It is clear from the above experiments that PCB's are susceptible to metabolic degradation in the goat and cow and are excreted in the urine. Since the toxicology and biological properties of hydroxylated PCB's are unknown this is, therefore, an area of potential environmental hazard and concern.

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Metabolism and Effects of Hexachlorobenzene on Hepatic Microsomal Enzymes in the Rat

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Seven days following a single oral dose of hexachlorobenzene (HCB-^{14}C) to adult male rats, approximately 16% of the dose was excreted in the feces and less than 1% in urine. Metabolites of HCB included pentachlorobenzene, tetrachlorobenzene, pentachlorophenol, and four unknowns; all were abstracted from urine and none was detected in the feces. Seventy per cent of the total dose remained in the body 7 days after administration, fat being the major depot. This residue consisted mainly of HCB with traces of the dechlorinated metabolites. Reductive dechlorination of HCB was catalyzed by an enzyme located in the microsomal fraction of liver, lung, kidney, and intestine. Added NADPH was required for the formation of pentachlorophenol. Hepatic glucuronyl transferase, aniline hydroxylase, ethyl mophine, and *p*-nitroanisol demethylases and cytochromes P-450 and b_5 were induced by HCB pretreatment. Urinary coproporphyrin levels more than doubled by the fourth day of treatment and remained relatively constant thereafter, although apparent signs of porphyria were not observed in these animals.

Hexachlorobenzene (HCB) is a selective fungicide for bunt of wheat and has been used as a general seed coating for cereal crops. It was introduced for agricultural use in 1945 (Yersin) and has since been reported to be a by-product of certain industrial processes (EPA, 1973). HCB has a low acute toxicity to mammals (Davis *et al.,* 1959; Savitskii, 1964), but is known to cause porphyria cutanea tarda, characterized by skin rashes and extreme sensitivity to sunlight (Schmid, 1960; Cam and Nigogosyan, 1963). A massive outbreak of human porphyria occurred in Turkey following consumption of wheat precoated with HCB (Schmid, 1960). There is some evidence for ecological magnification of residues of HCB (Metcalfe *et al.,* 1973) and residues have been reported in wild birds in the Netherlands (Vos *et al.,* 1968; Koeman *et al.,* 1969) and in North America (Gilbertson and Reynolds, 1973), in beef cattle in the U.S. (EPA, 1973), and in humans in Germany (Acker and Schulte, 1970), Japan (Carley *et al.,* 1973), and Australia (Brady and Siyali, 1972; Siyali, 1972). In the first reported study of HCB metabolism Parke and Williams (1960) administered a relatively large dose (0.4 g/kg) of unlabeled HCB to rabbits and reported that 75% remained in the gastrointestinal tract with only 6% excreted in feces. No HCB was detected in the urine and no metabolites were found. Since that time little has been reported on the metabolism of HCB. In view of the ubiquitous nature of the HCB residues and the forementioned distressing effects of its chronic ingestion by humans, it was of interest to study the distribution, metabolism, and excretion of a small dose of HCB. Such a study was carried out in rats using HCB-^{14}C. In addition, the effect of HCB pretreatment on some hepatic enzyme systems and

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Table I. Storage and Excretion of HCB-^{14}C[a] Administered Orally in Rats

Organ or tissue	% of total radioact. administered
1. Fat[b]	42.81 ± 6.14
2. Muscle[c]	9.41 ± 1.17
3. Skin[d]	8.64 ± 1.21
4. Liver	3.01 ± 0.23
5. Small intestine	2.43 ± 0.47
6. Bone[e]	1.04 ± 0.09
7. Kidney	0.76 ± 0.11
8. Large intestine	0.43 ± 0.08
9. Stomach	0.36 ± 0.04
10. Blood	0.24 ± 0.04
11. Lung	0.24 ± 0.04
12. Testes	0.21 ± 0.04
13. Heart	0.18 ± 0.03
14. Brain	0.17 ± 0.03
15. Spleen	0.04 ± 0.002
Total in tissues	70.09 ± 5.48
Excretion	
Feces	16.02 ± 2.31[f]
Urine	0.85 ± 0.13[f]
Gut contents	2.48 ± 0.45
Total recovery	89.44 ± 10.57

[a] A single orally administered dose of 5 mg/kg per animal. Mean ± standard deviation of 11 animals. Total radioactivity was determined by oxidizing samples as described in the text. [b] Based on 9% body weight as fat; Reed *et al.* (1930). [c] Based on 50% body weight as muscle; Bischoff *et al.* (1971). [d] Based on 16% body weight as skin; Matthews (1973). [e] Based on 10% body weight as bone, ICRP (1960). [f] Cumulative total for 7 days.

the excretion of coproporphyrins in the urine were also investigated.

MATERIALS AND METHODS

Uniformly labeled hexachlorobenzene-^{14}C (sp act. 4.02 mCi/mmol) was purchased from Mallinckrodt Chemical Works, St. Louis, Mo., and the purity was established by thin-layer chromatography. White male rats (Sprague-Dawley, CD) weighing about 180–230 g were obtained from Charles River Breeding Labs, Inc., Wilmington, Mass. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), NADP, uridine diphosphate glucuronic acid (UDPGA), UDPG, and reduced glutathione (GSH) were obtained from Sigma Chemical Co., St. Louis, Mo.

In Vivo **Metabolism.** Eleven male rats were administered HCB-^{14}C by oral intubation in arachis oil (16.2 μCi per animal, $\simeq$5 mg/kg). Treated animals were maintained in metabolism cages (Model E 110, Maryland Plastics) and fed Wayne Lab Blox and water *ad libitum*. Feces and urine were collected daily and, after 7 days, the animals were sacrificed and major organs and tissues were removed. Aliquots of urine (0.2 ml) were radioassayed directly in a liquid scintillation counter using Aquasol (New England Nuclear Corp.). The daily fecal samples were dried and powdered and two 100-mg samples of each day's collection were oxidized in a Biological Sample Oxidizer (Harvey Instrument Corp., Hillsdale, N.J.). The tissues were homogenized 1:1 in water in a Sorval Omnimixer and two 0.2-ml aliquots of each homogenate were oxidized and radioassayed similarly. Pooled urine and powdered feces samples were extracted three times with a 2:1 mixture of benzene and isopropyl alcohol. This extraction method yielded an average of 87% recovery of the radioactivity. Organic layers were pooled and concentrated and the residue was taken up in a small quantity of benzene and subjected to tlc analysis along with authentic standards. Prepored silica gel G plates (Anal Tech, Inc., Newark, Del.) were spotted and developed in (i) chloroform, (ii) benzene–acetone (4:1), and (iii) benzene–acetone (1:1). Radioactive spots were visualized by exposing the developed plates to medical X-ray films (Eastman Kodak Co.). Organs and tissues were homogenized using a micro attachment on a Virtis Homogenizer and extracted with 2:1 benzene–isopropyl alcohol and the extract subjected to tlc analysis as above.

Isotope Dilution. Radioactive metabolites isolated from rat urine or tissues were dissolved in a suitable solvent, ethanol for the chlorobenzenes and carbon tetrachloride for the chlorinated phenols, with a known amount of unlabeled standard. The mixtures were then repeatedly recrystallized, by chilling the solutions, removing the mother liquor, and redissolving the crystals in fresh solvent. The recrystallization was repeated until a constant specific activity was obtained for four successive recrystallizations.

In Vitro **Metabolism.** Liver, lung, kidney, and small intestine from control rats were homogenized in 3 vol of ice-cold Tris-HCl (0.15 *M*, pH 7.4) buffer. These homogenates were centrifuged at 10,000*g* for 20 min and the supernatant was further centrifuged at 105,000*g* for 60 min to obtain microsomal and post-microsomal supernatant fractions. These subcellular fractions of each tissue (1 ml of enzyme) were then incubated with HCB-^{14}C (2×10^6 dpm in 20 μl of dimethyl sulfoxide) with and without various cofactors (2 μmol of NADH, NADPH, UDPGA, UDPG, and reduced glutathione) in combination and individually in a total volume of 3 ml in a 25-ml flask, at 37° for 1 hr in a shaking incubator. The reactions were terminated by the addition of 4 ml of a 2:1 benzene–isopropyl alcohol mixture and extracted twice with the same solvent.

Effect of HCB Pretreatment on Hepatic Enzymes and Urinary Coproporphyrin. Two groups of six male rats were treated daily with HCB by oral intubation in 0.5 ml of arachis oil, 10 or 25 mg/kg per day for 6 days. Another group of six male rats received 0.5 ml of arachis oil per day and served as controls. These animals were maintained in metabolic cages with food and water *ad libitum*. The animals were sacrificed on the eighth day after noting the body weights. Livers were removed, washed, blotted, and weighed. Liver homogenates were made in 4 vol of Tris-HCl buffer (0.15 *M*, pH 7.4) and microsomes were prepared as described above. Cytochromes b_5 and P-450 contents of the liver microsomes were measured according to Omura and Sato (1964). Aniline hydroxylase activity was assayed by the method of Schenkman *et al.* (1967). Ethyl morphine *N*- and *p*-nitroanisol-*O*-demethylase activities were determined by the method of Kato and Gillette (1965). Glucuronyl transferase activity was determined using 1-naphthol-^{14}C as substrate as described elsewhere (Mehendale and Dorough, 1971; Mehendale *et al.*, 1973). Protein determinations were carried out according to Lowry *et al.* (1951). Daily urine samples were collected from treated and control animals from days 4 through 8 and urinary coproporphyrin was extracted from 5-ml aliquots according to Schwartz *et al.* (1963). The ethyl acetate extract of urine was extracted with four 5-ml volumes of 1.5 *N* HCl and the optical density at 400 mμ of a 10-ml aliquot of the 1.5 *N* HCl extract was measured and used as a relative measure of the level of coproporphyrin.

RESULTS

In Vivo **Metabolism of Hexachlorobenzene.** Seven days after the administration of a single oral dose of HCB-^{14}C (5 mg/kg), over 70% of the dose remained in the animal's body. HCB residues were present in every major organ and tissue (Table I). By far the most radioactivity,

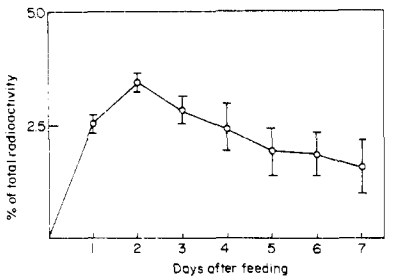

Figure 1. Daily excretory pattern of radioactivity in feces. Rats were treated with HCB-^{14}C at a dose of 5 mg/kg. Feces collected for 7 days.

Table II. Effect of HCB Pretreatment on Body and Liver Weight of Male Rats[a]

Dose, mg/kg	Gain in body wt,[b] g	Liver wt,[b] g	Liver/body wt × 100
0	49.08 ± 5.16[c]	11.65 ± 0.43	6.13
10	46.33 ± 2.89[c]	13.04 ± 0.64[c]	5.49[c]
25	45.75 ± 3.13[c]	14.33 ± 0.23[c]	5.22[c]

[a] Orally administered to male rats in arachis oil for 6 days and the animal sacrificed on eighth day after a day's lapse. [b] Average of seven determinations ± standard deviation. [c] Not significant at 0.05P confidence.

42.8% of the total dose, was found in the fat and lesser amounts were found in the muscle, skin, and liver. On a concentration basis, small intestine, stomach, liver, and skin contained considerably more radioactivity than the muscle.

The primary excretory route was fecal and accounted for 16% of the total dose in 7 days (Table I). Over 50% of the total fecal excretion occurred within the first 3 days (Figure 1). Extraction and analysis of fecal radioactivity failed to reveal the presence of a metabolite. Although total urinary excretion of HCB was less than 1% (Table I) of the total dose, the only evidence of HCB metabolite excretion was detected in the urine (Figure 2). In Figure 2, the spot immediately below HCB represents a mixture of chlorinated benzenes as evidenced by cochromatography on tlc and by isotope dilution. The other polar spots have not been identified. Extracts of fat, liver, intestines, kidney, lung, and brain contained primarily HCB along with trace amounts of chlorinated benzenes. Effective separation of the chlorinated benzenes or the chlorinated phenols from one another was not possible by tlc. Thus, it was necessary to use preparative tlc to eliminate most of the biological material, and identify the metabolites by isotope dilution using authentic unlabeled standards. Isotope dilution suggested the presence of pentachlorobenzene and tetrachlorobenzene in spot 7 and pentachlorophenol in spot 4. Authentic 2,4,5-trichlorophenol standard co-chromatographed with spot 6. Isotope dilution is an accurate qualitative analytical technique and hence the quantities of various metabolites were not estimated. However, in no instance did any of the metabolites make up more than a trace of the total radioactivity extracted from an organ, tissue, or the urine.

In Vitro **Metabolism of HCB.** Homogenates of liver, lung, kidney, and small intestines produced trace amounts of the chlorobenzenes, when incubated with HCB-^{14}C in the presence or absence of added cofactors. Of these, kidney appeared to be the most active on a per gram wet weight basis, but the amount of the metabolite(s) produced was too small for accurate quantitation. Microsomal preparations from liver, but not from the other tissues, also produced amounts of one or more chlorophenols when fortified with NADPH. The pentachlorophenol produced by liver microsomes was not observed when post-mitochondrial supernatant was used or when UDPGA was added to the microsomal incubation mixture. This disappearance of pentachlorophenol was presumably due to the formation of glucuronide or other conjugates since corresponding radioactivity was present in the aqueous phase. Fortification of kidney homogenates with glutathione resulted in the appearance of unextractable radioactivity in the aqueous phases indicating that glutathione conjugates of polar HCB metabolites might also be formed.

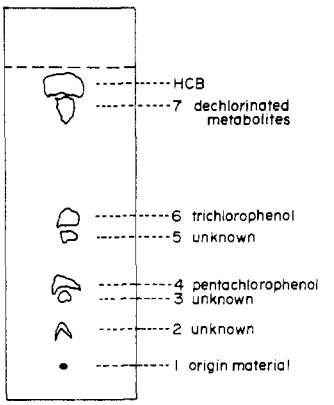

Figure 2. Autoradiography of a tlc of an organic solvent extract of urine on a silica gel g plate. Rats were treated with HCB-^{14}C at a dose of 5 mg/kg. Urine was collected for 7 days.

Table III. Effect of HCB Pretreatment on Hepatic Cytochromes P_{450} and b_5

Dose, mg/kg	nmol of P_{450}/ mg of protein[b]	% change	nmol of b_5/ mg of protein	% change
0	0.543 ± 0.058		0.118 ± 0.009	
10	0.669 ± 0.061	23[c]	0.144 ± 0.013	22[c]
25	0.816 ± 0.041	50[c]	0.158 ± 0.008	34[c]

[a] HCB was orally administered in arachis oil to male rats for 6 days. Animals were sacrificed on the eighth day. [b] Average of seven determinations ± standard deviation. [c] These differences are not statistically significant; $P < 0.20$.

Effect of HCB Pretreatment on the Hepatic Enzyme Systems. Male rats orally pretreated with 10 mg/kg of HCB for 6 days showed a slight liver enlargement and this effect was approximately doubled in the rats which received 25 mg/kg (Table II). There was no difference in body weight gained among the treated and control animals; thus the body to liver weight ratio decreased as the HCB dose increased. Oral pretreatment with 10 or 25 mg of HCB/kg appeared to result in a dose-related induction of the microsomal cytochromes b_5 and P-450 (Table III). In addition, some individual enzyme activities of the hepatic microsomal preparations were studied. Both ethyl morphine *N*- and *p*-nitroanisol *O*-demethylases were induced by HCB pretreatment (Table IV). A greater effect was observed on a third mixed-function oxidase, aniline hydroxylase (Table IV) than on demethylases. At 25 mg/kg treatment, aniline hydroxylase activity was more than doubled.

In addition to the above mixed-function oxidase activities, the hepatic microsomes contain UDP glucuronyl transferase activity. This is often the final reaction in the biotransformation of a xenobiotic. Increases in glucuronyl transferase activities were 92.9 and 202.9% in animals treated with 10 and 25 mg/kg of HCB, respectively (Table IV).

Urinary Coproporphyrin. Although rats orally preexposed (HCB at 25 mg/kg) for 6 days did not show any ap-

Table IV. Effect of HCB Pretreatment on Hepatic Microsomal Enzymes[a]

Dose, mg/kg	Aniline hydroxylase *p*-aminophenol formed, nmol/mg of prot. per 30 min[b]	% change	Ethyl morphine *N*-demethylase formaldehyde formed, nmol/mg of prot. per 30 min[b]	% change	*p*-Nitroanisole *O*-demethylase *p*-nitrophenol, nmol/mg of prot. per 30 min[b]	% change	Glucuronyl transferase naphthyl glucuronide formed, nmol/mg of prot. per 15 min[b]	% change
0	38.25 ± 0.75		3.44 ± 0.23		1.73 ± 0.19		8.75 ± 0.66	
10	61.01 ± 1.27	59.5	4.17 ± 0.18	21.2	2.26 ± 0.17	30.8	16.89 ± 0.98	92.0
25	80.83 ± 1.65	110.2	5.72 ± 0.25	65.7	2.90 ± 0.15	68.0	26.52 ± 2.56	202.9

[a] HCB was orally administered to male rats in arachis oil for 6 days and the animals were sacrificed on the eighth day. [b] Average of seven determinations ± standard deviation. All pairwise differences are significant ($P < 0.01$). All dose-response relationships are also significant ($P < 0.02$).

Table V. Effect of HCB on Coproporphyrin Excretion in Daily Urine Samples from the Male Rat (OD at 400 mμ)[a]

	Days after treatment					
	4	5	6	7	8	Av/day[b]
Control	0.20 ± 0.17	0.17 ± 0.14	0.18 ± 0.14	0.17 ± 0.15	0.17 ± 0.13	0.179 ± 0.137
Treated	0.43 ± 0.28	0.46 ± 0.30	0.45 ± 0.28	0.51 ± 0.32	0.45 ± 0.28	0.460 ± 0.292

[a] Male rats weighing 200 g were treated with HCB at 25 mg/kg per day in 0.5 ml of arachis oil by oral intubation for 6 days. Controls received 0.5 ml of arachis oil. Starting on the fourth day urine samples (5 ml) were extracted and analyzed for coproporphyrin at 400 μ*M* of 10-ml 1.5 *N* HCl extracts. Figures in table are mean optical density ± standard deviation of six animals. [b] Statistically significant difference at 0.05 level.

parent signs of porphyria, urinary excretion of coproporphyrin was more than doubled within the fourth day (Table V). Thereafter, the level of coproporphyrin remained relatively constant through the duration of the experiment. The average coproporphyrin excretion was 0.179 ± 0.137 for controls and 0.460 ± 0.292 (OD at 400 mμ) for the treated animals. This difference is statistically significant ($P < 0.05$). Variability in urinary coproporphyrin among the animals within the groups was large while day to day concentrations of urinary coproporphyrin were relatively much less variable in each group.

DISCUSSION

In view of its potential for widespread dispersal as a fungicide and through industrial wastes and the reports of its residues in wild birds, beef cattle, and humans, the questions of HCB metabolism and unknown effects on health become of important concern. Although this and one additional report (Metcalfe *et al.*, 1973) indicate that HCB is degraded in animals (rats), it is obvious that such degradation occurs very slowly. Less than 1% of the total dose given to rats was eliminated in the urine in 7 days and only a small percentage of that was in the form of HCB metabolites. The low rate of elimination of HCB from the body by the fecal and urinary routes, along with the fact that residues of HCB and dechlorinated metabolites are found in every tissue examined, indicates the great potential for HCB storage. The observations of Parke and Williams (1960) indicate that a large portion of an ingested dose of HCB may remain in the gut for several days. Thus, that portion of the dose excreted in the feces may represent material which was never absorbed from the gut. The fact that no metabolites of HCB were detected in the feces tends to support this hypothesis.

Elucidation of the route of the metabolism of HCB needs further attention. From the results presented here, HCB is reductively dechlorinated to a very small extent. HCB and these dechlorinated products are stored in the body. A small amount of dechlorinated material is further metabolized to polar metabolites, such as pentachlorophenol, and excreted as conjugates and free form in the urine. Reductive dechlorination of HCB by enzyme preparations does not seem to require added cofactors. Formation of polar metabolites requires added NADPH.

Porphyria cutanea tarda, a disease caused by repeated exposure to HCB and other agents, is characterized by skin rashes and extreme sensitivity to sunlight (Schmid, 1960). Since 8.64% of a single oral dose remained in the skin after 7 days, repeated exposure to HCB could result in increased levels of its residue in the skin. This might be related to this physiological abnormality. In addition, HCB residues found in the bone after a single dose may or may not be related to disturbances in heme synthesis. Disturbances in the heme metabolism are apparent by the fourth day of HCB treatment as indicated by the increased concentrations of coproporphyrin in the urine.

Hepatic microsomal enzyme activities studied in this report were generally increased by HCB pretreatment of animals; of the mixed-function oxidases studied, aniline hydroxylase was increased to the greatest extent. Treatment with other chlorinated pesticides may result in decreases in aniline hydroxylase activity (Mehendale *et al.*, 1973; Sell *et al.*, 1972). DDT pretreatment reduced aniline hydroxylase activity in hens and Japanese quail, but this effect was attributed to inhibition by residues of DDT and DDE (Sell *et al.*, 1971, 1972). Reduction of aniline hydroxylase activity by mirex pretreatment in rats was not attributable to enzyme inhibition by mirex (Mehendale *et al.*, 1973) and seemed to be a function of physiological response. Pretreatment with DDT elevated aniline hydroxylase in rats (Hart and Fouts, 1963).

While HCB pretreatment elicited significant induction of several mixed-function oxidase enzymes, it is also a good inducer of glucuronyl transferase activity, also associated with the microsomal fraction. Another potent inducer of glucuronyl transferase activity of rat liver is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Lucier *et al.*, 1973). However, unlike HCB, this substance has a high acute toxicity. Thus, HCB might be the better choice as an inducer in rats in studies of glucuronyl transferase. HCB induction of glucuronyl transferase activity more than other microsomal drug metabolizing enzymes would seem to be of interest since very few potent inducers of this conjugating system are known. Selective induction of UDPG-

transferase might be of value in studying the relationship between the oxidative and conjugative reactions of the hepatic microsomes as related to the drug metabolism. Relationships between this finding and porphyria caused by long term exposure to HCB, if any, need to be investigated.

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Retention and Excretion of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin by Rats

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Rats were fed 7 or 20 ppb of 2,3,7,8-[^{14}C]tetrachlorodibenzo-*p*-dioxin (TCDD) in their diets for 42 days. Dose-related effects on feed consumption and growth were more severe in males than in females. Both levels of TCDD significantly increased liver weights, but 7 ppb caused the greater increase. Total retention of TCDD was closely related to total intake at any given time period. Males and females did not differ significantly in total retention, but 85% of the TCDD was in the liver of males, whereas only 70% was in the liver of females. Total retention was 5.5, 7.5, and 10.0 times daily intake at 14, 28, and 42 days, respectively. Kinetic analysis indicated that at steady state, total retention would be approximately 10.5 times the average daily intake. When feeding stopped, TCDD residues were eliminated from the body with half-lives of 12 and 15 days for males and females, respectively.

Concern about the potential health hazards associated with the chlorinated dibenzo-*p*-dioxins has been expressed in recent years. Dioxins may arise as by-products in the manufacture of certain chlorinated phenols. Interest is mainly focused on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which appeared as a trace contaminant in some samples of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). TCDD has been associated with chloracne in humans and numerous pathological effects in laboratory animals (Environmental Health Perspectives, 1973). In addition, its single-dose oral LD_{50} is quite low for many species (Schwetz *et al.*, 1973). Because of its high potential for adverse effects, it is important to obtain information on the retention and elimination of TCDD in animals. Obtaining this information has been hindered because of the high toxicity of TCDD and the lack of an available analytical method that could be routinely used when animals are fed sublethal quantities.

Piper *et al.* (1973) studied the excretion and tissue distribution of [^{14}C]TCDD in the male rat after oral administration of a single dose approximately twice the LD_{50}. Among the tissues examined the liver had the highest concentration and contained about 50% of the ^{14}C activity during the first week after dosing. The concentration in liver was somewhat greater than in fat and both were about 10 times higher than any other tissue. Over 50% of the dose was eliminated *via* the feces in 21 days, with much smaller amounts eliminated *via* urine and expired air. Vinopal and Casida (1973) studid the fate of TCDD in mice injected with a single LD_{50} dose. As in rats, liver was the major site of concentration and no water-soluble products were detected. Feces was the major route of elimination, probably *via* the bile.

Single-dose studies indicated the major sites of retention and routes of elimination. However, they do not necessarily provide a good basis for evaluating steady-state burdens or rates of elimination resulting from continuous low-level exposure. In this study, we determined the rates

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