Carbaryl Metabolism in the Rat. A Comparison of in Vivo, in Vitro (Tissue Explant), and Liver Perfusion Techniques

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An investigation of the individual metabolic activity of the liver, lung, and the kidney of rat for carbaryl (Sevin) was conducted utilizing the tissue explant maintenance technique. Hepatic tissue of the rat incubated with carbaryl actively performed demethylation, hydrolysis, hydroxylation, and oxidation, followed by sulfate and glucuronide conjugations. Both kidney and lung tissues made most of the liver-generated metabolites in lesser quantities. A study of the metabolism of carbaryl by rat liver perfusion was undertaken for comparison with the in vitro technique and with in vivo metabolism of carbaryl by rats. Chromatographic profiles of anionic metabolites of carbaryl from in vivo 24-h urines, from 18-h in vitro liver explants and from 1-h liver perfusion agreed qualitatively and semiquantitatively. Since the in vitro tissue-maintenance technique faithfully reproduced the metabolites of carbaryl found in urine samples of similarly dosed animals and is rapid, simple, and reproducible, it offers great promise as a method to determine metabolic pathways in man without resorting to direct dosing of the human subject.

The explant-maintenance technique (Sullivan et al., 1972a) using liver tissues reflects the in vivo metabolic processes of animals, including man, and thus eliminates the need for dosing human beings. Significant conjugation of carbaryl (1-naphthyl *N*-methylcarbamate) was found during in vitro studies with the kidney, liver, and lung from human subjects (Chin et al., 1974). The in vitro metabolic products are species and tissue specific and do not depend upon the addition of cofactors for their production. Analyses of urinary metabolites showed that marked quantitative differences occur between the rat and man in the metabolism of carbaryl (Knaak et al., 1965; Knaak and Sullivan, 1967). Therefore, these three organs from the rat were studied using carbaryl to delineate their individual contributory metabolic roles.

The extensive metabolic studies of insecticide carbaryl in man and other species have been reviewed by Knaak (1971). However, the metabolism of carbaryl by rat liver perfusion has not been reported, in spite of the growing acceptance of the organ perfusion technique in metabolic studies. Therefore, the metabolic study of carbaryl by the liver perfusion technique was conducted. The liver perfusate was subjected to column chromatographic analyses and the results were compared with those obtained in vivo (Sullivan et al., 1972b) and in vitro (Sullivan et al., 1972a).

The designation of major metabolities of carbaryl in this study was based on chromatographic behavior and fluorescence characteristics rather than actual isolation and structural verification of the metabolites.

MATERIALS AND METHODS

The chromatographic profile technique reported by Knaak et al. (1965) as modified by Sullivan et al. (1972a,b) was used throughout the study.

In all the following studies, 1-[^{14}C]naphthyl *N*-methylcarbamate ([*naphthyl*-^{14}C]carbaryl) of specific activity 30 μCi/mg and 1-naphthyl-*N*-[^{14}C]methylcarbaryl ([*methyl*-^{14}C]carbaryl) of specific activity 11.3 μCi/mg were provided by Union Carbide Corporation Technical Center, South Charleston, WV.

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In Vivo Technique. An in vivo 24-h urine sample was obtained after peroral administration of [*naphthyl*-^{14}C]carbaryl to a 150-g male rat at a dosage of 30 mg/kg body weight (Sullivan et al., 1972b). The animal was placed in a glass metabolism cage which permitted the separate but simultaneous collection of urine, feces, and respiratory CO_2.

In Vitro Tissue Explant Technique. In all of the in vitro studies, a ratio of 100 μg of [*naphthyl*-^{14}C]- or [*methyl*-^{14}C]carbaryl/500 mg of tissue was used. This dosage level approximates the 2-year chronic no ill-effect feeding level of the compound to rats (Carpenter et al., 1961).

In brief, the method of preparation and incubation for the in vitro study was as follows: Trowell (1959) T8 medium was oxygenated by passing carbogen (95% oxygen/5% carbon dioxide mixture) at the rate of 100 mL/min through 100 mL of the medium in a 125-mL beaker for 30 min. Freshly oxygenated medium was used for each experiment. About 500 mg of fresh liver was cut aseptically into 2-mm cubes under oxygenated medium, rinsed twice with oxygenated medium, and transferred to a 60 × 15 mm petri dish containing 3 mL of the medium. The tissue was cut so that about one-half of its bulk was in the medium and the remainder exposed to the oxygen–CO_2 mixture. The petri dish was placed in a 9-L vacuum desiccator and the entire unit flushed with carbogen at 1.5 L/min for 30 min and then sealed. After 90 min at room temperature, the dessicator was opened and the tagged carbaryl was added to the dish, which was gently swirled to mix. Then the dish was returned to the chamber which was flushed again with carbogen, sealed, and placed in an incubator at 37 °C for 18 h.

At the end of the incubation period, the culture medium was removed and subjected to diethylaminoethyl (DEAE)–cellulose column chromatography (Sullivan et al., 1972b) to develop the profile. The three linear elution gradients for the column consisted of 0.005–0.01, 0.01–0.05, 0.05–0.1 N ammonium formate. Three hundred milliliters of each concentration was used per gradient. Four-milliliter fractions were collected and every fifth fraction was analyzed by a liquid scintillation counting technique. When greater resolution between peaks was required, every fraction was counted.

Liver Perfusion Technique. Perfusion of isolated livers from 200-g male Harlan-Wistar rats was carried out as described by Scholz et al. (1969). At the time of sacrifice the rats were injected intraperitoneally with 15 mg of

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aqueous pentobarbital. Livers were perfused with Krebs-Henseleit bicarbonate buffer (1932) containing 90 mg % glucose in a recirculating system. To 150 mL of buffer was added 3 mL of a penicillin–streptomycin mixture (catalog no. 12603 F) from Microbiological Associates, Bethesda, MD. The final concentration of the penicillin–streptomycin mixture in this buffer was 100 units/mL. Prior to the perfusion, the medium was passed through a 1.2-μm millipore filter to remove particles which might plug hepatic sinusoids. The perfusate was saturated with carbogen by use of a rotating disc oxygenator. A Sigma peristaltic pump was used to move perfusate through rubber-insulated polyethylene tubes from the oxygenator and then through a bubble and particle trap (nylon screen with 0.006-in. holes) to the liver. The perfusate at pH 7.4 was introduced into the liver at a rate of 32 mL/min via the portal vein and collected via a cannula in the inferior vena cava. Hepatic venous oxygen tension was monitored with a Beckman pO_2 electrode. The disc oxygenator was heated by an internal water jacket to maintain the hepatic venous effluent at 37 ± 0.5 °C.

After 20–30 min of equilibration, 10 mL of perfusate was removed from the system and used to dilute 60.3×10^6 dpm (1.21 mg of carbaryl) of [*naphthyl*-^{14}C]carbaryl which was previously solubilized in 690 mg of Carbowax 400. The resulting solution was then added back to the oxygenator over a time period corresponding to the total perfusate volume divided by the flow rate (5 min for 150 mL) in order to assure uniform distribution of the chemical in the perfusate. A ratio of 200 μg of carbaryl/g of tissue was used and the final concentration of carbaryl was 3.9×10^{-5} M. At various times after introduction of the carbaryl, 5-mL samples of perfusate were removed and immediately frozen for later analysis.

During the perfusion, the status of the liver was monitored by recording the venous oxygen tension. Since the flow rate was constant and the arterial oxygen tension was constant, the venous oxygen tension was inversely proportional to the hepatic oxygen consumption rate. The oxygen consumption rate was constant before and after the addition of the carbaryl.

The nonenzymatic hydrolysis of carbaryl at pH 7.4 during 5 h of perfusion was determined under the same experimental conditions without liver, using 27.4×10^6 dpm (0.55 mg of carbaryl) of [^{14}C]carbaryl which was dissolved in 310 mg of Carbowax 400.

The liver perfusate was subjected to DEAE–cellulose column chromatography to develop the carbon-14 metabolic profile. The elution gradients for the column and the analysis of the fractions was the same as for in vitro samples. Fractions of peak D from DEAE–cellulose perfusate were subjected to β-glucuronidase treatment (Sullivan et al., 1972b). The reaction mixture was incubated in the dark for 4 h at 38 °C in a water bath with constant stirring. Then the reaction mixture was extracted with ether overnight and the ether extract concentrated to dryness for thin-layer chromatography.

Thin-Layer Analysis. When the perfusate or incubated medium was chromatographed on a DEAE–cellulose column, materials chromatographing within one or two void volumes of the column were designated as "neutrals". These neutral fractions (peaks A and B) were combined and continuously extracted with diethyl ether using Soxhlet apparatus. The ether phase was concentrated and dissolved in 0.5 mL of acetone and 10–20 μL of this solution was applied to a silica gel G thin-layer plate (Brinkman 5 × 20 cm precoated plates). Chromatography was carried out in 4:1 (v/v) ether–hexane (Dorough and Casida, 1964) or in a 1:1 (v/v) isopropyl ether–acetonitrile by a vertical elution. For tentative identification purposes, 1-naphthol, carbaryl, and 4- and 5-hydroxycarbaryl were spotted on either side of the ^{14}C-labeled sample and chromatographed simultaneously in the 4:1 (v/v) ether–hexane system. Authentic 5,6-dihydro-5,6-dihydroxycarbaryl was used as a standard with the 1:1 (v/v) isopropyl ether–acetonitrile system. When sufficient radioactivity (in excess of 5000 dpm) was available, the plates were scanned on a Nuclear Chicago Actigraph III. When lesser activity was present, radioactive materials on the plate were located by removing successive 0.5-cm strips for direct counting in scintillation vials. The standards were spotted next to the unknown material and only the section containing the radioactivity was removed for counting. After removal of the labeled materials, the remaining nonradioactive compounds were located using an aqueous spray reagent composed of 5.0 g of potassium dichromate in 100 mL of 40% (v/v) sulfuric acid.

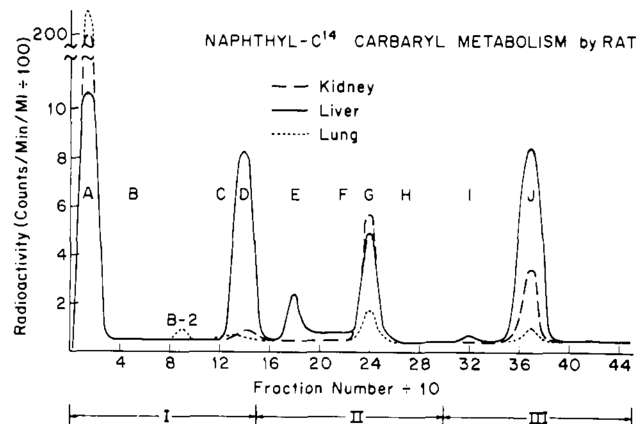

Figure 1. DEAE–cellulose chromatogram of [*naphthyl*-^{14}C]-carbaryl metabolism by kidney, liver, and lung of the rat: (- -) kidney, (—) liver, and (···) lung. Ammonium formate (pH 6.5) gradient elution program: (I) 0.005–0.01 N, (II) 0.01–0.05 N, and (III) 0.05–0.1 N.

RESULTS

Quantitative data obtained from the metabolic profile analyses of carbaryl using the tissue-explant technique for kidney, liver, and lung of the rat are summarized in Table I for the naphthyl and methyl labels. Peaks A and B are neutral to the DEAE–cellulose anion-exchange system and no conjugates have been found in this area. Peak B-1, B-2, C, and E are unknown weakly acidic metabolites. Peak D was determined (Sullivan et al., 1972b) to be a group of metabolites, the major component of which was the 5,6-dihydro-5,6-dihydroxycarbaryl glucuronide. Peak F has been shown to be glucuronides of ring hydroxylated carbaryls, while peak G is naphthyl glucuronide (Knaak et al., 1965; Knaak and Sullivan, 1967). Peaks I and J are sulfate conjugates of ring hydroxylated carbaryls and 1-naphthol, respectively (Knaak et al., 1965; Knaak and Sullivan, 1967).

Carbaryl metabolism by rat liver, in vitro, qualitatively and semiquantitatively reproduced previous in vivo results (Knaak and Sullivan, 1965; Sullivan et al., 1972b). Based upon the total anionic metabolites formed from each tissue using naphthyl-^{14}C, the quantitative metabolic activity in descending order was liver, kidney, and lung. Figures 1 and 2 are chromatographic profiles for the kidney, liver, and lung of the rat using naphthyl-^{14}C and methyl-^{14}C labels, respectively, of carbaryl. As shown in Table I, all tissues produced peaks A, D, G, and J with [*naphthyl*-^{14}C]carbaryl. Formation of peaks G and J shows that all

Table I. Comparison of in Vitro Metabolites of Carbaryl by Kidney, Liver, and Lung of the Rat[a]

tissue	A neutrals to DEAE	B	B-1	B-2 U.K.[b]	C	D dihydrodihydroxycarbaryl glucuronide, major aglycon	E U.K.[b]	F hydroxycarbaryl glucuronide	G naphthyl glucuronide	I hydroxycarbaryl sulfate	J naphthyl sulfate	pooled fractions[c]
						[*naphthyl*-^{14}C]Carbaryl						
kidney	80	0	0	0	0	1.3	0	0	8.4	0	5.0	1.4
liver	26	0	0	0	0	19	4		7.5	0.4	20	6
lung	93	0	0	0.9	0	0.4	0	0	3.4	0	0.9	1.3
						[*methyl*-^{14}C]Carbaryl						
kidney	80	0	0	0		4.8	0	0	0	0	0	2.5
liver	51	0	0	0		17	0	0.8	0	2	0	5
lung	84	0	2.0	0	0	6.3	0	0	0	0	0	3.1

[a] Calculated as percent of ^{14}C applied on the column. [b] U.K., unknown. [c] Combined fractions from areas of chromatogram not containing a distinguishable peak.

Table II. Carbaryl Metabolites: Rat Liver Perfusion[a]

perfusion time, h	A B neutrals to DEAE	C U.K.[b]	D H_2Hy_2-carb-GlcU,[d] major aglycon	E U.K.[b]	F Hycarb-GlcU[d]	G naph-GlcU[d]	H U.K.[b]	I Hycarb sulfate[d]	J naphthyl sulfate	K	L U.K.[b]	M	pool fraction[c]
					Perfusion without Albumin								
0.5	36.9	0	20.7	4.2	1.5	2.2	0.6	2.2	14.6	7.5	0	0	4.8
1.0	24.9	0	27.0	5.3		4.5	0.5	2.2	11.6	5.7	0	0	7.4
3.0	19.9	0	24.1	7.8		11.3	0	2.0	9.5	6.1	2.4		6.9
5.0	17.0	0	19.9	9.6		9.6	0	0.9	9.1	2.1	1.5	1.4	8.7
					Perfusion with Albumin								
3.0	8.0	0	22.0	4.3	6.8		0	4.5	13.8		39.3		1.2
7.0	12.0	0	14.8	6.1	5.2		0	2.6	14.4		42.1		2.7
					Control without Liver								
5.0	91.1	0	0	0	0	0	0	0	0	0			8.9

[a] Calculated as percent of ^{14}C applied on the column. [b] U.K., unknown. [c] Combined fractions from areas of chromatogram not containing a distinguishable peak. [d] H_2Hy_2-carb-GlcU, dihydrodihydroxycarbaryl glucuronide; Hycarb-GlcU, hydroxycarbaryl glucuronide; naph-GlcU, naphthyl glucuronide; Hycarb, hydroxycarbaryl sulfate.

the rat tissue studied hydrolyzed the carbaryl and then conjugated the hydrolysis product to form the sulfate as well as the glucuronide. Formation of peak D in the liver is significantly higher than that found in either the kidney or lung. Hydroxylation of carbaryl and sulfate conjugation of ring hydroxylated carbaryl was accomplished only in the liver, as evidenced by the production of peak I by this tissue. Unknown peak E was made by the liver, whereas unknown peak B-2 was produced by the lung.

Table I data using methyl-^{14}C indicate that all tissues produced the major metabolite peaks A and D. Larger values were obtained for peak A with the kidney and lung tissues than with the liver. Minor peaks F and I were produced only by the liver. Peak B-1 with methyl-^{14}C was formed only by the lung. Peak C, not resolved from peak D, was found in both the liver and kidney.

Figure 3 illustrates the DEAE–cellulose chromatogram of [*naphthyl*-^{14}C]carbaryl metabolites by 1-h perfusion of rat liver. Total radioactivity recovered in the perfusate was 84%. The quantitative results obtained from the metabolism of [*naphthyl*-^{14}C]carbaryl by liver perfusion at various times after introduction of the carbaryl are given in Table II. These data are given as percent of radioactivity applied on the column. The identity of the peak component in Table II is the same as in vitro rat liver

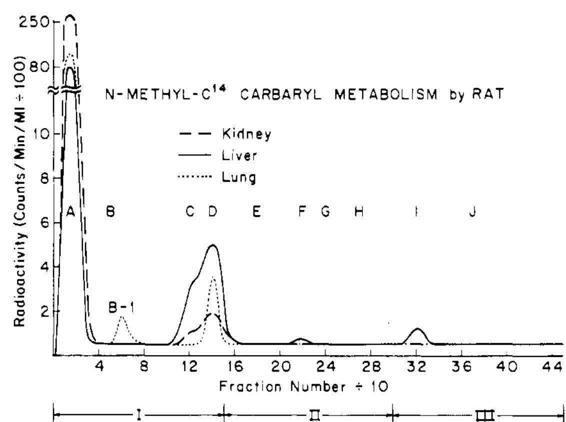

Figure 2. DEAE–cellulose chromatogram of [*methyl*-^{14}C]carbaryl metabolism by kidney, liver, and lung of the rat: (- -) kidney, (—) liver, and (···) lung. Ammonium formate (pH 6.5) gradient elution program: (I) 0.005–0.01 N, (II) 0.01–0.05 N, and (III) 0.05–0.1 N.

studies. A time dependence study showed that the percentage of neutrals, hydroxycarbaryl sulfate, naphthyl sulfate, and peak K all decreased with perfusion time, while the percentages of hydroxycarbaryl glucuronide,

Table III. Carbaryl Metabolites of Rat via Liver Perfusion, In Vitro Liver (Tissue Explant) and In Vivo[a]

	tentative identification by chromatographic position										
	A B	C	D	E	F	G	H	I	J	K	
exper. condit.	neutrals to DEAE	U.K.[b]	H_2Hy_2-carb GlcU,[d] major aglycon	U.K.[b]	Hycarb-GlcU[d]	naph-GlcU[d]	U.K.[b]	Hycarb sulfate[d]	naphthyl sulfate	U.K.[b]	pool fraction[c]
liver perfusion (1 h)	24.9	0	27.0		5.3	4.5	0.5	2.2	11.6	5.7	7.4
in vitro (18 h)	25.2	0	22.0		4.4	6.4	0	Tr	19.8		6.4
in vivo (24 h urine)	11.5	0	37.2		6.8	11.3	0	6.0	9.4	4.0	

[a] Calculated as percent of ^{14}C applied on the column. [b] U.K., unknown. [c] Combined fractions from areas of chromatogram not containing a distinguishable peak. [d] H_2Hy_2-carb-GlcU, dihydrodihydroxycarbaryl glucuronide; Hycarb-GlcU, hydroxycarbaryl glucuronide; naph-GlcU, naphthyl glucuronide; Hycarb sulfate, hydroxycarbaryl sulfate.

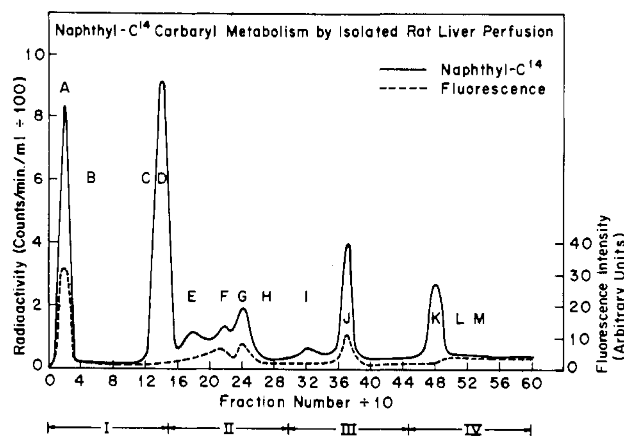

Figure 3. DEAE–cellulose chromatogram of [*naphthyl*-^{14}C]-carbaryl metabolism by isolated rat liver perfusion: (—) naphthyl-^{14}C and (- -) fluorescence. Ammonium formate (pH 6.5) gradient elution program: (I) 0.005–0.01 N, (II) 0.01–0.05 N, (III) 0.05–0.1 N, and (IV) 0.1–0.5 N.

naphthyl glucuronide, and peaks E, L, and M increased. The percentage of dihydrodihydroxycarbaryl glucuronide increased at 1 h and declined thereafter.

In another experiment when 4 g of bovine serum albumin (Pentex, Fr.V. powder; dialyzed Krebs-Henseleit buffer) was added to the 100 mL of perfusion medium, separation of peaks I and J and also peaks K, L, and M was poor. Total radioactivity recovered in the perfusate was 78%. Quantitatively, a slightly higher value was found for peak D after 3 h than in the 7-h perfusate. The percentage of radioactivity in peaks L and M from the perfusate with albumin was approximately five times greater than without albumin. This is believed to be an analytical artifact, due to the interference of albumin with the chromatography, rather than a metabolic difference. Based upon the profile analysis of the perfusates in Table II, it would appear that a 1-h perfusion without albumin is adequate for the quantitative study of anionic metabolites.

Table III shows the comparative metabolism of carbaryl by liver perfusion, in vitro liver explant (Sullivan et al., 1972a), vs. an in vivo 24-h urine sample (Sullivan et al., 1972a) using [*naphthyl*-^{14}C]carbaryl. Radioactivity in the neutral fractions from the 1-h liver perfusion and from the 18-h in vitro incubation of tissue amounted to approximately twice the in vivo results.

The percentage of ^{14}C found in the major metabolite (peak D) and all minor metabolites (peaks E, F, G, I, and J) in perfusate, growth medium, and 24-h urine agreed semiquantitatively (Table III). Naphthyl sulfate produced after 18-h in vitro incubation of tissue was significantly higher than that of perfusate.

The disagreement between the explant and perfusion values would be minimized if proper correction factors were applied to account for nonenzymatic hydrolysis (Chin et al., 1974). The reported nonenzymatic hydrolysis of carbaryl occurred to the extent of 12.6% at pH 7.2 with incubation at 37 °C for 18 h.

DISCUSSION

The designation of major metabolites of carbaryl in this study was based on the chromatographic behavior and fluorescence characteristics rather than the actual isolation and structural verification of the metabolites. Based upon total anionic metabolites formed from each tissue using [*naphthyl*-^{14}C]carbaryl, the quantitative tissue metabolic activity in descending order is liver, kidney, and lung. The comparative metabolic profiles from selected organs clearly show that the metabolic products found in vivo are not necessarily limited to liver-generated metabolites but rather that they represent a composite of metabolites formed from the liver, lung, and the kidney and possibly from other organs as well. The investigation of metabolic activity of individual organs for a given compond can be important in the interpretation of toxicological effects as they relate to a target organ.

The pharmacodynamic parameters of both the perfusion and the tissue-maintenance techniques cannot be delineated currently. The liver perfusion technique, as utilized in this study, does not reproduce certain aspects of the in vivo system such as biliary excretion, and the effects of blood serum components, namely, albumin, vitamins, and endocrine hormones. Because of the difficulties of acquiring whole viable human liver for perfusion we must rely on use of tissue maintenance for comparative metabolism. The latter approach has been utilized as a means for comparative metabolism among fish species (Sullivan et al., 1973). Additional research applications include sequential organ metabolism, the effect of the metabolic fate of one chemical upon another, and the contribution of organs other than the liver on total metabolism. The metabolic rate data of the tissue-maintenance technique would be valid only in the case where transfer of the test chemical and its metabolites by diffusion was not the rate-limiting factor.

Because the in vitro tissue-maintenance techniques faithfully reproduce the metabolites found in 24-h urine samples of similarly dosed animals and is rapid, simple, and reproducible (Sullivan et al., 1972a), it offers great promise as a method to determine metabolic pathways in man without resorting to direct dosing of human subjects.

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Chlorinated Hydrocarbon Insecticide Residues in Tissues of Rats Before and After Reduction of Body Fat by Dietary Restriction

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Male weanling rats were fed ad libitum until 250 days of age one of three nutritionally adequate diets with or without the addition of 2.80 ppm of a chlorinated hydrocarbon insecticide (CHI) mixture. The mixture comprised DDT, TDE, DDE, lindane, dieldrin, and perthane. Between 251 and 300 days of age, half of the rats in each group were restricted to 50% of their ad libitum food intake. Concentration and total amounts of each insecticide were determined in adipose tissue, liver, and brain. Deposition, and mobilization during weight reduction, of insecticides varied with diet and tissue examined. When food intake was reduced, total DDT amount did not change in the three tissues regardless of diet. However, total amounts of DDT metabolites and dieldrin were altered with reduced intake. Differences associated with diet occurred primarily in adipose and liver tissues. Total DDT amount was not affected by diet.

Although the general use of chlorinated hydrocarbon insecticides (CHI) is prohibited in several countries, the persistence of these compounds in the food chain and their use on a limited scale in agriculture indicate the need for continued research on their effects at low levels. Surveys have shown that all the chlorinated hydrocarbon insecticides are present in humans, but their concentrations vary among tissues (Kutz et al., 1974) and are affected by the physiological status of the tissue (Radomski et al., 1968; Oloffs et al., 1974). The food supply has been the major source of insecticide contamination for the human population as indicated by the report of Duggan and Corneliussen (1972) on typical human diets. Deposition, accumulation, and mobilization of individual chlorinated hydrocarbon insecticides have been examined extensively with the rat as an experimental model. However, information is limited on the effects of feeding two or more insecticides for an extended time (Adams et al., 1974). The use of a mixture of CHI provides a realistic approach for the study of the effects of diet on storage of CHI in the body. For a variety of reasons many individuals are subjected to dietary restriction at intervals during their lives and information is sparse on the fate of a mixture of insecticides in the body during weight reduction. The present report provides data on the influence of diet on the deposition of several chlorinated hydrocarbon insec-

Table I. Composition of Diets[a]

high-fat diet (HF-L)	low-fat diet 1 (LF-PB-BS)	low-fat diet 2 (LF-BS-CO)
20.0% casein	12.8% casein 6.4% lactalbumin	27.0% casein
69.2% lard	6.4% beef suet 20.0% peanut butter	2.0% corn oil 14.0% beef suet
0.0% carbohydrate	40.4% sucrose	50.0% sucrose
1.4% vitamin mix[b]	8.0% yeast	1.0% vitamin mix[b]
0.2% methionine		
0.2% additional choline		
7.0% salt mixture[c]	4.0% salt mixture[c]	4.0% salt mixture[c]
2.0% celluflour	2.0% celluflour	2.0% celluflour

[a] In addition, each animal received a weekly dose of percomorph liver oil containing about 3000 units of vitamin A, 400 units of vitamin D, and 36 mg of α-tocopheryl acetate (in 0.01 mL of cottonseed oil). [b] The vitamin mix provides the following amounts per kilogram of final diet: 4.0 mg of menadione, 10.0 mg of thiamin hydrochloride, 10.0 mg of riboflavin, 5.0 mg of pyridoxine hydrochloride, 60.0 mg of niacin, 40.0 mg of calcium pantothenate, 300.0 mg of choline, 0.4 mg of vitamin B_{12}, 0.4 mg of folic acid, 200.0 mg of *p*-aminobenzoic acid, and 400.0 mg of inositol. [c] Jones and Foster (1942).

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