incubation Time (Hours)	Strain	Sample[a]	% Recovery 1,2,4-TCB	1,2,3-TCB	1,2,4,5-TetCB	1,2,3,4-TetCB	γ-PCCH	Iso-PCCH[b]	Lindane	Total	
	WHO/IN	H	0	0	0	0	1.4	0	0.2	98.4	
		HT	0	0	0	0	1.1	0	0.1	76.3	77.5
1	G	H	0	0	T	0	0.8	0	T	99.2	
		HT	0	0	T	0	0.7	0	T	93.1	93.8
	RL	H	0.5	0	0.3	0.1	1.0	T	1.0	97.1	
		HT	0.4	0	0.2	0.1	0.7	T	0.7	69.5	72.0
	WHO/IN	H	0	0	T	0	1.4	0	0.3	98.1	
		HT	0	0	T	0	1.4	0	0.3	88.1	89.9
2	G	H	0	0	T	0	1.1	0	0.1	98.9	
		HT	0	0	T	0	1.1	0	0.1	104.5	106.0
	RL	H	0.2	T	0.5	0.2	1.2	0.2	1.6	96.6	
		HT	0.2	T	0.3	0.1	1.0	0.1	1.2	75.4	78.1
	WHO/IN	H	0	0	0.1	0	1.4	0	0.1	98.4	
		HT	0	0	T	0	1.0	0	0.1	68.3	69.4
4	G	H	0	0	0.1	0	1.7	0	0.1	98.1	
		HT	0	0	T	0	1.4	0	0.1	83.3	84.9
	RL	H	0.3	T	0.4	0.2	1.4	T	0.8	96.8	
		HT	0.2	T	0.4	0.2	1.0	T	0.6	67.8	70.0

[a] For abbreviations, see Table I. [b] Quantitation based on γ-PCCH.

following both the injection and topical application of lindane, suggesting superior degradation capabilities in the RL's. Following topical application (Table I) there was an increase with time in the lindane recovered internally, as would be expected, since penetration of the insecticide through the cuticle was still taking place. The RL's contained less than the other strains, implying slower penetration and/or more rapid metabolism. An examination of the lindane recoveries in the external rinses did not support the hypothesis of decreased penetration, and injection data (Table II) did not support more rapid metabolism; however, it was believed that the relatively large amount of insecticide used would have masked a subtle difference in penetration and metabolism.

Smaller doses (sublethal) of lindane were used to determine

Table III. The Recovery of Lindane Following the Topical Application and Injection of Lindane (0.015 μg per fly)

Application	Incubation Time (Hours)	Strain	% Recovery		
			Homogenate[a]	Unabsorbed	Total[b]
Topical	1	WHO/IN	50.4	10.3	61.9
		G	51.0	13.2	67.6
		RL	21.7	30.5	56.4
	2	WHO/IN	57.4	4.3	61.7
		G	46.6	11.1	57.7
		RL	18.2	38.4	59.7
	4	WHO/IN	41.8	2.8	46.9
		G	44.6	5.1	52.5
		RL	23.0	6.9	33.1
Injection	1	WHO/IN	81.7	...	81.7
		G	88.6	...	88.6
		RL	50.6	...	50.6
	2	WHO/IN	65.3	...	65.3
		G	73.3	...	73.3
		RL	58.5	...	58.5
	4	WHO/IN	58.4	...	58.4
		G	59.8	...	59.8
		RL	29.3	...	29.3

[a] Recovery of lindane found in homogenate based on total applied dose.
[b] For topical application, includes sum of homogenate, external rinse, and petri rinse. For injection, it includes only homogenate.

if more rapid metabolism and slower penetration through the cuticle were factors in resistance. Topical and injection application of 0.015 μg per fly was sublethal to all strains at 24 hr and produced no visible toxic symptoms throughout incubation periods. The amount of lindane remaining on the outside of the cuticle (Table III) demonstrated that penetration occurred faster in the WHO/IN strain than in the G strain, and faster in the G's than in the RL's at all three incubation periods. Although the amount found inside differed little between the incubation times, the RL's always had considerably less (Table III). This was most likely because of more rapid metabolism and slower penetration in this strain. The concept of more rapid metabolism was supported by the injection data (Table III) which showed that the RL's contained less lindane in the homogenates than the other strains, and that the amount of lindane decreased with time in all three strains.

While no quantitatable amounts of metabolites were found at this low level of lindane injection, trace amounts of γ-PCCH occurred in all strains at the three incubation periods. Iso-PCCH was present in the three strains at the end of 4 hr incubation, and in the RL strain following 2 hr incubation. No metabolites were detected following the topical application of 0.015 μg of lindane per fly.

In order to demonstrate that the total recoveries determined by glc following lindane application were an accurate measurement of the organic-soluble materials present and that the remainder of the lindane derivatives were in the water-soluble fraction, ^{14}C-labeled lindane was used. Since the recovery of lindane was lowest at 4 hr following the application of 0.015 μg per RL strain fly, this dosage, topically applied, was used. The organic-soluble (acetone-soluble) fraction contained 34% of the applied radiation; this was in close agreement with the total recovery of 33.1% found using GLC (Table III). The Soxhlet extractor recovered only an additional 10% of the applied radiation from the residue, indicating that the majority of the remaining material was either not soluble in methanol or was too tightly bound to be extracted in this manner. The aqua regia digestion produced only 34% of the applied radiation; however, the large crystals formed in the planchet during evaporation caused sample absorption of ^{14}C β's, causing an artificially low count. The radioactive part of this residue seemed only sparingly soluble upon reextraction with acetone. These data were interpreted as confirmation for total recoveries of organic-soluble materials determined by glc. Also, a significant fraction of the material not recovered by this method was probably water-soluble metabolite(s).

γ-PCCH Metabolism. To determine whether γ-PCCH was a short-lived intermediate, 4-μg doses (sublethal to all strains) were administered topically and by injection; the results of the metabolism to organic-soluble products are presented in Tables IV and V, respectively.

The penetration of γ-PCCH, presented in terms of percent unabsorbed, followed a pattern similar to that observed for lindane (0.015 μg per fly), in that penetration occurred more rapidly in the WHO/IN's than in the G's and more rapidly in the G's than in the RL's, but it differed in that penetration in general was much faster.

The only organic-soluble metabolites found internally were 1,2,4-TCB and 1,2,4,5-TetCB, following the topical application or injection of γ-PCCH. No metabolites were present in the external rinses. While the percentage (based on the homogenate) of 1,2,4-TCB following the topical application indicated that increasing amounts were formed with time and that the RL strain formed more than the less-tolerant strains, the data based on the total material applied did not support this. Following injection however, the RL strain did produce more 1,2,4-TCB than the more susceptible strains using both methods of calculation, but there was no increase with time. Following γ-PCCH applications, TCB was never present in appreciable quantities (0.5 % of applied dose).

The production of 1,2,4,5-TetCB followed a pattern from that of 1,2,4-TCB. After topical application of γ-PCCH, the RL strain produced more than the other strains. While the percent (based on homogenate) became sizable after 4 hr (48.1%) the amount based on the total applied dose did not exceed 2.1%. Similar data were produced following injection, except that the percent recovery based on the material found in the homogenate was much lower, never exceeding 6%. In none of the tests did the WHO/IN strain differ appreciably from the G's.

It was apparent that γ-PCCH was metabolized quite rapidly, considering its high rate of disappearance. This was probably the reason for the high percentage of 1,2,4,5-TetCB in the homogenate after topical γ-PCCH application. The fact that the amount of 1,2,4,5-TetCB never exceeded 2.6% of the total material applied indicated its presence as a short-lived intermediate, or that it was the product of a side reaction and the principal metabolic pathway of γ-PCCH was through different, more polar, intermediates. Concerning the apparent disparity in metabolic rates following topical application and injection, evaporation of the relatively volatile compound from the cuticle could have accounted for the faster disappearance of the γ-PCCH following topical application.

Also, all strains seemed to be adversely affected for the first 1 or 2 hr following injection of this concentration of γ-PCCH, but not after topical application, and thus may not have been able to metabolize as rapidly. Possibly, the relatively large amount of DMSO introduced into the hemolymph also slowed down the metabolism for a short period of time. The rapid metabolism of γ-PCCH, especially in the RL's, may have accounted for the lack of correlation between the quantity of PCCH produced following lindane application and the degree of resistance.

Table IV. The Recovery of Organic-Soluble Products Following the Topical Application of γ-PCCH (4 μg per fly)

Incubation Time (Hours)	Strain	Sample[a]	% Recovery 1,2,4-TCB	1,2,4,5-TetCB	γ-PCCH	Total[b]
	WHO/IN	H	0.5	2.5	96.9	
		HT	0.2	0.8	29.7	
		UA	0	0	5.0	35.6
1	G	H	0.3	1.0	98.6	
		HT	0.1	0.4	34.0	
		UA	0	0	5.7	40.1
	RL	H	1.2	12.3	86.5	
		HT	0.2	2.1	14.7	
		UA	0	0	11.9	28.9
	WHO/IN	H	0.9	3.7	95.4	
		HT	0.1	0.6	15.1	
		UA	0	0	0.5	16.3
2	G	H	1.1	3.4	95.5	
		HT	0.2	0.6	18.1	
		UA	0	0	1.2	20.8
	RL	H	2.8	25.2	71.9	
		HT	0.2	1.8	5.0	
		UA	0	0	4.6	11.6
	WHO/IN	H	2.8	14.4	82.8	
		HT	0.2	0.8	4.7	
		UA	0	0	0.1	5.8
4	G	H	2.2	6.6	91.2	
		HT	0.3	6.9	12.4	
		UA	0	0	0.4	14.0
	RL	H	5.0	48.1	46.9	
		HT	0.2	2.1	2.0	
		UA	0	0	0.6	5.1

[a] For abbreviations, see Table I. [b] Represents the sum of all materials found in the petri rinse, external rinse, and homogenate.

Table V. The Recovery of Organic-Soluble Products Following the Injection of γ-PCCH (4 μ/g per fly)

Incubation Time (Hours)	Strain	Sample[a]	% Recovery 1,2,4-TCB	1,2,4,5-TetCB	γ-PCCH	Total
	WHO/IN	H	0.2	1.0	98.8	
		HT	0.2	0.8	76.3	77.2
1	G	H	0.2	0.4	99.4	
		HT	0.2	0.4	84.7	85.2
	RL	H	0.7	3.6	95.7	
		HT	0.5	2.5	66.1	69.1
	WHO/IN	H	0.3	0.7	99.0	
		HT	0.2	0.5	72.0	72.7
2	G	H	0.3	0.8	98.9	
		HT	0.3	0.7	91.7	92.6
	RL	H	0.5	3.2	96.2	
		HT	0.4	2.5	75.7	78.7
	WHO/IN	H	0.4	1.2	98.4	
		HT	0.2	0.7	54.9	55.8
4	G	H	0.4	0.9	98.7	
		HT	0.3	0.7	78.4	79.4
	RL	H	1.2	5.8	93.0	
		HT	0.5	2.3	37.0	39.8

[a] For abbreviations, see Table I.

Iso-PCCH Metabolism. Iso-PCCH was injected into the three strains of flies at a dosage of approximately 0.1 μg per fly. This low rate was used because of the extremely small amount of material available. The method described for recovering Iso-PCCH for mass-spectral analysis was utilized to obtain the material for these tests; a total of approximately 50 μg was amassed for the metabolism studies. To conserve the compound, the 1-hr incubation period was eliminated.

No γ-PCCH was detected following the injection of Iso-PCCH; however, 1,2,4-TCB, 1,2,3-TCB, 1,2,4,5-TetCB, 1,2,3,4-TetCB, and PCB were found (Table VI). There appeared to be no relationship between the formation of 1,2,4-TCB, 1,2,3-TCB, 1,2,4,5-TetCB, and 1,2,3,4-TetCB and resistance. More PCB was found in the highly-resistant RL's, however, in terms of both the material found in the homogenate and the total applied. The data also indicated the RL's metabolized Iso-PCCH faster than the more-susceptible strains, as less of the applied material was found in the homogenates of the RL's following Iso-PCCH application. Compared with γ-PCCH (Tables IV and V), Iso-PCCH was metabolized at a slower rate, although greater amounts of organic-soluble metabolites were formed.

The flies used for the 2-hr study were much larger than those in the 4-hr one. To determine if this could have been responsible for the drop in the amount of metabolites at 4-hr, especially PCB, two different weight groups of RL flies were compared, *i.e.*, flies weighing less than 17 mg, and ones weighing more than 19. They were injected with Iso-PCCH, incubated for 2 hr, and analyzed. The metabolic pattern of the larger flies was similar to that obtained previously (Table VI) at 2 hr, whereas, the results with the smaller flies resembled those obtained at 4 hr. The large flies produced approximately three times as much PCB as the smaller ones, although there was little difference among the remaining metabolites.

Chlorinated-Benzene Metabolism. To determine whether the chlorinated benzenes were short-lived intermediates, doses of these compounds previously found as metabolites were injected into flies which were then incubated for 1, 2, and 4 hr. All three strains were injected with 4-μg-per-fly doses

Table VI. The Recovery of Organic-Soluble Products Following the Injection of Isomeric PCCH (0.1 μg per fly)

Incubation Time (Hours)	Strain	Sample[a]	% Recovery 1,2,4-TCB	1,2,3-TCB	1,2,4,5-TetCB	1,2,3,4-TetCB	PCB	Iso-PCCH	Total
	WHO/IN	H	8.3	T	11.7	6.2	4.6	69.1	
		HT	7.0	T	9.8	5.2	3.9	58.1	84.0
2	G	H	7.2	T	7.5	5.5	4.2	75.7	
		HT	6.0	T	6.4	4.7	3.5	64.4	85.0
	RL	H	7.0	T	8.0	7.4	20.5	57.1	
		HT	5.6	T	6.4	5.9	16.3	45.4	79.6
	WHO/IN	H	4.5	T	5.7	2.7	3.4	83.7	
		HT	4.1	T	5.2	2.5	3.1	76.7	91.6
4	G	H	3.2	T	3.9	3.4	3.0	86.5	
		HT	2.9	T	3.5	3.1	2.7	78.5	90.8
	RL	H	4.2	T	8.5	6.2	7.8	73.4	
		HT	3.1	T	6.4	4.6	5.8	55.0	75.0

[a] For abbreviations, see Table I.

of 1,2,4-TCB, 1,2,4,5-TetCB, and 1,2,3,4-TetCB. The RL strain was also injected with 0.055-μg-per-fly doses of 1,2,4,5-TetCB and 0.3-μg-per-fly PCB. In no case was conversion to organic-soluble products detected. The recoveries of the applied materials over the three incubation periods were as follows: 1,2,4-TCB at 4 μg per fly, 51–64%; 1,2,4,5-TetCB at 4 μg per fly, 97–110%; 1,2,3,4-TetCB at 4 μg per fly, 82–102%; 1,2,4,5-TetCB at 0.055 μg per fly, 87–96%; and PCB at 0.3 μg per fly, 81–105%. In all but the 1,2,4-TCB application, this was considered 100% recovery with random variation. Concerning the loss of 1,2,4-TCB, this highly volatile compound codistilled with the hexane during the vacuum evaporation of the solvent in the preparation of the samples for glc; this was considered the reason for the low recoveries.

Failure to demonstrate metabolism of the chlorinated benzene metabolites contradicts the concept of their being short-lived intermediates at a steady state concentration during the metabolism of Iso-PCCH and especially γ-PCCH. One explanation for this disparity is differences in physical properties from the PCCH's, such as solubility, which may have prevented the chlorinated benzenes from reaching the metabolic site. Another explanation is that these benzene derivatives were the result of side reactions or were byproducts of a more important reaction metabolizing lindane and/or the PCCH's to more polar products not detected by the analytical methods employed. Upon reaching a certain concentration, the chlorinated benzenes would then have applied enough feed-back pressure to halt further formation and, not being metabolized, would have appeared to have reached a "steady-state" concentration. If γ-PCCH is an important intermediate, its metabolism most likely does not take place through TetCB or TCB. Bridges (1959) indicated that γ-PCCH was not a major metabolite, because prior treatment with γ-PCCH did not cause an accumulation of ^{14}C γ-PCCH following application of ^{14}C-lindane.

Relationship to Resistance. At least two different reaction mechanisms were definitely occurring in the metabolism of lindane to organic-soluble products, *i.e.*, dehydrochlorination and dehydrogenation, along with combinations of the two. Generally, the highly resistant strain possessed greater metabolic capabilities than either the moderately resistant strain or the standard susceptible strain, in terms of both the disappearance of lindane and the formation of metabolites. The differences were best observed following injection tests where the initial internal concentration of substrate was the same for all strains, so that the metabolic rates were not obscured by differences in penetration (Tables II, III, V, and VI). Selection with lindane amplified the ability of a strain to metabolize the insecticide. In several instances, the smaller, less vigorous but moderately resistant strain possessed lower metabolic capabilities than the larger, more vigorous standard susceptible strain; however, the highly-resistant strain, selected from the moderately resistant by topical application of lindane, had increased metabolic capabilities over both strains.

It was equally apparent, however, that the metabolism to organic-soluble products did not account for the resistance differences between the three strains. Following lindane application, none of the metabolites was present in appreciable amounts. Since none of the chlorinated benzenes appeared to be metabolized further, their role as intermediates seemed to be refuted. Along with penetration differences, the metabolism of lindane to organic-soluble metabolites might have been a factor in the resistance of the housefly strains used, but it was not the major factor. However, consistently lower lindane recoveries in the highly-resistant strain indicate that metabolism to undetected products, most likely water-soluble, may be an important resistance mechanism.

ACKNOWLEDGMENT

We thank Robert K. Cruver for his capable technical assistance and Fred Gollob for obtaining and interpreting the mass spectra.

LITERATURE CITED

Bradbury, F. R., *J. Sci. Food Agr.* **8**, 90 (1957).
Bradbury, F. R., Standen, H., *J. Sci. Food Agr.* **6**, 93 (1955).
Bradbury, F. R., Standen, H., *J. Sci. Food Agr.* **9**, 203 (1958).
Bradbury, F. R., Standen, H., *Nature* **183**, 933 (1959).
Bridges, R. G., *Nature* **184**, 1337 (1959).
Clark, A. G., Hitchcock, M., Smith, J. N., *Nature* **209**, 103 (1966).
Forgash, A. J., *J. Econ. Entomol.* **60**, 1596 (1967).
Forgash, A. J., Hansens, E. J., *J. Econ. Entomol.* **60**, 1244 (1967).
Ishida, M., Dahm, P. A., *J. Econ. Entomol.* **58**, 383 (1965).
Moats, W. A., *J. Ass. Offic. Agr. Chem.* **46**, 172 (1963).
Nakajima, M., Okulio, T., Katamura, Y., *Botyu-Kagaku* **14**, 10 (1949).
Oppenoorth, F. J., *Nature* **173**, 1000 (1954).
Oppenoorth, F. J., Nasrat, G. E., *Entomol. Exp. Appl.* **9**, 23 (1966).
Reed, W. T., Forgash, A. J., *Science* **160**, 1232 (1968).
Reed, W. T., Forgash, A. J., J. AGR. FOOD CHEM. **17**, 896 (1969).
Sims, P., Grover, P. L., *Biochem. J.* **95**, 156 (1965).
Sternburg, J., Kearns, C. W., *J. Econ. Entomol.* **149**, 548 (1956).

Received for review September 15, 1969. Accepted March 2, 1970. Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers—The State University, New Brunswick, N.J. Research supported by the T. J. Headlee Fellowship and U.S. Public Health Service Grant No. CC-00102.