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Effects of Dietary Hexachlorobenzene on in Vivo Biotransformation, Residue Deposition, and Elimination of Certain Xenobiotics by Rats

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The effect of hexachlorobenzene (HCB) pretreatment of male albino rats on the in vivo biotransformation, residue deposition, and elimination of radiocarbon-labeled aldrin, 1-naphthol, DDT, HCB, and mirex was investigated. In rats pretreated with 250 ppm of dietary HCB for 4 weeks, the percentage of ^{14}C associated with the more polar urinary metabolites was either increased (aldrin; mirex), decreased (HCB), or unchanged (DDT; 1-naphthol). There was no evidence of qualitative changes in biotransformation of any of the five ^{14}C-labeled test compounds that could be attributed to HCB pretreatment. Rats fed the HCB diet and subsequently treated with [^{14}C]aldrin retained less radiocarbon residues in adipose and kidney tissue than comparably treated control rats retained, whereas rats fed the HCB diet and subsequently treated with [^{14}C]DDT or [^{14}C]mirex retained more radiocarbon residues in their adipose tissue than control rats retained. There were no differences due to HCB pretreatment in tissue radiocarbon residues of rats treated with [^{14}C]HCB or [^{14}C]-1-naphthol. Excretion rates of radiocarbon in HCB-diet rats were enhanced after treatment with each of the radiocarbon-labeled compounds. Rats fed the HCB diet gained more body weight than controls during the 4-week pretreatment period but subsequently lost more weight than controls during the 2 weeks posttreatment.

Since 1945, hexachlorobenzene (HCB) has been used worldwide as a fungicidal seed treatment on wheat and other small grains. In addition to its uses in agriculture, HCB is used as an additive for certain military pyrotechnic compositions, as a porosity controller in the manufacture of electrodes, as a chemical intermediate in dye manufacture and organic synthesis, and as a wood preservative (Mumma and Lawless, 1975). The primary commercial use of HCB in 1974 was as a peptizing agent in the manufacture of nitroso- and styrene-type rubber automobile tires. HCB is produced as a byproduct of industrial chlorination processes (including the manufacture of carbon tetrachloride, perchloroethylene, and trichloroethylene) and the production of chlorine by the electrolysis of brine (Mumma and Lawless, 1975).

HCB is a relatively stable, widespread environmental contaminant (U.S. EPA, 1973; National Academy of Sciences, 1975), and its misuse has resulted in serious human health problems as well as a major incidence of livestock contamination (U.S. EPA, 1973). In the early 1960s, an outbreak of cutaneous porphyria affecting over 5000 persons in Turkey resulted from consumption of bread prepared from HCB-treated seed (Schmid, 1960). In late 1972, HCB residues resulted in the quarantine of over 20 000 cattle in Louisiana. Apparently HCB spilled or blown from open trucks hauling industrial waste from a perchloroethylene plant to a dump was the primary source of the HCB contamination (U.S. EPA, 1973; Himbry et al., 1975).

Because HCB is an environmental contaminant of significance and is a potent inducer of the hepatic mixed function oxidase enzyme systems, (Rajamanickam and Padmanaban, 1974; Stonard and Nenov, 1974; Turner and Green, 1974; Koss and Koransky, 1975; Mehendale et al., 1975; Stonard, 1975; Iverson, 1976), we need to obtain information regarding its interactions with various components of the environment. The current study was undertaken to evaluate certain aspects of the interactions of HCB with mammals: specifically, its effects on in vivo biotransformation, residue deposition, and excretion of certain other chemicals by the laboratory rat. The chemicals selected for this study, aldrin, DDT, HCB, mirex, and 1-naphthol, represent a range of polarity and lipid solubilities and have functional groups that are subject to various biotransformation pathways (Gunther et al., 1968; Menzie, 1969, 1974).

MATERIALS AND METHODS

Chemicals. Unlabeled HCB (>99% purity) was obtained from Chem Service, Inc., West Chester, PA. Uniformly labeled [^{14}C]HCB (99% radiochemical purity,

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4.61 mCi/mmol) and uniformly labeled [^{14}C]mirex (dodecachlorooctahydro-1,3,4-metheno-1*H*-cyclobuta[*cd*]-pentalene; 99% radiochemical purity, 6.34 mCi/mmol) were obtained from Mallinckrodt Chemical Works, St. Louis, MO. Uniformly ring labeled [^{14}C]-*p,p'*-DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; 98% radiochemical purity, 1.92 mCi/mmol) was obtained from California Bionuclear Corp., Sun Valley, CA. [^{14}C]-1-Naphthol (98% radiochemical purity, 20.8 mCi/mmol) and uniformly labeled [^{14}C]aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-*endo*,*exo*-5,8-dimethanonaphthalene; 99% radiochemical purity, 79 mCi/mmol) were obtained from Amersham/Searle, Arlington Heights, IL. Each of the radiolabeled compounds gave a single spot upon analysis by thin-layer chromatography (TLC) in an appropriate solvent system, with detection by radioautography. Unlabeled chemicals used to dilute ^{14}C-labeled compounds and to identify metabolites were of the highest purity available from chemical supply houses. Glass-distilled solvents were used for extraction and for TLC.

Preparation of Feed. Pellets of Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) were ground in a small feed mill and then transferred to a large stainless steel food mixer in 10-kg batches. One liter of distilled water was added to the mixer to facilitate repelleting, and the mixing process was continued until the mixture appeared homogeneous. As the mixing continued, 100 mL of benzene containing 2.5 g of HCB was added slowly. Mixing was continued for 20–30 min to assure evaporation of the solvent. The feed was then repelleted in a Laboratory pelleter (Model CL Laboratory Pellet mill, California Pellet Mill Co., San Francisco, CA) and stored at <0 °C until fed. Control feed was prepared in the same manner as the treated feed but with only benzene added. In subsequent experiments (Clark and Elissalde, 1978) in which feed was prepared as described here, we were unable to detect benzene at the detection limit of 1 ppm.

Animals and Treatment. One-hundred male albino Sprague–Dawley outbred rats (200–250 g each; Timco Breeding Laboratories, Houston, TX) were randomly divided into groups of five rats per cage and were housed in molded plastic animal cages (Health-Guard System Research Equipment Co., Bryan, TX) containing steam-cleaned hardwood chip litter (Ab-sorb-dri, Garfield, NJ). Each group of five rats was housed together throughout the study. Cages were cleaned and litter was changed weekly. Water was provided ad libitum by an automatic watering system, room temperature was maintained at ~23 °C, and relative humidity was held at ~50% throughout the study.

The study consisted of 5 experiments; in each, 20 rats and 2 diets (control diet and HCB diet; 2 cages of 5 rats per cage per diet) were tested. All five experiments followed a similar design, but each was distinguished by the ^{14}C-labeled test compound investigated. The experiments and test compounds are designated as follows: experiment I, [^{14}C]aldrin; experiment II, [^{14}C]-1-naphthol; experiment III, [^{14}C]DDT; experiment IV, [^{14}C]HCB; experiment V, [^{14}C]mirex.

Within each experiment there were three time designations; the acclimation period, phase I, and phase II. The acclimation period was the 1–3-week period before phase I during which the rats were allowed to adjust to laboratory conditions; they were fed untreated feed and housed in the plastic cages described above. Phase I was the 4-week feeding period designed to effect microsomal induction in HCB-diet rats prior to treatment with the radiolabeled compounds. Control rats were fed the control diet during phase I and were maintained in a room separate from HCB-diet rats. Phase II was the period (2 weeks or less) that began immediately after phase I and upon treatment with the ^{14}C-labeled compounds. During phase II, urine and feces samples were collected periodically, and the rats continued to receive either the HCB diet or the control diet, as appropriate, until the experiment was ended. All rats were weighed at the beginning of phase I and at the beginning and end of phase II.

At the beginning of phase II, each rat from both diet groups was treated via a single intraperitoneal (ip) injection with 5 mg/kg of the appropriate ^{14}C-labeled test compound in ~0.5 mL of peanut oil; the ^{14}C content was equivalent to ~1.0 μCi. After injection of the radiochemical, the rats were transferred to stainless steel metabolism cages (Hoeltge Inc., Cincinnati, OH) designed for separate collection of urine and feces. The HCB diet or control diet (reground to minimize "carry-back" into the cages) and water were provided ad libitum.

Sample Collection (Phase II). Total urine and feces were collected from each cage at 3, 6, 12, and 24 h posttreatment and then at 24-h intervals until radioactivity was no longer detectable, or up to 14 days. In an effort to minimize microbial activity, each urine sample was collected in a flask containing 5 mL of 0.015% formaldehyde. The urine and feces samples were stored frozen until analysis. Phase II lasted for 14 days with each of the ^{14}C-labeled test compounds, with the exception of experiment II with [^{14}C]-1-naphthol, which, due to the rapid excretion of radioactivity, was ended 72 h posttreatment. After the final urine and feces collection of each experiment, the rats were individually weighed and then killed by asphyxiation with carbon dioxide. Brain, liver, kidney, testes, muscle, and adipose tissue from each animal were collected, placed in individual plastic bags, and stored frozen for subsequent radiocarbon assay.

Analytical Procedures. A Beckman LS-235 spectrometer (Beckman Instruments, Inc., Fullerton, CA) equipped for external standardization was used for liquid scintillation counting (LSC). A toluene-based scintillation cocktail was used in which 2-methoxyethanol was incorporated to improve miscibility with aqueous samples. All LSC measurements were corrected for background, quench, and instrument efficiency.

From each urine sample, duplicate 0.2-mL aliquots were added to vials containing 20 mL of scintillation cocktail. The vials were held in the dark for 24 h for depletion of photoluminescence, and then the radioactivity was quantitated by LSC. Air-dried samples of adipose tissue (~0.05 g), nonfatty tissue (~0.5 g), or feces (~0.5 g) were combusted under 1 atm of oxygen in a modified Packard Tri-Carb sample oxidizer (Packard Instrument Co., Downers Grove, IL). Combustion gases were bubbled through a carbon dioxide trapping solution (equal volumes of 2-aminoethanol and 2-methoxyethanol), and the trapped radiocarbon was then quantitated by LSC. Data were corrected for combustion efficiency and quench.

Urinary metabolites were identified by cochromatography on TLC of urine extracts with authentic potential metabolites obtained commercially. For each diet group (HCB or control) within each experiment, usually only the urine sample containing the highest level of radiocarbon activity was analyzed for metabolites; but in some experiments, urinary radiocarbon excretion was high enough to permit analysis of additional samples also.

The urine samples (5–40 mL) were acidified to pH 1 with HCl and then extracted 4 times with equal volumes of ethyl acetate. Aliquots of the combined ethyl acetate

Table I. Body Weight[a] of Male Rats Fed the HCB Diet or Control Diet[b] and Treated with either [^{14}C]Aldrin, [^{14}C]-1-Naphthol, [^{14}C]-*p,p'*-DDT, [^{14}C]HCB, or [^{14}C]Mirex[c]

	body wt, g					
	initial[d]		phase I[e]		final[f]	
experiment	control diet	HCB diet	control diet	HCB diet	control diet	HCB diet
I ([^{14}C]aldrin)	248 ± 18.3	236 ± 10.0	351 ± 21.9	330 ± 23.1	309 ± 24.5	320 ± 10.5[g]
II ([^{14}C]-1-naphthol)	317 ± 29.8	305 ± 22.2	366 ± 18.3	365 ± 22.6	359 ± 14.9	335 ± 16.0[i]
III ([^{14}C]-*p,p'*-DDT)	328 ± 18.9	325 ± 12.7	432 ± 21.0	424 ± 21.5	413 ± 21.6	363 ± 22.2[i]
IV ([^{14}C]HCB)	214 ± 29.8	190 ± 14.9[j]	327 ± 25.3	359 ± 26.4[j]	342 ± 26.8	290 ± 44.4[i]
V ([^{14}C]mirex)	274 ± 8.3	281 ± 21.8	386 ± 17.5	416 ± 38.7[j]	383 ± 33.1	350 ± 27.1[h,i]

[a] Mean (grams) ± SD, $N = 10$ (five rats per each of two cages). [b] HCB diet = 250 ppm of HCB added; control diet = no HCB added. Rats were maintained on the HCB diet or control diet for 4 weeks pretreatment and for 2 weeks posttreatment. [c] Each rat was treated by ip injection of the indicated radiolabeled compound (5 mg/kg; radiocarbon activity, ~1.0 μCi in 0.5 mL of corn oil). [d] Rats weighed at the start of the 4-week pretreatment period (phase I). [e] Rats weighed at the end of phase I/beginning of phase II. [f] Rats weighed at the end of phase II (phase II lasted 2 weeks for experiments I and III-V and 72 h for experiment II). [g] $N = 5$; 5 of 10 rats died during phase II. [h] $N = 8$; 2 of 10 rats died during phase II. [i] Less than the control at $\alpha \leq 0.05$. [j] Greater than the control at $\alpha \leq 0.05$.

extracts and of the aqueous phase were subjected to LSC to determine partitioning characteristics; then the organic phase was dried over anhydrous sodium sulfate, concentrated, and applied to TLC.

Silica gel F-254 precoated chromatoplates (gel thickness 0.25 mm; 20 × 20 cm glass plates; Merck Ag, Darmstadt, Germany) were used for TLC analyses. Radiolabeled compounds were visualized by radioautography (Mangold, 1969). The developed thin-layer plates were exposed to a no-screen type medical X-ray film (Eastman Kodak Co., Rochester, NY) for 3–30 days, depending upon the levels of radiocarbon present. After development of the film and location of the radioactive areas on the plate, the corresponding gel was scraped into scintillation vials for direct LSC quantitation of individual metabolites. For metabolite cochromatography studies the appropriate authentic standard was mixed with the extracted radioactive metabolite and the plate was developed single dimensionally in an appropriate solvent system. Unlabeled standards were located by viewing the plate under shortwave UV light or, for certain chlorinated hydrocarbons, by spraying the plates with a diphenylamine reagent, followed by exposure to shortwave UV light (Ivie and Casida, 1971). In tests to confirm the identity of metabolites that cochromatographed with authentic standards in the primary solvent system, the radioactive regions were scraped, extracted from the silica gel with ethyl acetate, and then rechromatographed in a second solvent system. Identical chromatographic behavior of the ^{14}C-labeled compound and the authentic standard in each of the solvent systems constituted tentative metabolite characterization. We did not attempt to isolate or characterize feces or tissue metabolites. The following solvent systems were used for TLC of the various metabolite mixtures: A, benzene–hexane, 5:2; B, 1% acetone in *n*-heptane; C, isooctane; D, benzene–dioxane–acetic acid, 220:62:3; E, benzene saturated with formic acid; F, heptane–chloroform–methanol, 9:4:1; G, chloroform–methanol–acetic acid, 8:2:1; H, *n*-butanol–ethanol–water, 17:3:20.

Statistical Analyses. Cumulative excretion data were analyzed by three-way analysis of variance (ANOVA). Liver weight and tissue residue data were analyzed by one-way ANOVA to determine the effect of HCB diet in each of the five experiments. In each analysis, the *F* test was used to determine statistical significance. For the present study, $\alpha \leq 0.05$ was established as the minimum level that would be considered significant.

RESULTS AND DISCUSSION

Hepatic Microsomal Enzyme Induction. At the end of the study, the livers of the HCB-diet rats were significantly larger ($\alpha < 0.01$) than those of the control-diet rats (7.13 ± 1.08% of body weight for HCB-diet rats and 3.77 ± 0.50 for control-diet rats). Increased liver weight is associated with hepatic microsomal enzyme induction (Parke, 1968) and has previously been observed in rats treated with HCB (Rajamanickam and Padmanaban, 1974). Additional evidence of hepatic microsomal enzyme induction was provided by a single experiment in which pooled liver samples from rats taken from the present study were used. In addition to larger livers, the HCB-diet rats had more microsomal protein (58 mg of protein/g of liver) than the control-diet rats (32 mg/g) had, and more cytochrome P-450 (2.01 nM/mg microsomal protein) than the control rats (0.9 nM/mg microsomal protein) had. Electron microscopic examination of liver samples from these rats confirmed proliferation of the smooth endoplasmic reticulum in the HCB-diet rats (Mollenhauer et al., 1975).

Body Weight. With exception of experiment IV in which the initial body weight (body weight at start of phase I) of the HCB-diet rats was less than their respective controls ($\alpha < 0.05$; Table I) there were no within-experiment differences in initial body weight between rats fed the HCB diet or control diet. Analysis of variance showed that the source of ~94% of the variation in initial body weight was due to differences in weight between experiments. About 4% of the total variation was due to within-experiment variation and was associated primarily with the differences observed in experiment IV.

At the end of phase I (and beginning of phase II), between-experiment variation was still the primary source (87%) of differences in body weight. The HCB-diet rats in experiments IV and V weighed significantly more ($\alpha \leq 0.05$) than their respective controls at the end of phase I. Analysis of data from all five experiments showed that, although there were some differences between the experiments, rats fed the HCB diet gained significantly more ($\alpha \leq 0.01$) weight than controls during the 4 weeks of phase I. Other investigators have reported either no effect (Grant, et al., 1974; Turner and Green, 1974; Mehendale et al., 1975) or decreased weight gain (Campbell, 1963) in male rats fed diets containing HCB.

Significant changes in body weight were observed during the 2-week postinjection period (phase II). Weight loss was observed both among controls and among HCB-diet rats; however, the HCB-diet rats lost significantly more ($\alpha < 0.01$) body weight than controls during phase II. During this period, the HCB-diet rats and control-diet rats lost 12.7 ± 9.16 and 2.8 ± 8.67% of their body weight, respectively. This pattern was consistent throughout all five experiments. Analysis of variance of these data in-

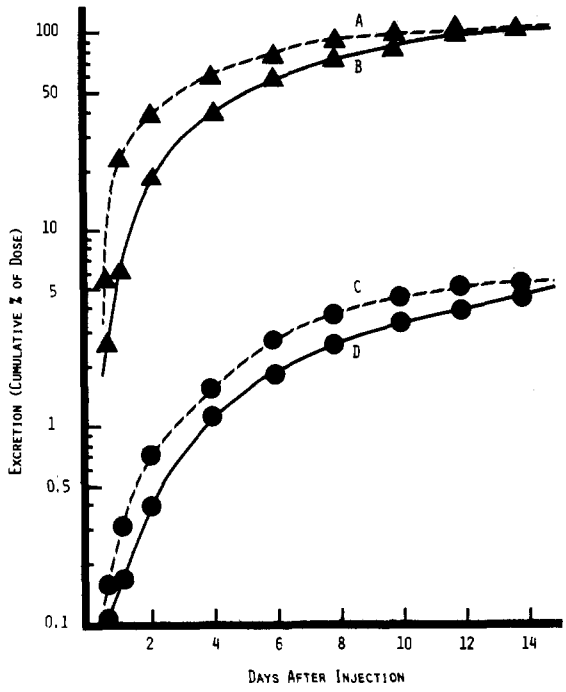

Figure 1. Effect of the HCB diet on the excretion of ^{14}C by male rats treated with [^{14}C]aldrin. (A) ^{14}C in the feces of HCB-diet rats. (B) ^{14}C in the feces of control-diet rats. (C) ^{14}C in the urine of HCB-diet rats. (D) ^{14}C in the urine of control-diet rats.

dicated that the source of ~75% of the variation was associated with HCB diet and was highly significant ($\alpha < 0.01$). Variation associated with the injected compound was ~2.5% of the total and was not significant ($\alpha > 0.05$). The "cage" effect was not significant ($\alpha > 0.05$), and a significant ($\alpha < 0.05$) diet X treatment interaction was detected.

The purpose of this study was to investigate the effect of the HCB diet on the pharmacodynamics of five exogenous chemicals. Thus, body weights were recorded primarily for calculation of liver weight:body weight ratios. The dramatic changes observed during phase II were not anticipated; thus, the study was not designed to answer the questions produced by these observations.

Apparently some factor associated with living in the metabolism cages resulted in weight loss since weight loss was observed in controls as well as HCB-diet rats and since rats in both diet groups gained weight during phase I. We do not know whether weight loss during phase II was due to anorexia and/or adypsia, to inability of the rats to locate food and water, or to some other factors. However, it is clear that weight loss asssociated with living in the metabolism cages was increased by the HCB diet.

Excretion, Residue Deposition, and Biotransformation. *[^{14}C]Aldrin.* The effects of the HCB diet on the distribution of radiocarbon residues in tissues of the rats given ip injections of 5 mg/kg [^{14}C]aldrin are shown in Table II. Rats fed the HCB diet retained significantly less radiocarbon in their adipose tissue and kidneys than did rats fed the control diet, but differences due to HCB diet were not significant in ^{14}C-labeled residue levels in brain, liver, muscle, or testes.

Total ^{14}C excretion in urine during the 14-day collection period was 5.1% for the HCB-diet rats and 4.1% for the control-diet rats (Figure 1). More than 90% of the total injected ^{14}C was eliminated in feces by the rats, regardless of diet. During the 14-day period after [^{14}C]aldrin treatment, 5 of the 10 HCB-diet rats died. The calculation of excretion data was appropriately adjusted for the rats to account for the reduced number of animals. In the group

Table II. Radiocarbon Residues in Tissues of Male Rats Fed the HCB Diet or Control Diet[a] and Treated with either [^{14}C]Aldrin, [^{14}C]-*p,p'*-DDT, [^{14}C]HCB, [^{14}C]Mirex,[b] or [^{14}C]-1-Naphthol[c]

	residues, ppm							
	[^{14}C]aldrin		[^{14}C]-*p,p'*-DDT		[^{14}C]HCB		[^{14}C]mirex	
tissue	control diet	HCB diet[d]	control diet	HCB diet	control diet	HCB diet	control diet	HCB diet[e]
brain	0.35 ± 0.16	0.31 ± 0.27	0.25 ± 0.14	0.17 ± 0.09	1.35 ± 0.54	2.35 ± 3.57	0.64 ± 0.51	0.76 ± 0.57
adipose	2.80 ± 2.02	0.43 ± 0.39[f]	6.91 ± 7.84	17.5 ± 14.9	66.2 ± 23.4	49.7 ± 20.2	8.19 ± 8.22	108 ± 157[e,g]
kidney	6.78 ± 4.21	1.26 ± 1.25[f]	0.26 ± 0.21	0.35 ± 0.21	2.02 ± 0.51	2.33 ± 2.37	0.92 ± 0.73	1.22 ± 1.17
liver	2.40 ± 1.27	1.57 ± 0.56	0.63 ± 0.38	1.47 ± 0.76[g]	2.15 ± 0.63	4.82 ± 5.60	2.50 ± 2.91	7.51 ± 10.18
muscle	1.79 ± 2.47	2.01 ± 1.72	0.61 ± 0.51	0.94 ± 1.08	1.34 ± 1.55	1.58 ± 1.46	0.69 ± 0.16	1.01 ± 0.95
testes	1.82 ± 4.43	0.57 ± 0.44	0.99 ± 2.40	0.51 ± 1.08	1.04 ± 0.63	1.44 ± 2.09	0.42 ± 0.16	1.43 ± 2.37

[a] See footnote *b*, Table I. [b] Each rat was treated by ip injection of the appropriate radiolabeled compound (either [^{14}C]aldrin, [^{14}C]-*p,p'*-DDT, [^{14}C]HCB, [^{14}C]mirex, or [^{14}C]-1-naphthol; 5 mg/kg; radiocarbon activity, ~1.0 μCi in 0.5 mL of corn oil). Tissue samples were collected at 2 weeks posttreatment. Residues are reported in ppm equivalents (mean ± SD) of the injected compound. Tissues collected from 10 rats. [c] Rats treated with [^{14}C]-1-naphthol were sacrificed 72 h posttreatment, and no ^{14}C-labeled residue was detected in any tissues of rats from either HCB-diet or control-diet rats. [d] Tissues collected from five rats (five rats died during phase II). [e] Tissues collected from eight rats (two died during phase II). [f] Differs from the control at $\alpha < 0.01$. [g] Differs from the control at $\alpha < 0.05$.

Table III. Distribution of Radioactive Metabolites in Urine[a] of Male Rats Fed the HCB Diet or Control Diet[b] and Treated with [^{14}C]Aldrin[c]

metabolite	R_f[d]	radioactivity, % control diet	radioactivity, % HCB diet
water soluble[e]		0	0
1	origin	3.6	27.3
2	0.09	7.5	13.9
3	0.18	26.1	35.7
4	0.30	32.5	3.6
5	0.50	21.0	6.6
6 (dieldrin)[f]	0.61	8.5	11.1
7 (aldrin)[f]	0.70	0.8	1.8

[a] Pooled samples from two cages of HCB-diet rats or control-diet rats; collected on the 5th day posttreatment. [b] See footnote *b*, Table I. [c] Each rat was treated by ip injection of [^{14}C]aldrin (5 mg/kg; radiocarbon activity, ~1.0 μCi in ~0.5 mL of corn oil). [d] Solvent system A = benzene–hexane (5:2). [e] Acidified urine sample was extracted with ethyl acetate. Radioactivity not extracted is reported as "water-soluble metabolites", percentage of total radioactivity in the urine sample. [f] Chromatographic behavior identical with that of authentic compounds.

treated with [^{14}C]aldrin and fed the control diet, none of the rats died.

The reduction of residue levels in the HCB-diet rats was probably the result of microsomal enzyme induction by HCB which led to an increased rate of biotransformation and excretion of aldrin and its metabolites. Interactions in which one chemical induces the microsomal enzyme system that results in reduction of residues of another compound are well-known and were first reported by Street et al. (1966a,b). Marked depression of dieldrin storage in the adipose tissue of young female rats fed dietary HCB has previously been reported; this depression was attributed to enhanced elimination of the insecticide and was directly related to the concentration of HCB in the diet (Avrahami and Gernert, 1972).

In the present study, elimination of over 90% of the radioactivity through the feces after treatment with the highly lipophilic [^{14}C]aldrin suggests that biliary excretion was involved to a large extent in the elimination of aldrin or its metabolites from the body. These data are in agreement with those of Ludwig et al. (1964), who reported that [^{14}C]aldrin fed to rats is excreted in the feces and urine (90% and 10% of the dose, respectively, in 12 weeks) as a mixture of hydrophilic metabolites together with small amounts of unchanged aldrin and its epoxide, dieldrin.

Urine samples collected on the 5th day posttreatment from HCB-diet and control-diet rats were used to study the effect of the HCB diet on the biotransformation of [^{14}C]aldrin. Essentially 100% of the radioactivity was extractable from acidified urine into ethyl acetate. Aldrin and dieldrin, along with several unidentified metabolites, were found in the urine of these rats (Table III). There was no evidence of qualitative differences between the two diet groups in the nature of radioactive metabolites extracted from the urine of the rats. However, there was evidence that the HCB diet caused most of the radioactivity to be associated with metabolites of greater polarity (Table III). For the HCB-diet rats, ~75% of the urine radioactivity was found below R_f 0.2, and for control-diet rats, ~35% was found in the same region. Other investigators (Kunze and Laug, 1953; Heath and Vandekar, 1964; Ludwig et al., 1964; Menzie, 1969) have also reported that [^{14}C]aldrin is metabolized by rats to dieldrin and a mixture of unidentified polar metabolites.

Table IV. Distribution of Radioactive Metabolites in Whole Urine of Male Rats Fed the HCB Diet or Control Diet[a] and Treated with [^{14}C]-1-Naphthol[b]

metabolite	R_f[c]	radioactivity, % control diet 3 h	6 h	12 h	HCB diet 3 h	6 h	12 h
unknown	origin	2	4	5	6	5	5
1-naphthyl glucuronide[d]	0.12	42	39	45	50	42	38
1-naphthyl sulfate[d]	0.35	56	57	50	44	53	57

[a] See footnote *b*, Table I. [b] Each rat was treated by ip injection of [^{14}C]-1-naphthol (5 mg/kg; radiocarbon activity, ~1.0 μCi in ~0.5 mL of corn oil). [c] Solvent system G = chloroform–methanol–acetic acid (8:2:1); whole urine spotted directly onto the TLC plate. [d] Chromatographic behavior identical with that of authentic compounds.

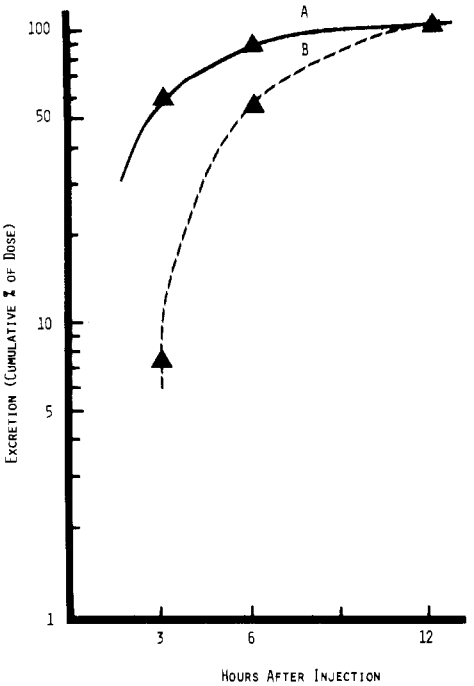

Figure 2. Effect of the HCB diet on the excretion of ^{14}C in the urine of male rats treated with [^{14}C]-1-naphthol. (A) ^{14}C in the urine of HCB-diet rats. (B) ^{14}C in the urine of control-diet rats.

[^{14}C]-1-Naphthol. Rats in both diet groups rapidly metabolized the injected [^{14}C]-1-naphthol to the glucuronide and sulfate conjugates which were eliminated rapidly through the urine (Table IV). Essentially all the administered radiocarbon was excreted in the urine within 12 h postinjection from both diet groups (Figure 2). The only observed difference between the diet groups in excretion of ^{14}C was the appearance of radioactivity in earlier urine samples of HCB-diet rats than of control-diet rats. After 3 h the HCB-diet rats had excreted ~60% of the total injected ^{14}C whereas the control-diet rats had excreted less than 10% at this time. After 6 h, excretion was ~87% by the HCB-diet rats and 55% by the control-diet rats. Likely, the HCB diet resulted in the induction of the enzyme systems involved in the synthesis of glucuronide and sulfate esters of 1-naphthol, which led to more rapid synthesis of these polar metabolites and thus more rapid excretion. Induction of hepatic microsomal UDP-glucuronyl transferase in rats after HCB treatment has been shown by Mehendale et al. (1975). Lin and Dorough (1974) reported no differences in the excretion or biotransformation

of [^{14}C]-1-naphthol by rats previously treated with either DDT or carbaryl. However, these workers collected only a single 24-h urine sample and thus could not have observed the possible early changes in excretion described in the study reported here.

Radiocarbon levels in the urine were high enough so that whole urine could be spotted directly onto the TLC plate without compromising separation. There was no evidence of qualitative differences between the two diet groups in the radioactive metabolites eliminated in the urine of rats injected with [^{14}C]-1-naphthol and no evidence of changes in the relative distribution of radioactive metabolites (Table IV). Three areas of radioactivity, including the origin, were resolved by solvent system G. The two major areas were identified by identical chromatographic behavior with authentic standards of 1-naphthyl glucuronide and 1-naphthyl sulfate. About 5% of the radioactivity remained at the origin, and free 1-naphthol was not detected. There was nearly equal distribution between the two conjugates for both diet groups. The metabolite characterizations were confirmed in solvent system H. The results reported here are in agreement with those of Berenbom and Young (1951), who found that the glucuronide and sulfate conjugates are the only major metabolites of 1-naphthol produced by the rat.

No radiocarbon residue was detected in any tissues of the HCB-diet rats or the control-diet rats that were collected upon sacrifice of the animals 72 h after the [^{14}C]-1-naphthol treatment. Likewise, analysis of feces samples collected during the 72-h posttreatment period showed that none contained detectable ^{14}C-labeled residue.

[^{14}C]-p,p′-DDT. Radiocarbon residues were sequestered primarily in the adipose tissue of both diet groups after treatment of rats with [^{14}C]-*p,p′*-DDT (Table II). Residues of [^{14}C]DDT or its metabolites were significantly higher in livers of HCB-diet rats than of control-diet rats. The same pattern was also observed with adipose tissue, although the differences were not statistically significant. The HCB diet did not affect the level of ^{14}C-labeled residues in any other tissues.

Urinary excretion was the predominant elimination route of ^{14}C by rats from both diet groups. Elimination of ^{14}C was relatively slow by rats in both diet groups, but urinary excretion was enhanced in rats fed the HCB diet (Figure 3). After 14 days, the total urinary excretion of ^{14}C in the urine was 5.1% of the injected ^{14}C for the HCB-diet rats and 1.9% for the control-diet rats. Total excretion of ^{14}C in the feces was less than 1% of the dose for each diet group and was negligible after the 4th day posttreatment. The slow excretion of DDT and metabolites, predominantly in the urine, is in agreement with the work of Negherbon (1959). Fries et al. (1969) compared whole body radiocarbon residue in rats pretreated with phenobarbital and given subsequent injections of either [^{14}C]-*p,p′*-DDT, [^{14}C]-*o,p′*-DDT, or [^{14}C]aldrin. Tissue radiocarbon residues in phenobarbital-pretreated rats were lower than those in rats treated with [^{14}C]aldrin or [^{14}C]-*o,p′*-DDT but were unchanged in rats injected with [^{14}C]-*p,p′*-DDT. Fries et al. (1969) concluded that microsomal induction has little effect on the pharmacodynamics of compounds such as *p,p′*-DDT that are strongly retained within the body and are slowly metabolized and excreted. These findings are consistent with those in the present study in which the HCB pretreatment did not greatly alter the excretion patterns of [^{14}C]-*p,p′*-DDT and its metabolites by the rat.

Ethyl acetate extraction of acidified urine collected 2 days after the [^{14}C]-*p,p′*-DDT treatment resulted in ~85%

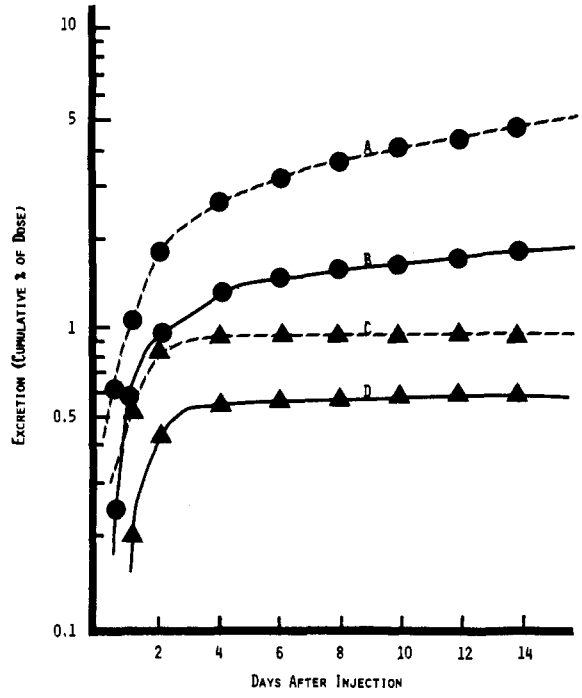

Figure 3. Effect of the HCB diet on the excretion of ^{14}C by male rats treated with [^{14}C]-*p,p′*-DDT. (A) ^{14}C in the urine of HCB-diet rats. (B) ^{14}C in the urine of control-diet rats. (C) ^{14}C in the feces of HCB-diet rats. (D) ^{14}C in the feces of control-diet rats.

partitioning of ^{14}C into the organic phase from the HCB-diet urine samples and 93% for the control-diet samples. There were no apparent differences due to the HCB diet in the relative distribution of the radioactive DDT metabolites resolved by TLC with several solvent systems. Six areas of radioactivity, including the origin, were separated by TLC with solvent system B. *p,p′*-DDT, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (TDE), and 4,4-dichlorobenzophenone (DCBP) were identified as very minor components in the urine extracts on the basis of identical chromatographic behavior with authentic standards (solvent systems B and C). However, ~97% of the extracted radioactivity from both the HCB-diet urine and the control-diet urine remained at the origin after TLC with solvent system B. These areas were scraped from the plates and extracted from the silica gel with ethyl acetate, and the extracts were chromatographed in more polar solvent mixtures [solvent system D (Wallcave et al., 1974) or solvent system E]. This procedure resulted in the resolution of at least 12 metabolites, including a product (17% of the total ^{14}C in control-diet urine and 12% in HCB-diet urine) that cochromatographed with authentic *p,p′*-DDA. These analyses gave no conclusive evidence of qualitative or quantitative changes due to the HCB diet in the biotransformation of [^{14}C]DDT. These findings contrast with those of Alary et al. (1971), who reported increased urinary excretion of *p,p′*-DDA after *p,p′*-DDT treatment of female Holtzman rats pretreated with phenobarbital. Differences in the strain and sex of the experimental animals, in the amount of DDT injected, and in the microsomal inducer used may account for the differences observed between the two experiments.

[^{14}C]HCB. [^{14}C]HCB or its metabolites were retained to a far greater extent by the adipose tissue of the [^{14}C]-HCB-treated rats than by other tissues examined, and no differences in tissue distribution of radioactivity due to the HCB diet were observed (Table II). Enhanced depletion of body burdens of certain organochlorine com-

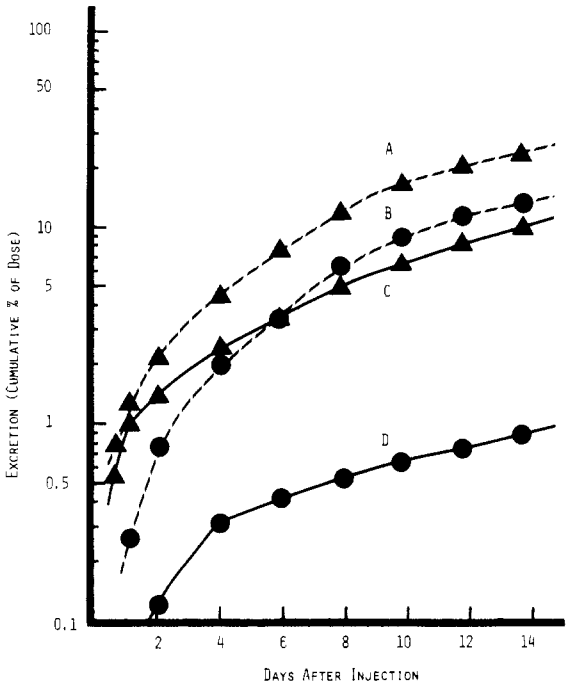

Figure 4. Effect of the HCB diet on the excretion of ^{14}C by male rats treated with [^{14}C]HCB. (A) ^{14}C in the feces of HCB-diet rats. (B) ^{14}C in the urine of HCB-diet rats. (C) ^{14}C in the feces of control-diet rats. (D) ^{14}C in the urine of control-diet rats.

pounds has been caused by microsomal induction (Street et al., 1966a,b; Alary et al., 1971). However, Villeneuve et al. (1974) reported a decreased rate of depletion of HCB residues from the livers and no effect on HCB residues in adipose tissue of male rats pretreated with phenobarbital. These workers were unable to correlate induction of microsomal enzyme activity with rate of disappearance of HCB residues from the liver. Their findings are consistent with the data reported here.

Excretion of radiocarbon after [^{14}C]HCB injection was greatly altered in the HCB-diet rats (Figure 4). Total elimination was increased in both urine and feces of the HCB-pretreated rats. Combined excretion of ^{14}C through the urine and feces totaled about 35% of the injected radioactivity by the HCB-diet rats and 10% by the control-diet rats. Elimination of ^{14}C in the urine was markedly enhanced in the HCB-diet rats as compared to that in the controls. Elimination of ^{14}C in the feces predominated in spite of the markedly increased elimination of ^{14}C in the urine (Figure 4).

The fact that control-diet rats eliminated ~90% of the excreted radioactivity through the feces suggests that biliary excretion is important in the elimination of HCB or its metabolites from the body. These findings agree with those of Koss and Koransky (1975), who found that female rats given single ip injections of 4 mg/kg [^{14}C]HCB eliminated 5% (urine) and 34% (feces) of the dose during a 14-day posttreatment period. The rats in the study by Koss and Koransky (1975) were not pretreated with HCB.

Total excretion of ^{14}C in urine and feces was greater in the HCB-diet rats than in the control-diet rats. Total urinary radiocarbon excretion during the 14-day collection period was 12.7% and 0.9% of the injected dose for the HCB- and control-diet rats, respectively. For the same time period total ^{14}C elimination in feces was 22.5% for the HCB-diet rats and 9.4% for the control-diet rats. The ratio of total ^{14}C excreted in urine to the total ^{14}C excreted in feces was ~1:2 for the HCB-diet rats and 1:10 for the control-diet rats.

Table V. Distribution of Radioactive Metabolites in the Urine[a] of Male Rats Fed the HCB Diet or Control Diet[b] and Treated with [^{14}C]HCB[c]

metabolite	R_f[d]	radioactivity, % control diet	radioactivity, % HCB diet
water soluble[e]		20.0	12.0
1	origin	30.4	20.2
2	0.03	16.2	4.0
3	0.06	12.1	9.1
4	0.09	2.2	1.0
5	0.28	4.7	2.2
6	0.42	1.8	7.5
7 (PCP)[f]	0.58	10.6	41.8
8 (HCB)[f]	0.71	2.0	2.2

[a] Pooled samples from two cages of HCB-diet rats or control-diet rats; collected on 3rd day posttreatment. [b] See footnote *b*, Table I. [c] Each rat was treated by ip injection of [^{14}C]HCB (5 mg/kg; radiocarbon activity, ~1.0 μCi in ~0.5 mL of corn oil). [d] Solvent system E (benzene saturated with formic acid). [e] Acidified urine sample was extracted with ethyl acetate. Radioactivity not extracted is reported as "water-soluble metabolites", percent of total radioactivity in the urine sample. [f] Chromatographic behavior identical with that of authentic compounds.

Urine samples collected on the 3rd day posttreatment from HCB-diet and control-diet rats were used to study the effect of the HCB diet on the biotransformation of [^{14}C]HCB. HCB-diet rats excreted a lower percentage of polar metabolites in the urine than control-diet rats excreted. This lower percentage was shown by both the relative percentage of water-soluble metabolites and the relative distribution of extracted metabolites (Table V). Ethyl acetate extraction of acidified urine showed that about 20% of the urine radioactivity in the control diet and 12% in the HCB diet were not extracted from the aqueous phase. Although microsomal induction generally results in enhanced production of more polar metabolites (Conney, 1971), the data reported here indicate a reduction in the relative percentage of water-soluble [^{14}C]HCB metabolites excreted by HCB-diet rats.

Eight areas of radioactivity, including the origin, were separated by TLC with solvent system E (Table V). In the present study HCB and pentachlorophenol (PCP) were detected in the extract of urine from rats in both diet groups and were identified by identical chromatographic behavior with authentic standards. However, pentachlorobenzene, isomers of tetrachlorobenzene, or 2,4,5-trichlorophenol was not among the radioactive metabolites in any of our extracts. Authentic standards of tetrachlorohydroquinone, pentachlorothiophenol, and tetrachlorothiophenol were not available during this study; therefore, we do not know whether these compounds were among the metabolites of [^{14}C]HCB separated by solvent system E. The ^{14}C-labeled components that cochromatographed with HCB and PCP in solvent system E were confirmed in solvent system F.

In solvent system E, ~42% of the radioactivity extracted from the urine of HCB-diet rats cochromatographed with PCP, and ~34% of the radioactive compounds remained at or near the origin ($R_f < 0.1$). About 11% of the radioactivity extracted from urine of control-diet rats was contributed by PCP, and 61% of the radioactivity was located below R_f 0.1. Only ~2% of the radioactivity in the urine of rats in either diet group was in the form of unmetabolized HCB.

Mehendale et al. (1975) identified traces of pentachlorobenzene and tetrachlorobenzene in urine of [^{14}C]-

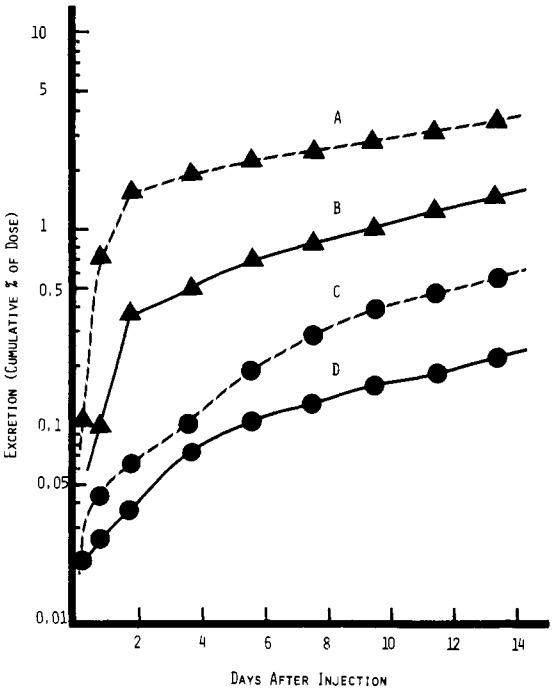

Figure 5. Effect of the HCB diet on the excretion of ^{14}C by male rats treated with [^{14}C]mirex. (A) ^{14}C in the feces of HCB-diet rats. (B) ^{14}C in the feces of control-diet rats. (C) ^{14}C in the urine of HCB-diet rats. (D) ^{14}C in the urine of control-diet rats.

HCB-treated rats, and, more recently, Renner and Shuster (1977) isolated and identified another HCB metabolite, 2,4,5-trichlorophenol, from rat urine. Koss et al. (1976) identified PCP, tetrachlorohydroquinone, and pentachlorothiophenol as major metabolites and an isomer of tetrachlorothiophenol as a minor metabolite in the urine of female rats administered multiple ip injections (total dose 260 and 390 mg/kg) of [^{14}C]HCB. They did not report the presence of either pentachlorobenzene or tetrachlorobenzene.

[^{14}C]*Mirex*. The effects of HCB diet on the distribution of ^{14}C-labeled residues in tissues of male rats given a single intraperitoneal injection of [^{14}C]mirex are shown in Table II. In both diet groups, residues were localized primarily in body fat. Levels of radiocarbon were higher in the HCB-diet rats than in the control-diet rats in all of the tissues studied, although adipose tissue was the only tissue for which a difference due to the HCB diet was statistically significant. The failure to show statistically significant differences for other tissues was attributed to the large variations in residue levels between animals within the same groups.

Elimination of ^{14}C by rats injected with [^{14}C]mirex was very slow, although HCB pretreatment caused more radioactivity to appear in both urine and feces (Figure 5). Total excretion (feces plus urine) was only about 4% of the injected dose for the HCB-diet rats and 1.6% for the control-diet rats. Urinary excretion during the 14-day postinjection period totaled 0.54% for the HCB-diet rats and 0.23% for the control-diet rats. Total feces excretion of radiocarbon was 3.4% for the HCB-diet rats and 1.4% for the control-diet rats (Figure 5). One rat from each of the two cages of HCB-diet rats was found dead on the 11th day postinjection. Appropriate corrections were thus made in subsequent calculations of excretion data.

The increased elimination of ^{14}C by the HCB-diet rats may have resulted from enhanced metabolism of mirex due to microsomal enzyme induction. Increased elimination and the indication of possible mirex metabolism by HCB-diet rats (vide infra) support this hypothesis.

Only the urine collected from the HCB-diet rats contained sufficient ^{14}C to permit analysis of its chemical nature. About 60% of the ^{14}C present was extracted from acidified urine into ethyl acetate. TLC (solvent system C) of the concentrated extract produced two areas of radioactivity, the origin (85%) and a spot that cochromatographed with the authentic mirex standard. The low level of ^{14}C remaining at the origin on TLC was insufficient to allow further study of its nature.

Other investigators (Gibson et al., 1972; Mehendale et al., 1972; Ivie et al., 1974) found no evidence of mirex metabolites in the urine of orally treated rats. Recently, Stein et al. (1976) reported a mirex metabolite in the feces of monkeys given intravenous injections of [^{14}C]mirex. However, they concluded that the metabolite probably resulted from the action of gastrointestinal bacteria rather than biotransformation by tissue. Although evidence in the present study is apparently the first reported of a possible biotransformation of mirex by a mammalian system, the evidence is marginal because the "metabolite" observed here could represent trace impurities in the [^{14}C]mirex injected.

CONCLUSIONS

Results in these studies have shown that exposure to HCB can alter the pharmacodynamic characteristics of other exogenous compounds in male rats. The response varies and depends upon the second compound involved. Results have also shown that because of the complexity of the living organism, the interactions of chemical combinations in vivo are difficult to predict accurately. Because of the unanticipated response of HCB-diet rats to the metabolism cage environment, it was not possible to determine the effect of the HCB diet alone on the biotransformation, excretion, or residue deposition of the carbon-14-labeled compounds or of the organ weight changes. Nor was it possible to determine whether the differences observed in the biotransformation, residue deposition, and excretion of the injected compounds were the result of microsomal enzyme induction or of some factor related to stress and weight loss. The mortality associated with the HCB-diet rats that were treated with either [^{14}C]aldrin or [^{14}C]mirex suggests a possible synergistic interaction resulting in increased toxicity. Whether these effects resulted from enhanced chemical toxicity or from some other factor(s) is not clear and will be the subject of further investigations.

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Biokinetics and Metabolism of *N*-(2,3-Dichlorophenyl)-3,4,5,6-tetrachlorophthalamic Acid in Rats

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The biokinetics and metabolism of the bactericide *N*-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalamic acid, techlofthalam, have been studied in rats after repeated oral doses of the ^{14}C-labeled compound for 7 days. Up to 6 days after the last of seven daily doses, means of 92 and 66% of the total dose were excreted in the feces of male and female rats, respectively, and means of 5 and 25%, respectively, in the urine. Only 0.2% dose was retained in the carcasses at this time. There was no extensive accumulation of radioactivity in tissues. Techlofthalam was metabolized by hydroxylation in the dichloroaniline group, and this metabolite was eliminated as a conjugate in bile and also partly unconjugated in the urine of female rats. No metabolites resulting from amide hydrolysis were detected. An oral dose of techlofthalam imide was excreted almost entirely unchanged in feces, indicating a very low absorption of this compound.

The compound *N*-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalamic acid, techlofthalam, is a new systemic bactericide for the control of bacterial leaf blight (*Xanthomonas oryzae*) in rice (Nakagami et al., 1980). Techlofthalam possesses low acute mammalian toxicity, and the oral LD 50 for both sexes of rats and mice is in the region of 2000 mg/kg (M. Ishida, unpublished results). In rice straw, but not grain, two major residues have been identified as techlofthalam and the imide, *N*-(2,3-dichlorophenyl)-3,4,5,6-tetrachlorophthalimide (M. Ishida, unpublished results). This paper describes the syntheses of [^{14}C]techlofthalam and [^{14}C]imide and studies on their fate in rats. Since hydrolysis of the amide group in techlofthalam could occur resulting in the formation of 2,3-dichloroaniline and tetrachlorophthalamic acid, these

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