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Toxicological Manifestations of Hexachlorobenzene Exposure in the Mink (Mustela vison) and the European Ferret (Mustela Putorius Furo)

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TOXICOLOGICAL MANIFESTATIONS OF HEXACHLOROBENZENE EXPOSURE IN THE MINK (*MUSTELA VISON*) AND THE EUROPEAN FERRET (*MUSTELA PUTORIUS FURO*)

By

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ABSTRACT

TOXICOLOGICAL MANIFESTATIONS OF HEXACHLOROBENZENE EXPOSURE IN THE MINK (*MUSTELA VISON*) AND THE EUROPEAN FERRET (*MUSTELA PUTORIUS FURO*)

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The mink and ferret are among the most sensitive mammalian species to the toxic effects of HCB. Anorexia, weight loss, gastrointestinal bleeding, and bloody droppings were observed prior to death. The acute toxicity of HCB was low in these two mustelids. The mink and ferret were, however, very susceptible to chronic HCB exposure.

No indications of porphyria were observed in the mink and ferret following the consumption of HCB-supplemented diets. Since the mink and ferret do not develop porphyria, it would appear that the toxic effects seen must be independent of the porphyrin cycle.

Ferrets on the 125 ppm HCB-diet were hyperexcitable and displayed signs of neurological impairment. The analysis of brain biogenic amines in mink and ferrets revealed alterations in neurotransmitter concentrations. Changes were also found in the mink kits.

HCB was readily absorbed (98.5% of the HCB dose) by female ferrets. A half-life of 32 days was calculated for ferrets raising kits and of 41 days for unbred ferrets. Offspring were an important route through which the lactating female excreted HCB. Exposure via the milk for the growing ferret kit was 31 times greater than the exposure occurring *in utero*.

HCB caused an increase in the gestational length of HCB-exposed female mink and a lower whelping percentage than was seen in control

females. A decrease in total litter size was seen in the mink. No change in litter size was found in the ferrets, but an increased incidence of kits born dead was observed. Kits born to dams consuming HCB exhibited depressed birth weights and poor survivability to weaning when compared to control offspring. In utero exposure caused the same kit mortality as in utero and lactational exposure combined. Mink kits receiving HCB only via the milk showed a poorer survivability than control kits, but better than kits experiencing HCB during gestation.

Chronic exposure to HCB was determined to be immunosuppressive to adult mink and ferrets at 25 ppm of the diet. The lymphocyte responsiveness of mink kits produced by HCB-treated dams was also subnormal.

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INTRODUCTION

Since World War II, an ever increasing array and quantity of chemicals have been introduced into the agricultural and industrial fields. In the fight against plant and animal pests, many products have been used to improve crop yields and/or livestock production. Unfortunately, some of the pesticides employed have also been found to be persistent in the environment and toxic to nontarget species. One such chemical is hexachlorobenzene (HCB), a chlorinated aromatic hydrocarbon. HCB is used as a pre-emergent fungicide on cereal grains, an additive for rubber products, an intermediate in the synthesis of many organic compounds, a wood preservative, a porosity controller in the manufacture of electrodes, and an additive for certain military pyrotechnic compositions. However, the single most important source of HCB contamination of the environment is its presence as an undesirable by-product in the manufacture of numerous economically important chemicals.

While HCB is a relatively simple molecule (C_6Cl_6), it is resistant to biological and chemical degradation. Additionally, HCB is lipophilic and readily traverses cell membranes. This chemical therefore shows a significant degree of bioaccumulation and biomagnification, as well as widespread dispersal through the environment. Residues of HCB are found in a wide range of animal species, including domestic livestock and humans. The accidental or environmental contamination of food, animals, and the human population is a common occurrence with numerous instances

of low level insult and occasional outbreaks that have led to overt toxicosis. The adipose tissue acts as a reservoir for HCB and so an animal's body burden of this chemical will be significant for a period of time long after exposure to HCB has ended.

HCB is a volatile chemical that sublimes at air-soil interfaces and then enters the atmosphere. While HCB is virtually insoluble in water, it is nevertheless regarded as a major oceanic pollutant. The levels of HCB found in the earth's waters can be attributed to either air-water interface diffusion or the direct dumping of HCB-contaminated wastes into streams and seas.

There are relatively few effects seen following acute HCB exposure and in most species thus far tested, the LD_{50} value is in excess of 1g/kg of body weight. While acute toxicological manifestations may be minimal, chronic low level exposure presents a greater hazard. Porphyria, cutaneous lesions, liver changes, and hyperexcitability are a few of the general indications of HCB toxicity. An outbreak of HCB-related porphyria was reported in several villages in Turkey between 1955-1960. This incidence of human exposure resulted in the death of many young children, especially those still being nursed by their mothers.

In the studies reported here, the mink (Mustela vison) and the European ferret (Mustela putorius furo) were used to ascertain HCB-related effects on the reproduction and immunological function of these upper echelon carnivores. Since both the mink and ferret occupy a position at the apex of the food chain, they represent species most likely to be subjected to high levels of a chemical such as HCB. The mink and ferret are also among the most sensitive test animals available to the effects of other halogenated aromatic hydrocarbons.

REVIEW OF LITERATURE

Hexachlorobenzene (HCB) is a white crystalline solid with a molecular weight of 284.79 and a melting point of 231°C (Merck 1976). The chemical has a log octanol/water partition coefficient of 6.43 and is the most lipophilic of the chlorinated benzenes (Weast 1975). The HCB molecule is a benzene ring with all available positions occupied by chlorine atoms (Figure 1). HCB is also known as perchlorobenzene, Anticarie®, Bunt-cure®, Bunt-no-more®, and Julin's carbon chloride (Merck 1976). Several of these names reflect the agricultural uses of HCB. It is a registered pre-emergent fungicide for the control of smut diseases on seed grains such as barley, wheat, oats, and rye. When used in agricultural situations, HCB is mixed with a blue dye to give the seeds a distinctive color (EPA 1980), thus making the grain obviously altered. The technical grade HCB used in agriculture contains 98% HCB, 1.8% pentachlorophenol, and 0.2% 1,2,4,5-tetrachlorobenzene. Several commercial HCB products have been shown to contain trace quantities of octachlorodibenzofuran and octachlorodibenzo-*p*-dioxin (Villeneuva et al. 1974). In 1974, only Stauffer Chemical Co. was producing HCB in the United States and its entire production capacity was committed for use as a peptizing agent in the manufacture of rubber used in automobile tires (Mumma and Lawless 1975). The domestic intentional production of HCB was probably an insignificant source of the chemical entering the environment in 1974 and commercial production of HCB in the

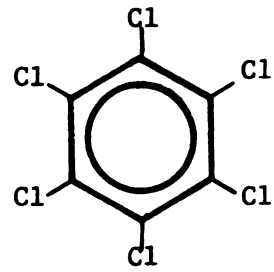


Figure 1. Structure of the Hexachlorobenzene Molecule.

United States was discontinued in 1976 (EPA 1980).

The HCB found in the environment is not the direct product of commercial manufacturing processes, even though a number of synthesis reactions are known (Mumma and Lawless 1975). HCB's entrance into the world's ecosystems appears to be primarily as a contaminant in pesticides, industrial wastes, and as an undesirable by-product in the synthesis of organochlorine compounds. Residues of HCB have been detected in perchloroethylene, trichloroethylene, carbon tetrachloride, chlorine, vinyl chloride, Dacthal®, Atrazine®, Propazine®, Simazine®, Mirex®, and pentachloronitrobenzene (Mumma and Lawless 1975). Industrial manufacturing procedures have been reported to produce 4.3 million pounds per year of HCB as an undesirable by-product (Quinlivan and Chassemi 1977; Courtney 1979). The production of perchloroethylene alone has been estimated to account for 72% of the total HCB entering the environment. At present, three major methods of HCB disposal are employed: 1) placement into sanitary land fills, 2) high temperature incineration (+1300° F with a residence time of approximately ¼ second), and 3) deep well injection. Of these three techniques, high temperature incineration is the preferred method as it is the most effective and safest, with virtually complete destruction of the HCB (Mumma and Lawless 1975). It is

important however, to insure the incinerator temperature is sufficiently high to destroy the chemical. When heated only to decomposition, HCB produces toxic fumes (Sax 1963) of greater toxicity than the parent molecule. Sanitary land fills are the method of second choice and represent a greater proportion of the chemical's ultimate disposal than does incineration. Deep well injection is a third alternative, although not a preferred technique (Mumma and Lawless 1975), due to questions of permanent containment. Although it is not a naturally occurring chemical (Courtney 1979), HCB shows great persistence once it enters the environment (Beck 1974; Beall 1976; Isensee et al. 1976; Plimmer and Klingebiel 1976). HCB is volatile (Beall 1976) and tends to sublime at air/water interfaces (NAS 1975; Beall 1976). Aerial dispersion, via volatilization, appears to be the major mechanism for HCB to enter the marine environment. The solubility of HCB in water is very low, generally less than 2 μg of HCB per kg of water. In spite of this, HCB has been the only organic chemical designated as being present in the world's oceans at concentrations likely to result in serious problems (Morse 1975; NAS 1975; Seltzer 1975).

HCB has been detected in surface waters, soils, and animal species (Leoni and D'Arca 1976), as well as in finished drinking water (EPA 1975). It readily bioconcentrates and bioaccumulates, showing a greater potential for the accumulation in fat of animal species than the polychlorinated biphenyls (PCBs) (Hansen et al. 1979). The biomagnification of HCB from the aquatic environment has been shown for algae, invertebrates, and fish species (Lu and Metcalf 1975). Bioconcentration factors of from 1,964 to 23,000 for fish and shellfish (EPA 1980) have been calculated. HCB residues have also been found in a variety of freshwater

fishes (Johnson et al. 1974; Niimi 1979), including alewives, smelt, salmon, and trout. When terns consumed fish contaminated with HCB, they showed tissue residues in excess of those found in the food fish. Predatory birds have been shown to likewise have high concentrations of HCB (Vos et al. 1969; Best 1973; Cromartie et al. 1975), concentrations much higher than are found in the prey species they feed upon. The residues of HCB were also shown to be greater in the fox (carnivore) and wild boar (omnivore) than the herbivorous deer (Koss and Manz 1976), showing HCB concentrations to increase progressively up the food chain.

The acute toxicity of HCB is low (WHO 1970; Booth and McDowell 1975), greater than 1g/kg of body weight in several species. However, the subdividing of a large dose of HCB resulted in greater accumulations of the chemical in the adipose tissue of beagles and appeared to enhance the toxic effects (Sundlof et al. 1981). The consumption of HCB has usually been the result of ignorance, carelessness, or a general lack of concern (Courtney 1979). This is especially true in impoverished/ developing countries where there is a strong tendency by the human population to feed excess seed grains to livestock, or even consume it directly.

HCB has been found in chemical plant workers (Burns and Miller 1975), as well as people with no known direct contact with HCB (Baaken and Seip 1976). Residues of this organic chemical have been detected in humans throughout the world (Siyali 1972; Brady and Siyali 1972; Curley et al. 1973; Leoni and D'Arca 1976; van hove Holdrinet et al. 1977) and in the breast milk of women (Newton and Greene 1972; Stacey and Thomas 1975; Baaken and Seip 1976; Strassman and Kutz 1977; van hove Holdrinet et al. 1977). Most of the human exposure occurs through the consumption of contaminated food products. HCB has been found in the adipose tissue

of domestic animals and poultry (Booth and McDowell 1975; Laska et al. 1976) destined for human use. It has also been detected in plant species commonly used for human and/or livestock feed (deVos et al. 1974; Dejonckheere et al. 1975, 1976). In market basket surveys of typical American food products conducted by the Food and Drug Administration, 5% of the samples contained HCB residues during the years of 1972-1974 (Johnson and Manske 1976; Manske and Johnson 1977). Similar incidences of contamination have been reported in fresh and processed milk products from eastern Bohemia (Cerna et al. 1977).

The absorption of HCB from the diet is somewhat dependent on the nature of food consumed. The feeding of high fat diets to rats resulted in higher HCB residues than equivalent concentrations fed in low fat diets (Zabik and Schemmel 1980). Broiler chickens absorbed virtually all of the HCB present in their diet in a study by Reed et al. (1977). The HCBresidue profile of rats showed that there was a dose-dependent accumulation (Villeneuve 1975) from the diet. The quantity of HCB absorbed from a single oral or dietary dose was also dependent on the nature of the carrier used. Rats absorbed approximately 80% of the chemical present when the HCB was dissolved in oil (Albro and Thomas 1974; Koss and Koransky 1975), but only 6% when the HCB was suspended in water (Koss and Koransky 1975). HCB is absorbed slower from the gastrointestinal tract of rats than the pesticide dieldrin or dichlorobiphenyl (Iatropoulos et al. 1975). It also shows a variable degree of tissue accumulation in different species. The rat accumulates a greater amount of HCB than does the guinea pig (Villeneuve and Newsome 1975), perhaps due to differences in absorption and/or metabolism between these two rodents.

The major portion of ingested HCB appears to be absorbed by the lymphatic system of the upper regions of the small intestine (Iatropoulos et al. 1975; Turner and Shanks 1980). The portal venous system plays a lesser role (Iatropoulos et al. 1975) in the passive transport of HCB across the gut wall (Richter and Schäfer 1981). Chemicals which enter the body tissues through lymphatic absorption bypass the liver, at least initially, and so may be stored in the body without being metabolically altered (Turner and Shanks 1980) and are less likely to be rapidly excreted. Metabolites accounted for 10% of the HCB in the blood of female rats and less than 0.1% of the chemical in adipose tissue (Koss et al. 1976). The distribution of HCB within an individual is closely correlated with the fat content of the tissues (Avrahami and Steele 1972b; Sundlof et al. 1982). Those tissues with the highest fat concentration generally exhibit the highest HCB residues. The tissues may contain not only HCB, but also traces of its dechlorinated metabolites (Mehendale et al. 1975). Richter et al. (1981) found pentachlorophenol, pentachlorothiophenol, 2,3,4,6- and 2,3,5,6-tetrachlorophenol, and pentachlorobenzene in all rat tissues analyzed for HCB. Differences have also been reported between sexes of the same species. Male birds show significantly higher HCB residues than females (Vos et al. 1971; Ingebrigtsen et al. 1981), indicating the importance of elimination via the egg yolk in avian species (Hansen et al. 1978; Ingebrigtsen et al. 1981). The reverse situation is seen in mammals with females accumulating higher HCB concentrations than males (Richter et al. 1981). This phenomenon is probably linked to the greater metabolic activity, and therefore more rapid rate of excretion, generally seen in male mammals when compared to non-lactating females. The tissue concentrations of HCB generally increase rapidly

upon first exposure to the chemical and then reach a plateau where intake and excretion are approximately equal (Kuiper-Goodman et al. 1977; Koss et al. 1978).

The excretion of HCB occurs predominantly through the feces (Koss and Koransky 1975; Yang et al. 1978; Richter and Schäfer 1981; Rozman et al. 1982; Sundlof et al. 1982). Fecal excretion in beagle dogs was demonstrated to be the result of two separate processes (Sundlof et al. 1982), the major contributor of HCB to the intestinal bolus being biliary excretion with intestinal wall excretion playing a lesser role. However, Rozman et al. (1982), in a study utilizing the rat, found intestinal wall passage to be the primary route of excretion in this species. The elimination of HCB with bile was insignificant and enterohepatic recirculation was not a major phenomenon in a concurring study of rats (Richter and Schäfer 1981). HCB was excreted by each of the three regions of the small intestine, with the jejunum excreting more HCB per unit length of gut than the segments (Richter and Schäfer 1981). Urinary excretion of HCB is much lower than fecal quantities, representing one-eighth or less of the total HCB eliminated by the rat (Koss and Koransky 1975), dog (Sundlof et al. 1982), and monkey (Yang et al. 1978). The egg yolk represents the main excretory route in laying female birds (Hansen et al. 1978; Ingebrigtsen et al. 1981). Yang et al. (1978) have proposed the underlying reason for the relatively slow elimination of the animal's total body burden of HCB to be due to the long-term storage in the adipose tissue. Food restriction caused a mobilization of the HCB in fat stores (Villeneuve 1975). The chemical was, however, only transferred into the plasma and other tissues of the rats, with no increase in the rate of excretion. The total body burden was therefore not altered and the HCB

was redistributed into depots where it may present a greater hazard to the animal than it represented while stored in the adipose tissue.

Various agents have been added to the diets of laboratory animals in attempts to increase the fecal and urinary excretion of HCB. Hexadecane, a liquid paraffin, enhanced fecal elimination of HCB and/or its metabolites 4 to 13 fold in rats and Rhesus monkeys (Rozman et al. 1981, 1982). This compound did not affect the urinary excretion of HCB, although bile duct ligation tripled elimination by this route. Mineral oil similarly resulted in an increase of from 6 to 9 times the normal rate of HCB excretion in monkeys (Rozman et al. 1981). Richter et al. (1982) also found dietary treatment of rats with a light liquid paraffin, squalone (a nonabsorbable hydrocarbon), or sucrose polyester improved fecal excretion of HCB without altering urinary excretion. Squalone was likewise shown to stimulate fecal HCB elimination in mice (Richter and Schäfer 1982). These compounds appear to act as a lipophilic compartment in the intestinal lumen (Richter and Schäfer 1981). The equilibrium between the gut wall and the bolus traveling along the intestinal tract for strongly lipophilic compounds such as HCB, would thereby be shifted in favor of the bolus. Cholestyramine, an anion binding resin, and sesame oil did not elicit any improvement in the excretion of HCB and/or its metabolites in rats or monkeys (Rozman et al. 1981).

Koss et al. (1976) found the majority (90%) of the HCB excreted in the urine of rats to be in the form of metabolites. The probable biotransformation of HCB is represented in Figure 2. The three major metabolites were identified as pentachlorophenol (45%), pentachlorothiophenol (30%), and tetra-chlorohydroquinone (17%). Due to the small quantity of HCB excreted via the urine, these metabolites actually comprise only a

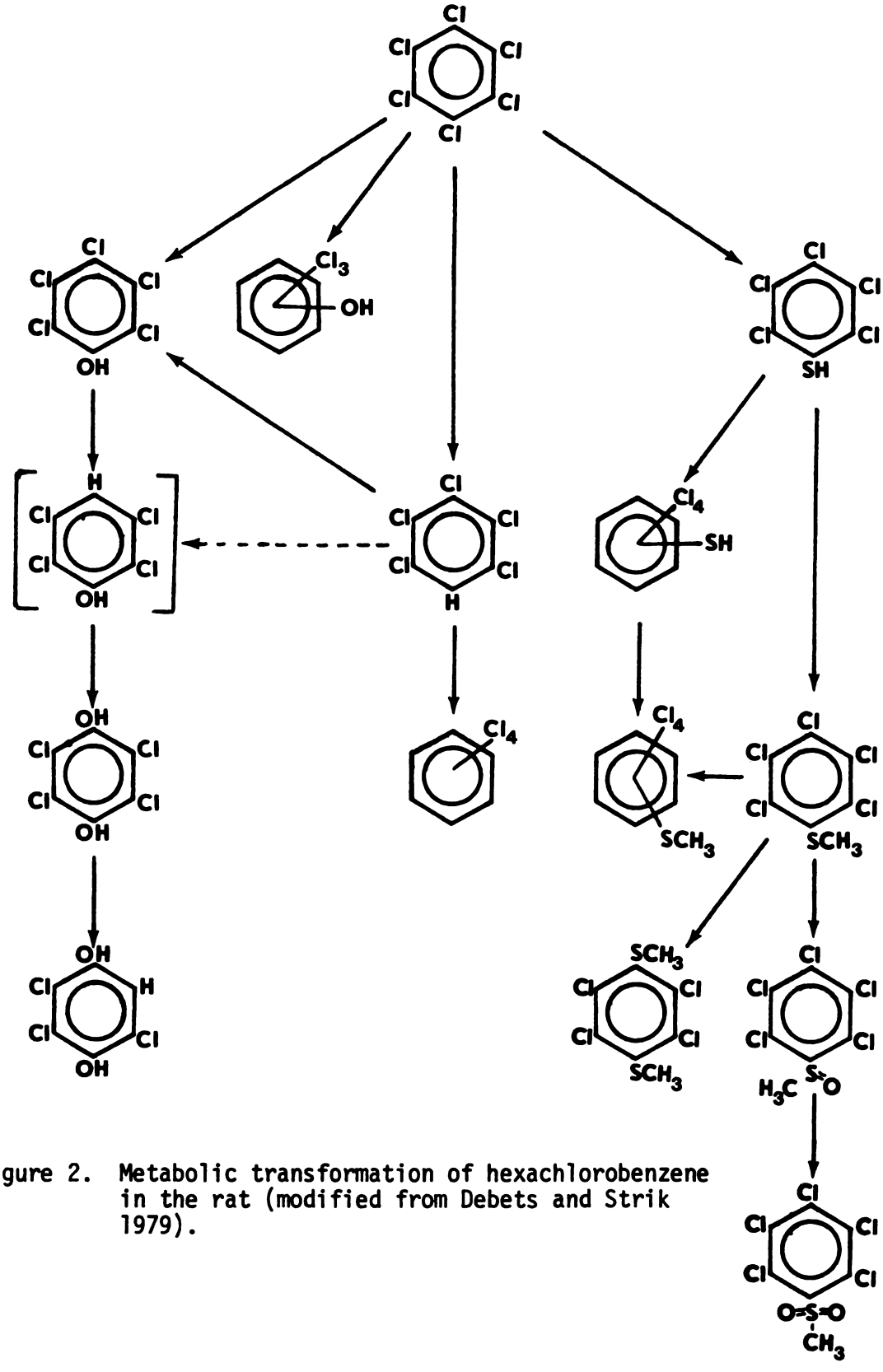


Figure 2. Metabolic transformation of hexachlorobenzene in the rat (modified from Debets and Strik 1979).

small quantity of the total HCB ingested.

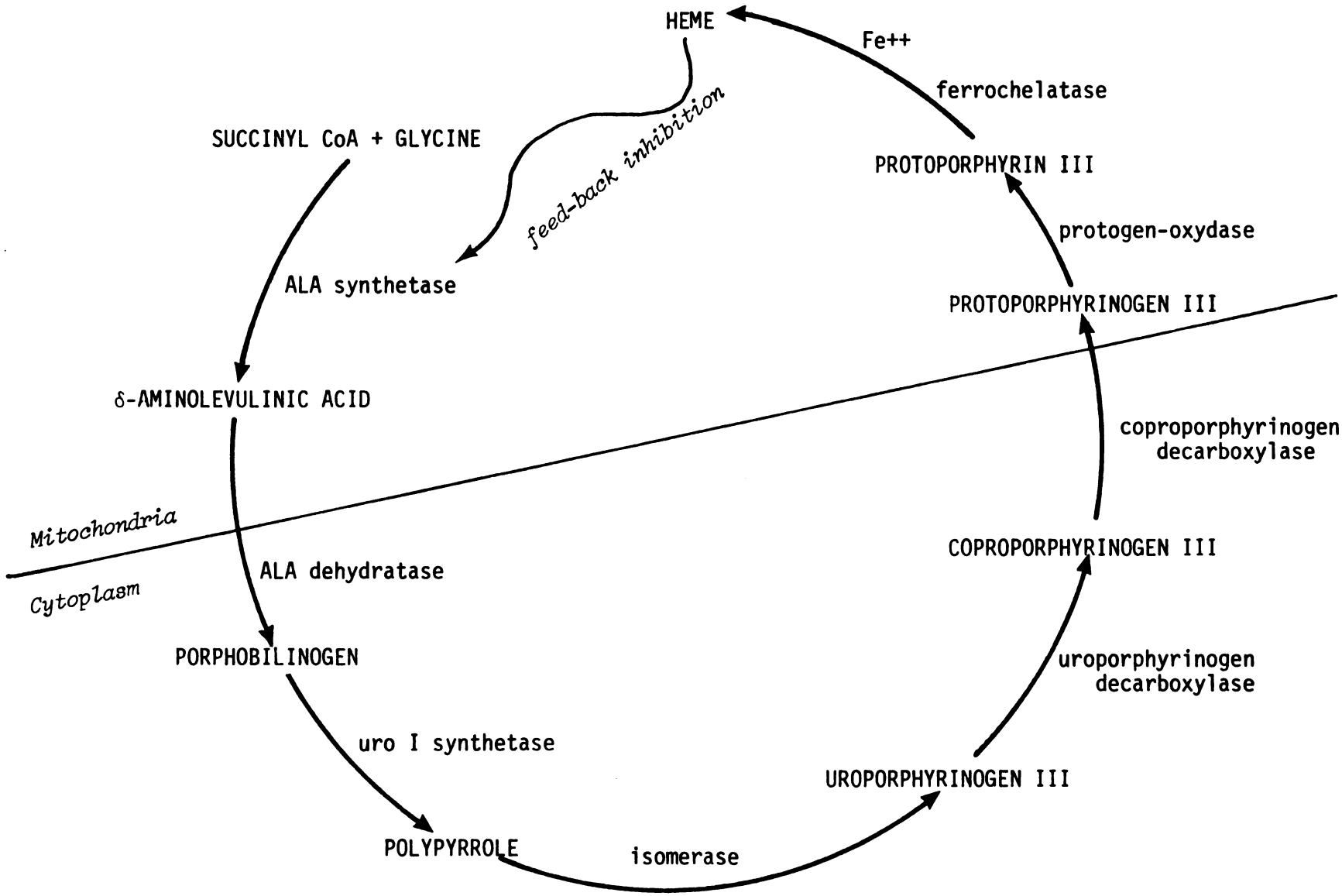
The biological half-life of HCB has been calculated in several laboratory, livestock, and fish species. Values of 60 days (Morita and Oishi 1975), 110 days (Richter et al. 1982), and 120 to 150 days (Koss et al. 1978) have been reported for the rat. The half-life in dogs was shown to be six weeks by Sundlof et al. (1982) and 10 to 18 weeks in the domestic sheep (Avrahami and Steele 1972a). Rainbow trout (Salmo gairdneri) were studied by Niimi and Cho (1981) and a biological half-life arrived at of from 61 to 117 days.

The clinical signs of HCB intoxication include anorexia, tremors, and hyperexcitability (Kimbrough and Linder 1974; Villeneuve 1975; Clark et al. 1981). HCB potentiated the severe weight loss seen in rats subjected to crowded conditions (Clark et al. 1981). The toxicity of mercuric chloride ($HgCl_2$) was also increased by HCB treatment (Renner 1980). Tremors and hyperexcitability have been reported in mice, guinea pigs, rabbits (DeMatteis et al. 1961), and Japanese quail (Vos et al. 1971) fed HCB. An increased incidence of tumors has also been observed in mice (Cabral et al 1978; Shirai et al. 1978) and hamsters (Cabral et al. 1977) consuming HCB in long-term carcinogenicity trials. Cabral et al. (1977) found the average number of tumors per hamster and the percentage of animals with multiple tumors to show a dose-response relationship. In this study, numerous neoplasms were identified including hepatomas, liver hemangioendotheliomas, spleen hemangioendotheliomas, adrenal neoplasms, and alveolar adenomas of the thyroid. HCB alone did not result in hepat- carcinogenicity in mice (Shirai et al. 19780), but potentiated the induction of tumors following treatment with PCBs. Upon histological examination, Iatropoulos et al. (1976) found thymic cortex atrophy and

degenerative changes of the liver and kidney in Rhesus monkeys treated with HCB.

Human exposure to low level dietary HCB has resulted in examples of HCB-induced porphyria cutanea tarda (PCT) in Turkey and Saudi Arabia (Courtney 1979). In both of these incidences, seed wheat that had been treated with HCB was used to make bread. Approximately 5000 people were estimated to have developed PCT (Schmid 1960; Dogramaci et al. 1962; Dogramaci 1964) during the course of the Turkish outbreak, with a mortality rate of 10%. A loss of appetite, cutaneous photosensitivity, muscle weakness, hypertrichosis, hyperpigmentation, and porphyrinuria were seen in many of the people exposed to HCB (Courtney 1979). PCT, also known as porphyria turcica, is a metabolic disorder of porphyrin production characterized by an increased excretion of porphyrins and their precursors. Cutaneous lesions are a visible manifestation of this porphyria, inducible by chemicals such as HCB (Courtney 1979). The repeated exposure to sunlight resulted in erythema, erosion, crusting, skin thickening, and scarring of the skin of rats with HCB-induced porphyria (Torinuki et al. 1981). The control mechanism underlying the normal synthesis of porphyrins (Figure 3) can break down when an animal is exposed to HCB. In HCB-related PCT, excessive quantities of porphyrins are produced which are not incorporated into hemoproteins, and so are excreted in the urine and feces. HCB-induced porphyria has been reported in rats (Lissner et al. 1975; Elder et al. 1976; Torinuki et al. 1981), rabbits (DeMatteis et al. 1961), Japanese quail (Vos et al. 1971), chick embryos (Debets et al. 1981a), and pigs (Hansen et al. 1977). In a separate study by denTonkelaar et al. (1978), pigs were observed to develop porphyria-like symptoms, but the liver fluorescence characteristic of PCT was not

Figure 3. Synthesis pathway of the heme molecule (modified from Courtney 1979 & Hammond and Beliles 1980).


HEME
Fe++
ferrochelatase
SUCCINYL CoA + GLYCINE
feed-back inhibition
PROTOPORPHYRIN III
protogen-oxydase
ALA synthetase
PROTOPORPHYRINOGEN III
δ-AMINOLEVULINIC ACID
coproporphyrinogen decarboxylase
Mitochondria
Cytoplasm
ALA dehydratase
COPROPORPHYRINOGEN III
PORPHOBILINOGEN
uroporphyrinogen decarboxylase
uro I synthetase
UROPORPHYRINOGEN III
POLYPYRROLE
isomerase

detected. The development of PCT following HCB exposure is not universal among the animal species tested. It was not possible to induce porphyria in Beagle dogs with dietary HCB (Gralla et al. 1977) and no liver fluorescence was seen at necropsy. The dog may lack the synthetic and excretory pathways which respond to HCB insult and result in porphyria. Differences were also observed between sexes of porphyria sensitive animals. Female rats exhibit PCT sooner after exposure to HCB and to a more severe degree than males (Kuiper-Goodman et al. 1977; Richter et al. 1981). This differential sensitivity may result from inherant differences in the basal levels of enzymatic biotransformation (Richter et al. 1981) or from some estrogen-related pathway (Kuiper-Goodman et al. 1977).

The mechanism by which HCB produces its porphyrinogenic effects is poorly understood (Debets and Strik 1979). However, the metabolic transformation of HCB appears to be a requirement for the induction of PCT (Kerklaan et al. 1979; Debets et al. 1980). A metabolic product, presumed to be a reactive intermediate, appears to be responsible for the alterations of heme synthesis seen in HCB-induced toxicity. Koss et al. (1980) have identified reactive conversion products of HCB in the livers of rats. The simultaneous administration of HCB and phenobarbital in the rat produces an earlier disturbance of porphyrin synthesis than HCB alone (Puzynska et al. 1978), probably as a result of mixed function oxidase enzyme induction. Diethyl maleate, a compound which depletes liver stores of glutathione S-transferase, also causes an earlier disturbance of the porphyrin pathway when given in conjunction with HCB (Puzynska et al. 1978; Kerklaan et al. 1979). The resulting lower concentrations of glutathione available for the detoxification of porphyria-inducing agents probably allows the more rapid onset of clinical signs seen in these rats.

Many porphyrinogenic chemicals, including HCB, cause the de novo synthesis of aminolevulinic acid (ALA) synthetase (Granick and Urata 1962). The elevated ALA-synthetase levels result in excesses of aminolevulinic acid, monopyrrole, porphobilinogen, tetrapyrroles, uroporphyrin, coproporphyrin, and protoporphyrin. The porphyrinogenic effects of HCB are therefore not due to a direct alteration of heme synthesis (Debets and Strik 1979). HCB exposure causes an inhibition or inactivation of the liver enzyme uroporphyrin decarboxylase (Elder et al. 1976; Debets and Strik 1979; Strik et al. 1979; Koss et al. 1980), and so uroporphyrins accumulate. The disturbance of uroporphyrin decarboxylase function may be a consequence of covalent binding of HCB-reactive conversion products to the enzyme (Koss et al. 1980). Rats treated with HCB also showed a reduced glucuronyl transferase activity and altered steroid metabolism with the increased production of unconjugated 5β-steroids (Graef et al. 1982).

The duration of time a chemical resides in the body and its intensity of action is largely dependent upon the speed at which it is metabolized by the enzymes of the mixed function oxidase (MFO) system in the liver (Conney 1967; Reed et al. 1977). The induction of these enzymes can thereby result in significant changes in the metabolism of xenobiotics. HCB has been reported by denTonkelaar and vanEsch (1974) not to be a potent inducer of the microsomal enzymes. Carlson (1978) found HCB to be a phenobarbital-type inducer of MFO activity, while others have reported this chemical to be a mixed inducer (Stonard 1975; Stonard and Greig 1976), inducing both 3-methylcholanthrene and phenobarbital type enzymes. Increased concentrations of liver cytochrome P-450 (Lissner et al. 1975; Puzynska et al. 1979) and of aniline hydroxylase activity

(Lissner et al. 1975; Grant et al. 1977) have been reported for rats exposed to HCB. Mehendale et al. (1975) likewise found the pretreatment of rats with HCB to lead to significant induction of the MFO system. A dose-related induction of liver enzymes was also observed in domestic pigs (denTonkelaar et al. 1978). The induction of MFO enzymes may represent an increase in the rate of synthesis, as well as a diminished rate of enzyme destruction (Conney 1967). Mehendale et al. (1975) have determined that the reductive dechlorination of HCB seen in the rat is catalyzed by enzymes located in the liver, lung, kidney, and intestine. Phenobarbital treatment (by MFO induction and therefore increasing HCB metabolic transformation) and diethyl maleate (by competing for hepatic glutathione) result in a more rapid production of HCB metabolites and the toxic effects they exert than is seen with HCB alone (Puzynska et al. 1979). Piperonyl butoxide, an inhibitor of MFO activity, decreased the irreversible binding of HCB metabolites to the cellular proteins in chick embryo liver cells (Debets et al. 1981a), suggesting a reduced rate of biotransformation of the parent compound by this enzyme system. One physiological effect that may be observed from phenobarbital-type inducers is the increased hydroxylation of steroids. By reducing steroid hormone concentrations, such as estradiol and estrone, an inhibition of their action on the reproductive tract of the female occurs and can result in reproductive disturbances or complete reproductive failure. These changes represent an undesirable side effect of the body's attempt to metabolize and/or detoxify xenobiotics.

HCB has a definite adverse effect on reproductive ability (Mendoza et al. 1975; Grant et al. 1977). Dietary HCB resulted in female rats producing fewer litters, an increased incidence of still born pups, and

poor postnatal viability (Grant et al. 1977). Young rats raised by mothers consuming HCB-supplemented diets died seven to eight days after birth in a study by DeMatteis et al. (1961). Additionally, rat pups from unexposed dams that were cross-fostered onto these HCB females died in convulsions after three to four days of nursing, as had the female's own litter. The percent mortality of rat pups exposed to HCB via their dams from two different laboratories is compiled in Table 1. From the study by Grant et al. (1977), it was concluded that 20 ppm HCB in the maternal diet was the no-effect level for reproduction in the rat. Reduced birth weight, a general indication of toxicosis, was also reported for rat pups born to HCB-exposed dams (Khera 1974; Grant et al. 1977), as was a reduced growth rate.

Table 1. Offspring mortality of rat pups produced by dams consuming control and HCB diets.

Dietary concentration	% Mortality	
	Grant et al. 1977	Kitchin et al. 1982
0 ppm	8	9.2
10 ppm	1	-
20 ppm	8	-
40 ppm	7	-
60 ppm	-	19.8
80 ppm	5	30.0
100 ppm	-	45.4
120 ppm	-	93.1
140 ppm	-	92.6
160 ppm	43	-
320 ppm	100	-

Transplacental passage of HCB from the mother to the fetus has been shown in rats (Villeneuve and Hierlihy 1975; Andrews and Courtney 1976; Courtney et al. 1979; Svendsgaard et al. 1979), mice (Courtney et al. 1976; Courtney and Andrews 1979), rabbits (Villeneuve et al. 1974), monkeys (Bailey et al. 1980), and humans (Siyali 1974). The placenta offers little resistance to the passage of small lipophilic molecules such as HCB. The so called "placental barrier" provides only limited protection to the developing fetus against chemicals it may be exposed to during gestation (Goodman et al. 1982). Svendsgaard et al. (1979) observed a sixfold increase in fetal concentrations of HCB in mice and rats that were multiply dosed during late gestation, as compared to animals similarly dosed during early pregnancy. This phenomenon probably results from normal changes in placental permeability (Moya and Smith 1965). As the placental membranes become thinner and the flow rate of the umbilical circulation increases with advancing pregnancy, fetal contact with substances in the dam's blood is enhanced. Since most foreign substances have been shown to cross the placenta by simple diffusion (Eckhoff 1972a,b), changes in placental surface area available for transfer, thickness of placental membranes, and the rate of maternal blood flow with progressing gestation would increase the rate of diffusion, resulting in near-term exposed fetuses possessing greater concentrations of HCB than those fetuses exposed during the early stages of development. The maternal tissue stores of HCB act as a readily available reservoir of chemical for continued exposure to the developing and growing offspring (Courtney and Andrews 1979). The potential of chemical-induced fetotoxicities to be the result of placental and/or fetal biotransformation must also be considered (Goodman et al. 1982). The placenta does, however, appear to offer some degree of

protection to the fetus. Andrews and Courtney (1976) found the HCB content of the placenta of mice and rats to always be greater than its respective fetus. HCB has also been found to cause grossly observable teratogenic effects in rats (Khera 1974) and mice (Courtney et al. 1976), including cleft palates and kidney malformations.

The presence of HCB in the milk of lactating females has been shown for humans (Siyali 1973; Baaken and Seip 1976), Rhesus monkeys (Bailey et al. 1980), cattle (Fries and Marrow 1971), and rats (Grant et al. 1977). Siyali (1973) found HCB to be present in the breast milk samples of all the women tested. Bailey et al. (1980) determined the milk concentrations of monkeys to be 17 to 20 times the concentration of maternal serum. High concentrations of HCB have been reported in weanling rats, compared to a relatively low dietary intake of the compound by their dams (Grant et al. 1977). This indicates the excretion of HCB via the milk to be an important route of offspring exposure and of maternal excretion. This finding is supported by the determination by Bailey et al. (1980) that nursing infant Rhesus monkeys were at greater risk than their mothers when the dam was exposed to HCB. Human infants have likewise been shown to be sensitive to HCB when exposure was limited to that coming from their mothers. A 95% incidence of infant mortality (as compared to 10% mortality in adults) was seen in some villages in Turkey when mothers consumed HCB-contaminated bread (Courtney 1979). Pembe yara, or "pink sore" was also seen in the nursing children. Porphyria was not noted in these children, suggesting that pembe yara may be a forerunner of porphyria in young children (Peters et al. 1966).

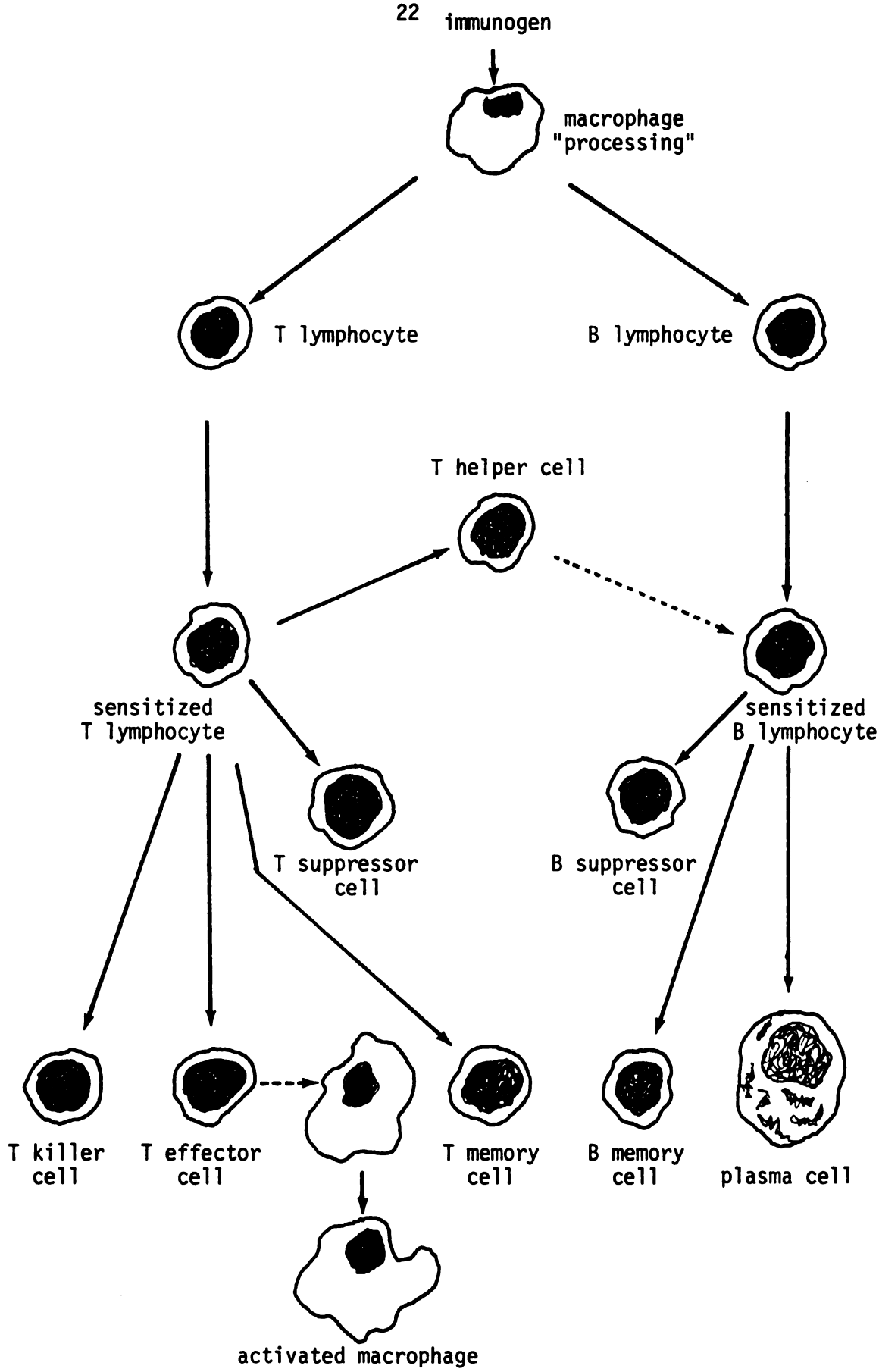
HCB can adversely affect reproduction in avian species as well. At high doses of HCB a delay in the onset of full egg production was seen in

chickens (Hansen et al. 1978). It has also been linked with poor embryonic survival and reduced hatching success in gulls (Gilbertson and Fox 1977) and terns (Gilbertson and Reynolds 1972).

The normal immunological defense mechanisms involve the participation of four major aspects: 1) T lymphocytes (cell-mediated immunity), 2) B lymphocytes (humoral immunity), 3) phagocytic cells, and 4) complement (Dean et al. 1979; Luster and Faith 1979; Heise 1982). A proper immune response following challenge is dependent upon the cooperative interaction of these factors. Different subpopulations of T lymphocytes and mononuclear cells are interconnected to form regulatory circuits (Figure 4) which determine the quality, extent, and duration of an immune response (Hahn and Kaufmann 1981; Litwin 1981). The cells of the immune system originate from pluripotent stem cells located in the yolk sac and liver of the fetus and in the bone marrow and spleen of the adult (Phillips and Melchers 1976; Abramson et al. 1977; Owen et al. 1977). The bone marrow serves as a source of cells to populate and proliferate in the thymus (Cantor and Weissman 1976), as well as the body's other lymphoid tissues. In the adult mammal, the proliferation of these stem cells provides for the constant renewal of the circulating pool of lymphocytes and phagocytic cells (Hume and Weidemann 1980). Liver, lung, and spleen contain the major populations of fixed tissue macrophages (Loose et al. 1977) which may be the initiators of the immune response. Cell-mediated immunologic competence provides the organism with protection against foreign tissue transplants, certain viral, bacterial and fungal infections, intracellular parasites and neoplastic cells (Eisen 1974; Dean et al. 1979; Luster et al. 1979).

The lymphocyte transformation microculture techniques, using plant

Figure 4. Interrelationship of T lymphocytes, B lymphocytes, and macrophages in the response, memory, and regulation of the immune system (modified from Vos 1977).


immunogen
macrophage
"processing"
T lymphocyte
B lymphocyte
T helper cell
sensitized
T lymphocyte
sensitized
B lymphocyte
T suppressor
cell
B suppressor
cell
T killer
cell
T effector
cell
T memory
cell
B memory
cell
plasma cell
activated macrophage

lectins (mitogens), provide a sensitive and reliable assessment of cell-mediated immunity (Thilstead et al. 1979; Dean et al. 1982a; Luster et al. 1982). These primary cell culture assays allow the monitoring of the immune system following chemical exposure. The immune system cells, having been exposed to the toxicant in vivo can be readily removed from the body and their function examined in vitro. Thilstead et al. (1979) have pointed out the further suitability of these techniques in that they allow repeated testing without altering the antigen-specific aspects of cell-mediated immunity. With the development and improvement of gradient separation techniques for the isolation of lymphocytes, lymphocyte activation/transformation tests have become simpler and more reliable. By taking advantage of differences in the size and density of the formed elements of blood, a large percentage of the total viable lymphocyte population from a given sample of whole blood can be isolated, relatively free of contamination (Perper et al. 1968). Antigens normally induce transformation in only a small responsive population of immune cells and such changes must be gauged against changes going on in a much larger background population of cells. These small alterations are not generally detectable by standard biochemical assays, but through the use of mitogens a large proportion of the lymphocytes can be stimulated, regardless of their antigenic specificity (Stanton et al. 1978; Hume and Weidemann 1980). The mitogen-induce lymphocyte subpopulations can then express their various functions. Hume and Weidemann (1980) have reported cellular proliferation not to be merely a simple reaction between a lymphocyte and the mitogen, but rather a complex interaction between several types of differentiated cells. The binding of the mitogen to the lymphocyte's surface receptor appears to initiate activation (Resch et al.

1981). This stage does not seem to require the actions of accessory cells such as macrophages. A second signal, independent of the mitogen, results in the progression of activated lymphocytes to proliferative cells. It is this step which is dependent on the macrophage and acts to modulate the size of the proliferating lymphocyte population (Resch et al. 1981). Unlike the lymphocytes, macrophages do not exhibit immunologic specificity (Rosenberg and Lipsky 1981) and so are capable of responding to a wide range of immunogens. The macrophage-mediated enhancement is probably the result of soluble factors released by the macrophages (Gery et al. 1972).

The plant lectin Concanavalin A (Con A) is considered to be capable of stimulating T lymphocytes, but not B lymphocytes (Janossy and Greaves 1972). More specific studies have shown Con A to selectively activate T-helper and T-suppressor subpopulations (Stanton et al. 1978; Hume and Weidemann 1980; Tsokos and Balow 1981). The T lymphocyte response to this mitogen is also dependent upon the presence of macrophages (Wedner and Parker 1976; DeVries et al. 1979; Resch et al. 1981) and their metabolic products (Persson et al. 1978).

Chronic low level or acute high level exposure to a toxicant can impair immunologic function and result in an increased susceptibility to disease and opportunistic microorganisms (Bick 1982; Heise 1982). The immunosuppressive effects of a chemical may or may not be the result of direct action upon the immune system cells. Other factors such as stress can alter immune function and produce an animal incapable of properly responding to challenge (Street and Sharma 1975; Kelley 1980; Luster et al. 1982). The abnormal immune profile may be the representative of a generalized toxic response rather than the immune system

being singled out as a specific target. This is an important consideration since the impaired immunologic responses seen are often occurring at toxicant levels which also result in toxic effects in other organs (Vos 1977). Other indirect effects (nutritional deficiencies, pathogenic organisms, endocrine inbalances) can similarly lead to functional abberations in the immune system (Vos 1977; Loose et al. 1979). The direct altering of the immune system, as measured by lymphocyte responsiveness, can result from: 1) a failure in the activation of the lymphocytes, 2) tolerance to the immunogen, and/or 3) the production of soluble suppressor factors by macrophages or the regulatory subpopulations of T lymphocytes (Dean et al. 1982a). Street and Sharma (1975) have reported that the immune responses most easily damaged are those portions of the system dependent on sensitized lymphocytes (Figure 4) to carry out the cell-mediated reactions. The immune system also shows a considerable ability to rebound after insult. Harmful effects may be temporary (Moore 1979; Bick 1982), even though the period of time required to reestablish competency may be long. The in vitro function assays may over estimate the degree of cellular damage. As stated by Archer (1982), cells which have been injured by exposure to a chemical may appear nonfunctional in the somewhat hostile in vitro environment, but might have been able to regain normal function had they been left in vivo. Although there exists a direct relationship between the chemical suppression of the immune system and resistance to infectious diseases, any relationship between immunosuppression and cancer and autoimmune disease is probably only an indirect link (Vos 1977).

The immune system is a sensitive indicator of toxic insult. This is probably the result of several different, but interconnected

components. Normal immune function is a tightly regulated and organized series of events with the cells involved acting in cooperation with each other (Litwin 1981; Dean et al. 1982a) to protect the host. Also, the numerous cell types participating in an immune response undergo rapid proliferation and differentiation to produce their actions. The high metabolic demands and elevated activity in the synthesis of proteins and DNA, makes these cells especially vulnerable to toxicant exposure.

MATERIALS AND METHODS

The mink (Mustela vison) used in these studies were from the Michigan State University Experimental Mink Ranch. The European ferrets (Mustela putorius furo) were bred from stock obtained from Marshall Research Animals, North Rose, NY. All animals were housed and cared for at the MSU Experimental Mink Ranch. The mink and ferrets were confined in individual pens of uniform size and each pen was equipped with a water cup and nest box. Except where stated otherwise in the text, the following procedures pertain to all experiments.

Standard ranch procedures were followed in the care, feeding, and breeding of the animals. The mink were vaccinated for canine distemper, botulism, and viral enteritis in July. Ferrets were similarly vaccinated in August. In alloting animals into various groups for experimentation, littermates were divided between groups in an attempt to balance genetic differences in growth, reproduction, and response to treatments.

The mink and ferrets were weighed individually at the start of the experiments and at specified intervals thereafter, except during the gestational periods. The females were mated to males within their respective dietary group. Each female mink was given an opportunity to remate either the day after the initial mating or eight days later. The female ferrets were mated beginning with their first estrus cycle in April. Any females that recycled were once again mated, up to three separate times. All matings were verified by the presence of motile spermatozoa in a

vaginal smear taken after copulation. The females were checked daily for the birth of young during the whelping period. Kits were counted and weighed the day of birth, at three weeks of age, and at six weeks of age.

Gross necropsies were performed on the animals that died during the course of the experiments. Organ weights were recorded and selected tissues collected for HCB analysis and histopathologic examination. Tissues for histology were fixed in 10% neutral formalin and processed by routine histological procedures. Neural tissues were sectioned at 15 microns and all other tissues cut at 5 microns. Sections were stained with Harris's Hematoxylin and differentiated for 10 seconds in 1% HCl and 80% ethyl alcohol. Each was then counterstained in 0.5% Eosin Y and differentiated in two chages of absolute ethyl alcohol. The slides were cleared in xylene and coverslipped using Flo-Texx® (Lerner Laboratories, Stamford, CT).

In the preparation of the experimental diets employed in these studies, the HCB was incorporated into 500 g of commercial mink cereal (XK Mink Foods, Inc., Thiensville, WI) and mixed with the other ingredients of the diet to yield a ration that contained the desired amount of chemical. The HCB used in the feeding trials was lot #H02550, technical grade, purchased from Pfaltz & Bauer, Inc. Stamford, CT. The composition of the basal (control) diet is shown in Tables 2 and 3. After February 10, 22 I.U. of vitamin E per kg of feed were added to all diets and continued until July 1. Corn oil was added to the diet after April 20 at a concentration of 1%. This supplementation was continued through lactation.

Unless stated otherwise, the $P<0.05$ level of probability was used as the criteria for significant differences between treatment means. The

Table 2. Composition of the basal diet.

Ingredient	Weight (lbs)	Percentage
Whole chicken	240	20.0
Commercial mink cereal*	200	16.7
Ocean fish scrap+	150	12.5
Beef tripe	40	3.3
Cooked eggs	35	2.9
Beef liver	80	6.7
Beef trimmings	40	3.3
Beef lungs	80	6.7
Powdered milk	13	1.1
Added water	322	26.8

*XK-40 Grower, XK Mink Foods, Inc., Thiensville, WI.

+Cod, haddock, and flounder.

Table 3. Moisture, protein, fat, and ash content of basal diet[1].

Component	Percentage of diet	
	As fed	Dry matter
Moisture	68.4	-
Protein	14.3	45.26
Fat	7.91	25.03
Ash	2.54	8.04

[1] As determined by Rosner/Runyon Laboratories, Inc., Chicago, IL.

standard error of each mean (S.E.) was calculated as $\sqrt{MS_E/r_i}$ (homogeneous variance) or as $\delta/\sqrt{n}$ (heterogeneous variance).

Experiment I. ACUTE TOXICITY OF HEXACHLOROBENZENE TO MINK

Purpose

The acute toxicity of HCB is not known in the mink and so this experiment was undertaken to determine the relative toxicity as shown by the 96 hour LD_{50}.

Materials and Methods

Sixteen adult male ranch-bred mink were randomly divided into four groups of four mink each. Treatment groups were 0, 500, 1000, or 2000 mg of HCB per kg of body weight. The HCB was suspended in corn oil at concentrations of 0 mg, 500 mg, 1000 mg, or 2000 mg HCB per ml. The appropriate dose of chemical was administered per os, via stomach tube, to each animal. The mink were observed four times on the day of dosing and twice daily on each subsequent day. Since no mortality was seen in the initial 96 hour period, all animals were observed until 30 days post-dosing.

Results

No mink died or showed clinical signs of toxicity at any of the treatment levels of HCB. All were judged to be in good condition and health throughout the 30 day observation period. The animals did not show indications of distress even immediately following dosing. When returned to their cages, the mink began grooming and eating as if they had experienced no ill effects from the treatment.

Discussion

Based on the results of this study, it can be concluded that the oral LD_{50} of HCB for mink is greater than 2000 mg/kg of body weight. At the concentration of 2000 mg of HCB per ml of corn oil, the suspension was a viscous sludge. This quantity of chemical represents a far greater

amount of HCB than a carnivore, or any other animal, is likely to come in contact with in an acute exposure situation.

HCB has generally shown a low order of toxicity in a variety of animal species when the exposure was of short-term duration (Booth and McDowell 1975). Its acute toxicity is quite low (>1 g/kg of body weight) in numerous laboratory animals (WHO 1970). The dangers to animal and human populations, therefore, appears to be small when exposures are limited to acute periods, even if the level of exposure is high.

Experiment II. EFFECTS OF CHRONIC DIETARY HEXACHLOROBENZENE EXPOSURE IN MINK AND EUROPEAN FERRETS

Purpose

This experiment was conducted to determine the chronic toxicity of HCB to mink and ferrets. The effects of chronic dietary exposure to this halogenated aromatic hydrocarbon on reproductive performance, mortality, and various physiological parameters were monitored during an 11 month feeding trial.

Materials and Methods

Seventy-eight standard dark mink were divided into five treatment groups and a control group. Each treatment group consisted of ten females and three males. Seventy-eight adult fitch-colored European ferrets were likewise assigned to five treatment groups and a control group. The animals were fed the basal diet supplemented with either 0 (control), 1, 5, 25, 125, or 625 ppm HCB. Standard ranch procedures were followed in the care, feeding, and breeding of the animals. The mink and ferrets were weighed and blood samples taken periodically during the course of the study. From the blood sample, obtained by toe clip, the following parameters were measured or calculated: 1) red blood cell(RBC) count, 2) white blood cell (WBC) count, 3) hematocrit (Hct), 4) hemoglobin (Hb) concentration, 5) leukocyte differential count, 6) mean corpuscular hemoglobin concentration (MCHC), and 7) mean corpuscular volume (MCV). Animals were observed daily for signs of toxicity and gross necropsies performed on those mink and ferrets dying prior to the termination of the study. Tissue samples were also taken from these animals for histological examination and HCB residue analysis. Reproductive parameters were monitored

during the mating and whelping periods (whelping date, number of kits/ litter, number of live and dead kits, birth weight, three week body weight, six week body weight, and kit mortality to weaning) of the mink and ferrets. The kits from both species were weaned at seven weeks of age and placed on unsupplemented basal diet.

Upon termination of the study (mink on 16 December 1981, ferrets on 17 December 1981), blood samples were collected from each animal and it was then killed by cervical dislocation, and a gross necropsy performed. Brain, heart, kidney, liver, spleen, and lung were dissected from the carcass and weighed. In addition to the previously named six tissues, samples were taken of ileum, pancreas, adrenal gland, muscle, stomach, and skin and placed in formalin for histological examination.

Results

No difference was found in initial body weight of the female mink used in this study (Table 4). However, after 22 days on the contaminated feed, the body weight of the mink consuming 125 ppm HCB was significantly lower than that of controls. Since only a single animal was still alive on the 625 ppm treatment, it was not possible to include this group for statistical analysis. The body weight of this one mink was, nevertheless, lower than any animal in the control group. No significant differences were seen in the body weights of the female mink in any of the treatment groups following 149 days of exposure (17 June 1981) to HCB. At the termination of the study no differences were found between the four surviving groups. The initial body weights of female ferrets were not significantly different from those of control ferrets. The 625 ppm HCB group showed significantly lower weights after 22 days of treatment than control animals, and the 125 ppm ferrets exhibited the same result

Table 4. Body weights[†] (g) of adult female mink and ferrets consuming control and HCB diets in a chronic feeding trial.

Treatment	Weigh date							
	n	1-20-81	n	2-11-81	n	6-17-81	n	12-17-81
Mink								
0 ppm	10	859 ± 33.0	10	894 ± 33.4	10	799 ± 31.1	9	957 ± 47.9
1 ppm	10	909 ± 33.0	10	953 ± 33.4	10	813 ± 31.1	9	923 ± 47.9
5 ppm	10	906 ± 33.0	10	950 ± 33.4	10	852 ± 31.1	10	995 ± 45.5
25 ppm	10	934 ± 33.0	10	979 ± 33.4	9	880 ± 32.8	8	1061 ± 50.8
125 ppm	10	855 ± 33.0	10	767 ± 33.4*	6	741 ± 40.2	-	---
625 ppm	10	868 ± 33.0	1	614	-	---	-	---
Ferret								
0 ppm	10	714 ± 26.4	10	751 ± 27.0	10	793 ± 29.9	10	792 ± 32.3
1 ppm	10	692 ± 26.4	10	763 ± 27.0	10	741 ± 29.9	9	771 ± 34.0
5 ppm	10	687 ± 26.4	10	746 ± 27.0	9	792 ± 31.5	8	797 ± 36.1
25 ppm	10	675 ± 26.4	10	726 ± 27.0	10	750 ± 29.9	8	783 ± 36.1
125 ppm	10	693 ± 26.4	10	705 ± 27.0	7	683 ± 35.7*	-	---
625 ppm	10	731 ± 26.4	10	617 ± 27.0*	-	---	-	---

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

after 149 days of dietary exposure. No differences were observed between the four treatment groups of ferrets still alive on the study's termination date (17 December 1981).

When the feeding trial was begun, the 5 ppm HCB group male mink weighed significantly less than, and the 125 ppm HCB group males weighed significantly more than, the control males (Table 5). All the mink were in good condition at this time and no significant differences were seen in the body weights of male mink at any point during the course of the experiment, except for the initial variation. No differences were found in body weights between control and HCB-treated ferrets at the beginning of the trial and only the 625 ppm males exhibited significantly depressed weights, following 22 days of HCB exposure. No other changes in body weight were seen during the length of the study for the HCB-treated ferrets.

The hematologic parameters of RBC count, Hb concentration, Hct., MCHC, MCV, and WBC count were not significantly different from control values for mink or ferrets on any of the HCB-supplemented diets (Tables 6 and 7) after 22 days of treatment. However, one female mink consuming HCB at 625 ppm of the diet exhibited RBC, Hb, Hct., and WBC values considerably lower than the untreated mink. Following the death of this mink two days after the blood was collected, blood was found throughout the gastrointestinal tract which probably accounts for these reduced values.

Determinations made on blood collected after 149 days of feeding the 125 ppm HCB-supplemented diet showed a significant increase in the WBC count in the female mink, but no alterations in the parameters measured in these animals (Table 8). No significant differences from control were

Table 5. Body weights[†] (g) of adult male mink and ferrets consuming control and HCB diets in a chronic feeding trial.

Treatment	Weigh date							
	n	1-20-81	n	2-11-81	n	6-17-81	n	12-17-81
Mink								
0 ppm	3	1588 ± 34.4	3	1698 ± 116.4	3	1585 ± 46.5	3	1839 ± 125.9
1 ppm	3	1608 ± 34.4	3	1626 ± 116.4	3	1596 ± 46.5	3	1707 ± 125.9
5 ppm	3	1432 ± 34.4*	3	1596 ± 116.4	3	1497 ± 46.5	3	1755 ± 125.9
25 ppm	3	1557 ± 34.4	3	1654 ± 116.4	3	1610 ± 46.5	3	1939 ± 125.9
125 ppm	3	1733 ± 34.4*	3	1514 ± 116.4	-	---	-	---
625 ppm	3	1558 ± 34.4	-	---	-	---	-	---
Ferret								
0 ppm	3	1292 ± 83.8	3	1399 ± 74.2	3	1427 ± 183.8	2	1647 ± 162.6
1 ppm	3	1498 ± 83.8	3	1563 ± 74.2	2	1559 ± 225.1	2	1902 ± 162.6
5 ppm	3	1470 ± 83.8	3	1521 ± 74.2	3	1444 ± 183.8	3	1750 ± 132.8
25 ppm	3	1238 ± 83.8	3	1338 ± 74.2	3	1414 ± 183.8	3	1543 ± 132.8
125 ppm	3	1335 ± 83.8	3	1227 ± 74.2	-	---	-	---
625 ppm	3	1257 ± 83.8	2	1153 ± 90.0*	-	---	-	---

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

Table 6. Hematologic profile[†] of adult mink and ferrets consuming control and HCB diets for 22 days in a chronic feeding trial.

Treatment	n	RBC ($x10^6$)	Hb (g %)	Hct (%)	MCHC (g/100 ml)	MCV (μ^3)	WBC ($x10^3$)
Mink							
0 ppm	10	9.61 ± 0.186	21.93 ± 0.412	55.6 ± 0.86	39.5 ± 0.61	57.9 ± 0.90	18.4 ± 1.90
1 ppm	10	9.49 ± 0.186	20.85 ± 0.412	54.2 ± 0.86	38.5 ± 0.61	57.2 ± 0.90	20.9 ± 1.90
5 ppm	10	9.95 ± 0.186	22.16 ± 0.412	55.1 ± 0.86	40.3 ± 0.61	55.4 ± 0.90	21.1 ± 1.90
25 ppm	10	9.30 ± 0.186	21.67 ± 0.412	54.7 ± 0.86	39.6 ± 0.61	59.0 ± 0.90	19.9 ± 1.90
125 ppm	10	10.11 ± 0.186	23.34 ± 0.412	57.3 ± 0.86	40.7 ± 0.61	56.8 ± 0.90	16.4 ± 1.90
625 ppm	1	4.55	10.75	27.0	39.8	59.3	13.2
Ferret							
0 ppm	10	10.01 ± 0.187	19.45 ± 0.425	47.4 ± 0.89	41.0 ± 0.56	47.4 ± 0.65	7.3 ± 0.68
1 ppm	10	9.93 ± 0.187	19.17 ± 0.425	47.3 ± 0.89	40.5 ± 0.56	47.7 ± 0.65	8.1 ± 0.68
5 ppm	10	9.97 ± 0.187	19.00 ± 0.425	46.8 ± 0.89	40.5 ± 0.56	47.1 ± 0.65	7.8 ± 0.68
25 ppm	10	9.88 ± 0.187	18.87 ± 0.425	47.5 ± 0.89	39.8 ± 0.56	48.1 ± 0.65	7.0 ± 0.68
125 ppm	10	9.83 ± 0.187	18.67 ± 0.425	45.5 ± 0.89	41.0 ± 0.56	46.4 ± 0.65	8.4 ± 0.68
625 ppm	10	9.98 ± 0.187	19.72 ± 0.425	46.7 ± 0.89	42.2 ± 0.56	46.9 ± 0.65	6.3 ± 0.68

[†]Mean ± S.E.

Table 7. Hematologic profile[†] of adult male mink and ferrets consuming control and HCB diets for 22 days in a chronic feeding trial.

Treatment	n	RBC ($x10^6$)	Hb (g %)	Hct (%)	MCHC (g/ 100 ml)	MCV (μ^3)	WBC ($x10^3$)
Mink							
0 ppm	3	9.68 ± 0.495	23.63 ± 0.654	56.8 ± 1.30	41.6 ± 0.85	58.8 ± 2.39	14.7 ± 2.51
1 ppm	3	10.03 ± 0.495	23.15 ± 0.654	57.5 ± 1.30	40.2 ± 0.85	57.3 ± 2.39	18.7 ± 2.51
5 ppm	3	10.00 ± 0.495	22.93 ± 0.654	57.8 ± 1.30	39.6 ± 0.85	58.3 ± 2.39	14.3 ± 2.51
25 ppm	3	10.07 ± 0.495	23.18 ± 0.654	56.2 ± 1.30	41.3 ± 0.85	56.1 ± 2.39	12.2 ± 2.51
125 ppm	3	10.90 ± 0.495	24.62 ± 0.654	61.5 ± 1.30	40.0 ± 0.85	56.5 ± 2.39	15.7 ± 2.51
Ferret							
0 ppm	3	10.68 ± 0.247	20.92 ± 0.656	50.0 ± 1.17	41.9 ± 0.97	46.8 ± 0.82	13.7 ± 4.30
1 ppm	3	11.26 ± 0.247	21.33 ± 0.656	53.3 ± 1.17	40.1 ± 0.97	47.4 ± 0.82	9.8 ± 4.30
5 ppm	3	11.24 ± 0.247	22.87 ± 0.656	53.0 ± 1.17	43.1 ± 0.97	47.2 ± 0.82	12.0 ± 4.30
25 ppm	3	10.31 ± 0.247	20.13 ± 0.656	48.2 ± 1.17	41.8 ± 0.97	46.7 ± 0.82	9.8 ± 4.30
125 ppm	3	10.12 ± 0.247	20.45 ± 0.656	50.5 ± 1.17	40.5 ± 0.97	49.9 ± 0.82	17.4 ± 4.30
625 ppm	2	10.77 ± 0.303	21.57 ± 0.803	51.3 ± 1.43	42.1 ± 1.19	47.7 ± 1.00	5.5 ± 5.27

[†]Mean ± S.E.

Table 8. Hematologic profile[†] of adult female mink and ferrets consuming control and HCB diets for 149 days in a chronic feeding trial.

Treatment	n	RBC ($x10^6$)	Hb (g %)	Hct (%)	MCHC (g/ 100 ml)	MCV (μ^3)	WBC ($x10^3$)
Mink							
0 ppm	10	8.94 ± 0.223	22.28 ± 0.437	54.2 ± 1.09	41.2 ± 0.75	60.9 ± 1.41	16.9 ± 4.05
1 ppm	10	9.06 ± 0.223	22.50 ± 0.437	54.2 ± 1.09	41.6 ± 0.75	60.0 ± 1.41	16.4 ± 4.05
5 ppm	10	9.72 ± 0.223	23.27 ± 0.437	57.1 ± 1.09	40.8 ± 0.75	58.9 ± 1.41	13.3 ± 4.05
25 ppm	9	8.50 ± 0.236	21.56 ± 0.461	52.6 ± 1.15	41.0 ± 0.79	62.1 ± 1.49	25.0 ± 4.27
125 ppm	6	8.46 ± 0.288	21.34 ± 0.565	51.7 ± 1.41	41.5 ± 0.96	61.3 ± 1.82	35.6 ± 5.23*
Ferret							
0 ppm	10	9.12 ± 0.245	19.99 ± 0.480	49.3 ± 0.94	40.7 ± 0.93	54.3 ± 1.23	12.6 ± 9.64
1 ppm	10	8.88 ± 0.245	19.77 ± 0.480	48.5 ± 0.94	40.8 ± 0.93	54.7 ± 1.23	24.2 ± 9.64
5 ppm	9	9.24 ± 0.258	19.39 ± 0.506	48.3 ± 0.99	40.3 ± 0.98	52.7 ± 1.29	14.4 ± 10.16
25 ppm	10	8.77 ± 0.245	20.03 ± 0.480	49.1 ± 0.94	40.9 ± 0.93	56.0 ± 1.23	31.7 ± 9.64
125 ppm	7	7.43 ± 0.292**	16.61 ± 0.574**	42.3 ± 1.12**	39.2 ± 1.11	57.1 ± 1.46	32.3 ± 11.52

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

**Significantly different from control ($P<0.01$).

seen in the female mink from any of the other treatment levels. Female ferrets consuming 125 ppm HCB showed significantly reduced RBC counts, Hb concentration, and Hct values at this point in the study. However, as noted in the mink, lower levels of dietary HCB did not produce significant changes in the hematologic profile.

Male mink did not show changes in the blood parameters measured after 149 days of HCB exposure, but the 125 ppm group had all died prior to this date (Table 9). The male ferrets on 25 ppm HCB had significantly lower RBC counts than control males. Other than the change in this one value, no differences were observed between control and treated animals. The one ferret still alive after consuming 125 ppm HCB for 149 days had a low RBC value as well, but did not differ much from control animals in any of the remaining aspects measured of its blood profile.

Leukocyte differential counts of adult female mink after 149 days of HCB exposure at 125 ppm in their diet, shown in Table 10 as arcsin transformed values, exhibited a significant decrease in segmented neutrophil count and a significant increase in lymphocyte numbers. No changes were found in the proportions of basophils, eosinophils, band cells, or monocytes. A small, but significant increase over control count was seen in the number of band cells detected in female ferrets on the 125 ppm HCB diet. The other leukocyte types were not different from control in these ferrets.

As shown in Table 11, no significant changes from control values were found in any of the blood parameters measured in female mink exposed to HCB for 331 days. A significant increase in RBC count was observed at 1 ppm dietary HCB in female ferrets. No alterations were detected in Hb and Hct values, but MCHC did show a significant reduction in the

Table 9. Hematologic profile[†] of adult male mink and ferrets consuming control and HCB diets for 149 days in a chronic feeding trial.

Treatment	n	RBC ($x10^6$)	Hb (g %)	Hct (%)	MCHC (g/ 100 ml)	MCV (μ^3)	WBC ($x10^3$)
Mink							
0 ppm	3	8.68 ± 0.428	22.08 ± 1.020	55.0 ± 2.02	40.2 ± 1.68	63.4 ± 2.23	19.1 ± 10.11
1 ppm	3	8.78 ± 0.428	21.65 ± 1.020	54.7 ± 2.02	39.7 ± 1.68	62.5 ± 2.23	13.3 ± 10.11
5 ppm	3	8.90 ± 0.428	22.43 ± 1.020	54.3 ± 2.02	41.3 ± 1.68	61.3 ± 2.23	11.7 ± 10.11
25 ppm	3	9.16 ± 0.428	22.92 ± 1.020	55.0 ± 2.02	41.7 ± 1.68	60.2 ± 2.23	29.1 ± 10.11
Ferret							
0 ppm	3	11.24 ± 0.203	23.75 ± 0.915	58.0 ± 1.20	40.9 ± 1.49	51.6 ± 0.61	13.0 ± 30.82
1 ppm	2	10.50 ± 0.249	22.07 ± 1.121	54.5 ± 1.47	40.5 ± 1.83	51.9 ± 0.75	21.4 ± 37.75
5 ppm	3	11.20 ± 0.203	23.88 ± 0.915	60.7 ± 1.20	39.4 ± 1.49	54.1 ± 0.61	19.9 ± 30.82
25 ppm	3	9.98 ± 0.203**	22.60 ± 0.915	53.7 ± 1.20	42.0 ± 1.49	53.7 ± 0.61	69.9 ± 30.82
125 ppm	1	8.87	21.95	49.5	44.3	55.8	98.1

[†]Mean ± S.E.

**Significantly different from control ($P<0.01$).

Table 10. Leukocyte differential counts[†] (arcsin transformed) of adult female mink and ferrets consuming control and HCB diets for 149 days in a chronic feeding trail.

		Cell type					
Treatment	n	Basophil	Eosinophil	Band	Segmented neutrophil	Lymphocyte	Monocyte
Mink							
0 ppm	10	0 ± 0.0	5 ± 1.2	15 ± 0.8	49 ± 1.7	35 ± 1.9	8 ± 1.1
125 ppm	6	0 ± 0.0	4 ± 1.8	16 ± 0.7	43 ± 1.1*	40 ± 1.5*	9 ± 1.8
Ferret							
0 ppm	9[‡]	2 ± 0.7	5 ± 1.3	15 ± 1.0	37 ± 1.7	46 ± 1.9	10 ± 1.1
125 ppm	7	0 ± 0.8	4 ± 1.4	19 ± 1.4*	38 ± 2.3	43 ± 2.4	10 ± 1.0

[†]Mean ± S.E.

[‡]Does not include one animal whose blood smear could not be read.

*Significantly different from control ($P<0.05$).

Table 11. Hematologic profile[†] of adult female mink and ferrets consuming control and HCB diets for 47 weeks in a chronic feeding trial.

Treatment	n	RBC ($x10^6$)	Hb (g %)	Hct (%)	MCHC (g/ 100 ml)	MCV (μ^3)
Mink						
0 ppm	9	9.03 ± 0.391	20.68 ± 0.784	53.7 ± 1.24	38.6 ± 1.75	56.8 ± 1.23
1 ppm	9	9.67 ± 0.391	22.10 ± 0.784	51.6 ± 1.24	43.3 ± 1.75	53.5 ± 1.23
5 ppm	10	9.88 ± 0.371	21.65 ± 0.743	54.9 ± 1.18	39.5 ± 1.66	55.7 ± 1.17
25 ppm	8	9.41 ± 0.415	22.26 ± 0.831	53.5 ± 1.31	41.7 ± 1.85	56.9 ± 1.31
Ferret						
0 ppm	10	10.65 ± 0.147	22.19 ± 0.440	51.7 ± 0.73	42.9 ± 0.75	48.6 ± 0.47
1 ppm	9	11.24 ± 0.155*	22.03 ± 0.464	52.2 ± 0.77	42.3 ± 0.79	46.5 ± 0.49*
5 ppm	8	10.68 ± 0.165	20.62 ± 0.492	49.2 ± 0.82	42.0 ± 0.83	46.1 ± 0.52**
25 ppm	8	10.79 ± 0.165	20.08 ± 0.492	50.1 ± 0.82	40.0 ± 0.83*	46.5 ± 0.52*
125 ppm	1	8.86	17.90	46.0	38.9	51.9

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

**Significantly different from control ($P<0.01$).

females consuming 25 ppm HCB. The MCV was significantly lower in female ferrets consuming 1, 5, and 25 ppm HCB. The single animal alive at this sampling time from the 125 ppm treatment group had lower values for each of the blood parameters measured than control, with the exception of MCV which was elevated.

No significant differences were found in the hematologic profile of male mink and ferrets after 47 weeks of HCB exposure with one exception (Table 12). The MCV of the ferrets consuming 1 ppm HCB in the diet was significantly greater than the control animals.

Leukocyte differential counts of female mink and ferrets consuming control and HCB-treated diets until the termination of the experiment (331-332 days) are shown in Table 13. Only the 1 ppm treatment group of mink exhibited differential counts significantly altered from that seen in the control females. Eosinophil counts were increased and monocyte counts were decreased in this group. The ferret females had a significantly lower eosinophil count at the 25 ppm HCB treatment than did controls. The ferrets exposed to 1 ppm of HCB had a decreased band cell count, but an increased number of segmented neutrophils (the more mature form of these phagocytic cells).

No change in leukocyte composition was seen in the male mink exposed to HCB for 331 days (Table 14). Band cell numbers were significantly reduced in the male ferrets at 1 ppm HCB at 332 days of exposure. The 25 ppm HCB ferrets showed a lower segmented neutrophil count than the control males. No other alterations were found in the leukocyte differential counts of the male ferrets.

The gestational length of mink and ferrets was not affected by dietary HCB in the treatment groups able to produce offspring (Table 15).

Table 12. Hematologic profile[†] of adult male mink and ferrets consuming control and HCB diets for 47 weeks in a chronic feeding trial.

Treatment	n	RBC ($\times 10^6$)	Hb (g %)	Hct (%)	MCHC (g/ 100 ml)	MCV (μ^3)
Mink						
0 ppm	3	10.23 ± 0.477	22.58 ± 0.614	56.7 ± 1.96	39.8 ± 0.92	55.4 ± 1.27
1 ppm	3	10.14 ± 0.477	23.57 ± 0.614	57.3 ± 1.96	41.2 ± 0.92	56.7 ± 1.27
5 ppm	3	9.76 ± 0.477	21.78 ± 0.614	54.2 ± 1.96	40.2 ± 0.92	55.5 ± 1.27
25 ppm	3	9.52 ± 0.477	21.90 ± 0.614	53.4 ± 1.96	41.0 ± 0.92	56.3 ± 1.27
Ferret						
0 ppm	2	11.01 ± 0.372	20.73 ± 0.798	49.5 ± 1.52	41.9 ± 1.86	45.0 ± 0.59
1 ppm	2	10.56 ± 0.372	22.07 ± 0.798	50.7 ± 1.52	43.7 ± 1.86	48.0 ± 0.59*
5 ppm	3	10.64 ± 0.304	20.55 ± 0.652	49.3 ± 1.24	41.6 ± 1.52	46.4 ± 0.48
25 ppm	3	10.09 ± 0.304	20.17 ± 0.652	47.7 ± 1.24	42.3 ± 1.52	46.6 ± 0.48

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

Table 13. Leukocyte differential counts[†] (arcsin transformed) of adult female mink and ferrets consuming control and HCB diets for 47 weeks in a chronic feeding trial.

		Cell type					
Treatment	n	Basophil	Eosinophil	Band	Segmented Neutrophil	Lymphocyte	Monocyte
Mink							
0 ppm	9	1 ± 0.8	4 ± 1.3	12 ± 1.2	52 ± 1.6	31 ± 1.8	14 ± 0.9
1 ppm	9	2 ± 0.8	8 ± 1.3*	11 ± 1.2	51 ± 1.6	32 ± 1.8	11 ± 0.9
5 ppm	10	2 ± 0.7	5 ± 1.2	15 ± 1.1	53 ± 1.5	28 ± 1.7	13 ± 0.9
25 ppm	8	0 ± 0.8	3 ± 1.4	15 ± 1.2	47 ± 1.7	36 ± 1.9	13 ± 1.0
Ferret							
0 ppm	10	2 ± 0.7	11 ± 1.3	15 ± 1.0	35 ± 1.5	45 ± 1.5	13 ± 1.0
1 ppm	9	1 ± 0.8	9 ± 1.4	12 ± 1.0*	41 ± 1.6*	42 ± 1.6	10 ± 1.1
5 ppm	8	0 ± 0.8	7 ± 1.5	13 ± 1.1	38 ± 1.7	46 ± 1.7	10 ± 1.1
25 ppm	8	2 ± 0.8	6 ± 1.5*	16 ± 1.1	36 ± 1.7	46 ± 1.7	12 ± 1.1
125 ppm	1	0	6	23	33	44	14

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

Table 14. Leukocyte differential counts[†] (arcsin transformed) of adult male mink and ferrets consuming control and HCB diets for 47 weeks in a chronic feeding trial.

Treatment	n	Cell type					
		Basophil	Eosinophil	Band	Segmented neutrophil	Lymphocyte	Monocyte
Mink							
0 ppm	3	2 ± 1.8	8 ± 2.2	14 ± 2.4	44 ± 4.2	38 ± 5.1	13 ± 3.3
1 ppm	3	3 ± 1.8	8 ± 2.2	13 ± 2.4	49 ± 4.2	34 ± 5.1	9 ± 3.3
5 ppm	3	2 ± 1.8	5 ± 2.2	13 ± 2.4	45 ± 4.2	37 ± 5.1	12 ± 3.3
25 ppm	3	0 ± 1.8	5 ± 2.2	12 ± 2.4	47 ± 4.2	35 ± 5.1	16 ± 3.3
Ferret							
0 ppm	2	3 ± 2.3	3 ± 2.7	18 ± 1.4	54 ± 5.9	28 ± 5.9	8 ± 2.9
1 ppm	2	4 ± 2.3	11 ± 2.7	11 ± 1.4**	37 ± 5.9	46 ± 5.9	11 ± 2.9
5 ppm	3	2 ± 1.9	10 ± 2.2	14 ± 1.2	42 ± 4.8	40 ± 4.8	12 ± 2.4
25 ppm	3	0 ± 1.9	5 ± 2.2	18 ± 1.2	35 ± 4.8*	45 ± 4.8	12 ± 2.4

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

**Significantly different from control ($P<0.01$).

Table 15. Length of gestation and litter size[†] of female mink and ferrets consuming control and HCB diets in a chronic feeding trial.

Treatment	Whelp/mated n	Gestation (days)	Litter size[‡] total	live	dead
Mink					
0 ppm	10/10	49.5 ± 1.23	6.4 ± 0.58	4.9 ± 0.72	1.5 ± 0.50
1 ppm	8/10	48.6 ± 1.38	5.3 ± 0.65	4.3 ± 0.81	1.0 ± 0.56
5 ppm	10/10	48.4 ± 1.23	5.9 ± 0.58	5.3 ± 0.72	0.6 ± 0.50
25 ppm	8/10	47.5 ± 1.38	3.4 ± 0.65**	1.9 ± 0.81*	1.5 ± 0.56
Ferret					
0 ppm	8/10	41.4 ± 0.35	8.8 ± 0.77	8.3 ± 0.82	0.5 ± 0.48
1 ppm	8/10	41.6 ± 0.35	9.7 ± 0.77	9.6 ± 0.82	0.1 ± 0.48
5 ppm	6/10	40.7 ± 0.40	11.3 ± 0.89	10.3 ± 0.95	1.0 ± 0.55
25 ppm	6/10	41.7 ± 0.40	7.2 ± 0.89	4.7 ± 0.95*	2.5 ± 0.55**

[†]Mean ± S.E.

[‡]Kits per female whelping.

*Significantly different from control ($P<0.05$).

**Significantly different from control ($P<0.01$).

In the mink, however, a significant decrease in total litter size was seen, along with a decrease in the number of kits born alive at 25 ppm HCB. This resulted in an increase in the proportion of stillborn fetuses. In several female mink of the 125 ppm HCB-treatment group that died during April, resorption sites were found in the uterus at necropsy. This demonstrates that although these females were unable to maintain their pregnancies, fertilization and implantation were not prevented. The ferrets also did not show changes in litter characteristics until fed 25 ppm HCB in the diet. Total litter size was unchanged in these ferrets, but the number of offspring born alive was lower than control females and a higher incidence of kits born dead was noted. The female ferrets consuming 125 ppm HCB failed to come into estrus, as determined by vulvar swelling, at any time during their normal breeding season. These animals were therefore never bred and could not be evaluated for other HCB-related reproductive effects.

As shown in Table 16, the mink kits born to dams consuming 1, 5, and 25 ppm HCB were smaller at birth than the kits born to untreated mothers. A similar relationship was seen in the ferret kit birth weights, although only in the 5 and 25 ppm treatment groups were they significantly smaller than controls. Three week old mink kit weights were significantly lower than control kits in the 5 and 25 ppm groups; at six weeks of age these two treatment group kits were still significantly smaller than the control mink kits. At three weeks of age, ferret kits on all HCB treatments weighed significantly more than untreated kits. By six weeks of age, only the 1 ppm HCB ferret kits continued to weigh more than control kits. A strong trend toward reduced biomass produced with increasing dietary HCB was seen in the female mink, with only the 5 ppm

Table 16. Birth, three week, and six week old body weights, and biomass[†] raised per lactating female mink and ferret consuming control and HCB diets in a chronic feeding trial.

Treatment	Kit weight (g)						n (litters)	Biomass[‡]
	n	Birth	n	3 week	n	6 week		
Mink								
0 ppm	49	9.1 ± 0.21	45	97.3 ± 3.37	45	244.8 ± 6.94	9	441 ± 55.5
1 ppm	34	7.9 ± 0.25**	23	86.6 ± 4.69	19	229.1 ± 17.13	6	301 ± 67.9
5 ppm	53	8.1 ± 0.20**	12	68.2 ± 6.24**	12	173.3 ± 20.04**	5	145 ± 74.4*
25 ppm	15	7.6 ± 0.38**	2	35.5 ± 1.50**	2	71.0 ± 4.00**	1	59
Ferret								
0 ppm	57[§]	9.0 ± 0.21	56	75.6 ± 2.93	42	256.6 ± 9.75	7	533 ± 60.6
1 ppm	77	9.4 ± 0.18	68	90.7 ± 2.66**	59	300.7 ± 8.23**	8	689 ± 56.7
5 ppm	62	7.9 ± 0.20**	49	87.0 ± 3.13*	42	262.5 ± 9.75	6	645 ± 65.4
25 ppm	28	6.9 ± 0.30**	16	94.6 ± 5.48**	10	262.2 ± 20.00	6	350 ± 65.4

[†]Mean ± S.E.

[‡]Biomass = average kit body weight gain between birth and three weeks of age x the average number of kits per lactating female.

[§]Does not include one female's litter that fell through the cage wire.

*Significantly different from control ($P<0.05$).

**Significantly different from control ($P<0.01$).

females raising a significantly lower biomass than the control mink. No significant difference was seen in the biomass produced by control and HCB-treated female ferrets.

HCB caused a dose related increase in kit mortality (Table 17) in both the mink and the ferret. The percentage of kits dying before three weeks of age accounted for the greatest proportion of the mortality seen in mink kits, with only occassional deaths occurring between three and six weeks. Although not quantitated, casual observation suggested the majority of the kit deaths to be occurring within three days of birth. The ferrets also demonstrated a dose-related increase in offspring mortality. At three weeks of age the percentage of kits dying was one-half to two-thirds lower in the ferrets than in the mink. The kit mortality to six weeks of age was still lower in the ferrets (except for the 0 ppm group) than in mink kits raised by dams consuming comparable dietary concentrations of HCB. A much greater portion of the kit mortality to six weeks of age occurred between three and six weeks after birth in the ferrets than in the mink.

Based on food consumption values reported by Bleavins and Aulerich (1981), the quantity of HCB consumed by the mink and ferrets on the various treatments was calculated (Table 18). No deaths directly attributable to the effects of HCB were seen in the animals consuming the 0, 1, 5, and 25 ppm HCB diets. The amount of HCB shown for these treatment levels in Table 18 therefore represents the quantity of the chemical eaten prior to the termination of the study rather than how much HCB resulted in death. The values reported for the 125 and 625 ppm HCB-treated animals are the mg of the chemical required to cause death at these two levels of dietary exposure. The actual quantity may be

Table 17. Kit mortality of the offspring produced by female mink and ferrets consuming control and HCB diets in a chronic feeding trial.

Maternal treatment	Kit mortality (%)	
	to 3 weeks	to 6 weeks
Mink		
0 ppm	8.2	8.2
1 ppm	32.4	44.1
5 ppm	77.4	77.4
25 ppm	86.7	86.7
Ferrets		
0 ppm	1.8	26.3
1 ppm	11.7	23.4
5 ppm	21.0	32.3
25 ppm	42.9	64.3

Table 18. Hexachlorobenzene consumed[†] (mg) by adult mink and ferrets in a chronic feeding trial.

	Male[ψ]				Female[ψ]			
Treatment	n	Mink	n	Ferret	n	Mink	n	Ferret
0 ppm	3	0	2	0	9	0	10	0
1 ppm	3	72	2	78	9	45	9	38
5 ppm	3	360	3	390	10	225	9	190
25 ppm	3	1800	3	1950	9	1125	8	950
125 ppm[‡]	3	1818 ± 205.8	3	3499 ± 1454.3	10	2649 ± 356.3	10	3088 ± 512.1
625 ppm[‡]	3	2629 ± 119.9	3	3920 ± 686.0	10	1546 ± 117.6	10	3031 ± 230.1

[†]Mean ± S.E.

[‡]All animals on these dietary treatments died prior to termination of the study.

[ψ]Based on daily food consumption values as reported by Bleavins and Aulerich (1981) x days alive on treatment.

slightly lower than reported since most animals refused to eat several days immediately preceeding death. The quantity of HCB consumed by male mink prior to death in the 125 and 625 ppm treatment groups was below the maximum dose tested (2000 mg/kg of body weight or 3600 mg for an average 1.8 kg male mink) during the LD_{50} trial reported in Experiment I. Unlike the acute exposure, the chronic effects included dramatic weight loss, anorexia, bloody droppings, gastrointestinal bleeding, and death. The male ferrets had a dietary intake of approximately 3500 mg and 3900 mg prior to death on the 125 and 625 ppm diets, respectively. This quantity of chemical was greater than the amount necessary to kill male mink. In both the male mink and ferrets, more HCB was ingested before death by the animals consuming the 625 ppm diet than by those eating the 125 ppm HCB feed. Female mink at the two highest dietary concentrations of HCB ate 2649 mg (125 ppm) and 1546 mg (625 ppm). The amount of HCB taken in by the female ferrets was roughly equal for the two highest diets (3088 vs. 3031 mg) and generally was greater than the quantity consumed by female mink on comparable levels of HCB.

The average survival times of mink and ferrets consuming 125 and 625 ppm HCB are shown in Table 19. Male mink fed 125 ppm HCB survived slightly longer than two months from the initiation of the study. One male died prior to the breeding season (March), one during the breeding season, and the final male died soon after the females had been mated. The males appeared to exhibit a compounding of the toxic effects of HCB with the added stress and increased activity levels of these animals in preparation for and during the breeding period. The male mink fed 625 ppm HCB survived somewhat less than three weeks at the high exposure level. The male ferrets showed a longer mean survival time than the

Table 19. Survival time[†] of adult mink and ferrets consuming HCB diets[‡] in a chronic feeding trial.

	Male			Female		
Treatment	n	Time (days)	Range (days)	n	Time (days)	Range (days)
Mink						
125 ppm	3	67 ± 7.6	53-79	10	156 ± 21.0	84-266
625 ppm	3	19 ± 0.9	18-21	10	18 ± 1.4	9-24
Ferret						
125 ppm	3	121 ± 50.2	46-216	10	221 ± 36.6	39-331
625 ppm	3	27 ± 4.7	18-34	10	42 ± 3.2	30-59

[†]Mean ± S.E.

[‡]No deaths directly attributable to HCB intoxication were seen in the 0, 1, 5, or 25 ppm treatment groups.

mink on both the 125 and 625 ppm HCB diets, but also exhibited a much greater variability within treatment groups. Female mink were able to survive longer on the 125 ppm treatment than the male mink, but were themselves outlived by the female ferrets on this diet. The female mink lived less than three weeks when consuming 625 ppm HCB and the ferret females at this exposure level lived approximately six weeks. Six of the female ferrets in the 125 ppm treatment group were very excitable and abnormally aggressive for this species. If these animals were handled, they began to shriek and acted as if the process of being picked up not only upset them, but was also a painful experience. Subsequently, four of the female ferrets were found immediately prior to death. Each animal was unable to stand, exhibited mild trembling of all four legs and appeared to lapse into occasional mild convulsions. These ferrets died one to three hours after being discovered and the above mentioned clinical signs observed.

At the end of the eleven months of HCB exposure (termination of the study), no significant change was found in the body weights of the 1, 5, and 25 ppm HCB-treated females from the control animals (Table 20). The females on the two highest treatment groups (125 and 625 ppm HCB) all died before the completion of the experiment and exhibited body weights that were significantly lower than untreated mink and ferrets. Brain weights of female mink consuming HCB diets were not significantly different from control females and only the 625 ppm treatment group of ferrets showed an elevated brain weight. Liver size, expressed as a percentage of brain weight, was not significantly influenced in any of the HCB-exposed mink, but was increased in the ferrets fed 125 ppm HCB. The livers of the mink and ferrets, both male and female, were placed

Table 20. Selected organ weights[‡] of adult female mink and ferrets consuming control and HCB diets for 47 weeks in a chronic feeding trial.

Treatment	n	Body weight (g)	Brain weight (g)	Tissue (% brain weight)				
				Liver	Spleen	Kidney	Lung	Heart
Mink								
0 ppm	9	957 ± 45.4	7.5 ± 0.20	422.6 ± 26.60	56.9 ± 5.27	61.7 ± 2.76	82.1 ± 5.59	74.6 ± 3.56
1 ppm	9	923 ± 45.4	7.5 ± 0.20	465.5 ± 26.60	64.3 ± 5.27	64.6 ± 2.76	82.4 ± 5.59	83.7 ± 3.56
5 ppm	10	995 ± 43.0	7.5 ± 0.19	464.8 ± 25.23	51.6 ± 5.00	63.7 ± 2.62	83.1 ± 5.31	72.2 ± 3.38
25 ppm	9	1014 ± 45.4	7.5 ± 0.20	515.4 ± 26.60	55.7 ± 5.27	68.6 ± 2.76	106.5 ± 5.59*	79.3 ± 3.56
125[†] ppm	9	486 ± 45.4**	7.6 ± 0.20	364.4 ± 26.60	37.2 ± 5.27*	70.4 ± 2.76	83.3 ± 5.59	51.4 ± 3.56**
625[†] ppm	9	526 ± 45.4**	7.5 ± 0.20	432.6 ± 26.60	26.1 ± 5.27**	75.7 ± 2.76**	122.6 ± 5.59**	58.8 ± 3.56*
Ferret								
0 ppm	10	792 ± 32.8	5.9 ± 0.12	459.2 ± 49.98	59.2 ± 5.44	70.1 ± 5.71	103.4 ± 4.59	75.5 ± 2.59
1 ppm	9	771 ± 34.6	6.0 ± 0.12	427.8 ± 52.68	59.8 ± 5.73	68.0 ± 6.02	96.6 ± 4.84	74.7 ± 2.73
5 ppm	9	766 ± 34.6	6.2 ± 0.12	500.3 ± 52.68	56.9 ± 5.73	73.7 ± 6.02	95.8 ± 4.84	71.1 ± 2.73
25 ppm	8	783 ± 36.7	5.8 ± 0.13	586.1 ± 55.88	72.3 ± 6.08	77.4 ± 6.39	95.6 ± 5.13	71.3 ± 2.89
125[†] ppm	10	445 ± 32.8**	6.2 ± 0.12	792.0 ± 49.98**	92.7 ± 5.44**	105.7 ± 5.71**	103.9 ± 4.59	62.1 ± 2.59**
625[†] ppm	10	423 ± 32.8**	6.4 ± 0.12*	548.6 ± 49.98	37.9 ± 5.44*	93.6 ± 5.71*	113.2 ± 4.59	56.7 ± 2.59**

[‡] Mean ± S.E.

[†] All died prior to termination date.

* Significantly different from control ($P < 0.05$).

** Significantly different from control ($P < 0.01$).

under ultraviolet light and observed for fluorescence. None of the animals exhibited the brilliant red fluorescence characteristic of hepatic porphyria. Spleen weight was significantly reduced in female mink at 125 and 625 ppm HCB, but significantly increased in ferrets fed 125 ppm HCB. The spleens of the 625 ppm HCB female ferrets showed a significant weight reduction, similar to the effect seen in the mink on this dietary concentration of the chemical (Table 20). Kidney weights were significantly higher than controls in the 625 ppm treatment groups and there was also a definite trend toward increased kidney weight with increased dietary HCB. In the 125 and 625 ppm HCB female ferrets, an increase in kidney weight was found. Lungs were significantly heavier in 25 and 625 ppm HCB female mink, but no differences from control were seen in the other treatment groups or in any of the HCB-exposed female ferrets. The 125 and 625 ppm female mink and ferrets had a significantly decreased heart weight when compared to controls of the same species.

The 125 and 625 ppm HCB male mink showed body weights that were significantly lower than control males (Table 21). The mink and ferrets on both of these high levels of HCB all died prior to the end of the study. Only the male ferrets fed 625 ppm HCB had significantly reduced body weights, although the 125 ppm animals did have an average weight which was significantly lower than the control at $P<0.10$. No difference in brain weight was seen in the HCB-treated mink and ferrets with the exception of the male mink consuming 125 ppm HCB. This treatment group showed significantly higher brain weights than control. An increased liver weight was observed in the mink at 25 ppm HCB and the ferrets at 125 ppm HCB. No other male treatment groups showed a significant change in liver weight. Spleen weight was not significantly lower than the

Table 21 . Selected organ weights‡ of adult male mink and ferrets consuming control and HCB diets for 47 weeks in a chronic feeding trial.

Treatment	n	Body weight (g)	Brain weight (g)	Tissue (% brain weight)				
				Liver	Spleen	Kidney	Lung	Heart
Mink								
0 ppm	3	1839 ± 119.0	8.6 ± 0.31	612.1 ± 50.88	44.4 ± 6.25	84.9 ± 5.84	102.4 ± 11.21	94.6 ± 6.26
1 ppm	3	1707 ± 119.0	8.8 ± 0.31	602.1 ± 50.88	55.3 ± 6.25	94.3 ± 5.84	103.7 ± 11.21	100.5 ± 6.26
5 ppm	3	1755 ± 119.0	9.6 ± 0.31	573.6 ± 50.88	45.3 ± 6.25	80.5 ± 5.84	97.5 ± 11.21	93.3 ± 6.26
25 ppm	3	1939 ± 119.0	8.7 ± 0.31	876.2 ± 50.88*	61.9 ± 6.25	96.0 ± 5.84	113.0 ± 11.21	95.3 ± 6.26
125† ppm	3	913 ± 119.0**	9.9 ± 0.31*	507.4 ± 50.88	39.0 ± 6.25	88.9 ± 5.84	102.7 ± 11.21	61.0 ± 6.26*
625† ppm	3	961 ± 119.0**	9.8 ± 0.31	533.9 ± 50.88	29.0 ± 6.25	96.5 ± 5.84	129.9 ± 11.21	74.2 ± 6.26
Ferret								
0 ppm	2	1647 ± 137.6	7.7 ± 0.37	678.4 ± 68.01	147.3 ± 31.64	102.6 ± 12.30	128.6 ± 13.50	106.5 ± 13.08
1 ppm	2	1902 ± 137.6	7.4 ± 0.37	846.4 ± 68.01	122.1 ± 31.64	115.0 ± 12.30	149.3 ± 13.50	111.9 ± 13.08
5 ppm	3	1750 ± 112.4	7.2 ± 0.30	843.1 ± 55.53	133.9 ± 25.84	122.5 ± 10.04	133.1 ± 11.02	114.6 ± 10.68
25 ppm	3	1543 ± 112.4	7.5 ± 0.30	911.6 ± 55.53	100.8 ± 25.84	104.5 ± 10.04	109.9 ± 11.02	104.7 ± 10.68
125† ppm	2	1209 ± 137.6	7.2 ± 0.37	1131.3 ± 68.01*	154.3 ± 31.64	151.9 ± 12.30	173.9 ± 13.50	133.8 ± 13.08
625† ppm	3	934 ± 112.4*	7.2 ± 0.30	798.5 ± 55.53	73.9 ± 25.84	147.4 ± 10.04	148.5 ± 11.02	84.8 ± 10.68

‡ Mean ± S.E.

† All died prior to termination date.

* Significantly different from control ($P < 0.05$).

** Significantly different from control ($P < 0.01$).

control in any of the HCB-exposed male mink and ferrets, but did tend to be somewhat reduced in the 625 ppm HCB animals. Kidney and lung weights were not significantly different from controls in the HCB-exposed animals, although the highest dietary concentration of HCB (625 ppm) did tend to show slightly higher weights than control for these two tissues. Heart weight was significantly lower in the 125 ppm HCB mink. No additional statistically significant alterations in the weight of this organ were seen in either the mink or the ferret.

Discussion

The results of this experiment have shown the mink and ferret to be sensitive to the toxic effects of HCB. A reduction in the body weight of adult animals consuming the two highest HCB diets (125 and 625 ppm) was a general indication of toxicosis in both species. The toxic signs of anorexia and eventual death are consistent with those seen in the rat (Villeneuve 1975). In addition, gastrointestinal bleeding and bloody droppings were observed in the mink and ferrets. The high fat content of the basal diet used in this study (25%) probably prompted excellent intestinal absorption of the HCB by these carnivores. The quantity of HCB absorbed by the rat has been shown to be highly dependent on the fat content of the diet (Koss and Koransky 1975; Zabik and Schemmel 1980), with high fat diets dramatically improving chemical uptake. Predatory birds, species also likely to be consuming diets with a high fat content (in addition to the biomagnification and bioconcentration effects by the prey animals), were found to have high body burdens of HCB (Vos *et al.* 1969; Best 1973; Cromartie *et al.* 1975).

An increased leukocyte count was seen in the HCB-exposed mink. The differential blood smear also indicated a reduction in the proportion of

phagocytic cells (segmented neutrophils) and an increase in lymphocytes. These conditions may be indicative of damage to theimmune system. HCB is immunosuppressive in conventional laboratory animals (Iatropoulos et al. 1976; Loose et al. 1977, 1978, 1979) and the mink and ferret (Experiment IV). The elevated WBC count seen could be the result of the stressed animal's attempting to fight off opportunistic and disease organisms.

In general, the male mink and ferrets died earlier in the course of the experiment than females of the same species. The ferrets consistently lived longer on the HCB diets than mink on a comparable treatment level. As has been observed with previous studies with PCBs (Bleavins et al. 1980), the ferret appears to be quite sensitive to the effects of halogenated aromatic hydrocarbons, but more resistant than the mink. A differential sensitivity to HCB has also been reported in rodents, with the rat being less susceptible to the toxic effects than the guinea pig (Villeneuve and Newsome 1975).

The female ferrets consuming the 125 ppm HCB diet failed to exhibit signs of estrus during the normal breeding season (April to September). This condition may have been produced through the increased biotransformation of endogenous hormones by steroid hydroxylating enzymes induced by the HCB. Levin et al. (1967) found an inhibition of the effects of estradiol and estrone on the female reproductive tract due to the increased metabolism of these estrogens. A similar condition may underlie the anestrus state of the ferrets used in the experiment being reported here. It is also possible that the HCB resulted in a stressed animal, in poor condition, which was not capable of initiating and proceeding with the normal reproductive cycle. Since the general state of these

ferrets was not observably different from untreated females, this second explanation does not seem likely.

Dietary exposure to HCB depressed reproduction in the rat (Mendoza et al. 1975; Grant et al. 1977). The females produced fewer litters and the number of pups surviving to weaning was reduced. At 25 ppm HCB, mink females produced fewer kits, but no increase was seen in the total number of kits born dead per litter. Rats exhibited an increased incidence of stillbirths, as did women in Turkey consuming HCB-contaminated bread (Courtney 1979). Ferrets on the 25 ppm HCB diet showed no change in total litter size, but did demonstrate an elevated number of kits born dead as was seen in other species.

The mink and ferret kits produced by females on the HCB diets were smaller at birth than control kits. This is consistent with the effects seen in rats (Khera 1974; Grant et al. 1977), although exposure levels were much higher in the rodents. The reduced rat pup birth weights were first seen at 80 ppm HCB; the depressed birth weight was observed at 1 ppm HCB in the mink and at 5 ppm HCB in the ferrets. A reduced rate of growth has also been reported in rats (Grant et al. 1977) and monkeys (Bailey et al. 1980) produced by HCB-treated dams. The HCB-mink kits exhibited lower three and six week old body weights than control kits. These effects were seen at 1 and 5 ppm of maternal dietary exposure which was dramatically less than was required to produce a similar condition in the rats and monkeys. Three week old ferrets from HCB-treated dams weighed significantly more than their control counterparts. This is probably the result of fewer kits per litter, since a corresponding increase in biomass would otherwise be expected and was not seen.

Offspring mortality showed a dose-dependent increase in both the

mink and ferret. Even low level exposure to HCB resulted in poor kit survival. Low postnatal viability was also seen in rats and humans (Courtney 1979) exposed to HCB. Mortality to weaning of 45.4% was seen at 100 ppm HCB in the rat (Kitchin et al. 1982), while 1 ppm HCB resulted in 44.1% mortality in the mink. Even taking into consideration the longer period of nursing in the mink than the rat (6 weeks vs. 3 weeks), the mink must still be considered much more sensitive to the effects of HCB. Ferret kit mortality at 25 ppm HCB was 64.3%, which is intermediate between the levels seen at 100 ppm (45.4%) and 120 ppm (93.1%) in rat pups born to dams consuming HCB diets (Kitchin et al. 1982). The ferret must also be classified as reproductively more sensitive to HCB than the rat. Grant et al. (1977) reported a 20 ppm HCB content in the maternal diet to be the no-effect level for reproduction in the rat, a level which had profound effects on the reproductive performance of both the mustelids used in this experiment.

Experiment III. EXCRETION, PLACENTAL, AND MAMMARY TRANSFER OF HEXACHLOROBENZENE IN THE EUROPEAN FERRET

Purpose

In this study the placental and mammary transfer of HCB was determined after a single dietary exposure to ^{14}C-labeled HCB. The importance of *in utero* and lactational excretion by the dam, in addition to the relative exposure levels to the developing/growing offspring, was calculated. Fecal and urinary elimination of HCB was also monitored in the adult female ferrets.

Materials and Methods

Eight adult female ferrets were individually housed in metabolism cages 61 cm L x 36 cm W x 33 cm H. All animals received the basal diet and water *ad libitum* throughout the study. The ferrets were divided into two treatment groups:

Group I - pregnant ferrets dosed on day 14 of gestation

Group II - unbred ferrets dosed on the same day as Group I.

These treatment groups allowed the investigation of differences between pregnant (and subsequently lactating) females and females not raising offspring. The females in Group I were mated, upon evidence of estrus, with untreated males. Five days prior to the calculated whelping date (41st day of pregnancy) wooden nest boxes and false cage bottoms (to prevent the kits from dropping through the wire mesh floor) were placed in the metabolism cages of Group I females to permit the raising of kits to five weeks of age.

On the day of dosing, feed was withheld from the animals for four hours prior to the presentation of the ^{14}C-labeled HCB-treated feed.

The single dose of HCB was prepared by mixing 7.15 μCi of ^{14}C-labeled HCB (New England Nuclear, Boston, MA, lot #852-058, 98% radiochemically pure, specific activity = 35.32 mCi/mmol.) into 7.5 g of the diet. Each ferret was observed on the day of dosing until the HCB-treated feed was consumed. As soon as the full amount of HCB-treated feed was eaten, the untreated basal diet was once again fed ad libitum.

Urine and fecal samples were collected daily for seven days following treatment of the ferrets with ^{14}C-labeled HCB feed and weekly thereafter until the termination of the study. The volume of urine and weight of fecal material produced during the 24-hour sampling period was recorded. The weekly excretion of labeled compounds via the urine or feces was calculated as: ^{14}C activity per gram or ml x weight or volume collected per day for each of the seven days immediately following dosing (initial week) or the ^{14}C activity per gram or ml x weight or volume for the 24-hour test period x seven (subsequent weeks). Milk samples were taken on weeks two through five post-partum. One ferret kit was collected from each litter at birth, one, two, three, and four weeks of age for whole kit residue concentrations. The fifth week after birth, the last two kits were taken from each litter, one for whole kit residue analysis and the other for individual organ residues. When the last kits were taken, at five weeks of age, the dams were also killed and selected organs taken for HCB residue analysis. All animals were killed with CO_2 gas.

The urine, feces, maternal and kit tissues were analyzed for ^{14}C activity using the Unisol-Complement system (Isolab, Inc., Akron, OH) for liquid scintillation counting. Each sample was counted for 10 minutes on an Isocap/300, model 6872 liquid scintillation counter (Searle

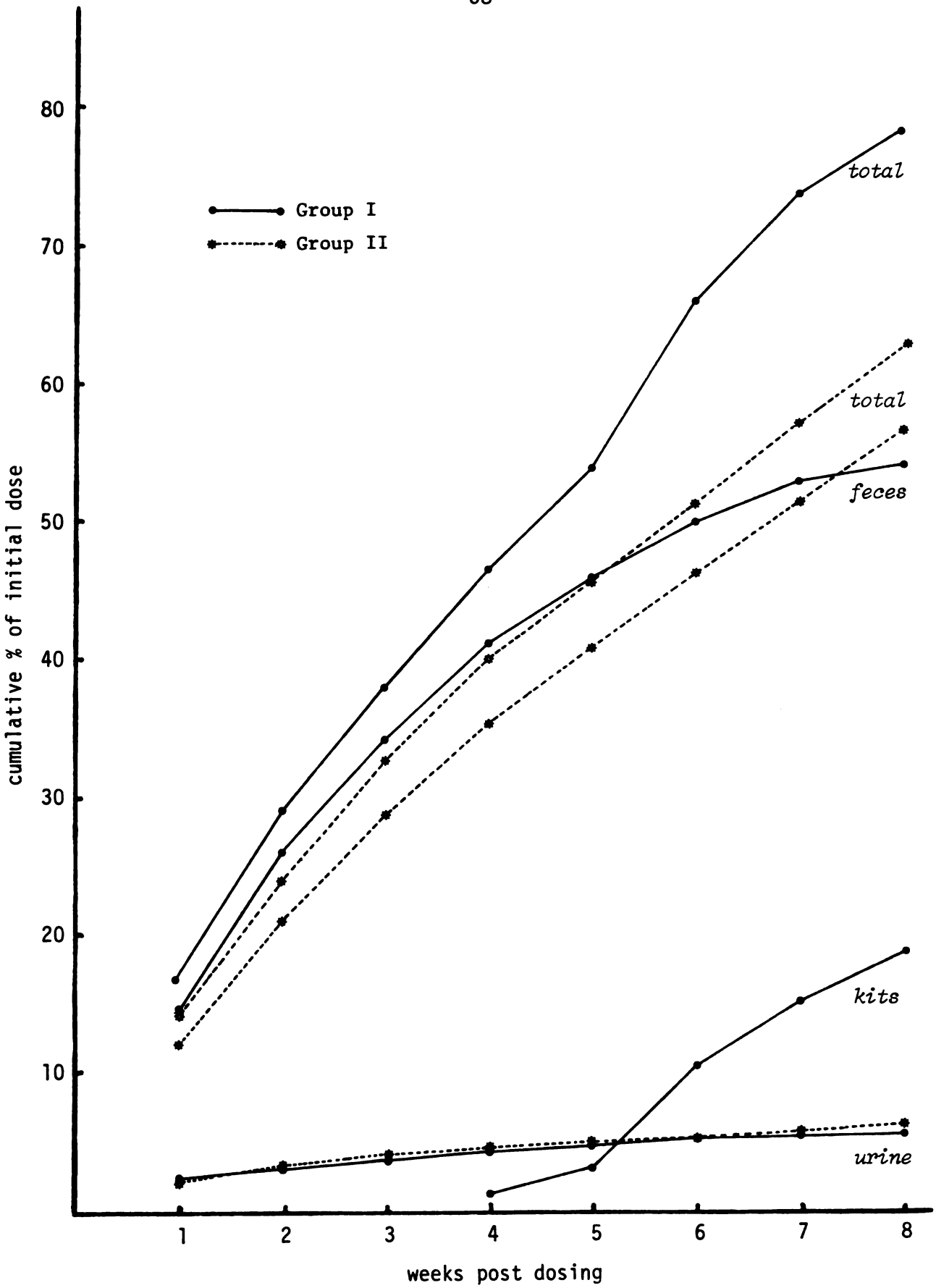
Analytic, Inc., Des Plaines, IL). The values, presented in the tables as disintegrations per minute (DPM), were corrected for background radiation and quenching by the sample-channels-ratio method (Searle 1974). The data were analyzed by using a Bonferroni t-test or the Student's t-test (Gill 1978).

Results

The percentage of initial dose of ^{14}C-labeled HCB absorbed by the ferrets used in this study was calculated to be 98.5%. This value was obtained by predicting the amount of radioactivity excreted and the amount absorbed during the first 24-hour period, based on the extrapolation of the daily excretion for days two through seven post-dosing. This estimation was used due to the food passage time in the ferret being just over three hours (Bleavins and Aulerich 1981).

The fecal and urinary excretion data, expressed graphically as a percentage of initial dose, are presented in Figure 5 for Groups I and II. No significant differences were found in weekly fecal excretion of HCB (or its ^{14}C metabolites) between pregnant/lactating female ferrets (Group I) and unbred ferrets dosed at the same time (Group II), or in the 8-week total fecal excretion between the two groups. Urinary excretion of the ^{14}C-labeled compound/metabolites was not significantly different in either group for individual weeks or the cumulative eight-week total. As shown in Figure 5, the females raising offspring (Group I) and the unbred females dosed at the same time (Group II) had virtually identical values for urinary excretion of HCB. The values for fecal excretion however, diverged somewhat during the middle weeks of the eight-week collection period. Although no statistically significant difference was determined between individual weeks, the graphic

Figure 5. Cumulative excretion of HCB by pregnant/lactating female ferrets (Group I) and unbred female ferrets (Group II).


80
70
60
50
40
30
20
10
cumulative % of initial dose
Group I
Group II
total
total
feces
kits
urine
1
2
3
4
5
6
7
8
weeks post dosing

representation suggest non-significant changes. Towards the end of the study, HCB fecal excretion by females in Group I appeared to be leveling off while values for Group II females continued to increase, suggesting a greater rate of fecal elimination by the Group II females.

As presented in Table 22, the ^{14}C-HCB concentration of the females raising kits to 5 weeks of age (Group I) was significantly lower in all tissues tested than in unbred females dosed at the same time (Group II). HCB residues in blood, muscle, and heart were significantly reduced ($P<0.05$), while adipose tissue levels (subcutaneous and visceral), kidney, spleen, liver, lung, and brain were significantly lower at $P<0.01$. The concentrations of HCBin liver and subcutaneous fat were significantly higher in the five-week old offspring than in the same tissues of the dams that raised them. No significant differences in HCB concentrations between maternal and kit tissues were found for muscle, kidney, and brain.

The ^{14}C-HCB concentration of kits produced by Group I females is shown in Table 23 for newborn through five weeks post-partum. On a per gram of kit basis, very little change was seen between the newborn (2984 DPM/g) and the five week old kit (2685 DPM/g). However, the total radioactivity per kit increased from 25,145 DPM to 805,661 DPM over the same period of time. This increase represents a ratio of 31:1 for the five week old kit to newborn kit [(5 week value - newborn value) ÷ newborn value]. Milk samples collected from Group I ferrets, during the second through fifth weeks of lactation, showed a continual decline in HCB concentration as lactation progressed (Table 23). However, HCB exposure via the milk was 31 times greater than by placental passage.

Table 22. Mean (±S.E.) radioactivity (DPM/g) of selected tissues 62 days post-dosing from adult bred (Group I) and unbred (Group II) female ferrets exposed to a single dose of 14C-labeled HCB and of the offspring born to the bred females.

Tissues	Group I (n = 3)	Group II (n = 3)	Kits† (n = 3)
Blood	49 ± 34.6*	166 ± 26.8	-
Subcutaneous fat	4472 ± 780.5**	19,525 ± 1503.9	11,678 ± 712.4‡‡
Visceral fat	4429 ± 867.6**	19,704 ± 1666.0	-
Muscle	53 ± 14.4*	384 ± 64.0	561 ± 204.8
Heart	34 ± 9.2*	310 ± 56.8	-
Kidney	105 ± 31.1**	611 ± 80.4	209 ± 37.2
Spleen	13 ± 7.5**	180 ± 24.8	-
Liver	248 ± 68.9**	1445 ± 145.2	1420 ± 185.6‡
Lung	1 ± 0.3**	241 ± 18.4	-
Brain	61 ± 30.0**	395 ± 48.5	130 ± 29.4

†Kit tissues, from 5 week old offspring, were contrasted only with maternal (Group I) tissues.

*Significantly different from Group II tissue of the same type ($P<0.05$).

**Significantly different from Group II tissue of the same type ($P<0.01$).

‡Significantly different from maternal tissue of the same type ($P<0.05$).

‡‡Significantly different from maternal tissue of the same type ($P<0.01$).

Table 23. Mean (± S.E.) radioactivity (DPM x 10^3) of kits born to female ferrets exposed to a single dose of 14C-labeled HCB and the milk produced by those dams.

		Weeks post-partum					
	n	0	1	2	3	4	5
Per gram of kit	3	2.9± 0.19	2.7 ± 0.57	4.3 ± 0.67	3.9 ± 0.73	3.5 ± 0.50	2.7 ± 0.14
Per whole kit	3	25.1± 1.43	76.7 ±14.35	311.4 ±63.39	492.5 ±92.22	672.8 ±117.63	805.7 ± 54.25
Increase over previous week		-	51.6	234.7	181.1	180.3	132.8
Milk (per ml)		†	†	6.1 ± 0.66	2.9 ± 0.45	1.8 ± 0.17	0.8 ± 0.20

†Ferrets were not milked during these weeks.

Discussion

In this study the ferrets absorbed 98.5% of a single dietary dose of ^{14}C-labeled HCB. This percentage of initial dose is higher than the 80% value reported for rats (Koss and Koransky 1975) following a single oral dose of HCB in oil. Since this carnivore has a digestive system well adapted to utilize a diet rich in fat, like that of the mink (Travis and Schaible 1960), the high fat content of the diet (22.2%; Bleavins and Aulerich 1981) probably promoted good absorption of HCB by the ferrets. In samples collected from wild populations (Koss and Manz 1976), carnivorous (red fox) and omnivorous (wild boar) species have been found to have higher HCB tissue residues than herbivorous species (deer). Their position nearer the top of the food chain puts the carnivore, and the omnivore to a lesser degree, in a situation more likely to be consuming a diet in which biomagnification and bioaccumulation of HCB have occurred than is seen for herbivorous animals.

HCB has been reported to be transported passively across the gut wall in rats (Richter and Schäfer 1981). The small molecular size and high lipid solubility of HCB would suggest the absorption across the gut wall and subsequent diffusion into the systemic circulation are the principal means by which orally ingested HCB enters the organism. Iatropoulos *et al.* (1975) have reported that the major portion of ingested HCB is absorbed via the lymphatic system of the gastrointestinal tract and deposited in the fat, thereby effectively by-passing the systemic circulation and an initial exposure to the excretory organs in the rat. Whether or not this is the mechanism of absorption in the ferret has not been determined, but ^{14}C-labeled HCB/ metabolites were detected in their blood and selected tissues at the termination of the experiment.

Studies with rats have shown that HCB concentrations in tissues decline rapidly at first and then fall more slowly (Kuiper-Goodman et al. 1977) with at least 70-80% of the initial dose being retained after seven days (Albro and Thomas 1974). Two weeks after intraperitoneal (IP) injection of ^{14}C-HCB, 34% of the dose administered was recovered from the feces and 5% in the urine (Koss and Koransky 1975). The whole body half-life for HCB in rats was estimated to be about 60 days following a single IP injection (Morita and Oishi 1975). In this study, ferrets raising kits excreted 50% of the initial dose by approximately 32 days after consuming HCB-treated feed, while the unbred ferrets achieved this same degree of HCB elimination in 41 days (Figure 5). The percentages of HCB excreted via the urine and feces were approximately 5% and 45%, respectively, at the 50% stage of elimination in unbred females and 5% in urine, 43% in feces, and 2% in the kits for pregnant/lactating females. It would therefore appear to be more efficient at ridding itself of HCB than the rat. The high fat content of the basal diet used in this study, coupled with the ferret's relatively short digestive tract and rapid food passage time may have provided this species with a more efficient method for the elimination of HCB than the rat. Although the circulating levels of HCB in the blood supplying the ferret's gastrointestinal tract would have been low in the weeks following the single exposure, a concentration gradient favoring the passage of HCB into the intestinal contents would nevertheless exist. The passive transfer of HCB into the bolus traveling through the intestine would occur in conjunction with the active excretion processes.

The adipose tissue was the most significant long-term repository of HCB in the ferret. Adipose tissue residues were many times the value

of any other tissue. Similarly, the fat concentrations of HCB were the highest of all tissues tested in rats (Iatropoulos et al. 1975), pigs (denTonkelaar et al. 1978), chickens (Hansen et al. 1978, 1979), and dogs (Sundlof et al. 1981). In other tissues, HCB residues were closely associated with the fat content of the tissue (Avrahami and Steele 1972b; Hansen et al. 1979). The same general relationship was seen in the ferret tissues analyzed in this study.

HCB can undergo metabolic transformation following absorption by the animal. The principal metabolites seen are pentachlorophenol, tetrachlorophenol, trichlorophenol, and pentachlorobenzene. In rats treated with HCB, 70-80% of the excreted compound in the feces was parent chemical (Koss and Koransky 1975). Engst et al. (1976) reported that the fecal material of HCB-treated rats contained small amounts of pentachlorobenzene, but the principal product was the parent compound. Only 4-10% of the urinary product was HCB (Koss and Koransky 1975; Koss et al. 1976), with the remainder of the ^{14}C-labeled compound present being the more polar metabolites. Koss et al. (1976) reported that approximately 16% of the dose of ^{14}C-labeled HCB given to rats was converted to metabolites that could be detected in the excreta and animal's body. The tissue concentrations of metabolites accounted for only a small proportion of the total dose (10% of the labeled compound in the blood and less than 0.1% in the body fat). Major organs of female rats treated with HCB generally showed less than 1% of the chemical form present to be the metabolic products of HCB (Richter et al. 1981). Similarly, Engst et al. (1976) reported that HCB metabolites were present in quite small concentrations in rat tissues after HCB exposure. Rozman et al. (1975) found 3% of the HCB ingested by Rhesus monkeys was

metabolized, primarily to pentachlorobenzene and trace amounts of tetrachlorobenzene.

Although the relative proportions of HCB and its metabolites were not quantified in the study reported here, based on the work of other researchers detailed above, it would appear that a similar profile of metabolism may occur in the ferret. Since the tissue residue and blood concentrations of HCB metabolites were low in all species studied, in utero and mammmary exposure of the developing/growing ferret kits would be almost exclusively to the parent compound, HCB.

Ferrets raising kits were able to significantly reduce their body burden of HCB compared to unbred females. By the fifth week of lactation, 20.3% of the dam's initial dose had been eliminated via the offspring. If the litter size had not been continuously decreasing as kits were taken for ^{14}C-HCB analysis, this level might well have been higher. The lactating mammary gland has been described as an organ of excretion of polybrominated biphenyls (Dent et al. 1978; Bleavins et al. 1981) and polychlorinated biphenyls. Nursing female rats have likewise been reported to significantly decrease their body burden of PCB through the secretion of milk (Takagi et al. 1976; Ando 1978).

Foreign substances, especially those of high lipid solubility and low molecular weight such as HCB, have been shown to traverse the placenta by simple diffusion (Eckhoff 1972a, b). The ferret has an endotheliochorial zonodiscoidalis type placenta during the first stages of gestation (Enders 1952), but develops an endotheliochorial bidiscoidal placenta in the latter stages of pregnancy (Enders 1957). This deciduate placenta establishes a close relationship between the maternal and fetal tissues with a large surface area for diffusion. Thus, the

developing ferret kits were subjected to HCB insult both in utero and through the dam's milk following birth.

A ratio of placenta to milk HCB exposure in the growing ferret kit was calculated to be 1:31 in this study. The milk represents a greater quantity of this aromatic hydrocarbon entering the young animal than does the placenta. However, placental exposure affects the embryo during organogenesis and so may represent a greater than or equally high risk as mammary exposure. High concentrations of HCB have also been reported in weanling rats, compared to a relatively low dietary intake of the compound by their dams (Grant et al. 1977). This indicates the excretion of HCB via the milk to be an important route in the nursing rat as well as the ferret. The ferret ratio falls between the 14.6:1 value reported by Ando (1978) for rats and the 100:1 ratio calculated by Masuda et al. (1978) for mice exposed to PCBs. Other researchers studying rats (Takagi et al. 1976) and humans (Masuda et al. 1978) have similarly noted a greater quantity of PCBs to enter the growing offspring via the milk than the placenta.

HCB is a widely dispersed environmental contaminant (Courtney 1979) and has been shown to depress reproduction in rats by decreasing the number of litters produced and the number of offspring surviving to weaning (Grant et al. 1977). It has also been found to be immunosuppressive in mice (Loose et al. 1977, 1979) and to reduce growth rate in chickens (Hansen et al. 1979). The maternal tissues act as a readily available supply of HCB to the offspring (Courtney and Andrews 1979). The placental and mammary transfer of HCB imparts a significant risk to the developing and growing animal, in addition to any toxic effects HCB may have on the adult reproducing population.

Experiment IV. NEUROTRANSMITTER CONCENTRATIONS IN FIVE BRAIN REGIONS OF ADULT MINK AND THEIR OFFSPRING, AND ADULT FERRETS CONSUMING HEXACHLOROBENZENE IN A CHRONIC FEEDING TRIAL

Purpose

One of the biological effects manifested by exposure to HCB is the development of hyperexcitability, tremors, and/or convulsions (DeMatteis et al. 1961; Ockner and Schmid 1961; Vos et al. 1971; Taljaard et al. 1972; Kimbrough and Linder 1974; Timme et al. 1974; Kuiper-Goodman et al. 1977). Despite the appearance of such clinical signs in intoxicated mammalian species, little work has been done to elucidate the etiology of these neurotoxic effects. The fact that certain organochlorine pesticides produce similar effects and these effects occur concomitantly with alterations in brain neurochemical parameters (Wagner and Greene 1974; Hrdina et al. 1974; Willhite and Sharma 1978; Heinz et al. 1980) suggests that examination of regional brain biogenic amine levels in animals exposed to HCB might provide important information pertaining to the manifestation of the clinical signs. Thus, the purpose of the present study was to determine the concentrations of norepinephrine (NE), dopamine (DA), serotonin (5-HT), and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) in five brain regions of adult mink and ferrets that had been exposed to HCB in the diet for 47 weeks and in 16-17 week old mink kits that had been exposed to HCB in utero and via nursing for 6 to 7 weeks after birth.

Materials and Methods

Twenty-eight adult standard dark female mink and twenty-eight adult fitch-colored European ferrets were used in this study. These animals

consumed HCB in a chronic feeding trial initiated on 20 January 1981 and described in greater detail in Experiment II. The 0, 1, 5, and 25 ppm supplemental HCB treatment groups were used, seven animals from each level. At necropsy (16 December 1981 for mink and 17 December 1981 for ferrets), the brain was removed, dissected into five regions (cerebellum, medulla, midbrain, hypothalamus, and cerebral hemispheres), and frozen on dry ice for subsequent neurochemical analysis. Dopamine, norepinephrine, and 5-hydroxytryptamine (serotonin) were determined by the method of Jacobowitz and Richardson (1978). The concentration of 5-hydroxyindoleacetic acid was measured in accordance with the technique described by Curzon and Green (1970).

Regional brain neurotransmitter concentrations were determined in 18 standard dark female mink, age 16-17 weeks. These animals were born to dams consuming diets with 0, 1, and 5 ppm of supplemental HCB as described in Experiment II. The mink kits were placed on non-supplemented feed for 10 weeks following weaning and prior to analysis. The young mink used in this study were therefore exposed to HCB via three routes: 1) in utero throughout gestation, 2) their dam's milk during nursing, and 3) any HCB-contaminated feed they may have consumed directly, before weaning.

The data from both the adult and young animals were analyzed using the Dunnett's t-test to determine differences between the control and HCB-treated groups (Gill 1978).

Results

Hypothalamic 5-HT concentrations in the mink were slightly, but significantly increased at 1, 5, and 25 ppm of supplemental HCB (Table 24). A significant elevation of serotonin was seen only at 1 ppm HCB

Table 24. Concentration (ng/gm of tissue) of 5-hydroxytryptamine‡ (serotonin) in selected brain regions of adult female mink and ferrets consuming control and HCB diets for 47 weeks.

Dietary concentration	Brain region									
	n	Cerebellum	n	Medulla	n	Midbrain	n	Hypothalamus	n	Cerebral hemispheres
Mink										
0 ppm	6	239 ± 104.2	7	755 ± 131.4	7	461 ± 37.7	7	528 ± 34.9	4	127 ± 15.6
1 ppm	7	149 ± 24.8	7	494 ± 77.5	7	480 ± 29.0	7	699 ± 34.9**	7	94 ± 11.8
5 ppm	5	183 ± 45.5	7	567 ± 91.7	7	491 ± 33.1	7	735 ± 34.9**	7	123 ± 11.8
25 ppm	7	191 ± 27.4	7	633 ± 116.9	7	561 ± 82.8	7	710 ± 34.9**	7	95 ± 11.8
Ferret										
0 ppm	7	159 ± 25.1	7	566 ± 48.6	6	460 ± 21.9	7	633 ± 37.5	7	130 ± 11.1
1 ppm	5	296 ± 53.3*	7	661 ± 65.5	7	391 ± 51.2	7	640 ± 37.5	7	134 ± 7.1
5 ppm	7	188 ± 16.6	7	629 ± 35.6	7	391 ± 27.7	7	728 ± 37.5	7	148 ± 15.4
25 ppm	7	138 ± 17.7	7	587 ± 38.4	7	408 ± 29.9	7	641 ± 37.5	7	119 ± 9.6

‡ Mean ± S.E.

* Significantly different from control ($P < 0.05$).

** Significantly different from control ($P < 0.01$).

in the cerebellum of the ferrets. Hypothalamic 5-HIAA concentrations were significantly higher than control in the mink consuming the 5 ppm diet (Table 25), while this catecholamine was significantly depressed in the midbrain of ferrets at 5 and 25 ppm HCB. No significant change was found in the concentration of NE (Table 26) or DA (Table 27) in any brain region of either the mink or the ferret.

No significant change was detected in the brain concentration of serotonin in the young mink (Table 28). However, 5-HIAA was elevated in the 5 ppm group. As shown in Table 29, no alteration in the concentration of NE was observed for any brain region of these animals. The hypothalamic DA concentrations were significantly depressed in the mink kits whelped by dams consuming 1 and 5 ppm HCB.

A treatment group of 125 ppm dietary HCBwas included in the original parent study (Experiment II), but none of these adult mink and ferrets survived until the termination date. However, behavioral changes were observed in six of the 10 female ferrets consuming this concentration of HCB. The animals were very excitable and abnormally aggressive for this species. If handled, they began to shriek and acted as if being picked up not only upset them more than usual, but was also painful. Later in the course of the study, four of these females were found immediately prior to death. Each animal was unable to stand or reposition itself, extremely weak, exhibited trembling of all four legs, and appeared to lapse into occassional mild convulsions. The ferrets died one to three hours after being discovered in this condition.

Discussion

Numerous studies have indicated that exposure to HCB can result in disruption of the nervous system clinically manifested as

Table 25. Concentration (ng/gm of tissue) of 5-hydroxyindoleacetic acid‡ in selected brain regions of adult female mink and ferrets consuming control and HCB diets for 47 weeks.

Dietary concentration	Brain region									
	n	Cerebellum	n	Medulla	n	Midbrain	n	Hypothalamus	n	Cerebral hemispheres
Mink										
0 ppm	6	302 ± 114.7	7	432 ± 57.4	7	400 ± 21.9	7	523 ± 32.9	4	105 ± 15.6
1 ppm	7	227 ± 74.1	7	455 ± 63.1	7	394 ± 29.6	7	522 ± 32.9	7	67 ± 11.8
5 ppm	6	224 ± 28.0	7	529 ± 31.4	7	494 ± 53.4	7	719 ± 32.9**	7	89 ± 11.8
25 ppm	7	394 ± 105.8	7	609 ± 77.7	7	352 ± 26.7	7	517 ± 32.9	7	57 ± 11.8
Ferret										
0 ppm	7	147 ± 30.3	7	601 ± 73.3	7	662 ± 46.9	7	929 ± 90.3	7	87 ± 6.9
1 ppm	7	180 ± 22.1	7	612 ± 87.0	7	559 ± 79.3	6	878 ± 42.6	7	98 ± 4.7
5 ppm	7	118 ± 10.1	7	677 ± 37.6	7	472 ± 21.7*	7	864 ± 32.1	7	100 ± 17.5
25 ppm	7	92 ± 8.0	7	600 ± 33.3	7	399 ± 22.2**	7	791 ± 30.2	7	88 ± 22.5

‡ Mean ± S.E.

* Significantly different from control ($P < 0.05$).

** Significantly different from control ($P < 0.01$).

Table 26. Concentration (ng/gm of tissue) of norepinephrine‡ in selected brain regions of adult female mink and ferrets consuming control and HCB diets for 47 weeks.

Dietary concentration	Brain region									
	n	Cerebellum	n	Medulla	n	Midbrain	n	Hypothalamus	n	Cerebral hemisphere
Mink										
0 ppm	7	55 ± 7.1	7	196 ± 20.8	7	190 ± 15.7	7	167 ± 8.2	6	44 ± 5.2
1 ppm	7	68 ± 15.5	6	179 ± 22.5	7	204 ± 13.5	7	253 ± 52.9	7	45 ± 3.1
5 ppm	6	60 ± 3.8	7	138 ± 20.8	7	189 ± 12.0	7	279 ± 29.6	7	45 ± 6.3
25 ppm	7	71 ± 12.1	7	206 ± 20.8	7	260 ± 61.0	7	219 ± 43.8	7	41 ± 3.6
Ferret										
0 ppm	7	87 ± 6.7	7	227 ± 22.9	7	216 ± 5.6	7	425 ± 33.5	7	75 ± 5.0
1 ppm	7	78 ± 5.6	7	245 ± 22.9	7	207 ± 18.5	7	377 ± 26.5	7	85 ± 5.0
5 ppm	7	70 ± 4.7	7	288 ± 22.9	7	216 ± 18.8	7	460 ± 28.8	7	77 ± 5.0
25 ppm	7	75 ± 2.6	7	252 ± 22.9	7	216 ± 14.0	7	428 ± 16.6	7	82 ± 5.0

‡ Mean ± S.E.

Table 27. Concentration (ng/gm of tissue) of dopamine‡ in selected brain regions of adult female mink and ferrets consuming control and HCB diets for 47 weeks.

Dietary concentration	Brain region									
	n	Cerebellum	n	Medulla	n	Midbrain	n	Hypothalamus	n	Cerebral hemisphere
Mink										
0 ppm	7	268 ± 24.4	7	418 ± 41.9	7	753 ± 184.4	7	306 ± 26.5	6	233 ± 23.9
1 ppm	7	326 ± 35.6	6	488 ± 54.4	7	1045 ± 181.0	7	476 ± 60.8	7	227 ± 22.1
5 ppm	6	317 ± 18.3	7	316 ± 21.7	7	855 ± 121.3	7	456 ± 84.3	7	227 ± 22.1
25 ppm	7	319 ± 33.7	7	466 ± 75.3	7	917 ± 241.5	7	352 ± 37.9	7	200 ± 22.1
Ferret										
0 ppm	7	353 ± 7	7	373 ± 58.7	7	1174 ± 180.4	7	779 ± 71.9	7	350 ± 14.4
1 ppm	7	409 ± 19	7	449 ± 38.7	7	1371 ± 238.9	7	608 ± 52.8	7	385 ± 37.1
5 ppm	7	386 ± 12	7	633 ±218.1	7	1662 ± 58.5	7	766 ± 47.4	7	318 ± 11.2
25 ppm	7	347 ± 19	7	509 ± 86.2	7	1703 ± 225.2	7	663 ± 37.4	7	335 ± 17.7

‡ Mean ± S.E.

Table 28. Concentrations (ng/gm of tissue) of 5-hydroxytryptamine‡ and 5-hydroxyindoleacetic acid‡ in selected brain regions of young female mink† perinatally exposed to HCB.

Dietary concentration	Brain region				
	Cerebellum	Medulla	Midbrain	Hypothalamus	Cerebral hemispheres
5-hydroxytryptamine					
0 ppm	245 ± 14.1	899 ± 102.3	844 ± 72.3	939 ± 41.2	133 ± 5.4
1 ppm	248 ± 18.3	870 ± 57.6	751 ± 79.5	880 ± 43.3	125 ± 9.8
5 ppm	277 ± 28.6	955 ± 97.5	928 ± 98.3	983 ± 48.5	143 ± 15.8
5-hydroxyindoleacetic acid					
0 ppm	76 ± 18.0	565 ± 49.0	495 ± 51.0	583 ± 75.6	69 ± 5.6
1 ppm	85 ± 12.8	525 ± 60.8	465 ± 30.1	518 ± 34.9	60 ± 4.0
5 ppm	126 ± 10.6	618 ± 39.7	674 ± 56.3*	699 ± 84.4	69 ± 5.7

‡ Mean ± S.E.

† n = 6.

* Significantly different from control ($P < 0.05$).

Table 29. Concentrations (ng/gm of tissue) of norepinephrine‡ and dopamine‡ in selected brain regions of young female mink† perinatally exposed to HCB.

Dietary concentration	Brain region				
	Cerebellum	Medulla	Midbrain	Hypothalamus	Cerebral hemispheres
norepinephrine					
0 ppm	63 ± 3.7	210 ± 18.6	233 ± 18.2	669 ± 60.7	33 ± 3.8
1 ppm	53 ± 3.8	189 ± 13.4	198 ± 13.1	573 ± 21.7	24 ± 1.7
5 ppm	48 ± 4.0	191 ± 10.2	189 ± 13.8	531 ± 29.5	23 ± 1.9
dopamine					
0 ppm	119 ± 13.4	228 ± 14.6	526 ± 78.2	652 ± 21.6	128 ± 9.0
1 ppm	128 ± 10.8	209 ± 7.3	714 ± 151.7	447 ± 30.8*	98 ± 5.9
5 ppm	121 ± 16.4	257 ± 21.1	527 ± 104.7	471 ± 29.0*	105 ± 8.0

‡ Mean ± S.E.

† n = 6.

* Significantly different from control ($P < 0.05$).

hyperexcitability, tremors, and/or convulsions. For example, Timme et al. (1974) fed rats a diet containing 0.3% HCB which resulted in an estimated daily dose of 250 mg/kg of body weight. Tremors became apparent in exposed rats shortly after the feeding trial began. Similarly, Taljaard et al. (1972) administered 0.3% HCB in the diet to rats and observed nervous system effects by 21 days. Thirteen of 33 rats fed 0.2% HCB died within the first month exhibiting terminal tremor, ataxia, weakness, and paralysis (Ockner and Schmid 1961). Kimbrough and Linder (1974) fed dietary HCB to rats for four months at levels of 100, 500, and 1000 ppm which corresponded to 5, 30, and 70 mg/kg/day, respectively. Those rats fed 500 and 1000 ppm displayed clinical signs of tremors and hyperexcitability while no toxic signs were observed in those rats receiving 100 ppm. Kuiper-Goodman et al. (1977) reported clinical signs of excessive irritability, ataxia with hindleg paralysis, and tremors in rats fed HCB at a level equivalent to 32 mg/kg of body weight over a 15 week period. Rabbits fed a diet containing 0.5% HCB for 8 to 10 weeks developed clinical signs of tremors and paresis within the last week of treatment, while guinea pigs fed 0.5% HCB in the diet displayed tremors with clonic convulsions within 8 to 10 days. These animals were also described as being hyperexcitable which increased with handling. Likewise, mice administered 0.5% HCB in the diet developed tremors within 8 to 10 days, as well as clonic contractions of the hind limbs (DeMatteis et al. 1961). Japanese quail fed 80 ppm HCB in the diet were adversely affected beginning three weeks after initiation of the treatment. Thirty percent of the birds at this level died between 18 and 62 days of the experiment after a short period of anorexia, ruffled feathers, tremor, and ataxia (Vos et al. 1971). The observation of clinical

signs indicative of neurological impairment was possible with six of the 125 ppm treatment group. These animals were judged to be very excitable and abnormally aggressive for the ferret. Four of the females were observed immediately prior to death. None of the animals was able to stand and all exhibited trembling of all four legs and mild convulsions. These data indicate that, at least in the ferret, death is preceeded by clinical signs indicative of nervous system disruption as has been reported for other species. In fact, that such signs and subsequent death were apparent at an approximate HCB dose of 17 mg/kg body weight/day (125 ppm in the diet) in both the mink and ferrets suggests that these two species are among the more susceptible mammals to HCB intoxication.

Despite the fact that clinical signs indicating nervous system involvement are consistently observed in HCB-intoxicated animals, little information exists concerning the mechanism of action. Kuiper-Goodman et al. (1977) reported that tremors started to appear soon after elevated porphyrin levels were detected although determination of a cause-effect relationship was beyond the scope of the experiment. However, Ockner and Schmid (1961) observed that none of the 13 rats dying within the first month of exposure to dietary HCB, after displaying clinical signs indicative of nervous system involvement, exhibited major disturbances in porphyrin metabolism. DeMatteis et al. (1961) suggested that elevated blood ammonia concentrations might be responsible for the neurological signs observed. No histopathological abnormalities have been found in brain, spinal cord, motor nerves, or sensory nerves of skeletal muscle of HCB-exposed animals (DeMatteis et al. 1961; Kuiper-Goodman et al. 1977).

Numerous studies with cyclodiene organochlorine pesticides have demonstrated that acute exposure to such compounds produces neurotoxic effects similar to those described for HCB such as hyperexcitability, tremors, and convulsions (Hrdina et al. 1974; Wagner and Greene 1974). Furthermore, it has been shown that both acute cyclodiene organochlorine exposure where clinical signs are evident and in chronic situations where clinical signs are absent, various brain neurochemical parameters are altered, suggesting a relationship between altered brain neurochemistry and the mechanism of action of the cyclodiene compounds. For example, following an acute dose of α-chlordane (200 mg/kg), endrin (50 mg/kg), heptachlor (200 mg/kg), or heptachlor epoxide (100 mg/kg) rats developed slight tremor paralysis of the hind legs and convulsions. In the chlordane-treated rats these signs were accompanied by a decrease in brain stem NE concentration and cortical and striatal acetylcholine (ACh) concentrations, Chronic treatment with these compounds produced no apparent signs of neurotoxicity, but did cause a significant drop in cerebro-cortical ACh and a significant increase in brain stem 5-HT (Hrdina et al. 1974). Similarly, Wagner and Greene (1974) reported that an acute oral dose of dieldrin (50 mg/kg) produced severe neurotoxic signs, as well as a significant decrease in whole-brain NE concentration in male rats. Chronic exposure to dieldrin via the feed (50 ppm) caused a significant decrease in midbrain, striatum, hippocampus, and medulla NE concentrations, as well as a significant depression in 5-HT concentrations in the midbrain, striatum, and medulla. Sharma (1976) reported that chronic administration of dieldrin (40 ppm) to mice caused a significant increase in the concentration of the serotonin metabolite 5-HIAA, but did not affect brain serotonin concentrations. This effect

was explained not as an increase in serotonin turnover, but rather as an impairment of 5-HIAA efflux from the brain. In a later study, Willhite and Sharma (1978) found that acute exposure of chickens and hamsters to dieldrin (10 mg/kg) caused an increase in brain serotonin concentrations and a decrease in the activity of monoamine oxidase (MAO) which is involved in the metabolism of 5-HT. While the neurochemical alterations resulting from cyclodiene organochlorine exposure are not consistent with respect to the parameter affected and the direction of the change, it is apparent that alterations in catecholamine and indoleamine dynamics do occur.

Results from the present study indicate that HCB also causes alterations in brain neurochemical parameters which are similar to changes induced by cyclodiene compounds. Female mink exposed to dietary HCB for 47 weeks had elevated 5-HT concentrations at all dose levels. The significant increase in 5-HT without a concomitant consistent change in 5-HIAA suggests that 5-HT turnover in the hypothalamus is being depressed by HCB. Concentrations of 5-HT and 5-HIAA in the cerebellum, medulla, midbrain, and cerebral hemispheres were unaffected. In female ferrets exposed to HCB for 47 weeks, cerebellar 5-HT concentration was increased at 1 ppm while 5-HIAA concentrations were increased at 5 and 25 ppm HCB, in the midbrain. The elevated concentrations of midbrain 5-HIAA with no consistent alterations in midbrain 5-HT concentrations suggest that serotonergic activity was increased in this brain area or that 5-HIAA efflux was impaired. Regional brain NE and DA concentrations were not altered by HCB exposure in either the adult mink or ferrets.

In mink kits that had been exposed to HCB _in utero_ and during

lactation, midbrain 5-HIAA was increased at 5 ppm without a concomitant change in 5-HT concentration, suggesting either an increase in serotonin turnover or an impairment of 5-HIAA efflux from this brain region. Hypothalamic DA concentrations were depressed at the 1 and 5 ppm levels while regional brain NE concentrations were unaffected by HCB exposure.

The results of this study suggest that chronic exposure to HCB does cause alterations in brain neurochemical parameters and that these changes are apparent weeks after in utero and perinatal exposure. Although the present study was not designed to establish a correlation between clinical signs of HCB-induced nervous system impairment and neurochemical alterations, such a relationship is suggested. Perhaps of greater importance is the fact that neurochemical alterations are apparent even in the absence of clinical signs, alterations that could have a subtle and possibly detrimental effect on the behavior and normal physiological functioning of the animal.

Experiment V. CELLULAR RECOVERY FROM WHOLE AND DILUTED MINK BLOOD AND LYMPHOCYTE-TO-MONOCYTE RATIOS OF DENSITY CENTRIFUGATION ISOLATED MONONUCLEAR LEUKOCYTES

Purpose

The use of diluted blood is a common technique in density centrifugation procedures for the isolation of mononuclear leukocytes. This study was initiated to determine if the number of cells recovered from a sample of whole blood could be increased by diluting the blood with Hank's Balanced Salt Solution (HBSS), prior to Ficoll-Paque isolation. A second trial was conducted to determine whether or not the ratio of lymphocytes to monocytes remains constant across a wide range of cellular concentrations obtained from the same original quantity of whole mink blood.

Materials and Methods

Effects of Dilution

Heparinized blood (11 ml) was collected, via cardiac puncture, from nine adult ranch-bred male mink. The 11 ml blood-heparin sample (9.9 ml blood: 1.1 ml of 200 U heparin per ml on physiological saline) was divided into three 16 x 125 mm sterile tissue culture tubes as follows: 1) 5 ml of whole blood solution, 2) 3.5 ml of blood + 1.5 ml of HBSS, and 3) 2.5 ml of blood + 2.5 ml of HBSS. Mononuclear leukocytes were isolated from each of the samples by density centrifugation using Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). Following the initial centrifugation (400 x g, 40 minutes), the cells at the Ficoll-Paque: plasma interface were collected and washed twice with HBSS and then resuspended in 1 ml of HBSS. The resulting mononuclear leukocytes were counted on a Coulter Counter (Coulter Electronics, Hialeah, FL). The

cell recovery per ml of the original heparinized blood sample was then calculated. The data were analyzed by a Student's t-test for comparisons of two means.

Lymphocyte-to-Monocyte Ratios

Five ml of heparinized blood was collected, via cardiac puncture, from 10 adult male and 10 adult female ranch-bred mink. The 5 ml sample (4.5 ml whole blood: 0.5 ml of 200 U heparin per ml of physiological saline) was layered onto Ficoll-Paque in 16 x 125 mm sterile tissue culture tubes and centrifuged for 40 minutes at 400 x g. The cells at the Ficoll-Paque:plasma interface were collected and washed twice with HBSS and then resuspended in sufficient HBSS to allow counting on a Coulter Counter. After the cells were counted, each tube was recentrifuged (200 x g, 10 minutes) and the supernate discarded. The remaining cell pellet was mixed with one drop of HBSS and used to make a smear for leukocyte differential counting. The leukocyte smears were stained with Wright's stain (Camco Quik Stain, Cambridge Chemical Products, Ft. Lauderdale, FL). The percentage of monocytes and lymphocytes was determined by counting 500 leukocytes per smear. The data were analyzed by a Student's t-test for differences between means (Gill 1978) and the correlation calculated between % monocytes and cell recovery.

Results

Effects of Dilution

To allow each animal to act as its own control, the values obtained per ml of diluted blood were expressed as a percentage of the value obtained per ml of undiluted blood from the same mink. The 3.5 ml blood: 1.5 ml HBSS dilution gave 80.7 $\pm$ 5.59 % of the whole blood number of cells and the 2.5 ml blood: 2.5 ml HBSS solution produced 71.8 $\pm$ 6.59 %

of the cells yielded from each ml of whole blood. The diluted blood samples consistently produced fewer mononuclear leukocytes than a comparable quantity of blood without HBSS.

Lymphocyte-to-Monocyte Ratios

The average total number of mononuclear leukocytes obtained from nine ml of whole blood was 125.5 $\pm$ 19.12 million, with a range of from 34.5 to 366.8 million cells. The isolated cell fraction was greater than 95% viable mononuclear leukocytes. Differential counting of these cells revealed them to be a population of 2.9 $\pm$ 0.32 % monocytes (range = 0.8 to 6.4) and 97.1 $\pm$ 0.32 % lymphocytes (range = 93.6 to 99.2). No relationship was seen between cell yield and percentage of monocytes. The correlation between % monocytes and cell yields was calculated to be 0.281.

Discussion

The separation of lymphocytes and monocytes via density gradient centrifugation was not improved by the dilution of the original blood sample with HBSS. The number of mononuclear leukocytes was greatest when whole blood was layered on the Ficoll-Paque and centrifuged. The use of diluted blood samples, as is commonly practiced with human and mouse blood, only resulted in a larger number of tubes to be spun and more reagents being necessary to isolate fewer cells from a given blood sample. The separation of lymphocytes from mink blood appears to be best accomplished through the centrifugation of whole blood, both in terms of the number of cells recovered and the efficient use of reagents.

The percentage of monocytes in the total population of mononuclear leukocytes isolated in these studies was found to be constant over a wide range of cell yields. There was no significant correlation between

cell yield and monocyte-to-lymphocyte ratio. This finding indicates that the cell suspensions obtained by Ficoll-Paque isolation can be diluted to a constant concentration for each experimental animal without affecting the relative proportions of lymphocytes and monocytes, regardless of the original cell concentration obtained.

The proliferative response of T lymphocytes to Con A is a macrophage (blood monocyte) dependent reaction (Persson et al. 1978; DeVries et al. 1979; Resch et al. 1981) and unless lymphocyte-to-monocyte ratios are kept constant, the incorporation of ^{3}H-thymidine can be highly variable following mitogen stimulation. Insufficent macrophages result in low responses which increase as macrophage numbers increase, until an excess of macrophages become suppressive (DeVries et al. 1979). The mononuclear cells isolated from mink during the course of this experiment showed a relatively constant percentage of monocytes (1 to 6 %) across a wide range of total cells recovered from the same quantity of blood. Variability between animals may well prove to be due, at least in part, to differences in monocyte concentration. However, these fluctuations in monocyte numbers cannot be accurately linked to the number of cells recovered from a whole blood sample.

Experiment VI. LYMPHOCYTE BLASTOGENIC RESPONSE OF ADULT FEMALE MINK AND FERRETS, AND THEIR OFFSPRING, AFTER CONSUMING HEXACHLOROBENZENE IN A CHRONIC FEEDING TRIAL

Purpose

The chronic exposure of organisms to low levels of toxic substances may lead to the impairment or alteration of normal physiological function. The cell-mediated immune system may be one such target for some toxicants. The effects of HCB on cell-mediated immunity in the mink and ferret was studied via the T lymphocyte mitogen Con A.

Materials and Methods

Study 1. Effects on the Response of Adult Mink and Ferrets

Adult standard dark female mink and adult female European ferrets were exposed to HCB in a chronic feeding trial. The mink and ferrets were immunized against canine distemper, botulism, and viral enteritis thirteen months prior to the collection of blood for blastogenesis. Mink and ferrets were from the treatment groups fed diets containing either 0, 1, 5, or 25 ppm of supplemental HCB in the trial initiated on 20 January 1981 (Experiment II).

Mitogenic lymphocyte blastogenesis was performed with mink after 34 weeks of HCB exposure and with ferrets after 35 weeks of consuming HCB-treated feed. Heparinized blood was collected, via cardiac puncture, following light ether anesthesia. Mononuclear cells were isolated from the whole blood by density centrifugation using Ficoll-Paque. The 10 ml blood-heparin solution sample was transferred to two 16 x 125 mm sterile tissue culture tubes and handled as separate samples until after the final rinse with Hank's Balanced Salt Solution (HBSS) without Ca^{++} or Mg^{++}. Following the initial centrifugation (400 x g, 40 minutes), the

cells at the Ficoll-Paque:plasma interface were collected and washed three times with HBSS and then resuspended in 1.5 ml of RPMI-1640 medium (GIBCO, Grand Island, NY). The final media used contained 25 mM HEPES, 2 mM l-glutamine, 100 U penicillin, 100 U streptomycin, 0.25 µg Fungizone, and 10% heat inactivated fetal calf serum. The two tubes per animal were then combined to yield the final cell suspension. The concentration of each cell suspension was measured by hemocytometer count and the percentage of live mononuclear leukocytes determined using the Trypan blue exclusion test (Kruse and Patterson 1973) on a phase-contrast microscope. Cell yields from the original blood sample were 3.4 to 25.6 x 10^6 total cells, with 90% or greater being live mononuclear cells. The concentration of live mononuclear leukocytes was adjusted to 3.5 x 10^6 cells per ml ($\pm$3%) with RPMI-1640 medium.

Con A (Miles Laboratories, Elkhart, IN), a T cell stimulator, was diluted using RPMI-1640 media to concentrations of 40, 20, 10, 5, 2.5, or 0 µg/ml. Triplicate samples, except in a few animals which had poor cell recovery and so only duplicate samples were made, 100 µl aliquots of the cell suspension from each animal were placed into wells of sterile U-bottom microtiter plates containing 100 µl of the various Con A suspensions; final mitogen concentrations in the microtiter wells were therefore 20, 10, 5, 2.5, 1.25, or 0 µg/ml.

The cultures were incubated at 38°C in a humidified, 5% CO_2 incubator for 48 hours. Each well was then pulsed with 0.25 µCi of ^{3}H-thymidine (New England Nuclear, Boston, MA, specific activity = 6.7 Ci/mM) in 25 µl of RPMI-1640 medium and incubated for an additional 24 hours. The cells were harvested onto glass fiber filter strips using a multiple automated sample harvester. The filter strip areas containing the

cellular fragments were cut out and placed into mini-scintillation vials containing 5 ml of Scintiverse (Fisher Scientific, Cincinnati, OH). Each vial was counted for 10 minutes on an Isocap/300, model 6872 liquid scintillation counter (Searle Analytic, Inc., Des Plaines, IL).

The stimulation index for each animal at the various mitogen concentrations was calculated as: (DPM of mitogen dosed culture - DPM of background culture) ÷ (DPM of background culture). The data were analyzed by the Dunnett t-test to examine differences between the control and treatment groups (Gill 1978).

Study 2. Effects on the Response of Young Mink and Ferrets

Twenty-four standard dark female mink and twenty-three fitch female ferrets, age 16 to 17 weeks, were used to assess lymphocyte blastogenic response of the kits exposed to HCB. The animals were born to dams consuming diets that contained 0, 1, and 5 ppm of supplemental HCB as described in Study 1. The treatment regime consisted of exposure of the mink to HCB for six weeks prior to the mating of the adult females, for approximately seven weeks of gestation, and seven weeks of lactation. The kits were placed on untreated feed for 10 weeks following weaning and prior to blastogenesis testing. As the ferrets do not come into breeding condition until approximately a month later than the mink, the adult ferrets were exposed to HCB for 10 weeks prior to mating, for six weeks of gestation, and seven weeks of lactation. The ferret kits were also fed non-HCB treated feed for 10 weeks after weaning and prior to blastogenesis testing. The young mink and ferrets used in this study were therefore exposed to HCB in utero, via their dam's milk, and from any HCB-contaminated feed they may have consumed before weaning

Lymphocyte blastogenesis was conducted in the young animals in the

manner described in Study 1 for their dams. Cell yields from these mink and ferrets were generally 5.5 to 20.3 x 10^6 total cells with 85% or greater being live mononuclear leukocytes. Due to the poor cell recovery from the blood of several mink kits, the 20 µg/ml Con A concentration was not used.

The data were analyzed using the Dunnett t-test on the ferret data and by a Dunnett-type procedure designed to compensate for heterogeneous variance between groups on the mink data (Gill 1978).

Results

Study 1.

At the 20 µg/ml Con A concentration (Table 30), no statistically significant ($P<0.05$) decrease in the blastogenic response was seen in ferrets fed 1 or 5 ppm HCB in the diet. The 5 ppm treatment group was ignificantly lower than control at $P<0.10$. The 25 ppm HCB-treated ferrets yielded a significantly ($P<0.05$) lower stimulation index than control ferrets. At this concentration of mitogen, Con A was inhibitory for all treatment groups. A general trend toward diminished blastogenic response was seen with increasing HCB concentration in the diet. No significant reduction was observed in the 10 µg/ml Con A microtiter wells until the 25 ppm HCB dietary level. Statistically significant changes from the control stimulation indices were not seen at 5 µg/ml of Con A, although the 25 ppm HCB group did show a reduction significant at $P<0.10$. At this mitogen dose, as well as the two mitogen concentrations below it, a trend toward elevated blastogenic response (above the control value) was observed in animals consuming 1 ppm HCB. Higher toxicant exposures resulted in stimulation indices lower than control values. HCB-treated ferrets did not differ significantly ($P<0.05$) from

Table 30. Stimulation indices[‡] of adult female ferrets[†] consuming control and HCB diets for 35 weeks.

Treatment	n	Mitogen concentration[ψ] (μg/ml) 20	10	5	2.5	1.25
0 ppm	6	32.51 ± 5.926	63.80 ± 10.247	57.65 ± 9.747	25.43 ± 6.897	10.11 ± 3.678
1 ppm	6	24.72 ± 5.926	60.53 ± 10.247	70.57 ± 9.747	44.09 ± 6.897	17.73 ± 3.678
5 ppm	6	15.21 ± 5.926	41.26 ± 10.247	37.38 ± 9.747	19.29 ± 6.897	4.89 ± 3.678
25 ppm	6	4.18 ± 5.926*	23.83 ± 10.247*	27.45 ± 9.747	18.76 ± 6.897	9.93 ± 3.678

[‡]Stimulation index = (DPM of mitogen dosed culture - DPM of background culture) ÷(DPM of background culture).

[†]Mean ± S.E.

[ψ]Concentration in the microtiter wells of Con A.

*Significantly different from control ($P<0.05$).

the untreated animals at mitogen concentrations of 2.5 and 1.25 μg/ml of Con A.

The stimulation indices of HCB-exposed mink are shown in Table 31. At all mitogen concentrations of Con A there was a trend for a low level of toxicant (1 ppm) in the diet to trigger a blastogenic response greater than the control was observed. There was no statistically significant decrease in blastogenesis at 20, 2.5, and 1.25 μg/ml of Con A. The 10 and 5 μg/ml Con A concentrations did not differ significantly from the control at 1 or 5 ppm HCB, but were significantly ($P<0.05$) reduced in mink that consumed 25 ppm HCB in the diet.

In both the mink and ferrets, the highest concentration of Con A (20 μg/ml) suppressed the lymphocyte blastogenic response as compared to the 10 μg/ml concentration. The optimal mitogen concentration for either species was 10 μg/ml of Con A, with doses above or below this value producing a lesser response. Animals consuming HCB diets had the same optimal mitogen concentration as was observed for control mink and ferrets.

Study 2.

The stimulation indices of the ferrets born to dams consuming HCB (Table 32) did not show a significant reduction in blastogenic response from control values at any mitogen concentration at $P<0.05$. At 20 μg/ml of Con A, a strong trend toward reduced blastogenic response was noted at the 1 and 5 ppm concentrations of HCB. HCB at 5 ppm in the dam's diet, led to a decrease in stimulation index that was significant $P<0.10$. There was no significant change in stimulation indices with increasing exposures to HCB at 5 or 10 μg/ml of Con A. At 2.5 and 1.25 μg/ml of this mitogen, the response was equal to or greater than that of the

Table 31. Stimulation indices[‡] of adult female mink[†] consuming control and HCB diets for 34 weeks.

Treatment	n	Mitogen concentration[ψ] (μg/ml) 20	10	5	2.5	1.25
0 ppm	9	21.16 ± 6.800	77.49 ± 11.203	51.79 ± 7.312	26.80 ± 4.633	12.39 ± 2.156
1 ppm	8	33.33 ± 7.212	85.61 ± 11.882	60.02 ± 7.756	34.97 ± 4.914	16.11 ± 2.287
5 ppm	6	12.66 ± 8.328	39.53 ± 13.720	29.24 ± 8.956	16.09 ± 5.674	9.66 ± 2.641
25 ppm	6	5.07 ± 8.328	28.42 ± 13.720*	22.43 ± 8.956*	15.52 ± 5.674	9.09 ± 2.641

[‡]Stimulation index = (DPM of mitogen dosed culture - DPM of background culture) ÷(DPM of background culture).

[†]Mean ± S.E.

[ψ]Concentration in the microtiter wells of Con A.

*Significantly different from control ($P<0.05$).

Table 32. Stimulation indices[‡] of young female ferrets[†] born to dams consuming control and HCB diets throughout pregnancy and lactation.

Treatment	n	Mitogen concentration[ψ] (μg/ml) 20	10	5	2.5	1.25
0 ppm	12	24.60 ± 7.035	56.30 ± 9.143	48.16 ± 6.820	20.15 ± 4.129	6.06 ± 1.957
1 ppm	5	11.09 ± 10.898	49.25 ± 14.164	42.62 ± 10.565	23.00 ± 6.396	6.80 ± 3.031
5 ppm	6	4.68 ± 9.949	43.39 ± 12.930	39.51 ± 9.645	25.73 ± 5.839	10.53 ± 2.767

[‡]Stimulation index = (DPM of mitogen dosed culture - DPM of background culture)÷ (DPM of background culture).

[†]Mean ± S.E.

[ψ]Concentration in the microtiter wells of Con A.

control animals.

As shown in Table 33, the ability of lymphocytes from mink exposed to HCB to respond to mitogenic stimulation was significantly reduced at 10 and 5 μg/ml of Con A. The reduction in stimulation index observed at 2.5 μg/ml of mitogen was not significantly ($P<0.05$) less than control values for either the 1 or 5 ppm HCB groups. It was significantly less however at $P<0.10$ for both HCB-exposed groups. The lowest mitogen concentration stimulation index, 1.25 μg/ml of Con A, was significantly lower ($P<0.05$) in kits from the 1 ppm HCB-treated animals, but not in the 5 ppm HCB mink kits. No mitogen concentration or treatment level of HCB gave a stimulation index greater than the control values at 10 μg/ml of Con A.

Discussion

An increasing number of compounds have been identified as being capable of altering normal immune function. Tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to possess both humoral and cell-mediated immunosuppressive properties in the mouse (Vos and Moore 1974; Hinsdill et al. 1980) and to depress cellular immunity in rats (Vos and Moore 1974). The polychlorinated biphenyls have been found to significantly reduce T cell function and humoral immunity in guinea pigs (Vos and VanDriel-Grootenhuis 1972) and polybrominated biphenyls have caused a depression in the cell-mediated immune response of Michigan dairy farmers (Bekesi et al. 1979a, b).

In this study, mink and ferrets were used to assess the effects of HCB on T lymphocyte function as measured by the incorporation of ^{3}H-thymidine following stimulation by Con A. Because of their position at the top of the food chain, these two carnivores are representative of a

Table 33. Stimulation indices[‡] of young female mink[†] born to dams consuming control and HCB diets throughout pregnancy and lactation.

Treatment	n	Mitogen concentration[ψ] (μg/ml) 10	5	2.5	1.25
0 ppm	12	80.35 ± 14.430	50.42 ± 10.149	24.42 ± 5.604	14.34 ± 3.899
1 ppm	6	32.80 ± 7.427*	22.63 ± 3.380*	12.02 ± 1.404	4.74 ± 1.304*
5 ppm	6	33.80 ± 5.708*	24.99 ± 2.670*	14.39 ± 1.828	7.11 ± 1.538

‡ Stimulation index = (DPM of mitogen dosed culture - DPM of background culture) ÷ (DPM of background culture).

† Mean ± S.E.

ψ Concentration in the microtiter wells of Con A.

* Significantly different from control ($P<0.05$).

class of animals with high risk from compounds capable of biomagnification. Additionally, both the mink and ferret have been shown to be extremely sensitive to the other halogenated aromatic compounds such as PCBs (Aulerich et al. 1973; Platonow and Karstad 1973; Bleavins et al. 1980) and PBBs (Aulerich and Ringer 1979). The cell-mediated aspects of the immune response have been considered to be especially sensitive to alterations by aromatic hydrocarbons (Moore 1979) such as HCB, TCDD, PCBs, and PBBs.

A significant reduction in the stimulation index was found in the adult mink and ferrets fed 25 ppm HCB in the diet. This decrease was seen following Con A stimulation, a general T cell mitogenic response (Janossy and Greaves 1972), and so indicates a depression of T cell function. At low dietary levels of HCB (1 ppm), an enhancement of lymphocyte response was seen in the mink at all mitogen concentrations and at several mitogen concentrations in the ferret. This phenomenon has been described by Luster and Faith (1979) for several laboratory species and man following exposure to a variety of halogenated aromatic hydrocarbons.

A suppression of the lymphocyte blastogenic response at the highest Con A concentration (20 μg/ml) was observed in the adult mink and ferrets on all treatments. The sub-optimal degree of lymphocyte activation at high mitogen concentrations has been likened to immunologic tolerance seen in vivo following large doses of antigen (Möller 1970). The depression of T lymphocyte proliferation by high concentrations of Con A was reported to be a reversible occurrence (Andersson et al. 1972) when the excess mitogen was removed. The degree of suppression (stimulation index at 20 μg Con A/ stimulation index at 10 μg Con A)

was greatest in the mink and ferrets consuming the 25 ppm HCB diet, presumably due to a decrease in the number of cells proliferating and/or a greater degree of blastogenic inhibition than was present in the other treatment groups.

The in utero and early postnatal exposure of mink to HCB resulted in a significant depression of the lymphocyte blastogenic response. The lowest level of exposure (mothers consuming diets containing 1 ppm HCB) caused reduced T cell response in the offspring following Con A stimulation of their isolated lymphocytes, in contrast to the enhanced effects observed in their dams. The cell-mediated immune system has similarly been shown to be depressed in mice and rats prenatally and postnatally exposed to TCDD without significant toxic effects being produced in their dams (Vos and Moore 1974; Luster et al. 1979). The young rats affected by TCDD (Luster et al. 1979) had however, returned to a normal lymphocyte blastogenic response by seven months of age. The immunosuppression resulting from perinatal toxicant exposure need not necessarily permanently impair the immune system. Luster et al. (1979) found the degree of immunosuppression to be less severe in rats treated only postnatally when contrasted to rats exposed in utero and postnatally. The in utero exposure of rats to alcohol has also been reported to create a prolonged depression in T lymphocyte reactivity to Con A stimulation (Monjon and Mandell 1980).

HCB has been shown to cross the placenta in the rat (Villeneuve and Hierlihy 1975; Courtney 1979), rabbit (Villeneuve et al. 1974), mouse (Courtney et al. 1976), and the mink and ferret (Bleavins, unpublished data). It has also been detected in the milk produced by cows (Fries and Marrow 1971), mice (Courtney et al. 1979), ferrets (Bleavins,

unpublished data), and humans (Miller 1973; Siyali 1973). Indirect exposure of developing/nursing offspring to HCB is, therefore not only possible, but virtually unavoidable when their mother is consuming a diet contaminated with this persistent halogenated hydrocarbon. The risk to which progeny are exposed was dramatically brought to light by the HCB-induced outbreak of porphyria cutanea tarda (PCT) in Turkey from 1955-1959 (Courtney 1979). Nursing infants showed a variety of histological abnormalities and an exceptionally high mortality rate (Courtney 1979) while the only cause of death consistently reported was secondary pulmonary infection. This secondary infection may have been the result of cell-mediated and/or humoral immune system suppression.

As proposed by Vos (1977), the subtle effects of immune responsiveness can most readily be measured by treating the test species with the chemical of interest during the early developmental stages of the immune system. Prenatal and early postnatal treatment of an animal may well provide the most sensitive means by which to detect impairment of the immune response (Vos 1977) in neonates. Although the immune system is very sensitive to chemical insult, it also possesses considerable potential for recovery (Luster et al. 1979). If the immunosuppressive nature of HCB is temporary, it may not represent a permanent threat to the animal's ability to survive. If no serious challenge to the cell-mediated or humoral immune system occurs during this time, the lowered host defenses may be sufficient to protect the animal. However, in this study immune responsiveness was significantly depressed 10 weeks after the animal's exposure to HCB had ended. This suggests a reasonably long period of lowered immune defenses even if the compound should be found

not to permanently impair immune function in future studies.

The immune system provides a sensitive indicator of toxicity. Effects on the immunocompetence of an animal can preceed related histopathologic changes (Loose *et al*. 1979). While immunosuppression may not be immediately or directly lethal, it does result in an increased susceptibility to infection and cancer. It is, therefore, important that immune function be incorporated into the routine toxicity testing procedures. The initial assessment of a compound's immunosuppressive potential can be made in conjunction with the standard 90-day toxicity study, even though this subchronic trial may not be of sufficient length for long-lived memory lymphocytes to show an effect following exposure to some toxicants (Menson *et al*. 1982).

The complex interactions of immune system cells with each other, with the various other tissues of the body and their response to challenge demands more detailed study. The development of *in vitro* lymphocyte activation techniques has made it possible for the researcher and clinician to study immune function in the intact animal or human. Although there are limitations in metabolic activation and pharmacodynamic interactions, these procedures do not require termination of the test animal, use limited quantities of blood and can be made directly with human peripheral blood lymphocytes. The immune system cells, having been exposed to a chemical *in vivo*, can be readily removed from the body and their function examined *in vitro*.

Experiment VII. EFFECTS OF *IN UTERO* AND EARLY POSTNATAL EXPOSURE TO HEXACHLOROBENZENE ON HEPATIC MIXED FUNCTION OXIDASES, *IN VITRO* RENAL FUNCTION AND HISTOLOGY, AND TISSUE RESIDUES IN MINK.

Purpose

This study was conducted to determine whether or not exposure of mink kits to HCB during gestation and nursing resulted in persistent or permanent alterations in their kidney and renal morphology, and function. In addition, tissue residue concentrations were measured in the kits from each of the treatment groups.

Materials and Methods

Eighteen female mink kits, born to the females described in Experiment II, were used in this study. Six kits were selected from each of the three treatments having sufficient numbers of kits surviving to weaning (control, 1 ppm and 5 ppm of HCB) to allow reasonable replication. The mink kits are described in greater detail in Experiment IV, Study 2. The animals were killed by cervical dislocation at 16-17 weeks of age and blood, adipose tissue, muscle, kidney, and brain samples were taken for HCB analysis. Livers and kidneys were removed and immediately placed into ice-cold physiological saline.

Microsomes from mink livers were prepared according to Dent *et al.* (1980). In a typical reaction, microsomes (0.25-0.5 mg/ml) were suspended in 1 ml of 66 mM Tris-HCl buffer (pH 7.8) containing 4.5 μM glucose-6-phosphate, 0.3 μM NADH, 0.3 μM NADP, 0.1 μM NADPH, 163 μM $MgCl_2$ and 1 unit of glucose-6-phosphate dehydrogenase. After a three minute preincubation at 37°C, the reaction was initiated by the addition of substrate. Enzymatic activities measured were ethoxyresorufin-O-deethylase (Johnson *et al.*

1979), ethoxycoumarin-O-deethylase (Aitio 1978), and benzphetamine-N-demethylase (Prough and Ziegler 1977). Cytochrome P-450 and cytochrome b5 were measured according to the method of Omura and Sato (1964). NADPH cytochrome-c reductase was measured by the method of Pederson et al. (1973). Microsomal protein was determined by the method of Lowry et al. (1951).

Thin renal cortical slices were prepared free-hand. Organic ion accumulation was determined by incubating 50-150 mg of slices in 4 ml of medium composed of 96.7 mM NaCl, 7.4 mM sodium phosphate buffer (pH 7.4), 40 mM KCl and 0.74 mM $CaCl_2$ (Cross and Taggart 1950). The medium was supplemented with 10 mM lactate (Sigma Chemical Co., St. Louis, MO) for one-half of the slice incubations and not supplemented for the other half of the slices. It also contained 7.4×10^{-5} M p-aminohippurate (PAH) and 1×10^{-5}M (1-^{14}C)-tetraethylammonium (TEA), specific activity 2.00 mCi/mmol (New England Nuclear, Boston, MA). After incubation for 90 minutes at 25°C under 100% O_2 in a Dubnoff metabolic shaker, slices were removed from the medium, blotted and weighed. Slices were homogenized in 6 ml of 5% trichloroacetic acid and brought up to volume of 10 ml with distilled water. A 2 ml aliquot of the incubation medium was treated similarly. After centrifugation, the supernatant was assayed for PAH by the method of Smith et al. (1945) and for TEA by liquid scintillation spectrometry. PAH or TEA accumulation in the kidney slices was expressed as the slice-to-medium or S/M ratio.

Glucose synthesis by renal cortical slices was determined by a method similar to that of Roobol and Alleyne (1974). The incubation medium consisted of a Krebs-bicarbonate medium containing 10 mM pyruvate as the substrate for gluconeogenesis. After gassing the medium with O_2-CO_2,

50-100 mg of the slices were placed into 5 ml of the medium in a 50 ml Erlenmeyer flask, flushed with O_2-CO_2, stoppered tightly and incubated at 37°C for 1 hour in a Dubnoff metabolic shaker. After incubation, the slices were removed from the medium, blotted and weighed. The medium glucose content was determined using glucose oxidase and peroxidase as described in Sigma Technical Bulletin No. 510 (Sigma Chemical Co., St. Louis, MO).

Concentrations of HCB in liver, kidney, fat, muscle, brain, and blood were determined by the following technique. Samples were weighed and ground with sufficient anhydrous sodium sulfate to render them completely dry and pulverized. Powdered tissues were extracted three times with 40 ml portions of hexane. Pooled extracts were reduced in volume to 1-2 ml and placed on a Florisil column (60-100 mesh) of 500 x 200 mm. HCB was eluted with 200 ml of hexane which was evaporated to a 1 ml volume and then brought up to the desired volume. Quantitation was by gas-liquid chromatography on a Varian Model 2100 gas chromatograph (Verian, Palo Alto, CA) with a Sc^3H electron capture detector using a 1.7 mm x 2 mm i.d. column packed with 3% OV-225. Carrier gas (N_2) flow and column temperature were 30 ml/min and 165°C, respectively. Concentrations were expressed as nanograms of HCB per gram of tissue (wet weight) or ppb.

Several thin slices of liver and kidney were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and processed for routine light microscopic observation, including fat staining with Oil red O. For electron microscopy, tissue slices were diced into 1 mm blocks, dipped in 4% glutaraldehyde/0.1 M cacocodylate buffer fixative and placed into fresh fixative for 3 hours. Tissue blocks were then washed in Zetterquists' buffer twice, placed in 1% OsO_4 solution for 1½ hours and embedded into

Epon-Alaldite plastic after dehydration through graded solutions of alcohol and propylene oxide. Thin sections were then cut on an LKB-III ultratome, stained with uranyl acetate/lead citrate and examined on a Zeiss Electron Microscope (9S2) operated at 60Kv.

Data were analyzed statistically by an analysis of variance, completely random design. Treatment differences were examined by the least significance difference test (Steel and Torrie 1980). The 0.05 level of probability was used as the criterion of significance.

RESULTS

Offspring of HCB exposed dams had a high degree of mortality (77.4% in the 5 ppm group) as reported in Experiment II. The present data are on the survivors of that study. At 17 weeks of age, kits from adult female mink fed 0, 1 or 5ppm HCB had no alterations in body weight, or kidney or liver weights (Table 34). Microsomes from control livers contained 0.08 $\pm$ 0.010 nmoles of cytochrome P-450 (P-450) per mg protein while kits from the 5ppm treatment group had elevated hepatic P-450 concentrations of 0.16 $\pm$ 0.014 nmoles/mg protein (Figure 6). Cytochrome b5 was not altered in any treatment groups. Correlating with an increased hepatic P-450, hepatic ethoxyresorufin-O-deethylase in the 5ppm group increased 1.5 fold over controls, but not significantly. No changes were observed in ethoxycoumarin-O-deethylase, benzphetamine-N-deethylase and NADPH cytochrome-c reductase (Table 35). No alterations in the *in vitro* renal function were observed as determined by the accumulation of PAH or production of glucose by renal cortical slices in any treatment group. TEA accumulation was significantly decreased in kidneys from the 5ppm treatment group (Table 36). HCB was primarily distributed in the adipose tissue in all treatment groups with detectable concentrations in brain,

Table 34. Effect of perinatal HCB exposure on body and organ weights[†] of 16-17 week old mink kits.

Maternal treatment	n	Body wt. (kg)	Tissue[‡]	
			Liver	Kidney
0 ppm	6	0.73 ± 0.02	2.6 ± 0.12	0.5 ± 0.03
1 ppm	6	0.75 ± 0.09	2.8 ± 0.07	0.5 ± 0.01
5 ppm	6	0.67 ± 0.05	2.7 ± 0.08	0.5 ± 0.03

[†]Mean ± S.E.

[‡]Expressed as a percentage of body weight.

Figure 6. The effect of perinatal HCB exposure on hepatic cytochrome P-450 (open bars) and cytochrome b5 (hatched bars). Asterick indicates a value significantly different from control ($P<0.05$).

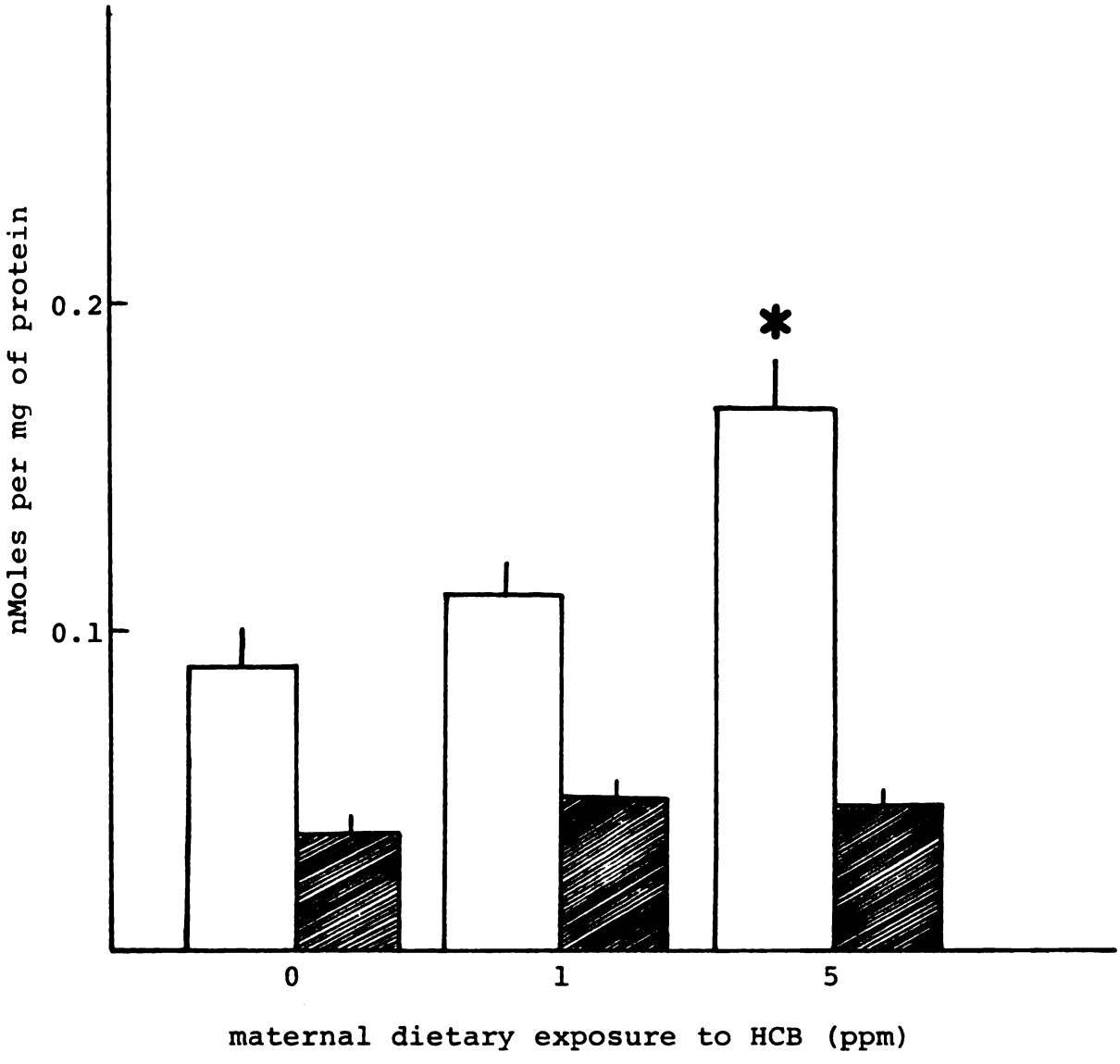

nMoles per mg of protein
0.2
0.1
*
0
1
5
maternal dietary exposure to HCB (ppm)

Table 35. Effect of perinatal HCB exposure on the hepatic mixed function oxidases[†] of 16-17 week old mink kits.

Maternal treatment	n	NADPH cytochrome-c reductase[‡]	Ethoxyresorufin O-deethylase[ψ]	Ethoxycoumarin O-deethylase[ψ]	Benzphetamine N-demethylase[ψ]
0 ppm	6	56.3 ± 12.00	1.6 ± 0.35	0.9 ± 0.11	4.2 ± 1.90
1 ppm	6	70.6 ± 5.41	2.4 ± 0.20	1.2 ± 0.14	6.1 ± 2.01
5 ppm	6	70.8 ± 4.20	2.5 ± 0.50	0.9 ± 0.09	6.2 ± 2.11

[†]Mean ± S.E.

[‡]nmoles of cytochrome-c reduced/ minute/ mg of protein.

[ψ]nmoles/ minute/ mg of protein.

Table 36. Effect of perinatal HCB exposure on renal gluconeogenesis and accumulation of *p*-amino-hippurate (PAH) and tetraethylammonium (TEA) by renal cortical slices† of 16-17 week old mink kits.

Maternal treatment	n	Gluconeogenesis‡	- Lactate		+ Lactate	
			PAH (S/M)	TEA (S/M)	PAH (S/M)	TEA (S/M)
0 ppm	6	4.3 ± 0.27	3 ± 0.1	13 ± 1.0	14 ± 2.1	20 ± 0.2
1 ppm	6	3.8 ± 0.18	3 ± 0.1	13 ± 1.0	17 ± 1.0	19 ± 1.0
5 ppm	6	4.0 ± 0.41	3 ± 0.2	13 ± 0.8	16 ± 1.0	17 ± 0.3*

†Mean ± S.E.

‡mg of glucose/ g of tissue/ hour.

*Significantly different from control ($P<0.05$).

liver, kidney, and muscle of kits from the 5ppm HCB group (Table 37). HCB could not be detected in blood from any treatment group.

Light microscopy of hepatic cells revealed numerous small cytoplasmic vacuoles which stained red with Oil red O, a differential stain for fat, however, no treatment differences were observed. Electron microscopy of liver showed almost the entire cell's cytoplasm was occupied by elipsoidal vesicular profiles of various sizes (250 mμm to 7 μm in diameter). These vesicles were largely empty except for occasional deposition of fine electron dense granules and were not membrane enclosed. There were no alterations in Golgi apparatus, mitochondria or other major cytoplasmic organelles including the endoplasmic reticulum due to HCB. No alterations were seen in either light or electron micrographs of kidney.

Discussion

Administration of the PCB mixture Aroclor® 1242 to mink at 5ppm of the diet has been shown to result in complete reproductive failure (Bleavins et al. 1980). The administration of equivalent doses of HCB did not interfere with reproductive success; however, 77.4% of the kits died prior to weaning (Experiment II). In the present study, surviving kits from dams fed 1ppm and 5ppm HCB displayed no overt indications of toxicosis. Ultrastructural changes have been observed in rat livers after the consumption of a 5ppm HCB-treated diet, including proliferation of the smooth endoplasmic reticulum (SER) and abnormal mitochondrial morphology (Mollenhaver et al. 1975). While proliferation of hepatic SER was not observed in any of the kit mink, hepatic P-450 and ethoxyresorufin-O-deethylase were induced in kits from dams fed 5ppm HCB. Phenobarbital and 3-methylcholanthrene induce mink hepatic cytochrome P-450, but there appears to be a very limited substrate specificity as few enzymatic

Table 37. Tissue residue concentrations (ppb) of HCB[†] following perinatal exposure of 16-17 week old mink kits.

Maternal treatment	n	Tissue					
		adipose	brain	liver	kidney	muscle	blood
0 ppm	6	35.7 ± 12.81	ND[‡]	ND	ND	ND	ND
1 ppm	6	94.6 ± 17.30	ND	ND	4.2 ± 0.98	1.4 ± 0.50	ND
5 ppm	6	626.1 ± 12.01	36.3 ± 3.52	36.7 ±13.48	14.6 ± 5.01	8.5 ± 0.75	ND

[†]Mean ± S.E.

[‡]ND = none detected.

activities increase (Shull et al. 1982). Ethoxyresorufin-O-deethylase, which was increased in this study, is a 3-methylcholanthrene inducible enzyme whereas benzphetamine-N-demethylase, which was not changed, is a phenobarbital inducible enzyme. In the rat, HCB increases enzymatic activity consistent with a phenobarbital type of induction, but results in a shift in spectral characteristics of P-450 to a lower wavelength similar to that seen after 3-methylcholanthrene induction (Stonard and Nenov 1974; Courtney 1979) and has been termed a "mixed" inducer. In the mink, HCB appears to behave predominantly as a 3-methylcholanthrene type mixed function oxidase inducer.

Pregnant mice administered pentachloronitrobenzene (PCNB) contaminated with 10% HCB demonstrated a high incidence of renal agenesis in offspring, whereas no alteration was observed after technical grade PCNB administration (Courtney et al. 1976). It was concluded that the renal teratogenic effects were due to HCB. In addition, female Rhesus monkeys exposed to HCB showed renal histological alterations (Iatropoulos et al. 1976). In contrast, perinatal HCB exposure had no effect on in vitro renal function or histology in the mink kits used in this experiment. Apparently no permanent compromise of renal morphology or physiology occurred, although temporary abberations cannot be ruled out. These kits were exposed to HCB throughout gestation and nursing, but were placed on clean feed for the ten weeks between weaning and the measurement of kidney function. Therefore, it is possible that renal effects, of a temporary nature, may have been present at birth or at some point during nursing, and had been compensated for prior to the end of the study.

The reason for the high mortality among nursing mink remains unclear. The apparent lack of toxicity among surviving mink may reflect their

relatively low body burden of HCB, as kits fed 5ppm had only 0.6 ppm in the body fat and very low levels in other tissues. It is likely that the kits received the majority of their body burden of HCB during nursing, since in the mouse toxic doses of HCB can readily be transmitted via the milk. Young, untreated mice cross-fostered onto lactating HCB-treated mothers showed a high rate of mortality (Courtney 1979). Similarly, mammary transfer of HCB has been shown to be 31 times greater than placental exposure to growing ferret kits (Bleavins et al. 1982). The 10 week period of time in which the mink kits were on diets not supplemented with HCB would allow the animals to actively metabolize and excrete this halogenated aromatic hydrocarbon. Their body burden would, thus, be less at the date on which the study was ended than was present at weaning. The rapid growth rate of mink kits from weaning to approximately 17 weeks of age would further act to dilute the concentration of HCB per gram of tissue, in addition to any changes in total body burden. Immunosuppression from HCB exposure may also have contributed to the high incidence of kit mortality seen prior to weaning (Experiment II) in the mink.

In conclusion, HCB, while causing a high degree of mortality in young mink prior to weaning, resulted in no significant hepatic or renal damage in the surviving mink ten weeks after exposure ended. The increased xenobiotic metabolism of these animals may alter their response to agents in the environment known to be metabolically activated. Alternately, induction of hepatic mixed function oxidases in these mink may also result in certain endocrine related disorders through altered metabolism of steroid hormones.

Experiment VIII. EFFECTS OF HEXACHLOROBENZENE ON REPRODUCTION, CELL-MEDIATED IMMUNITY, ORGAN WEIGHTS, AND MICROSOMAL ENZYMES OF MINK IN A CROSS-FOSTERING STUDY.

Purpose

This experiment was designed to attempt to differentiate the effects of *in utero* and mammary exposure to HCB from the effects seen following their combined insult. At birth, mink kits were transferred from HCB treated dams to control dams, and vice versa, to allow exposure during gestation only, and during nursing, only.

Materials and Methods

Forty standard dark female mink, proven second year breeders, were randomly assigned to two treatment groups of twenty animals each. The treatments consisted of an untreated basal diet and a diet that contained 2.5 ppm supplemental HCB. Standard ranch procedures were followed in the care, handling and feeding of all animals. Thirty-nine females were bred once and given an opporutnity to remate on the following day. All matings were made within a four day period and first matings were confirmed by a vaginal smear examined for the presence of motile spermatozoa. One female, later to be placed on HCB-treated feed, refused to breed and was thus excluded from the study. All animals were maintained on the basal diet until 17 March 1982. On this date, the 2.5 ppm HCB-treated group was placed on contaminated feed and remained on the HCB-treated diet throughout pregnancy and lactation.

Each female was checked twice daily for the birth of kits during the whelping period. On the day they whelped the female was weighed, the kits

counted and weighed, and the number of live and dead kits recorded. At this time, one kit was taken from each of five litters on the control and HCB diets for histologic examination of the thymus, liver, spleen, and kidney. On the days that female mink from both the control and HCB treatments whelped, their entire litter was transferred to a female of the opposite treatment (cross-fostered). Prior to the switching of litters, the kits, nest box and female's nasal region were dusted with talcum powder to reduce the risk of rejection or killing of the transferred kits. Fourteen litters were cross-fostered to yield the following treatments:

1) Control: Kits born to and nursed by females consuming the basal diet (n=11 litters)
2) Control → HCB: Kits born to females consuming the basal diet, but nursed by females consuming 2.5 ppm HCB (n=7 litters)
3) HCB → Control: Kits born to dams consuming 2.5 ppm HCB, but nursed by females consuming the basal diet (n=7 litters)
4) HCB: Kits born to and nursed by females consuming 2.5 ppm HCB (n=8 litters)

The kits were maintained on the maternal diet until weaning (seven weeks of age) and then fed the untreated basal diet until 13 July 1982.

On 13 July 1982 twenty-four male kits were selected, six from each treatment group, and the following procedures performed. A nine ml sample of blood was collected via cardiac puncture and lymphocyte blastogenesis performed as described in Experiment V. After the blood samples were collected, all animals were weighed and then killed by cervical dislocation. Brain, liver, spleen and thymus were weighed and sections taken for histological examination. Also taken for histology were kidney, ileum, and

adrenal gland. A sample of liver was collected for mixed function oxidase evaluation as described below.

After the fresh livers were weighed and rinsed in 150 mM KCl and blotted dry, 10 g portions were minced and then homogenized at 4°C in 20 ml of 20 mM Tris-HCl:1.15% KCl (pH 7.4) with a Polytron[1] tissue homogenizer. The homogenate was centrifuged at 12,000 x g for 20 minutes at 0-4°C, the supernatant filtered through four layers of cheesecloth and then ultracentrifuged at 105,000 x g for 75 minutes at 2°C. The microsomal pellet was suspended in 15 ml of 0.3 M sucrose:0.1 M sodium pyrophosphate (pH 7.5) with the tissue homogenizer, and recentrifuged as above. The resulting pellet was resuspended in 150 mM KCl to yield a concentration of approximately 30 mg of protein per ml. Protein was determined colorimetrically by the Biuret method using a bovine serum albumin standard (Gornall et al. 1949) on a Gilford Stasar II spectrophotometer[2] (model 1367X5).

The assay procedures for aminopyrine N-demethylase (Anders and Mannering, 1966) and benzo-a-pyrene (Van Canfort et al. 1977) were modified as follows. Each assay vessel contained a total volume of 0.5 ml which included 281 µl of NADPH regeneration system [250 µl of 200 mM HEPES[3] (pH 7.6) containing 0.5 mg NADP[4], 20 µl of 10% glucose-6-phosphate[4] solution (pH 7.0), 10 µl of 250 mM $MgCl_2.6H_2O$ solution, and 1 µl (0.5 units) of glucose-6-phosphate dehydrogenase[4]], 200 µl of microsomal suspension and 20 µl of substrate solution. The benzo-a-pyrene hydroxylase vessels contained 0.5 mg of NADH[4]. Substrate concentrations added to each vessel in a 20 µl volume were 20 µmoles of aminopyrine

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[1] Brinkman Instruments, Westbury, NY 11590.

[2] Gilford Instrument Laboratories, Inc., Oberlin, OH 44074.

[3] U.S. Biochemical Corp., Cleveland, OH 44122.

[4] Sigma Chemical Co., St. Louis, MO 63178.

in methanol or 0.88 μmoles of benzo-a-pyrene including 0.25 μCi of (G-^{3}H)-benzo-a-pyrene[5] in acetonitrile. The incubation phase for the aminopyrine N-demethylase was carried out in 16 x 125 mm disposable glass culture tubes and the benzo-a-pyrene hydroxylase assay in 8 ml glass mini-scintillation vials. Microsomes diluted to 20 mg protein per ml (aminopyrine) or 2 mg per ml (benzo-a-pyrene) with 150 mM KCl were added to vessels containing the NADPH regeneration system and preincubated for 3 minutes at 37°C in a Dubnoff metabolic shaker bath[6]. The reactions were initiated by the addition of 20 μl of substrate and allowed to incubate under air at 37°C for 20 minutes while oscillating. The aminopyrine N-demethylase assay included a microsomal blank (no added NADP or NADH) for every microsomal preparation. For the benzo-a-pyrene test, both NADP and NADH were included during the 20 minute incubation time and microsomes were withheld until after termination of the reaction. All assays, including blanks and standards, were conducted in duplicate. Both enzyme procedures were conducted under reduced light with the vessels placed in ice following termination of the reactions.

The aminopyrine N-demethylase assay was terminated by the addition of 1 ml of 20% $ZnSO_4$. After adding 1.0 ml of saturated barium hydroxide solution, vortexing, and centrifuging for 20 minutes, 1.0 ml of supernatant was added to 1.0 ml of Nash reagent[7]. The mixture was heated for 10 minutes at 60°C, cooled, and the optical density (OD_{412}) determined against a water blank and compared to a formaldehyde standard curve. Termination of the benzo-a-pyrene assay was accomplished by the addition of 1.0 ml

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[5] Amersham Corp., Arlington Heights, IL 60005.

[6] GCA Precision Scientific, Chicago, IL 60647.

[7] 15 g ammonium acetate, 400 μl acetylacetone, 300 μl glacial acetic acid brought up to a volume of 100 ml with distilled deionized water.

DMSO[8]-KOH solution (85% DMSO:15% 1.0 M KOH). The mixture was extracted twice with hexane, which was discarded. A 500 µl subsample of the aqueous phase was then added to 5.0 ml of counting cocktail (77.4% ACS[9], 15.5% absolute ethanol, 6.5% DMSO and 0.6% 1.0 M glacial acetic acid) and counted on an Isocap/300 liquid scintillation counter[10] for ten minutes.

Cytochrome P-450 (448) concentrations were measured with a Beckman Acta III dual-beam spectrophotometer[11] using the carbon monoxide difference spectral method of Omura and Sato (1964) in microsomes diluted with 100 mM Tris buffer (pH 7.4) to 1.0 mg/ml of protein.

Results

Both the control and 2.5 ppm HCB-treated females showed good whelping percentages, 90% and 79%, respectively (Table 38). The HCB-treated females did exhibit a significantly longer length of gestation than control females, however both groups fell within the normal term of pregnancy for mink of approximately 40-60 days (Travis and Schaible 1960). No difference was seen in total litter size or the average number of kits born alive vs. dead between the two groups. The control female mink showed a low level of kit mortality (5%), presumably due to their having raised offspring the previous year and having been selected on this criteria. Kits whelped by control females, but nursed by HCB treated dams (Control → HCB) showed an increased mortality rate of 13.6% while HCB → Control kits had a mortality rate of 22.5%. Kits whelped and nursed by HCB-treated females had a

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[8] Aldrich Chemical Co., Milwaukee, WI 53201.

[9] Aqueous counting scintillant, Amersham Corp., Arlington Heights, IL 60005.

[10] Searle Analytic, Inc., Des Plaines, IL 60018.

[11] Beckman Instruments, Inc., Fullerton, CA 92634.

Table 38. Gestation length, reproductive performance, kit mortality, and biomass[†] produced by female mink consuming control and HCB diets in a cross-fostering study.

Treatment	♀ whelped/ ♀ mated	Gestation (days)	Litter size at birth			Kit mortality %		litters	
			alive	dead	total	to 3 weeks	to 6 weeks	n	Biomass[‡]
Control	18/20	51 ± 0.8	6.1 ± 0.43	0.3 ± 0.14	6.4 ± 0.39	3.3	5.0	11	477 ± 57.8
Control→HCB	-	-	-	-	-	13.6	13.6	7	515 ± 72.4
HCB→Control	-	-	-	-	-	22.5	22.5	7	403 ± 72.4
HCB	15/19	54 ± 0.9*	5.8 ± 0.47	0.3 ± 0.16	6.1 ± 0.43	20.5	20.5	8	377 ± 67.7

[†]Mean ± S.E.

[‡]Biomass = average kit body weight gain between birth and 3 weeks of age x average number of kits per lactating female.

*Significantly different from control ($P<0.05$).

mortality comparable to the HCB → Control group (20.5 vs. 22.5%). No significant difference was seen in the biomass raised by females on any treatment.

No significant change was observed in birth weights between the two groups (Table 39). Female mink kits exposed to HCB in utero and via the milk weighed significantly more than control female kits at three weeks of age. At six weeks old, male kits on the Control → HCB treatment were significantly heavier than their control counterparts. The six week weights of female kits did not show any significant differences.

The lymphocyte blastogenic response was not significantly different from control for any of the three HCB-exposed groups, either on a disintegrations per minute or a stimulation index basis. There was also no change seen in body weight or brain weight of kits in any treatment group when contrasted to the control kits. Liver, spleen, and thymus (expressed as a percentage of brain weight) were likewise not significantly different from control values.

The in utero and/or mammary exposure of mink kits to HCB did not result in significant liver microsomal enzyme induction. No changes above control values were seen in aminopyrine N-demethylase, benzo-a-pyrene hydroxylase, cytochrome P-450 (P-448) or mg of protein per gram of liver.

Discussion

The lengthened gestational period seen in the dams consuming a HCB-treated diet during this study was not observed in Experiment II. However, in the work reported above, all females were mated within a four day period during the middle of the breeding season and only second year proven breeders were used. Experiment II utilized first year females bred throughout the approximately 21 day breeding period. Presumably, this may

Table 39. Birth, three week, and six week old body weights[†] of mink kits exposed to HCB in a cross-fostering study.

Treatment	Body weight (g)					
	n	Birth	n	3 weeks	n	6 weeks
Male						
Control	62	9.3 ± 0.21	31	104.4 ± 2.74	30	296.8 ± 11.41
Control→HCB	-	-	23	110.2 ± 3.18	23	340.2 ± 13.04*
HCB→Control	-	-	10	107.8 ± 4.82	10	308.6 ± 19.77
HCB	28	10.0 ± 0.31	11	111.5 ± 4.59	11	289.1 ± 18.85
Female						
Control	47	8.7 ± 0.23	27	94.0 ± 2.35	27	259.7 ± 8.33
Control→HCB	-	-	15	94.5 ± 3.15	15	274.3 ± 11.18
HCB→Control	-	-	21	97.0 ± 2.66	21	281.9 ± 9.45
HCB	59	9.1 ± 0.21	20	104.8 ± 2.73*	20	283.9 ± 9.68

[†]Mean ± S.E.

*Significantly different from control ($P<0.05$).

account for the greater clustering of whelping dates seen in this experiment (as evidenced by the smaller standard errors) than was found in the earlier study.

An elevated degree of kit mortality was seen in all treatments exposed to HCB. This is not unexpected since the maternal tissue stores act as a readily accessible source of HCB for the fetus (Courtney and Andrews 1979) and nursing offspring. Kit mortality of mink exposed to HCB via the milk (Control → HCB) was higher than for control mink, but lower than in the animals exposed only in utero (HCB → Control). These kits exposed to HCB in utero showed a mortality level comparable to the animals receiving HCB insult both during gestation and nursing (HCB). At the low dietary concentrations of HCB used in this study (2.5 ppm), in utero exposure appears to represent a more serious threat to offspring survival than exposure via the dam's milk. This phenomenon was seen even though the quantity of HCB received by a nursing animal via the dam's milk is many times the amount that passes across the placenta during fetal development (Grant et al. 1977; Bleavins et al. 1982). Hexachlorobenzene has also been reported to adversely affect reproduction in the rat (DeMatteis et al. 1961; Grant et al. 1974, 1977; Courtney and Andrews 1979; Kitchin et al. 1982). The effects seen included a decrease in the number of litters whelped, fewer pups surviving to weaning, increased incidence of stillbirths, reduced birth weight, and depressed rate of growth. The cross-fostering of rat pups born to untreated dams onto females consuming HCB in their diet, resulted in the death of all pups (DeMatteis et al. 1961). All of these effects were seen at exposure levels much higher than can be used in the mink and still have the females produce any

viable offspring. A percent mortality (to weaning) comparable to the level seen at 2.5 ppm HCB in the mink was not observed in the rat until maternal exposure was 60 ppm (Kitchin et al. 1982). Grant et al. (1977) have reported 20 ppm HCB in the maternal diet to be the no-effect level for reproduction in the rat, a dietary concentration comparable to the 25 ppm HCB treatment fed in Experiment II and which had a profound adverse effect on the reproductive performance of the mink. The improved survivability of mink kits seen during this study at 2.5 ppm of HCB than in Experiment II at 1 ppm (20.5% vs. 44.1% kit mortality) is probably the result of better maternal ability in the second year females and/or fluctuations in environmental factors from year to year.

The increased three week weights of the HCB-treated female kits over the control females is potentially due to a reduced number of kits per litter, since no change was found in the biomass raised per female. If a significant increase in body weight occurred without a reduction in the number of kits raised, it would be reflected by a concurrent elevation in the biomass value. The Control → HCB males showed a similar body weight increase at six weeks of age which may also be explained by the higher kit mortality seen in this group than in the control males, thereby reducing intralitter competition for the dam's milk and eventually the available solid food.

No significant effect was seen in the lymphocyte blastogenic response to Con-A in the young mink in this study, in contrast to the results observed during Experiment VI. The underlying cause for the different results is not known, but may involve the shorter period of maternal exposure to HCB. In Experiment VI, the females were placed on HCB-supplemented diets six weeks prior to mating. In the study reported above, HCB

treatment did not begin until immediately after the females had been bred, at the middle of the breeding season. These female mink, therefore, had a period of HCB exposure approximately eight weeks shorter than the animals in Experiment VI. This would result in lower tissue residue concentrations of HCB and so a lower level of this aromatic hydrocarbon in the systemic circulation and milk. This would in turn lead to fetal and neonatal exposures below those seen when blood or milk concentrations were higher.

Although an increase has been reported in the mixed function oxidases (MFO) of suckling rat pups raised by dams consuming HCB (Grant et al. 1977), no induction was seen in the mink kits cross-fostered in this experiment. Whether this is the result of the animals being maintained on non-HCB diets from weaning until the termination of the study (5 weeks) and, therefore, returning to a normal MFO profile or due to HCB not being a particularly potent inducer of microsomal enzymes (Carlson 1978), is not clear. Either or both of these possibilities may be involved, as may other factors, including the variability seen within different runs of the enzyme assays. The shorter duration and low level of maternal exposure may also have come into play in affecting the results of this study.

Kit mortality proved to be the most sensitive indicator of HCB toxicity in this cross-fostering experiment. The other physiological parameters monitored (lymphocyte blastogenic response, organ weights, and MFO induction) did not show significant changes, although such changes might have been detected if these tests had been run immediately after exposure to HCB ended, instead of allowing the animals to consume non-HCB diets for five weeks.

SUMMARY AND CONCLUSIONS

The results of these studies indicate that the mink and ferret are among the most sensitive mammalian species to the toxic effects of HCB. Anorexia, weight loss, gastro-intestinal bleeding, and bloody droppings were observed in these animals prior to death at dietary concentrations of 125 and 625 ppm HCB. Although these clinical signs are not specific for HCB toxicosis, they are consistent with halogenated aromatic hydrocarbon poisoning. In general, no change was found in the hematologic parameters measured (RBC, WBC, Hb, Hct, MCV, MCHC) until just before death. The changes in erythrocyte numbers and leukocyte proportions are probably secondary to gastro-intestinal bleeding and impaired immunocompetence, respectively, rather than a direct interference with cellular formation. As has been reported in other species, the acute toxicity of HCB was low (> 1000 mg/kg of body weight) in these two mustelids. The mink and ferret were, however, very susceptible to chronic HCB exposure.

No indications of abnormal porphyrin synthesis or metabolism (porphyria) were observed in either the mink or the ferret following the consumption of HCB-supplemented diets. Other carnivorous animals, dogs and some predatory birds, also do not develop prophyria as a toxic manifestation of exposure to HCB. These species are in contrast to the rat, mouse, monkey, human, chicken, and quail, each of which exhibits HCB-induced porphyria. The development of porphyria has been implicated as being involved in the underlying cause of HCB-related toxicity in the porphyrin sensitive species. Since the mink and ferret do not develop porphyria, it would appear that the toxic effects seen must be independent of the porphyrin cycle.

Female ferrets fed a 125 ppm HCB diet were hyperexcitable and displayed signs of neurological impairment. The analysis of biogenic amines in the brains of mink and ferrets on the control, 1, 5, and 25 ppm HCB diets revealed alterations in brain neurotransmitter concentrations. Changes were also found in neurotransmitter concentrations of the mink kits produced by females consuming 1 and 5 ppm HCB.

A single dietary dose of HCB was found to be readily absorbed (98.5% of the HCB given) by adult female ferrets. A total body HCB half-life of 32 days was calcualted for ferrets raising kits to 5 weeks of age and of 41 days for unbred female ferrets. Lactation was an important excretion route for HCB in the female. Greater than 20% of the HCB absorbed by the dam was detected in the kits she raised. The exposure to HCB via the milk for the growing ferret kit was determined to be 31 times greater than the exposure occurring in utero across the placenta. Although not studied directly, a similar scenario is presumably also true in the mink.

The reproductive performance of the mink and ferret was found to be a sensitive indicator of HCB toxicity. HCB caused an increase in the gestational length of HCB-exposed female mink and a lower whelping percentage than was seen in control females. A decrease in total litter size, without a change in the incidence of still births, was seen in the mink. No change in litter size was found in the ferrets, but an increased number of stillborn kits was observed. The mink and ferret kits born to dams consuming HCB diets exhibited depressed birth weights and poor survivability to weaning when compared to control animals. The HCB-treated mink kits also displayed a decreased growth rate. When the kits born to control and HCB-treated female mink were cross-fostered, HCB-exposed offspring had

a higher mortality rate than control offspring. In utero exposure alone to HCB resulted in the same level of kit mortality as in utero and lactational exposure combined. Mink kits receiving HCB only via their dam's milk showed a poorer survivability than control kits, but better than those kits exposed to HCB during gestation. The in utero stage of development is, therefore, more sensitive to the effects of HCB than the postnatal period. This phenomenon is seen in spite of the much lower quantity of the chemical crossing the placenta than is consumed by the growing offspring through the dam's milk.

Chronic exposure to HCB was determined to be immunosuppressive to adult mink and ferrets at 25 ppm in the diet. The lymphocyte blastogenic response of these animals to the mitogen Con-A was significantly impaired when compared to the response of control animals. The lymphocyte responsiveness of mink kits produced by HCB-treated dams was also subnormal. This impairment of immune function appears to be primarily regulatory in nature since Con-A selectively stimulated the T-helper and T-suppressor lymphocyte subpopulations.

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