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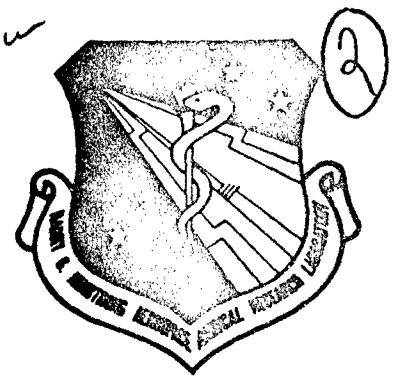

ASSESSMENT OF THE BEHAVIORAL AND NEUROTOXIC EFFECTS OF HEXACHLOROBENZENE (HCB) IN THE DEVELOPING RAT

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DECEMBER 1990

FINAL REPORT FOR THE PERIOD MAY 1989 TO DECEMBER 1990

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TECHNICAL REVIEW AND APPROVAL

AAMRL-TR-90-076

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

JAMES N. McDOUGAL, Maj, USAF, BSC
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Harry G. Armstrong Aerospace Medical Research Laboratory

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	DECEMBER 1990	FINAL, May 1989 - December 1990

4. TITLE AND SUBTITLE
ASSESSMENT OF THE BEHAVIORAL AND NEUROTOXIC EFFECTS OF HEXACHLOROBENZENE IN THE DEVELOPING RAT.

5. FUNDING NUMBERS
Contract F33615-85-C-0532
PE 62202F
PR 6302
TA 630202
WU 630202D2
Accession #316095

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
NSI Technology Services Corporation
101 Woodman Drive, Suite 12
Dayton, OH 45431

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
AAMRL, Toxic Hazards Division
HSD, AFSC
Wright-Patterson AFB, OH 45433-6573

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
AAMRL-TR-90-076

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Hexachlorobenzene (HCB) is a widespread environmental contaminant. Due to its highly lipophilic nature, HCB is stored in the body adipose tissue and is released with the milk during lactation. Female Sprague-Dawley rats were given 0 (control), 10, or 100 mg HCB/kg body weight, and dosing was completed two weeks prior to breeding. We evaluated the gestational and lactational transfer of HCB from the dams to fetuses and pups and determined that HCB is present in the developing rat brain. Throughout gestation, the HCB tissue concentrations for the 10 and 100 mg HCB/kg body weight groups differed by 10 fold. The maternal body burden of HCB was quickly depleted by lactational transfer of the HCB to the suckling pups as reflected by HCB concentrations in the milk and pups. However, across treatment groups, only a 2-3 fold difference existed between tissue concentrations of HCB in both dams and pups during lactation. Subsequently, we assessed the developmental neurotoxicity of HCB using a battery of behavioral tests. The negative geotaxic response and olfactory homing were assessed in two male and two female pups from each litter between 6 and 11 days of age. The development of exploration and locomotion was assessed in whole litters 14-21 days of age. Acoustic startle response was assessed in both young and mature offspring. Learning (swim T-maze), exploratory and locomotor activity were assessed in mature offspring. The significant effects observed in the (continued)

14. SUBJECT TERMS	15. NUMBER OF PAGES
Hexachlorobenzene; lactation; gestation; developmental neurotoxicity; behavioral teratogen	40
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
UNCLASSIFIED	UNCLASSIFIED	UNCLASSIFIED	UL

13. Abstract (Continued)

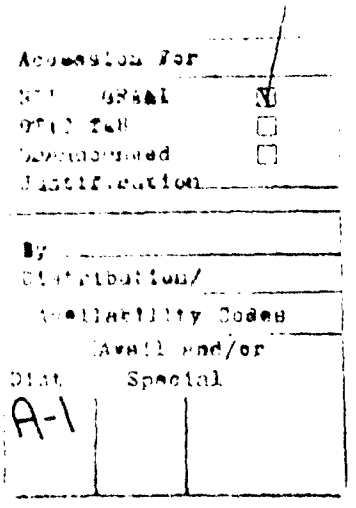
negative geotaxis and olfactory homing tests suggest hyperactivity in exposed young pups. Testing of mature offspring revealed significant effects only in the acoustic startle procedure indicating that HCB causes a reduced motor response. This study reveals the importance of testing more than one animal per litter, of using litter as the smallest unit of testing, and of following individuals throughout development. Our results demonstrate that HCB is a behavioral teratogen, and that the prebreeding dosing protocol used here may find wide applicability in teratological assessments of similar halogenated compounds. This work suggests that human fetuses and suckling infants may be at risk from HCB.

PREFACE

The research described in this report was completed and reported by Miami University (Oxford, OH) under subcontract to NSI Technology Services Corporation in support of the Toxic Hazards Research Unit (THRU) research program under Air Force Contract Number F33615-85-C-0532. The THRU is a contractor-operated effort of the Toxic Hazards Division of the Harry G. Armstrong Aerospace Medical Research Laboratory located at Wright-Patterson Air Force Base, OH. The research covered in this report was begun in May 1989 and was completed in December 1990. The research reported herein was sponsored by the U. S Air Force. During the initiation and conduct of this research Lt Col Harvey J. Clewell, III; Lt Col Michael B. Ballinger; and Maj James N. McDougal served consecutively as the Contract Technical Monitor for the U. S. Air Force, Harry G. Armstrong Aerospace Medical Research Laboratory

The opinions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Air Force The use of trade names in this report does not constitute an official endorsement or approval of the use of such commercial hardware or software. This report may not be cited for purposes of advertisement.

The animals used in this study were handled in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Uses of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services, National Institute of Health Publication #85-23, 1986, and the Animal Welfare Act of 1966, as amended.



Introduction

Humans are exposed to polychlorinated hydrocarbons, such as hexachlorobenzene (HCB), due to the ubiquitous presence of these chemicals in the environment. An estimated 4130 tons of hexachlorobenzene (HCB) are generated annually in the U.S. as a by-product or contaminant (Jacoff et al., 1986); 77% of this is generated in the production of carbon tetrachloride, perchloroethylene, and trichloroethylene (Menzie, 1986), and as an impurity in numerous other pesticides (Tobin, 1986).

The major route for human exposure to HCB is ingestion and absorption from the gastrointestinal tract, and it is very slowly eliminated from the body, primarily as the parent compound, in the faeces (Matthews, 1986). Little HCB is excreted in the urine since HCB binds to blood proteins and is not subject to glomerular filtration (Matthews, 1986). HCB is not readily metabolized and, over the lifetime of the individual, it may accumulate to relatively high levels in the body fat. HCB was detected in nearly 100% of the human adipose tissue samples collected from the U.S. population from 1970-1983 (Mack and Mohadjer, 1985). Other recent surveys of human adipose tissue samples from the US and Spain indicated average HCB concentrations of 53 ppb and 5550 ppb respectively, suggesting that certain populations may be at greater risk from the chemical than others (Robinson et al., 1986; To-Figueras et al., 1986).

While HCB remains sequestered in the adipose tissue of an adult, toxicity to the organism may be low. In rodents the only effective means of reducing HCB body burden is via lactation (Matthews and Anderson, 1975). As the body fat is broken down in the production of milk fat, the residues are released from the body in the milk (Poitrast et al., 1986). Numerous reports have documented the presence of polychlorinated compounds, including HCB, in the milk of nursing mothers (Jensen, 1983 review).

Although HCB is primarily stored in the adipose tissue of the adult animal, HCB remains in dynamic equilibrium with other organs via the blood. In humans, HCB levels in fetal cord blood were higher than maternal blood levels indicating a preferential placental transfer of the compound (Bush et al., 1984). The physiological changes occurring during gestation may cause an increase in the amount of HCB circulating in the blood. 2,4,5,2',4',5'-Hexachlorobiphenyl (6-CB) has been shown to associate with very low density lipoproteins (VLDL) which become elevated to facilitate milk triacylglycerol synthesis during late pregnancy (Spindler-Vomachka and Vodicnik, 1984). Therefore, the equilibrium may shift away from adipose stores to the circulation at this time. Thus, human offspring may be at risk not only due to the lactational transfer of the compound with the milk to the suckling infant, but also due to transplacental passage of the HCB via the mothers blood.

Developing organisms are more susceptible to the effects of HCB than adults since their lower body fat allows HCB to circulate in the blood for long periods, and target tissues may be more susceptible to the effects of the chemical before they are fully developed (Weisenberg, 1986). Ethological methods provide an objective tool for evaluating the effects of a wide range of toxicants at doses often below those of other methods utilized in toxicology (Silverman, 1988), and behavioral teratology testing is a useful method for determining nervous system toxicity in the developing organism (Sobotka and Vorhees, 1986). Behavioral disorders in both humans and laboratory animals exposed to HCB have been noted by researchers (Gocmen et al., 1986; Courtney, 1979, Kuiper-Goodman et al., 1979) although no objective assessment of the neurobehavioral teratology of HCB has been made. Many lipophilic chemicals easily cross the blood-brain barrier, especially before this barrier is fully developed. Since existing evidence indicated that HCB may be a neurotoxin, the current study was performed to objectively evaluate this possibility. Prior to assessing the neuroteratology of HCB, it was important to understand the disposition of the chemical

in the body of the pregnant and lactating animal and the accumulation and disposition of the compound in the offspring.

Methods

Virgin Sprague Dawley rats were purchased from Harlan Sprague Dawley, Columbus, Ohio. HCB dissolved in corn oil was administered by gastric intubation at a daily volume of 2 ml/kg at dose levels of 0, 2.5, and 25 mg HCB/kg body weight. Female rats were dosed for 4 days to achieve a total dose of 0, 10 and 100 mg HCB/kg body wt. Two weeks following dosing, the rats were pair bred, and the presence of a sperm plug denoted Day 0 of gestation. The HCB was obtained from Aldrich, and had a purity not less than 99%.

Hexachlorobenzene was extracted from the tissues by homogenizing the tissue in 10 vol. of hexane, centrifuging the homogenate and assaying the hexane layer of each sample in duplicate by direct injection onto a Hewlett Packard (5790 series) gas chromatograph equipped with a capillary column and a ^{63}Ni electron capture detector. HCB was not detected in tissues from control animals. Extraction efficiencies determined for all tissue type samples spiked with 100 ug HCB/g tissue were on average 91%.

Blood, fat, liver, brain and kidneys were collected from groups of three or four HCB treated dams on each of gestation days 9, 15 and 20 and on post-natal days 4, 7, 10 and 14. Milk samples were collected from the dams during lactation. During gestation, amniotic fluid, placentas and whole fetuses were collected for analysis. Additionally, brain, blood and liver tissues were collected from 20 day-old fetuses. Amniotic fluid, whole 9-day fetuses, and blood samples from 20 day fetuses were pooled within a litter for analysis, although all other tissues were analyzed separately, and a mean concentration value for each tissue was determined.

Blood, liver, brain and kidneys were collected from four pups from each dam sacrificed during lactation. Tissues from pups were assayed individually, and a mean concentration per litter was determined for each tissue. Tissues were also collected from the four remaining pups in each litter after aortic perfusion with saline to remove blood. In this way it was possible to determine how much HCB was present in the tissue itself, and how much HCB was in the blood circulating through the tissue.

Additionally, two groups of six female rats were given a total dose of either 10 or 100 mg HCB/kg body weight over four days as described above. Uptake of HCB from the gastrointestinal tract into the blood was determined from blood samples collected 30 min, 1 h, 2 h, 3 h, 4h, and 24h after the initial dose, and at 1 h, 4 h and 24 h intervals following dosing on subsequent days. Blood was collected from the tail vein of each rat after immobilizing the animal in a plastic restraint cylinder. A heparinized microhematocrit capillary tube was fitted into the base of a 25-gauge disposable needle, and the needle was inserted into the lateral vein of the tail. Blood filled the tube by capillary action (70 ul). Samples were taken from distal to proximal locations along the length of the tail.

Behavioral Testing Battery

Additional animals were exposed to HCB by the same dosing protocol described above and subjected to different tests in the following battery in a longitudinal design. The test battery utilized in this study consisted of seven tests selected to explore the effects of HCB on reflex function, locomotor activity, exploratory activity and learning. An attempt was made to investigate reflex response and activity patterns in both young animals and mature animals to determine if abnormal behaviors seen in young animals persisted into adulthood or, conversely, if abnormal brain function did not manifest itself until animals had reached some critical age (Johnson, 1978). Learning was assessed at only one age.

The negative geotaxis reflex response was tested in pups 6, 8 and 10 days of age. In this test a pup was placed in the downward position on a 25° incline plane. The time taken for the animal to turn 180° to reach the upward position was recorded. Two male and two female pups from a litter were tested individually. For this and the following two tests, treatment effects were determined by a repeated measures Analyisis of Varience (ANOVA) and mean separations by the Duncan New Multiple Range test. In all tests litter was the smallest unit of analysis, and a mean response per litter was utilized when pups within a litter were tested separately.

Olfactory discrimination/homing was assessed in pups 9, 10 and 11 days of age (Fig. 1). Bedding from the pups home cage was placed in one end of a clear cylindrical tube 1 m long, and clean bedding was placed in the other end. Perforated partitions retained the bedding samples in the ends of the apparatus. Air was pumped into both ends of the tube at a rate of 10 ml/min. A centrally located hole in the top of the tube allowed for the air to exit the tube, and a pup was placed into the center of the apparatus through this hole. Two male and two female pups were tested per litter. A correct response was defined as a pup successfully reaching the end of the cage containing bedding from the pup's home cage. The time to a correct response was recorded.

The development of exploratory and locomotor activity was monitored using an apparatus modified from Crofton et al. (1980) (Fig. 2). The cage is divided into two sections by a partition perforated with three holes that are large enough to allow the passage of pups between the sections, but too small to allow the dam to move through. At day 14 post-partum the dam and her litter were placed in the section containing bedding, food and water. As the pups moved back and forth between sections, their passage through the holes broke an infrared beam transecting the holes, and the event was recorded automatically by an Apple 2e computer. Activity was recorded continuously for seven days. Activity counts increased over the duration

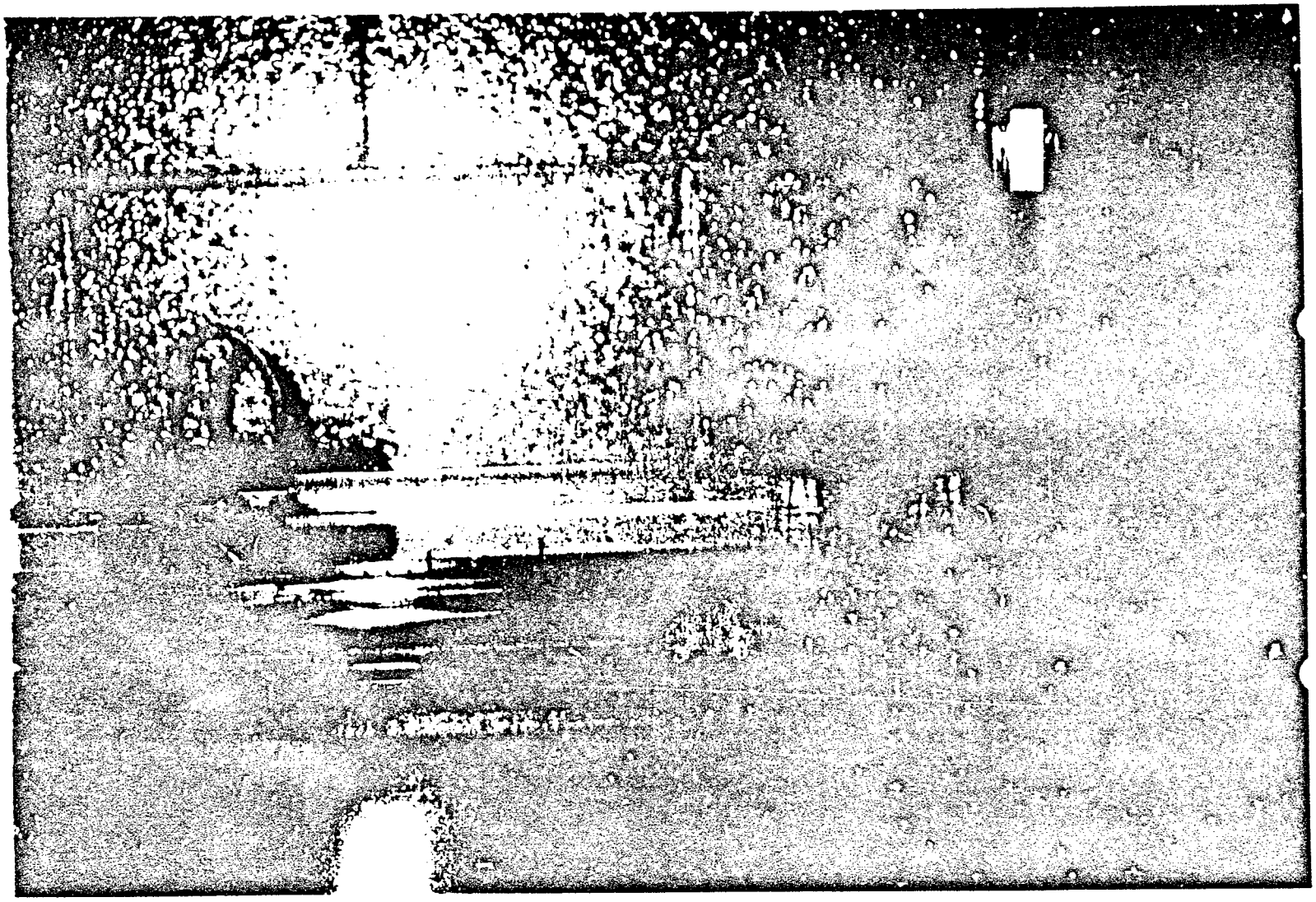


Figure 1: Olfactory homing apparatus. Bedding from the pup's home cage was placed in one end of the apparatus, and clean bedding was placed in the other end. A constant stream of air (10 ml/min) flowed from both ends. An animal was placed in the center of the apparatus, and the time for the pup to reach the home cage bedding was recorded. Two male and two female animals/litter were individually tested on post natal days 9, 10 and 11.

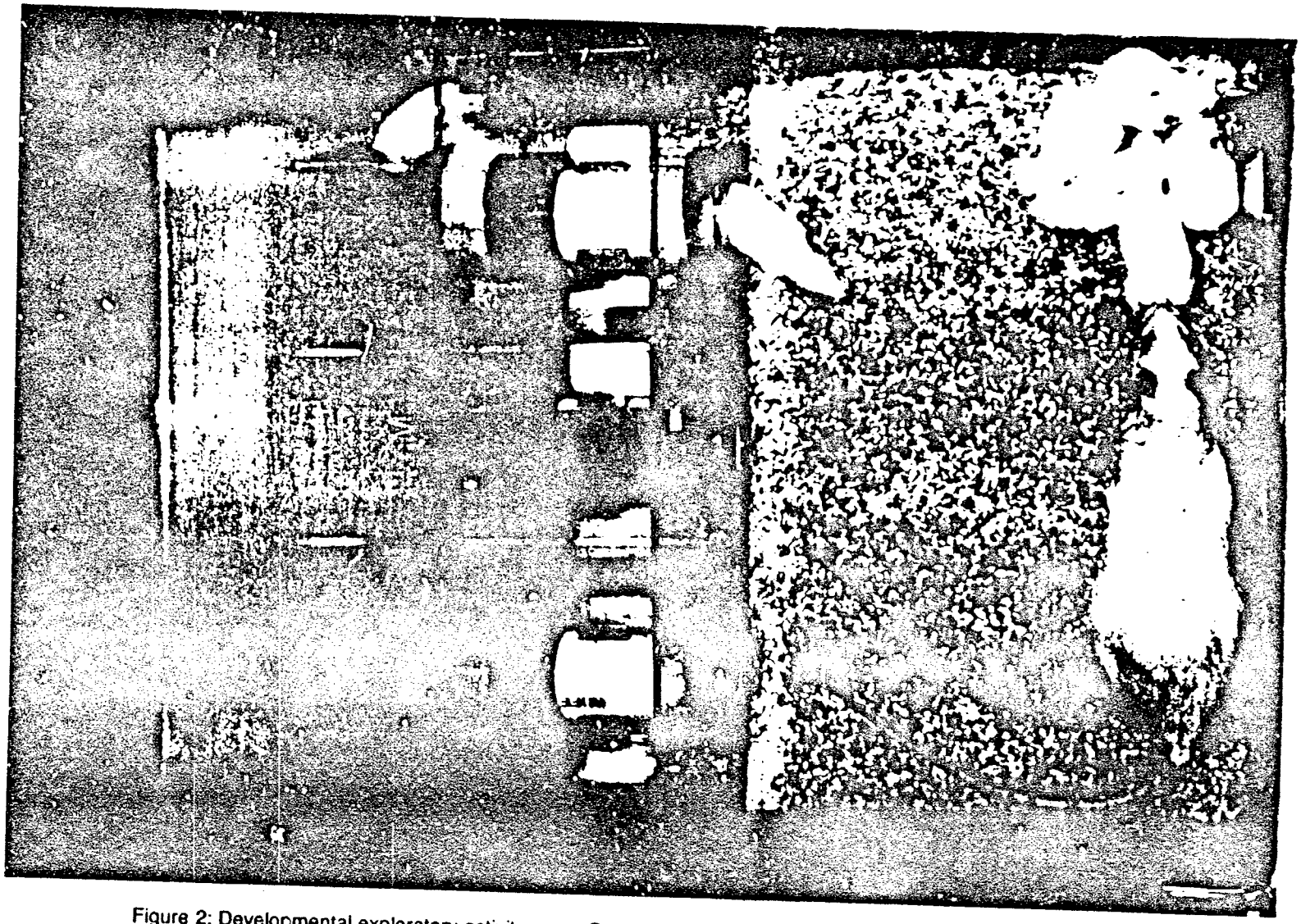


Figure 2: Developmental exploratory activity cage: On post-natal day 14, the dam and her litter were placed in one side of the cage with bedding, food and water. Three short tunnels through a central partition allow the movement of pups between the sections while restricting the dam to one side. The pups moved through the tunnels, interupting an infrared beam, an event recorded automatically by a computer. The data were collected continuously until the pups were 21 days of age.

of the test as pups moved away from the dam to explore their surroundings. Pups were weaned at the termination of this experiment at 21 days of age. The data were collapsed over the seven days and were subjected to a log + 1 transformation.

Locomotor activity was assessed in mature offspring, sixty days of age. This apparatus consists of an open area (60 cm X 50 cm) transected by 3 infrared beams evenly spaced at 15 cm intervals (Fig. 3 "B"). Two male and two female rats from each litter were tested individually in the apparatus for a period of 1 h. As the animal moved in the cage and broke the light beams, and event was recorded by an Apple 2e computer. This test evaluated overall activity and time to acclimation of animals placed in a novel environment.

Adult animals, 100 days of age, were tested for exploratory behavior in an apparatus 30 cm X 50 cm in which a rat explores any of 4 ports which are bisected with infrared beams (Fig. 3 "A"). Cups at the base of these ports contained clean bedding to provide an olfactory stimulus for exploration. Two male and two female rats from each litter were tested for a period of 15 minutes. Previous experience in our laboratory indicated that virtually all animals cease exploration after this time period.

Learning was assessed in 40 day-old animals using a water filled T-maze modified from Lochry et al. (1985) (Fig. 4). The pup was placed in the water at the stem of the maze facing away from the choice point. The pup was required to swim to one of the two goals where a submerged escape ramp was located. If, for example, on the first trial the animal chose the left goal, the left escape platform was removed and the right goal was the required response on subsequent trials. The pup was tested until a criterion of 5 errorless trials had been reached, or a maximum of 20 trials. The data were scored for the number of trials required to satisfy a learning criterion of either 5 consecutive errorless trials, 4 consecutive errorless trials, or three consecutive errorless trials. The latency for each trial (time to swim to the escape platform) was

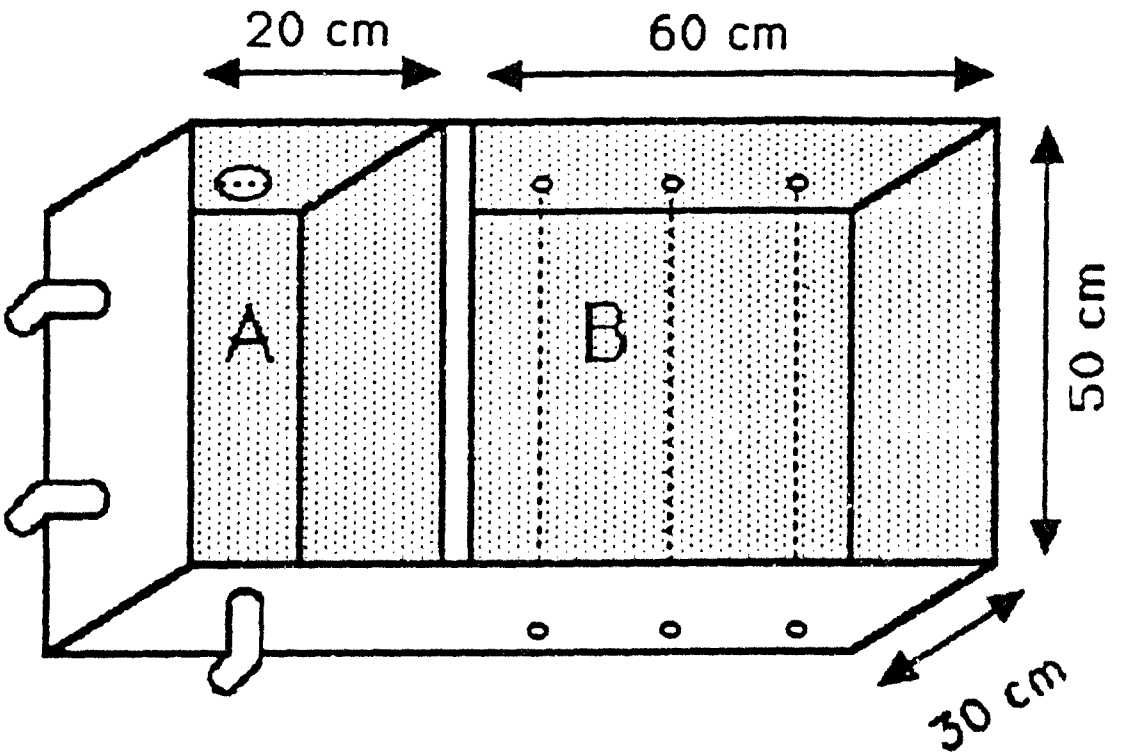


Figure 3: Locomotor and exploratory activity box. Two male and two female offspring/litter were tested individually in each side of this apparatus. Exploratory activity was assessed in compartment "A". In this alcove four exploratory ports are bisected with infrared beams. A rat explores these ports by inserting its head into the opening, breaking the beam. Exploratory activity was assessed for 15 min in offspring 100 days of age. Locomotor activity was assessed in compartment "B" for one hour/animal (30 day-old pups). The locomotor arena is crossed with 3 infrared beams. Interruptions of infrared beams are recorded automatically by a computer.

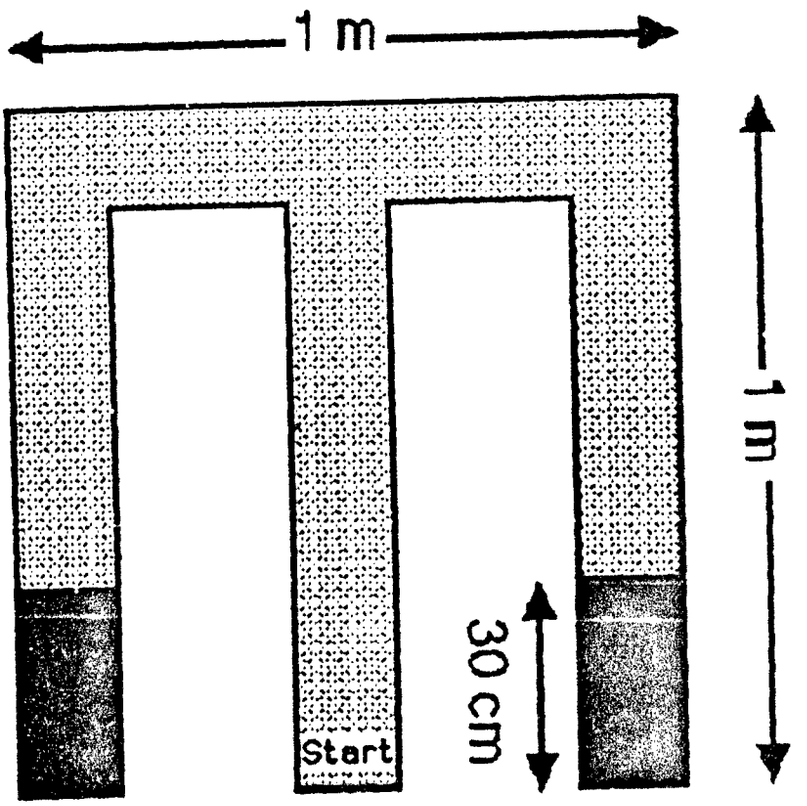


Figure 4. Water filled "T"- maze for the assessment of learning. Escape platforms are darkened in diagram above. See text for description of test.

also recorded. The mean number of trials to satisfy a criterion was determined for each litter.

The acoustic startle response (ASR) was measured in pups 23 days of age and in adult offspring 150 days of age. A rat was placed in a small wire cage sitting atop a force transducer platform. An acoustic stimulus (120 dB, white noise, 40 msec duration) was presented to the rat through a speaker situated at head height of the animal. The sound elicits a reflex extension of the forelimbs and hindlimbs which is detected by the force transducer and transmitted to a Macintosh SE/30 computer. Animals 23 days of age were submitted to 100 repetitions of the stimulus (20 s intertrial interval). Older animals (> 100 days of age) were submitted to 50 repetitions. The mean response amplitude was determined and compared across groups using ANOVA and mean separations by the Duncan New Multiple Range test..

Results

In an early series of experiments a total dosage of 365 mg HCB/kg body weight was administered to virgin rats over 4 days, and the animals were pair bred as indicated in the methods above. This experiment helped us to determine the range of dosages that we would evaluate in subsequent work. Offspring from these dams demonstrated signs of overt toxicity (severe body weight loss, organ malformations) whereas no evidence of toxic effects on the dam were observed. We evaluated pups from these dams in the negative geotaxic test. Pups maternally exposed to 365 mg/kg showed in a reduced time to criteria relative to control pups (Fig. 5).

Tissue analysis

The concentration of HCB in maternal tissues declined gradually throughout gestation (Fig. 6). The adipose tissue (Fig. 7) is the largest sink of HCB, although HCB is also stored in the liver, kidney and brain preferentially to being carried in the

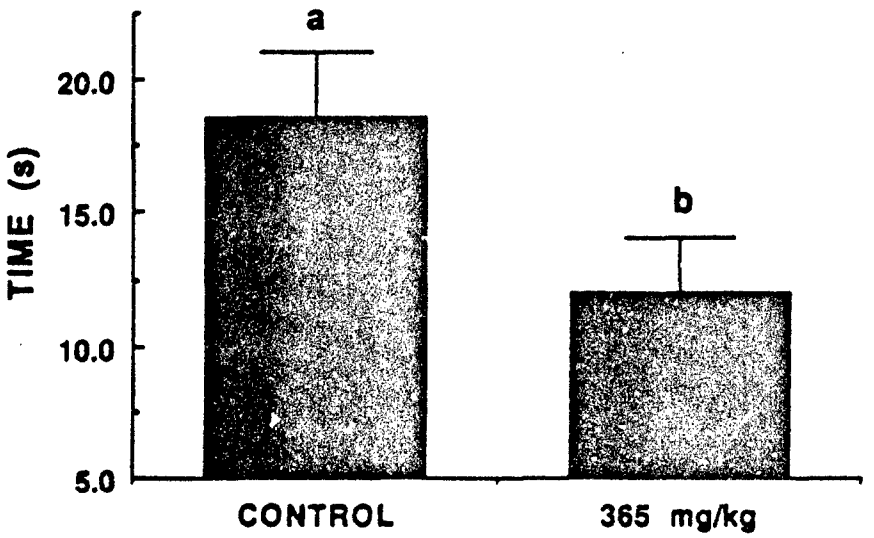


Figure 5: Mean negative geotaxic response (± S.E.) of pups 6, 8 and 10 days of age. N = 10 and 8 for the control and 365 mg/kg groups respectively, p = 0.0001.

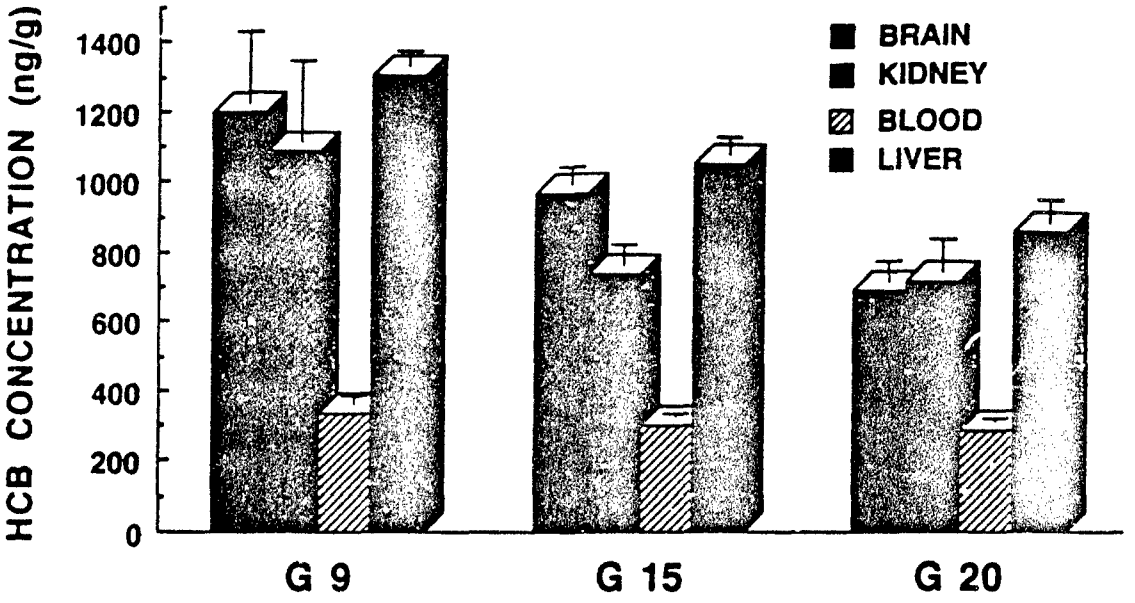


Figure 6a: HCB concentration (ng/g) ± S.E. in tissues collected 9, 15 and 20 days post conception from dams exposed to 10 mg HCB/kg body wt.

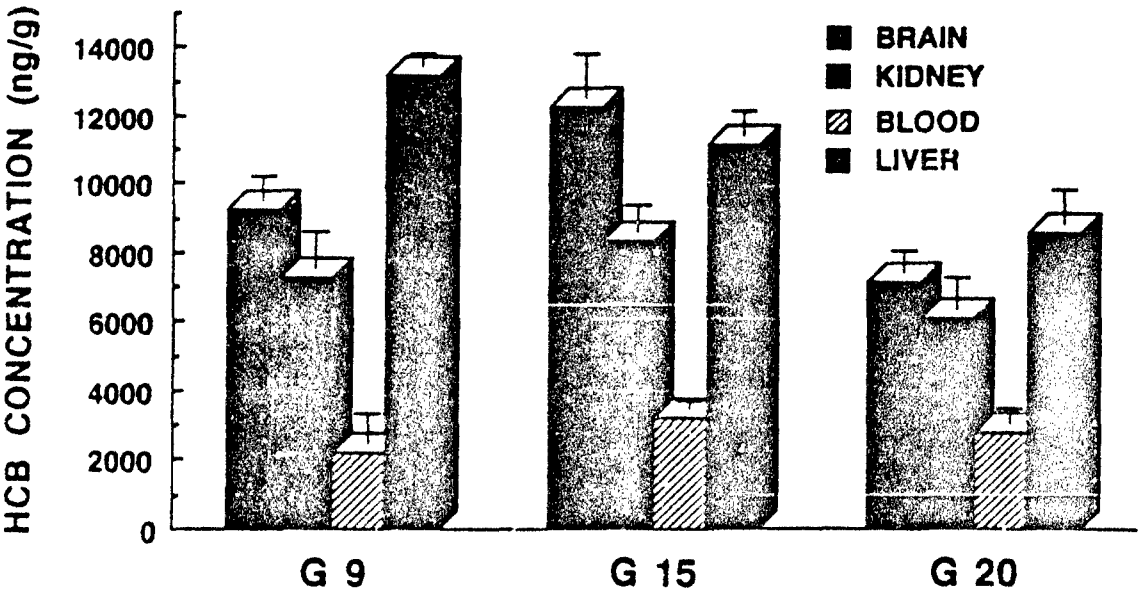


Figure 6b: HCB concentration (ng/g) ± S.E. in tissues collected 9, 15, and 20 days post conception from dams exposed to 100 mg HCB/kg body wt.

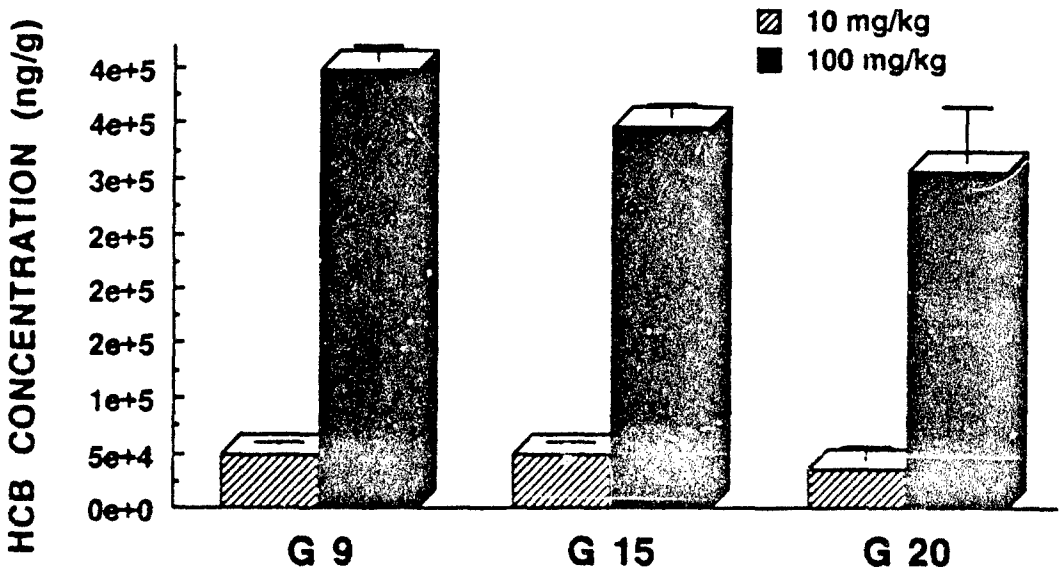


Figure 7: HCB concentration (ng/g) ± S.E. in adipose tissue samples collected 9, 15 and 20 days post conception from dams exposed to either 10 or 100 mg HCB/kg body weight.

circulation. It is of interest to note the expected 10 fold difference in HCB tissue concentration between the 10 and 100 mg/kg dosage groups for all tissues during gestation.

We showed that HCB is present in the fetus as early as day nine of gestation. In general, the HCB concentration in placental tissue and fetuses (Fig. 8) reflected the maternal blood concentration of HCB (see Fig. 6). The amount of HCB in amniotic fluid was low as would be expected for a lipophilic (hydrophobic) compound. HCB was below the limit of detection in amniotic fluid from the 10 mg HCB/kg body weight group (Fig. 8b), but was detected at low concentrations in the higher treatment group (Fig. 8d). Since we are particularly interested in the developmental neurotoxicity of HCB, the presence of HCB in the brain of 20 day fetuses suggests that the toxicity to the central nervous system may begin during gestation.

The maternal body burden of HCB was rapidly depleted during lactation at both dosage levels and tissue concentrations were at or near the level of detection by 14 days post partum (Fig. 9 and 10). Table 1 displays the relative concentration factor, RCF, (the HCB concentration in a tissue sample from animals dosed with 100 mg HCB/kg body weight divided by the HCB concentration in the same tissue from animals exposed to 10 mg HCB/kg body weight) for all tissues analyzed. The RCF values for dam tissues during gestation ranged from 6.64 to 12.72 with a mean RCF of 9.32, whereas the RCF values for dam and pup tissues during lactation ranged from 1.04-4.71 with a mean value of 2.77. Similarly, the RCF calculated from HCB concentrations in pup tissues (Fig. 11) ranged from 1.84 to 5.61, with a mean RCF = 2.61. Therefore, the expected ten-fold difference between the treatment groups (10 mg HCB/kg and 100 mg HCB/kg), in evidence during gestation, was eliminated during lactation.

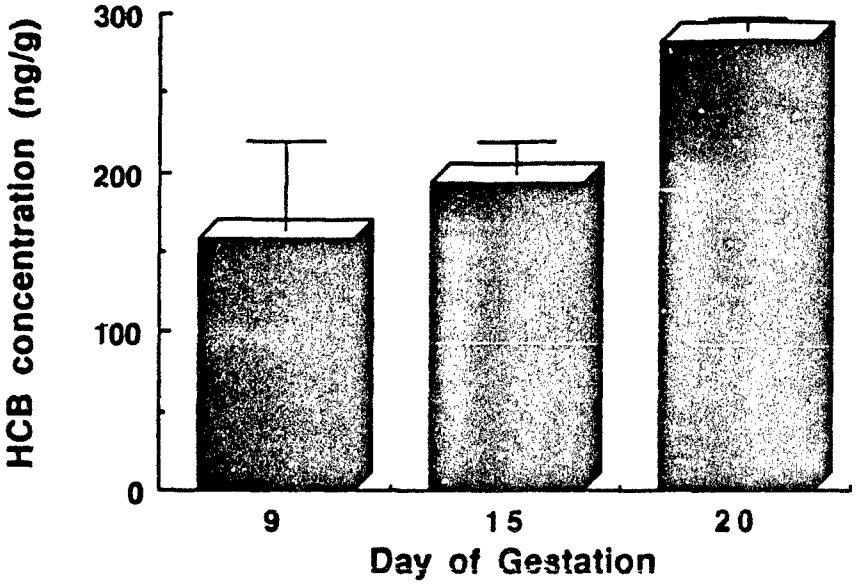


Figure 8 a: Hexachlorobenzene concentration in whole fetuses collected from dams dosed with 10 mg HCB/kg body weight.

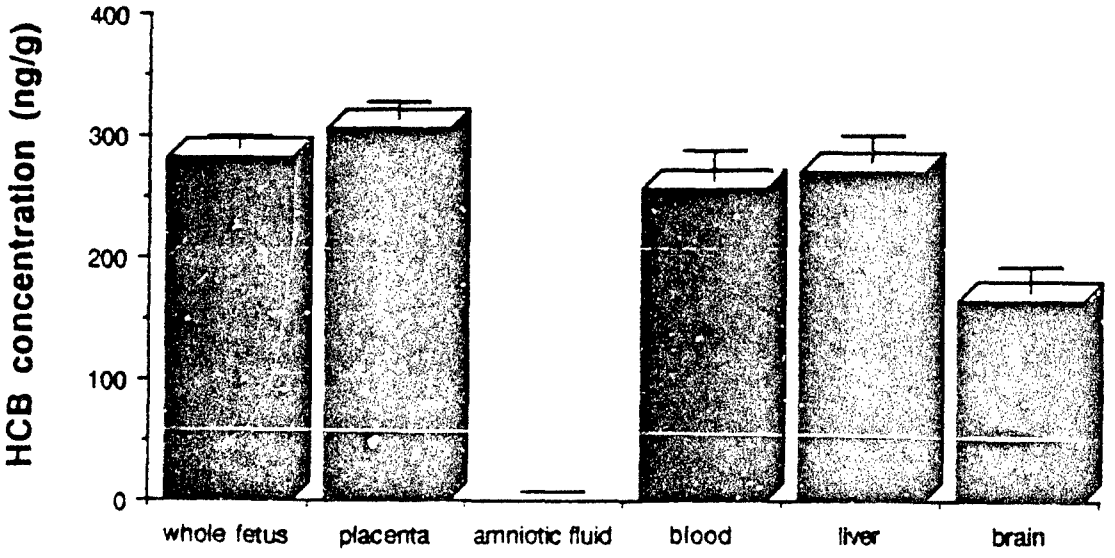


Figure 8 b. Hexachlorobenzene concentration (± S.E.) in tissues collected on gestation day 20 from dams dosed with 10 mg HCB/kg body weight.

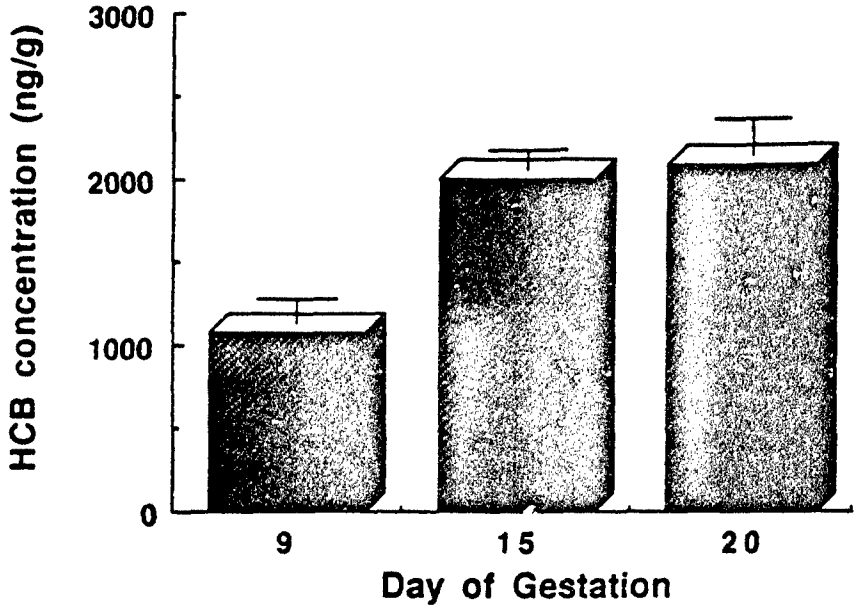


Figure 8 c: Hexachlorobenzene concentration in whole fetuses collected from dams dosed with 100 mg HCB/kg body weight.

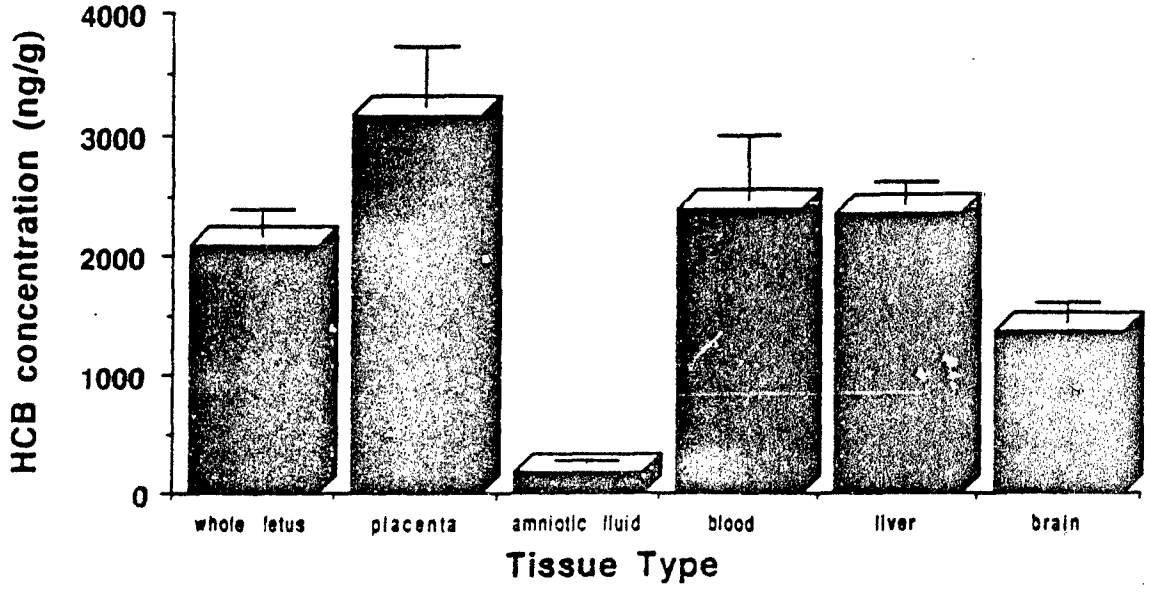


Figure 8 d: Hexachlorobenzene concentration (± S.E.) in tissues collected on gestation day 20 from dams dosed with 100 mg HCB/kg body weight.

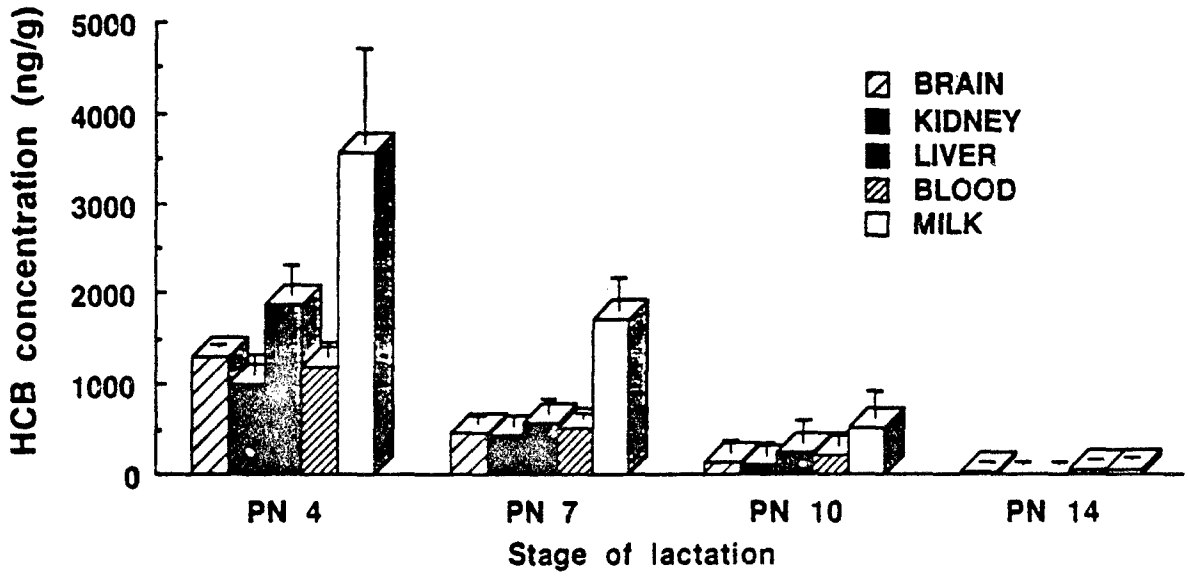


Figure 9 a: HCB concentration (±S.E.) in maternal tissues and milk from animals which received a total dose of 10 mg HCB/kg body weight 2 weeks prior to breeding. Tissues were collected on postnatal days 4, 7, 10 and 14.

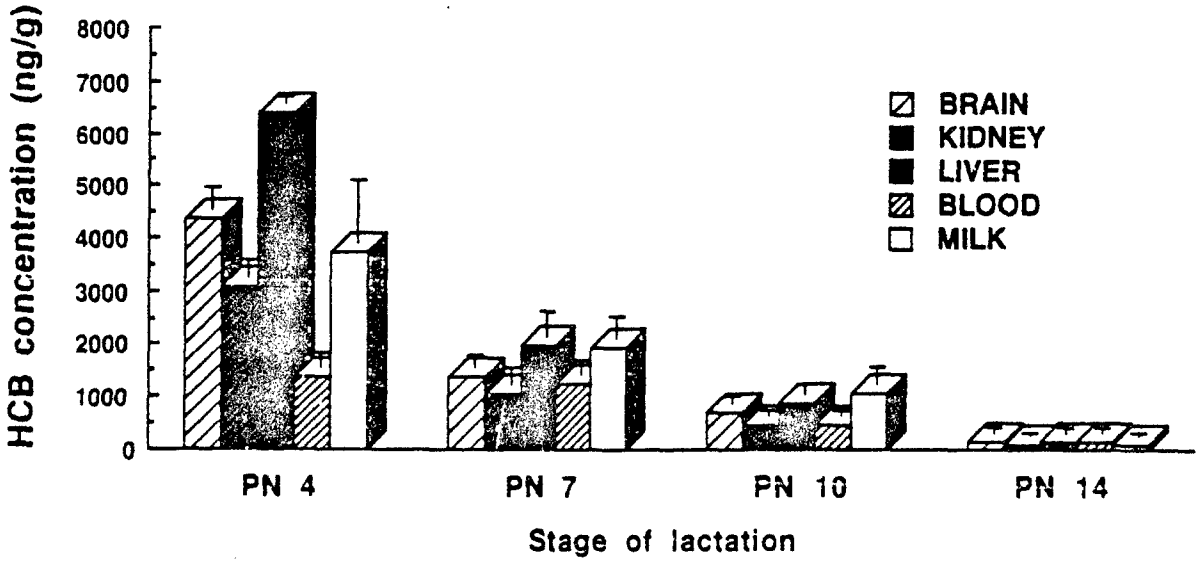


Figure 9 b. HCB concentration (±S.E.) in maternal tissues and milk from animals which received a total dose of 100 mg HCB/kg body weight 2 weeks prior to breeding. Tissues were collected on postnatal days 4, 7, 10 and 14.

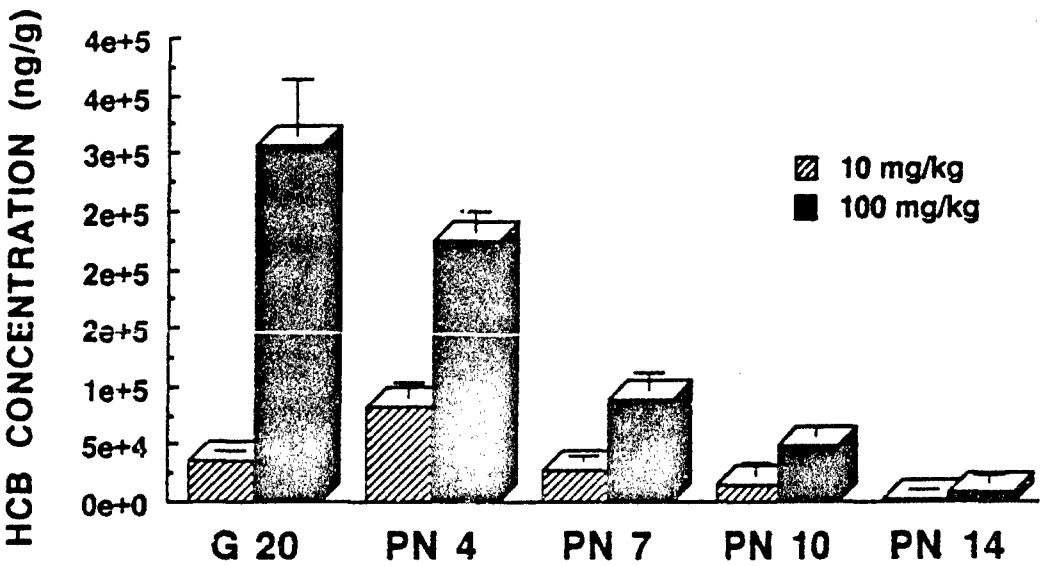


Figure 10: HCB concentration (ng/g) ± S.E. in adipose tissue samples collected on the last day of gestation (G 20), and on post natal days 4, 7, 10 and 14, from dams exposed to either 10 or 100 mg HCB/kg body wt.

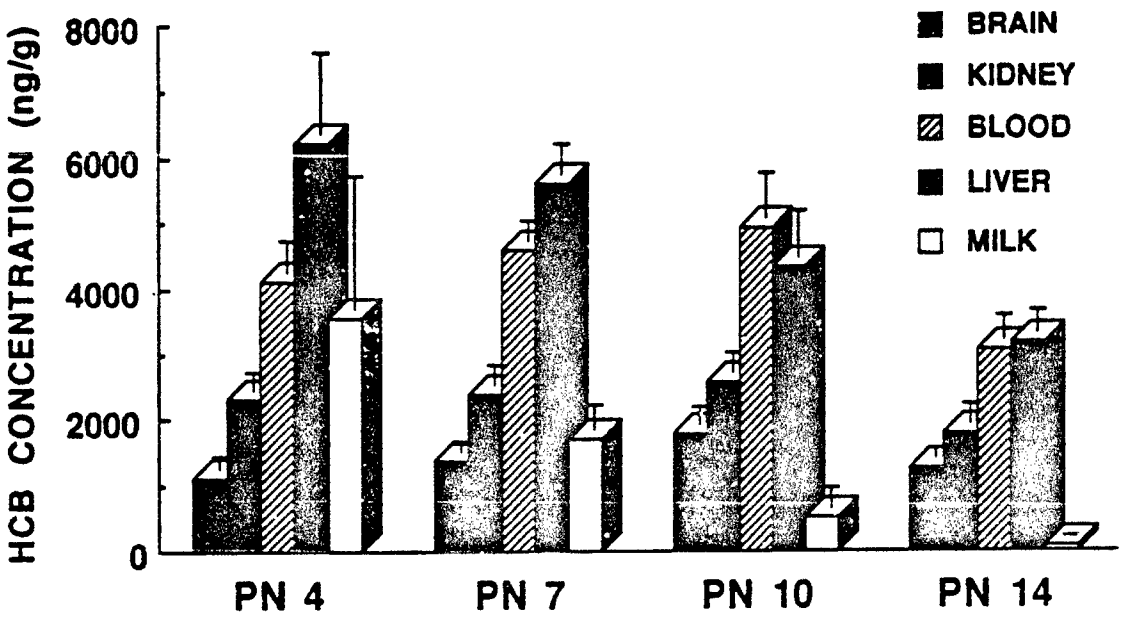


Figure 11a: HCB concentration (ng/g or ng/ml) ± S.E. in milk, and in tissues from pups maternally exposed to 10 mg HCB/kg body weight.

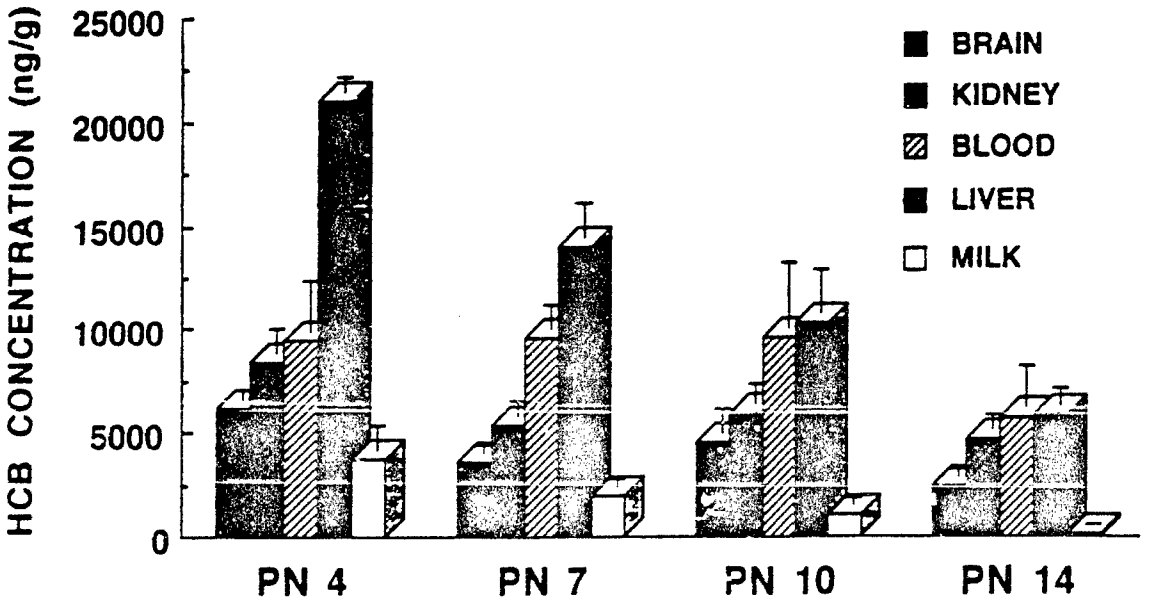


Figure 11b: HCB concentration (ng/g or ng/ml) ± S.E. in milk, and in tissues from pups maternally exposed to 100 mg HCB/kg body weight.

Table 2: The relative concentration factor, RCF = [tissue] $_{100}$ Π [tissue] $_{10}$, for dam, fetus and nonperfused pup tissues.

Dam tissues

	Brain	Kidney	Liver	Fat	Blood	Milk
Gestation 9	7.71	6.72	10.11	7.95	6.64	
Gestation 15		12.72	11.37	10.63	7.04	10.58
Gestation 20		10.42	8.60	10.62	8.67	9.95
Lactation 4	3.37	3.02	3.44	2.73	1.15	1.04
Lactation 7	3.00	2.54	3.49	3.32	2.39	1.14
Lactation 10	4.71	3.70	3.55	3.21	2.02	2.05

Fetal tissues

	Whole fetus	Placenta	Blood	Liver	Brain
Gestation 9	6.82	NA	NA	NA	NA
Gestation 15	10.38	13.45	NA	NA	NA
Gestation 20	7.38	10.33	9.41	8.72	8.41

Pup tissues (nonperfused)

	Brain	Kidney	Liver	Blood
Post natal 4	5.61	3.21	3.18	2.31
Post natal 7	2.40	2.49	3.25	2.07
Post natal 10	2.54	2.33	2.38	1.95
Post natal 14	1.85	2.64	1.84	1.85

There was little difference between the HCB concentration in tissues collected from pups perfused with saline and those that were not perfused, which suggests that blood circulating through the organ may contribute very little HCB to overall tissue levels.

The uptake of HCB from the intestine to the blood reaches asymptotic levels within four hours following dosage (Fig. 12). The blood HCB concentration appears to be relatively stable over the intervening hours until the next dose is given which suggests that it may take several days for HCB to move from the bloodstream to tissue storage compartments. A comparison of the blood HCB concentration in the 10 mg/kg dosage group 24 hours after the last dose is given (Fig. 12a) to the maternal blood concentration at day 9 of gestation (Fig. 6a) (2350 ng HCB/ml blood to 335 ng HCB/ml

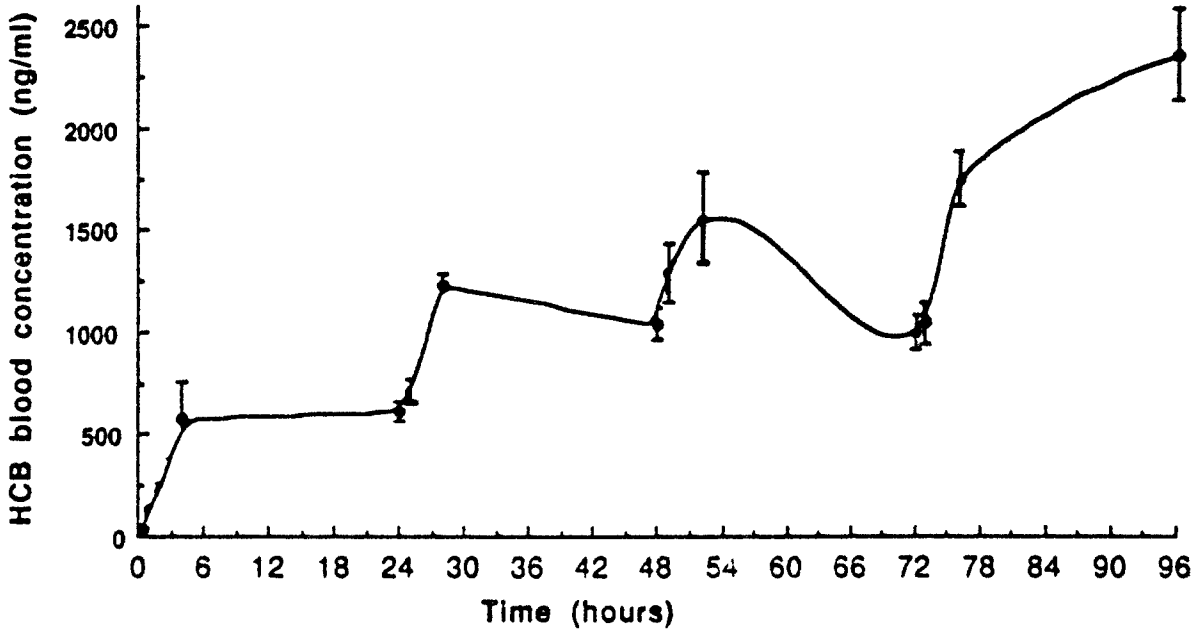


Figure 12 a: Blood concentration of HCB (±S.E.) after gavage dose of 2.5 mg/kg per day for 4 days. Total dose = 10 mg/kg.

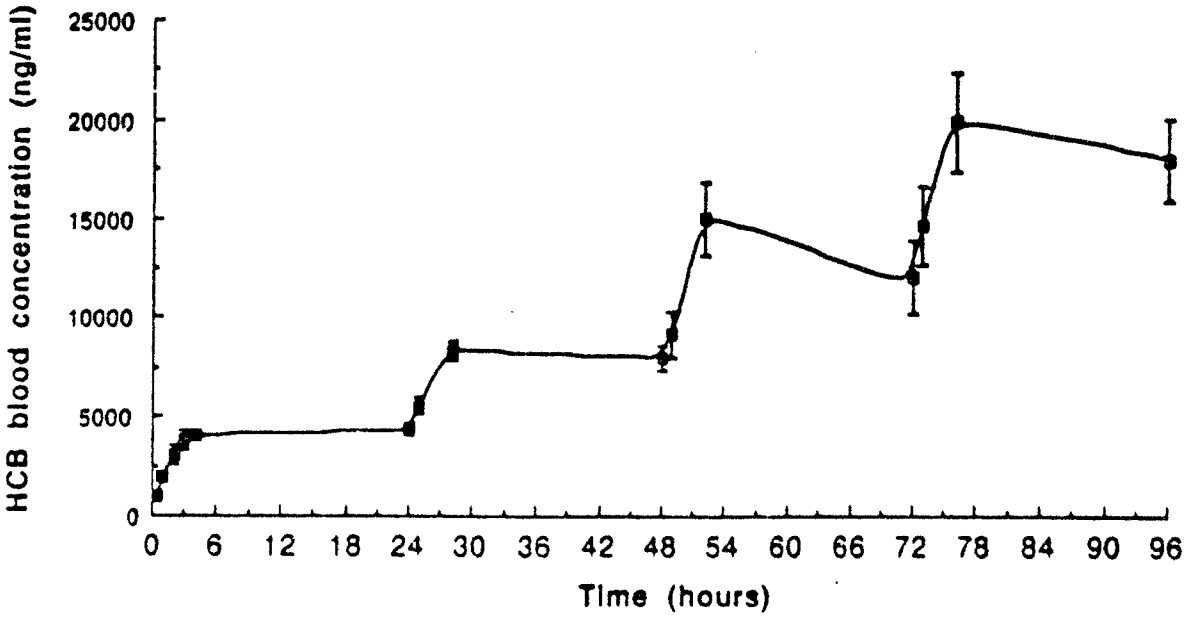


Figure 12 b: Blood concentration of HCB (±S.E.) after gavage dose of 25 mg/kg per day for 4 days. Total dose = 100 mg/kg.

blood, respectively) demonstrates that blood borne HCB is greatly reduced over the intervening weeks, presumably as it moves into lipid-rich tissues.

Behavior Results

Pups maternally exposed to HCB (10 and 100 mg HCB/kg body weight) showed significantly faster responses than control animals in both the negative geotaxis (Fig. 13) and olfactory homing tests (Fig. 14). Similarly, the results of exploratory cage tests indicated that litters exposed to the highest dose of HCB exhibited significantly increased exploratory behavior and/or hyperactivity during early life i.e., 15-21 days of age (Fig. 15) ($df = 2$, $F = 4.482$, $p = 0.0114$). Activity in the low treatment group also appeared to be elevated, although this trend was not significant.

Learning ability, as measured in the water filled T-maze, was not affected by maternal HCB exposure. There were no significant differences between control litters and treated litters in this task when compared across 3, 4 or 5 errorless trials or across average latency of escape.

Locomotor activity in offspring (30 days of age) measured in an open arena (Fig. 3 "B") was not significantly different between HCB treated litters and control litters. Similarly, exploratory activity (Fig. 3 "A") measured in mature, 100 day-old, offspring did not reveal significant effects between HCB-exposed litters and control litters.

Hexachlorobenzene significantly decreased the amplitude of the acoustic startle response in 23 day-old pups maternally exposed to 100 mg HCB/kg body weight compared to controls ($df = 2$, $F = 35.928$, $p = .0001$) (Fig. 16). When maternally exposed offspring were later tested as adults, 120 days of age, response amplitude was decreased in both treatment groups compared to the control animals ($df = 2$, $F = 49.587$, $p = .0001$) (Fig. 17).

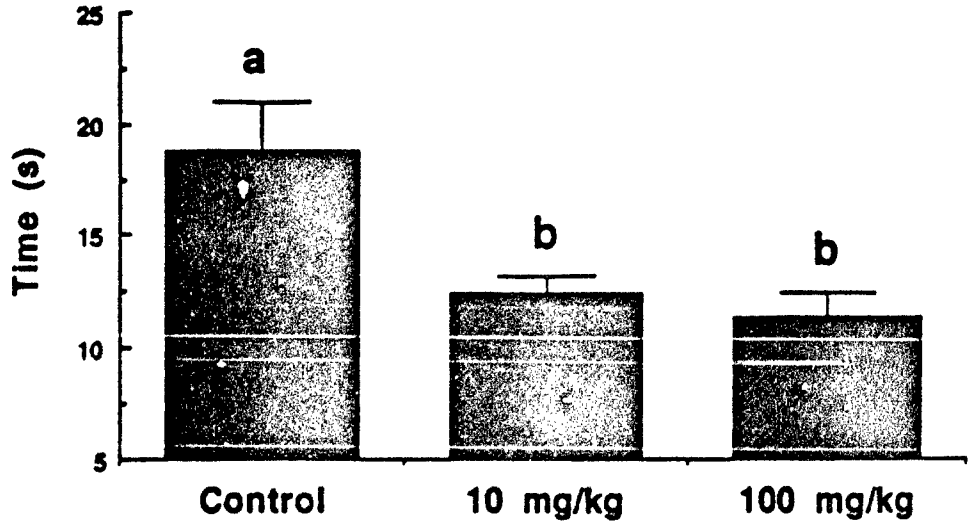


Figure 13: Mean negative geotaxic response of pups 6, 8 and 10 days of age. N = 10, 9, and 8 litters (4 pups/litter) for control, 10 mg/kg and 100 mg/kg groups respectively. DF = 2, F = 7.109, p = 0.0014.

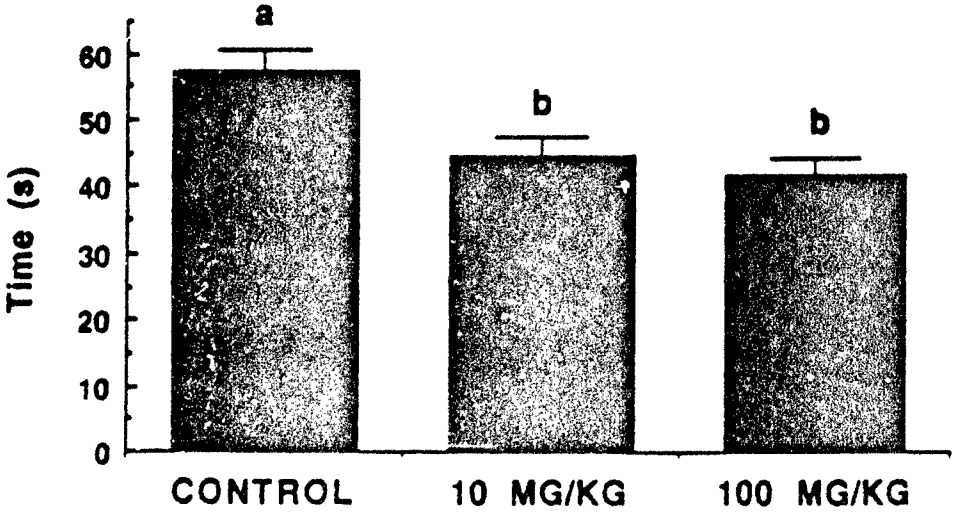


Figure 14: Olfactory homing. Mean time to reach home bedding. N = 10, 9 and 7 litters (4 pups/litter) for control, 10mg/kg and100 mg/kg groups respectively. DF = 2, F = 5.877, p = 0.0039.

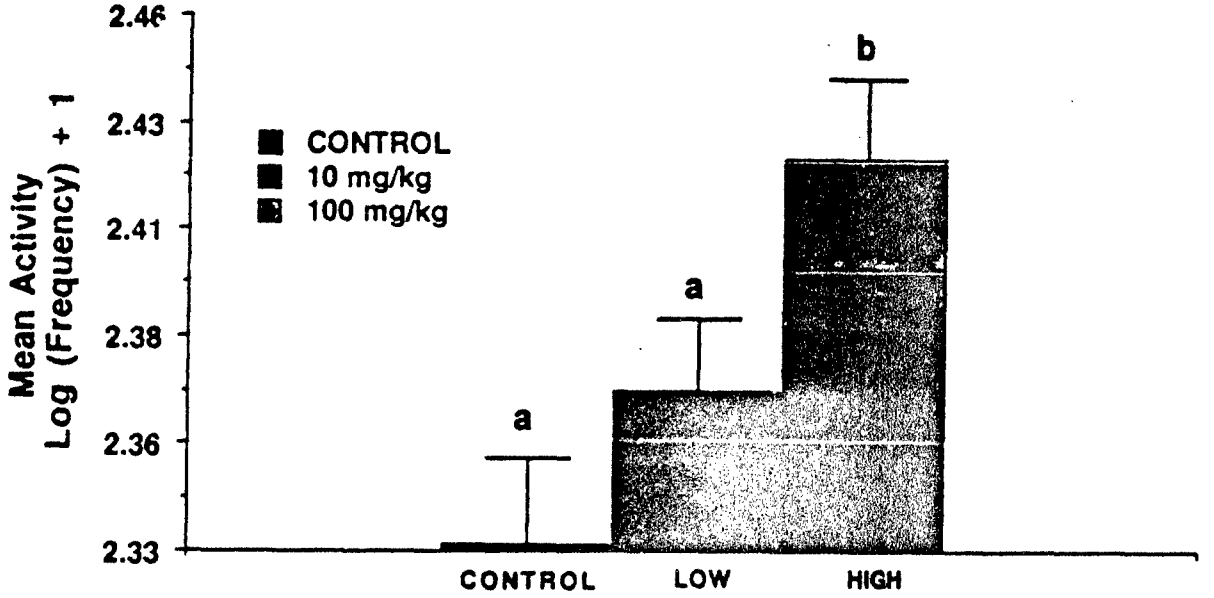


Figure 15. Exploratory cage activity (±S.E.) for pups from dams exposed to 0 (control), 10 (low) or 100 mg HCB/kg body weight (high) 2 weeks prior to breeding. N (# litters) =10, 11, and 11 for control, low and high groups respectively.

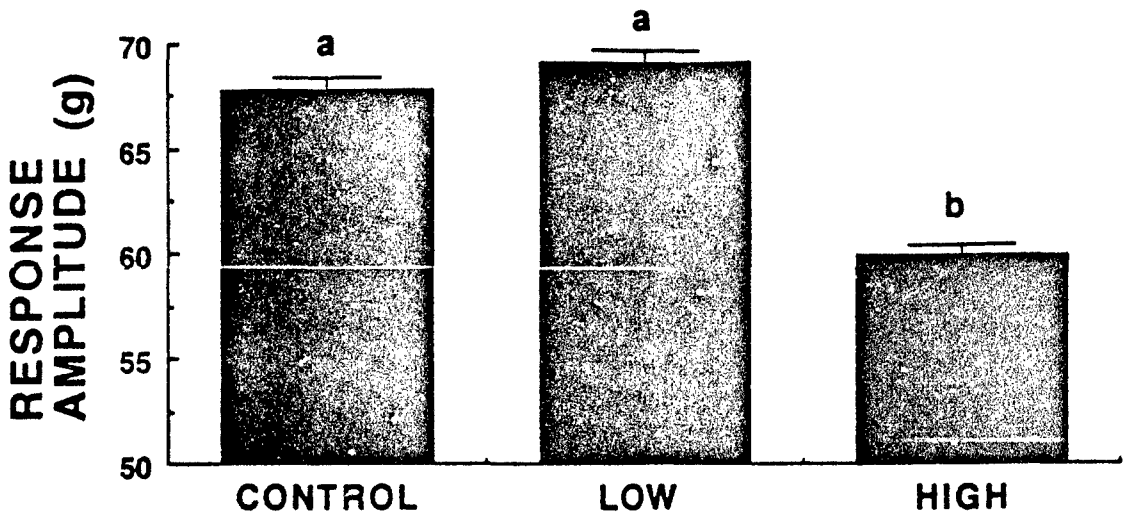


Figure 16: Amplitude (mean ± S.E.) of response to 120 dB white noise burst of 23 day-old rats maternally exposed to 0, 10, or 100 mg HCB/kg body weight. N = 8, 9, and 9 litters for control, low and high groups respectively.

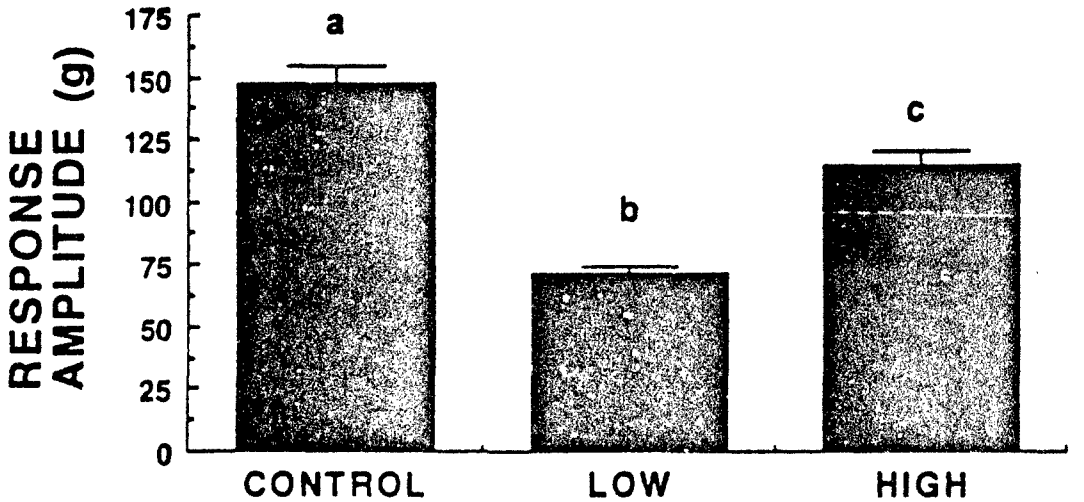


Figure 17: Amplitude (mean ± S.E.) of response to 120 dB white noise burst of adult rats (120 days of age) maternally exposed to 0, 10, or 100 mg HCB/kg body weight. N = 10, 7, and 9 litters for control, low and high treatment groups respectively.

Discussion

Dosing Protocol

One goal of the current study was to assess the uptake and storage of HCB in the tissues of the female rat, and subsequently to monitor the gestational and lactational transfer of the maternal body burden of HCB to the fetuses and suckling pups. Previous studies have monitored the maternal transfer of HCB in animals dosed with the chemical during gestation (Courtney and Andrews, 1985), however we believe that gestational exposure does not mimic the human situation in which accumulation of stable chlorinated compounds occurs over the lifetime of the individual. Therefore we utilized a prebreeding protocol that could be consistently used for risk assessments of the effects of metabolically stable, lipophilic compounds on the developing offspring. Prebreeding dosing protocols have also been utilized for assessments of other polychlorinated compounds such as 2,4,5,2',4',5'-hexachlorobiphenyl (6-CB) (Vodicnik and Lech, 1980).

Chernoff, et al. (1989) have shown that maternal toxicity may be an important consideration in teratogenicity investigations since maternal stress may reduce litter size and increase malformations in offspring. Prebreeding dosing greatly reduces the handling of animals and therefore reduces maternal toxicity to the developing offspring. The "last in-first out" phenomenon (Colburn and Matthews, 1979) may also be an important consideration in teratogenicity studies. Recent work demonstrated that a maternal dose of 6-CB given at the start of gestation is transfered to offspring more quickly than a dose given weeks prior to the onset of pregnancy (Gallenberg et al., 1990).

The analysis of the oral uptake of HCB showed that HCB concentrations in the blood show a stepwise increase over the 4 day dosing period. Twenty-four hours following the last dose, the blood HCB concentration was 8 times higher than it was approximately 4 weeks later (when the first tissue samples were collected at day 9 of

gestation). Dosing the animal during gestation, the currently utilized protocol by regulatory agencies, would result in blood HCB levels of the compound that are much higher and in a more dynamic state than blood HCB levels in animals in which the body burden of the chemical has been allowed to equilibrate in tissue storage compartments. It has also been shown that 6-CB is transported by low density lipoproteins (LDL) immediately following dosing but is subsequently carried by VLDL 24 hours after the dose is given (Spindler-Vomachka et al., 1984). The association of 6-CB with VLDL appears to be unique to this PCB congener, and less chlorinated congeners remain associated with LDL, high density lipoprotein (HDL) and the lipoprotein-poor (albumin) fractions (Borlakoglu et al., 1990). Since it is reasonable to assume that the blood transport mechanisms for HCB and 6-CB are similar, it is probable that the mode of action of these compounds on target tissues may be influenced by the lipoprotein fraction with which they are associated. Therefore, we believe that the widely used practice of dosing animals during gestation and/or lactation may yield misleading results in teratogenicity studies, and we advocate our method as an alternative. Crossfostering animals at birth makes it possible to separate the effects of gestational from lactational exposure in studies where it is important to make this distinction. The prebreeding dosing procedure utilized in our study has the potential for widespread use for teratogenicity assessments with other polyhalogenated compounds.

Disposition of HCB during gestation and lactation

We have shown that although HCB is preferentially stored in the adipose tissue of the female, HCB does remain in the circulation of the dam, and is readily transferred across the placenta to the fetus. Analysis of chlorinated residues in maternal and fetal cord blood in humans suggested that HCB preferentially crossed the placenta as indicated by higher HCB levels in the cord blood than maternal blood (Bush et al,

1984). Our study did not support this finding since the residue amounts in the rat placenta and fetus correlate with the amount in the maternal blood.

The disposition of HCB appears similar to that of 6-CB. An assessment of the gestational and lactational transfer of a single 100 mg/kg i.p. injection of 6-CB indicated that little 6-CB was transferred to the fetus, while lactating mothers transferred nearly all of their body burden to their suckling pups by 15 days post-partum (Vodicnik and Lech, 1980). Similarly, in the current study, the maternal body burden of HCB declined gradually during gestation whereas the lactational transfer of the chemical was rapid; and in both treatment groups HCB was virtually eliminated from the dam's body by 14 days post-partum.

During lactation, VLDL are metabolized primarily in the mammary gland to facilitate triacylglycerol (TG) incorporation into milk. It has been suggested that the increase in circulating 6-CB levels in animals sacrificed at birth (Vodicnik and Lech, 1980) were due to the concomitant increase in circulating VLDL from lipid stores during milk TG synthesis (Spindler-Vomachka and Vodicnick, 1984). Based on the assumption of similarity between these compounds, we expected that maternal blood levels of HCB to increase at the end of pregnancy, which did not occur. It is interesting to note, however, that in the 10 mg HCB/kg group, tissue concentrations of HCB were elevated in dams sacrificed on post-natal day four compared to tissues collected during gestation, which may be related to the findings of Vodicnik and Lech (1980).

One of the most striking results from the current study indicates that the rate and/or route of elimination differs between the treatment groups following the onset of lactation. We found that only a 2-3 fold difference between the treatment groups remained in dam and pup tissues after the onset of lactation. Since the levels in the pups reflect the difference in the dams, it is evident that, at least for the higher dose of HCB, milk is not the only route for HCB elimination from the body.

Alternative or additional metabolic processes and/or elimination routes may be more strongly affected by parturition in the higher dosage group than in the lower dosage group. One possible explanation is the mediating factor of hormones on the metabolism/elimination of HCB. For example, the rapid reduction of sex steroids at parturition and the concomitant induction of milk secretion by prolactin, and/or the increase in oxytocin which causes milk let down may influence the metabolism and/or excretion of HCB. To our knowledge no studies have been performed on the effects of pregnancy, parturition and lactation on the metabolism of HCB or related compounds. It would be of interest to pursue this line of research in future work.

Teratogenicity

Hexachlorobenzene caused no overt toxicity to the dams (no weight loss). Therefore we have demonstrated that the developing rat is much more sensitive to the effects of HCB than the adult. We feel that our data clearly indicates that HCB is a teratogen. Pups from dams exposed to high levels of HCB (365 mg HCB/kg body wt.) demonstrated overt signs of toxicity such as weight loss and organ deformities. Pups from this group also demonstrated significantly altered behavior in the negative geotaxis response which was also demonstrated at the lower doses in the absence of signs of overt toxicity.

Behavioral teratology

Our findings have demonstrated that behavioral testing is a sensitive tool for regulatory purposes. It is dubious that visible brain pathology would be evident at lower exposure levels, particularly due to the immense structural and chemical complexity of the nervous system, however behavior is a functional manifestation of a diversity of effects on this complex system (Weiss, 1988). Recently, the U.S. EPA proposed guidelines for assessing developmental neurotoxicity from exposure to

pesticides and other chemicals of concern (Francis, 1990). These guidelines call for behavioral testing at different ages throughout the animals' development. Recognition the U.S. EPA of the need for behavioral testing further emphasizes the importance of integrating behavioral tests in studies such as ours.

We have shown that young HCB-treated animals exhibit abnormal behaviors compared to controls in both low and high treatment groups. Three tests were performed on animals prior to weaning. Negative geotaxis, olfactory homing and the automated exploratory cage all revealed similar and significant effects of HCB. Results from these tests indicated that HCB causes hyperactivity in the developing rat. These findings demonstrate that neurotoxicity can be detected early in the life of the animal and reveal the importance of testing young animals in behavioral teratology assessments.

The acoustic startle apparatus detected reduced ASR in both young (23 day old) and adult rats maternally exposed to HCB. In this test, an acoustic stimulus elicits a well characterized, whole body reflex response in the animal. The neural circuitry that mediates the acoustic startle is contained entirely within the brainstem (Davis et al., 1985). Therefore effects on the ASR indicate effects in a well defined region of the central nervous system. Thus the ASR represents a sensitive tool for correlating abnormal functional output (motor response) with toxicant effects on a particular sensory-motor arc. Our results indicate that the ASR is sensitive to maternal HCB exposure. We tested both young and adult offspring and found significantly reduced response amplitude in HCB-treated animals at both ages. This finding suggests that HCB causes long-term and perhaps permanent effects on this sensory-motor pathway. Numerous compounds, including triethyltin, also cause decreased startle amplitude. Triethyltin induces demyelination in the nervous system resulting in a reversible decrement in motor function, and this has been suggested as the mechanism for reduction of ASR amplitude (Crofton, 1990). Previous work in our laboratory showed

that trichloroethylene also decreases myelin in the brain (Isaacson and Taylor, 1989). Since HCB is a lipophilic compound, and myelin has a high lipid content, it would be of interest to determine the effects of HCB on myelin in the ASR reflex pathway.

The results of this study demonstrate the sensitivity of behavioral testing and illustrate that such tests provide sensitive indicators of HCB-induced central nervous system (CNS) toxicity.

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PAPER PRESENTATIONS

Maternal transfer of Hexachlorobenzene in the rat: A preliminary report. 1989. E.S. Goldey, J.W. Fisher and D.H. Taylor. Presented at conference on "Methods in Behavioral Toxicology/Teratology, National Center for Toxicological Research, Little Rock, AR

Maternal transfer of Hexachlorobenzene in the rat. 1990. E.S. Goldey, J.W. Fisher and D.H. Taylor. Society of Toxicology Annual Meeting, Miami Beach, FL.

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• U.S. Government Printing Office: 1991 – 548-016/40016