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UPTAKE, EXCRETION, AND PHYSIOLOGICAL EFFECTS OF HEXACHLOROBENZENE IN GROWING LAMBS

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Summary and Conclusions

A 90 day feeding trial was performed in male crossbred (Targee/whiteface) lambs with hexachlorobenzene at 0.01, 0.1, and 1.0 ppm daily. The results showed:

1. There was no significant difference in the growth rates of any group of lambs compared with control over a 306 day observation period (HCB fed days 0-90);
2. The blood and blood forming tissues remained unaffected by the HCB at these dosages, as assessed by standard clinical blood parameters;
3. Plasma alkaline phosphatase, glutamic oxaloacetic transaminase, glucose-6-phosphate dehydrogenase, and succinic dehydrogenase activities were not elevated by the exposure regimen;
4. Antipyrine metabolims measured *in vivo* was not significantly increased from control after the 90 day trial;
5. *In vitro* N- and O-demethylase activities were significantly increased over control;
6. HCB accumulated in omental fat to a peak level approximately 10-12 times the intake level over 90 days feeding, and this fat concentration is 50-100 times the level seen in other tissues analyzed;
7. HCB decayed from fat after cessation of administration with a half-time of approximately 95 days;
8. No gross or microscopically visible lesions were caused by these levels of intake.

A parallel 19 day feeding trial at 100 ppm HCB for 19 days (daily) showed similar results, with the following exceptions:

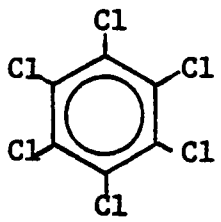
1. Growth rates were not measured;
2. *In vivo* antipyrine metabolism was significantly increased over control; and
3. *In vitro* O-demethylase activity was not significantly increased over control.

We concluded that HCB given daily at 0.01, 0.1, or 1.0 ppm for 90 days, or at 100 ppm for 19 days, causes no detectable harmful effects to growing male lambs.

I. Introduction

Hexachlorobenzene (HCB) has become of concern because of its widespread distribution as an environmental contaminant and a contaminant of food products used for human consumption (1). This compound was initially synthesized in France in 1946 (2). Since then, it has been used in many countries to control bunt fungi (Tilletia spp.) in cereal crops (3), seed-borne inoculum, soil-borne spores (4), and seed-borne flag smut (Urocystis agropyri) (5), perhaps through inhibition of spore germination (6).

HCB is an halogenated benzene. The chemical structure of this compound is shown below:



It is a white crystalline substance, water insoluble, easily sublimable and nearly odorless. Its melting point range is 226-230°C; synthesis is effected by direct catalytic halogenation of C_6H_6.

In order to investigate the metabolism, distribution and excretion of HCB, Mehendale and Matthews (7) administered ^{14}C-labeled HCB to male rats by the oral route. They found that less than 20% of the administered dose had been excreted by 7 days. Over 90% of the stored HCB was retained in fat, muscle, liver, and small intestine. These tissues each contained at least one dechlorinated metabolite. Urinary excretion accounted for less than 1% of the total dose administered, and the urine contained at least 7 metabolites, including pentachlorophenol, pentachlorobenzene and tetrachlorohydroquinone (7). It was also demonstrated that microsomal preparations of liver, lung, small intestine, and kidney metabolized HCB to dechlorinated products. These microsomal preparations produced penta-

chlorophenol in the presence of nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). In the presence of uridine diphosphoglyceric acid (UDPGA) and NADPH, the liver microsomal preparations resulted in disappearance of pentachlorophenol. It was also found that a slight amount of HCB was excreted in the feces (7). Parke and Williams (8) reported as early as 1922 that HCB does not conjugate to glucuronic acids, ethereal sulphates, or mercapturic acids.

Reports of HCB in Humans

There have been many reports of significant levels of HCB in the human body (fat, milk and blood), including serious cases of human poisoning. In the years 1955-1959, more than 3000 persons ate HCB treated wheat. These people developed a "Porphyria Cutanea Tarda Syndrome" with symptomatic photosensitization, porphyrinuria, hyperpigmentation and hypertrichosis, hepatomegaly, weight loss, osteoporosis and enlargement of the thyroid gland and lymph nodes (9,10,11 p. 463).

Significant levels of HCB have been found in human breast milk, as reported by investigators in Australia (12,13), the Netherlands (14), Germany (15), and Switzerland ((16). HCB has also been found in human blood (17), and in perirenal and other adipose tissue (15,18,19,20). In all of these studies HCB levels ranged from trace amounts to 8.2 ppm. The sources of HCB residues in the aforementioned cases were water, cereals (16), milk (21), milk products (22), eggs (18), wild birds (23), meat and poultry fat (24), and beans and potatoes (16).

Reports of HCB in Animals

The most serious cases of HCB residues in animal fat in the U.S. were reported in Central Louisiana (cattle) (25), and in Western Texas and

Eastern California, where the United States Department of Agriculture (USDA) detected the residue in slaughtered sheep (26). Appreciable residues of HCB have also been found in wild and domestic animals from all over North America (27). Because of these findings, the USDA asked the Environmental Protection Agency (EPA) to set an interim tolerance limit for HCB, which was set at 0.5 ppm for cattle, sheep, swine, goats and horses (26).

Experiments in Animals

Because of the importance of HCB, various experiments have been done to investigate its distribution, metabolism, excretion, toxicity, rate of accumulation, and placental transfer.

HCB has a relatively low toxicity to animals (3). In one study pigs fed HCB treated wheat for more than 12 weeks showed no apparent harmful effects (28). However, in these animals HCB was shown to accumulate with time (29).

Avrahami and Steele (30) carried out a study in sheep dosed orally for 18 weeks with 0.1, 1.0, 10, and 100 mg HCB per sheep per day. They found accumulation of the drug in fat to a maximum level of 0.9, 7.5, 75, and 650 ppm, respectively.

The same investigators reported that laying hens and growing chickens exposed to the same dietary levels of HCB (0.01, 1.0, 10, and 100 ppm) accumulated residues of HCB in the tissues according to the tissue fat content (body fat, egg yolk, liver, and muscle). However, feeding HCB up to 100 ppm did not effect the general health of the animals (31,32).

Studies have been carried out in the Netherlands involving broiler chickens and Japanese quail. The chickens were dosed with 0.05 to 0.3 ppm HCB for 7 weeks. This study demonstrated that the residue level in the

fat was directly proportional to the level of HCB fed in the ration, and that the concentration of HCB in fat leveled off by the end of the fourth week. The Japanese quail received dietary concentrations of HCB of 0, 1, 5, 20, and 80 ppm for 90 days. Results showed that 0 and 1 ppm HCB did not cause any effect, 5 ppm produced slight liver damage and caused excretion of porphyrins in feces, while 20 and 80 ppm caused extensive liver damage and death (33).

In feeding experiments with rats, death occurred when the HCB concentration in brain reached 300 ppm (34). Liver damage with intracytoplasmic inclusions was seen in rats fed a diet containing 0.2% HCB (35), and severe porphyria accompanied with liver damage was induced in rats fed (36) or injected (37) with HCB at 0.8 to 1.0 g/kg/day, and 20 mg/ml, respectively.

In 1973 placental transfer of HCB was demonstrated in pigs (38). It was shown that sows dosed with HCB transferred a considerable quantity of residue to their offspring before birth. In addition, sows secreted HCB in the milk in sufficient amount as to make the piglets accumulate significant HCB residue in their bodies (38). These findings were later confirmed by Villenueve et al. (39). These investigators dosed pregnant rabbits orally with subtoxic doses of HCB of 0, 0.1, 1.0, and 10 mg/kg over a period of 27 days. The fetuses showed HCB accumulation in fat, liver, heart, kidneys, brain, lung, spleen, and plasma. It was noted that no toxic effects were observed in these fetuses at any of these dose levels (39).

Since HCB is a commonly used fungicide, and has been demonstrated to localize and accumulate in mammals, it was felt advisable to conduct a controlled experiment to evaluate quantitatively the kinetics of HCB

uptake and excretion, and physiological effects. For this reason, we chose to study the effects of HCB on growing lambs. To accomplish this purpose, several characteristics were chosen for study. These included growth rate, plasma enzyme activities and associated blood parameters, liver drug metabolising enzyme activity, and HCB residue levels in animal fat. Associated with these determinations were gross and microscopic pathological examinations. It was felt that using this battery of analyses, we could best determine the effects of chronic HCB administration to growing lambs.

Some plasma enzymes have been used as indicators of organ integrity. A high level of activity of an enzyme in plasma not normally found in plasma is generally thought to be due to the release of intracellular enzyme from damaged tissue (40). Four plasma enzymes were chosen in this study as indicators in detecting tissue damage caused by HCB. These plasma enzymes were alkaline phosphatase (AP), glutamic oxalacetic transaminase (GOT), glucose-6-phosphate dehydrogenase (G6PDH), and succinic dehydrogenase (SD).

Phosphatases are enzymes which hydrolyse phosphoric esters, releasing inorganic phosphate. Two principal types of phosphatases are known in blood, alkaline phosphatase, which has a pH optimum range between 9 and 10, and acid phosphatase, with a pH optimum of approximately 5 (41). These are normally contained in the white blood cells. A second kind of alkaline phosphatase has been reported recently by Neuman et al. (42) in the serum of patients with lymphatic leukemia and infectious mononucleosis.

Plasma alkaline phosphatase is thought to be of liver origin, but is is widely distributed in high concentrations in bone, intestinal mucosa, and renal tubular cells as well. This enzyme is useful in the study of

hepatic diseases. An elevated AP activity has been associated with nucleic acid synthesis in reparative processes (43). AP also shows increasing levels of activity in the blood of apparently health persons as they age (44). This agrees with other studies carried out in different species (40).

GOT is found in high concentration in straited muscle. Therefore, elevated levels in plasma can be a valuable tool to confirm diagnosis of muscular degeneration (41,45). Additionally, serum GOT activity has been reported in sheep with liver flukes (46,47) and in some cases of copper poisoning (48). Thus, GOT activity can be used as a measure the level of liver damage if no evidence of other organ damage exists. Like AP, GOT also shows differences in activity associated with the age of sheep (49).

One characteristic of sheep is the absence of erythrocytic glucose-6-phosphate dehydrogenase. This enzyme, synthesized in the liver, can therefore also be used to assess liver integrity.

The last chosen plasma enzyme was succinic dehydrogenase, a mitochondrial enzyme found in the red or dark granular muscle fibers (50). It was felt that plasma succinic dehydrogenase could be used as an indicator of skeletal muscle degradation, since SDH would be released into the plasma with cellular lysis.

In addition to these plasma enzymes, the hematocrit and plasma proteins were periodically checked to assess any gross hematological effect of HCB.

The chronic administration of many drugs is characterized by gradual decline of the steady state level of the drug because of its ability to stimulate its own metabolism via enhanced enzyme activity (51). This can have special significance in chronic toxicity studies. Signs of toxicity present at the beginning of a drug administration regimen may disappear after repeated dosage because the drug might stimulate its own metabolism

and thus result in a decreased circulating level of the drug. This has been demonstrated in growing rats, by evaluation of microsomal cytochrome P-450 (52,53,54), and in dogs and monkeys by measuring antipyrine metabolism in vivo (55).

As this project was concerned with chronic effects of HCB administration, a concept we explored was whether HCB could act as an inducer of microsomal enzyme activity in growing lambs. To evaluate this, the activity of two microsomal enzymes, N- and O-demethylase, were measured in vitro. The plasma half-life of antipyrine, metabolised via N-demethylation, was also determined in these lambs in order to correlate in vitro findings with the in vivo ability of these animals to metabolize a drug.

In summary, the specific aims of the present investigation were as follows:

1. To evaluate the effect, if any, of HCB on the growth rate of lambs;
2. To observe the lambs closely for any signs of systemic toxicity from the drug;
3. To monitor standard clinical hematology parameters to determine any gross effects of HCB on the blood or hematopoietic systems;
4. To monitor the plasma activities of the enzymes alkaline phosphatase, glutamic oxaloacetic transaminase, succinic dehydrogenase, and glucose-6-phosphate dehydrogenase, and use these activities as indicators of any biochemical lesions that the drug might produce;
5. To measure the in vivo metabolism of antipyrine at various times;

6. To measure *in vitro* N- and O-demethylase activities, and to correlate any changes seen *in vitro* after chronic HCB administration to the *in vivo* metabolic studies:
7. To determine the rate of increase of HCB residue in omental fat in the growing lambs, and to also measure the maximal resulting concentration at each of several dosage levels;
8. To determine the decay characteristics of HCB residue levels after abrupt cessation of exposure; and
9. To examine for any gross or microscopic pathological changes in the lamb tissues by necropsy at various stages during and after the HCB administration.

Although we were interested in all the above aspects of HCB toxicity, our major emphasis was on the determination of uptake and elimination kinetics of HCB in the growing lambs. We felt that it was important to determine these kinetics at doses approximating what one might expect to find in an environmental contamination situation, as from pesticide misuse. It was also hoped that we could characterize any toxic effects of the HCB administration by careful biochemical and pathological examinations.

II. Methods and Materials - General

A. Source and care of the animals

50 neutered male cross-bred (Targee rams out of white-face ewes) weanling lambs (4-5-months old), representative of those grown in California's winter lamb industry, were purchased from the U.C. Hopland Field Station and housed during the initial 90 days of the project in runs which were divided between an indoor and an outdoor area. The indoor portion of the area was swept clean five times a week. During the final 210 days the sheep were housed in all indoor runs. These runs were divided in half. One part was bedded with sawdust, which was changed every other day; the unbedded portion was washed down everyday.

B. Preparation and administration of HCB

HCB technical grade (BDH Chemicals Ltd.) was recrystallized four times before dissolving it in Mazola corn oil previously analyzed to be free of HCB (detection limit 4 ppb), at three concentrations: 0.04, 0.4, and 4.0 mg/ml. The purity was calculated to be greater than 99.5 percent. The solution was placed in 000 size Lilly gelatin capsules.

All lambs were biopsed for HCB levels in omental fat (detection limit 4 ppb) prior to being included in the experiment. They were then randomly divided into 6 groups as follows:

1. Ten sheep in the control group, which was kept isolated from the others, and was given a daily dose of corn oil of equivalent volume to that received by the treated sheep;
2. Ten sheep which were given a calculated daily oral dose of HCB at 0.01 ppm of diet;

3. Ten sheep which were given a calculated daily oral dose of 0.1 ppm HCB;
4. An associate group of 5 sheep was placed with the 0.1 ppm group but did not receive any treatment except for a daily dose of corn oil of equivalent volume to that given the treated sheep. This was done because contamination of control animals from association with treated animals has been reported (30);
5. Ten sheep which were given a calculated daily oral dose of 1.0 ppm HCB; and
6. A second associate group of 5 sheep which was placed with the 1.0 ppm group. They were handled as was the first associate group described.

The groups were placed in four separate runs: 1) control group alone; 2) 0.01 ppm group alone; 3) 0.1 ppm group and associated 0.1 ppm group; 4) 1.0 ppm group and associated 1.0 ppm group.

All sheep were fed alfalfa pellets shown to be free of HCB (detection limit 4 ppb) by gas liquid chromatographic (GLC) analysis prior to use. The daily feed given to each group was calculated to increase the average weight 4.54 kg/lamb/month. The calculation was based on the following equation derived by Garret et al. (56):

$$TDN = 0.029\ w^{3/4}\ (1 + 5.072\ g)$$

TDN = Total Digestible Nutrients, kg/day

w = Average herd weight, kg

g = Desired weight gain, kg/day. In our experiment, g = 0.151 kg/day

TDN was then divided by 0.52, which was considered to be the average metabolisable energy content of the feed.

The amount of HCB administered was based on the calculated average weight of feed each sheep consumed per day. The HCB and/or the corn oil were given daily during the first 90 days of the experiment.

After 90 days, 20 sheep were slaughtered for necropsy and determination of HCB residues levels in various tissues. Liver samples were also obtained for N- and O-demethylation assays. Five sheep were sacrificed from the control group, 3 from the 0.01 ppm group (one lamb had died from infection in this group), 4 each from the 0.1 and 1.0 ppm groups, and 2 each from the control associates of the 0.1 and 1.0 ppm groups. HCB administeration on the remainder was stopped to determine the temporal characteristics of the disappearance of HCB.

In the final 19 days of the project the 6 remaining associate sheep (3 from each of the 1.0 and 0.1 ppm associate groups) were given HCB at 100 ppm. Due to the large volume needed (9-12 ml), it was necessary that the suspension of HCB be placed in the back of the mouth using a syringe. The 5 remaining control sheep were given 10.0 ml of corn oil in the same manner.

C. Blood parameters determination (57)

1. Hematocrit was determined by use of standard microhematocrit procedure on whole heparinized venous blood.
2. Plasma proteins were determined by measurement of total solids of plasma using a Goldberg refractometer (American Optical Company). This method has been reported to produce good agreement with the Biuret reaction for nitrogen (58).

3. The following additional blood parameters were determined according to standard procedures (57): total RBC, total WBC, differential WBC, total (Hb) and mean corpuscular (MCHC) hemoglobin, mean corpuscular volume (MCV), erythrocytic sedimentation rate (ESR), and clotting time.

D. Plasma Enzyme Analysis

Following venipuncture and collection of blood samples from the jugular vein in a heparinized vacutainer, the following assays were performed on plasma:

1. Alkaline phosphatase was determined using the method of Bessey, et al. (59).
2. Glutamic oxalocetic transaminase was determined spectrophotometrically using the method of Freedland, et al. (60).
3. Glucose-6-phosphate dehydrogenase was determined using the method of Löhr and Waller (61).
4. Succinic dehydrogenase was assayed using the method of Freedland (62).

E. In vivo metabolism of antipyrine

Following i.v. injection of 100 mg/kg antipyrine, blood samples were obtained by venipuncture at 15, 30, 60, 120 and 180 minutes. Plasma concentration was determined by the methods of Brodie, et al. (63,64) as modified by Welch, et al. (55).

F. In vitro liver metabolism studies

N- and O-demethylase activities were determined on the 10,000 xg supernatant of 12.5% whole liver homogenate. Assays were by the methods of Mazel (65, ch. 27). Microsomes were isolated according to the scheme of Mazel (65, ch. 27).

G. Determination of HCB residues in various tissues

1. Biopsy procedure

The group of sheep to be biopsed, fasted overnight, was placed in a holding pen and given 1 ml promazine HCl (i.m.) each as a tranquilizer. The sheep were then secured on their backs to a surgery board by tying their legs.

The abdominal area was shaved and washed with Septisol® and Betadine® scrubs. An area near the midline was chosen for the incision and 3.5 ml of lidocaine HCl was injected subcutaneously. An oblique incision 4-5 cm long was made through the skin and muscle layers. An incision 3 cm long was made through the peritoneum, and using a spay hook, the omental fat was pulled out through the incision and 10-20 grams excised. The peritoneum and muscle were then sutured, and the skin sutured separately. The area was sprayed with buffered iodine and the sheep were then returned to their runs.

For 3 days following surgery the temperature of each sheep was monitored. If temperature rose above 40°C, 4 ml of procaine penicillin G was administered IM. Six to eight days after surgery the skin sutures were removed and operated area cleaned and inspected.

Blood samples were taken every time fat samples were collected. In addition, at the termination of the chronic feeding, samples of brain, liver, and kidney were taken for HCB residue analysis.

2. Extraction procedure

HCB was extracted from fat according to the procedure of Collet and Harrison (66). Extraction from plasma was according to the method of Siyali (17).

Extraction from tissue was performed using the following method:

10 g of tissue was homogenized with 50 g of anhydrous sodium sulfate in 100 ml of hexane in a Polytron homogenizer. This homogenate was filtered (liver and kidney) or centrifuged (brain) and the filtrate/supernatant was hydrolyzed with 15-20 ml of concentrated sulfuric acid. Brain samples required a second hydrolysis due to the formation of emulsions. The mixture was allowed to sit overnight to permit separation of layers. A 50 ml (5 g) aliquot of the hexane phase was concentrated to approximately 5 ml and transferred to a 1.5 cm x 10 cm PR grade Florisil column prewashed with hexane. The column containing the sample was eluted with 30 ml of hexane into a round bottom flask. The sample was then diluted or concentrated depending on the amount of residue in the sample in preparation for GLC-EC analysis.

3. GLC Analysis

The samples were chromatographed on a Aerograph 204 gas chromatograph equipped with an electron capture (tritium foil) detector and a Honeywell strip chart recorder. The fat and tissue

samples were chromatographed on a 1.8 m glass column, 3.2 mm i.d., packed with 10% QF-1 on 80-100 mesh Gas Chrom Q. The injector and detector temperatures were 210°C and the column temperature was 175°C. The plasma samples were chromatographed on a 1.8 m glass column, 3.2 mm i.d., packed with 5% OV-210 on 80-100 mesh Gas Chrom Q. The injector, detector, and column temperatures were 200°C, 207°C, and 180°C, respectively. The reason for this change in columns was due to interferences associated with the plasma samples. The chosen plasma column was adequate for separating these extranous peaks from the desired peaks. The flow rate for the nitrogen carrier gas for both columns was 16 cm^3/min. HCB was quantitated using peak height calibrated from a standard curve. Control and fortified control samples were analyzed daily with the treated samples.

H. Histopathological procedures

Sheep were killed for necropsy by captive bolt or electrocution. Samples of the following tissues were taken for histopathological examination: brain, lung, myocardium, small and large intestine, liver, kidney, adrenal, and mesenteric lymph node.

Tissues were fixed by immersion in 10% formalin. After mounting in paraffin, sections 8-10 μ were taken. Stain was hematoxylin and eosin. The slides were examined by a veterinary pathologist.

III. Results and Discussion

Included in each section is a brief statement of specific procedures employed.

A. Effect of Chronic HCB Feeding on Body Growth Rate of Lambs.

1. Specific Materials and Methods

The lambs were fed HCB as described earlier.

During the initial 90 days the sheep were weighed on a commercial scale every week. Following this period the sheep were weighed every other week. The average weight of each group was then calculated.

2. Results

The group means $\pm$ standard deviation are presented tabularly in Table 1, and graphically in Figure 1. No significant difference ($p>0.05$) was found between growth rates of the experimental groups as compared to control.

3. Discussion

In the present study doses of HCB had no significant effect on the lambs' growth rate. Other investigators (31) have reported that the growth rate in sheep was affected by as much as one-third at a dose of 100 mg HCB per day for 18 weeks. This difference was reported as significant ($p<0.05$). However, when weight differences between their sheep and ours are taken into account, this dose of 100 mg HCB per day works out to approximately 110 ppm HCB per day for 18 weeks - a dose about 100 times as concentrated, and administered 150% as long, as our highest dosage level. In our experiment the lamb growth rate was not affected by dietary levels of HCB up to 1.0 ppm during a 90 day (13 week) feeding trial.

TABLE 1

Effects of HCB feeding at different dose levels on lamb weight

Body weight for each group[a] of animals expressed in kilograms as x ± s.d.

Time in days after beginning HCB feeding[b]	HCB fed (ppm) Control	0.01	0.1	0.1 Assoc.	1.0	1.0 Assoc.
0	41.7 ± 5.1	40.6 ± 1.9	43.7 ± 0.6	44.7 ± 2.0	39.6 ± 1.2	40.6 ± 1.5
17	44.3 ± 5.1	42.9 ± 1.6	45.2 ± 1.4	45.1 ± 1.5	41.6 ± 1.7	42.5 ± 1.6
24	45.7 ± 5.1	44.8 ± 1.4	46.0 ± 1.6	47.5 ± 2.8	42.9 ± 1.4	44.7 ± 1.3
31	47.1 ± 4.9	44.4 ± 1.8	46.5 ± 1.9	48.4 ± 2.4	42.9 ± 1.7	43.6 ± 0.8
38	49.0 ± 5.1	46.0 ± 2.0	48.0 ± 2.3	51.1 ± 4.0	43.9 ± 2.0	44.8 ± 0.9
45	46.0 ± 5.0	45.9 ± 1.9	47.7 ± 2.0	50.1 ± 3.1	45.0 ± 1.8	45.8 ± 0.7
52	46.9 ± 4.9	45.8 ± 1.9	49.3 ± 2.9	51.7 ± 5.2	45.1 ± 1.7	44.5 ± 0.6
58	47.7 ± 5.1	47.1 ± 2.8	49.9 ± 2.9	51.9 ± 4.9	46.0 ± 2.0	47.7 ± 0.8
67	48.4 ± 4.6	50.8 ± 2.0	50.2 ± 2.8	53.5 ± 4.5	46.5 ± 1.5	48.0 ± 2.3
80	50.3 ± 5.4	50.4 ± 2.1	53.2 ± 3.5	54.7 ± 5.5	48.5 ± 2.3	48.8 ± 1.6
87	51.0 ± 5.5	51.3 ± 1.7	54.1 ± 4.0	56.6 ± 5.2	50.3 ± 3.2	50.4 ± 2.6
94	52.9 ± 6.1	53.7 ± 2.0	57.2 ± 5.0	59.2 ± 4.9	52.0 ± 3.4	51.6 ± 3.1
109	54.7 ± 6.1	55.2 ± 3.2	58.7 ± 3.3	60.7 ± 5.6	52.3 ± 3.6	52.2 ± 2.3
122	60.4 ± 7.0	57.4 ± 2.7	61.3 ± 4.0	62.6 ± 6.6	55.5 ± 4.8	55.1 ± 3.0
136	61.8 ± 6.4	58.2 ± 3.6	63.5 ± 3.6	64.6 ± 7.1	56.6 ± 4.6	56.8 ± 4.7
151	62.5 ± 6.2	59.0 ± 3.6	63.5 ± 3.9	65.9 ± 7.0	58.2 ± 4.6	58.8 ± 4.6
165	64.0 ± 7.1	59.5 ± 4.1	63.7 ± 3.8	65.8 ± 6.6	59.6 ± 5.1	60.0 ± 5.6
175	66.5 ± 7.0	62.3 ± 4.0	66.4 ± 3.9	67.4 ± 7.4	60.2 ± 5.3	60.9 ± 5.7
193	69.3 ± 7.5	64.0 ± 3.8	67.5 ± 3.0	70.2 ± 6.9	61.8 ± 5.9	62.2 ± 6.0
208	71.5 ± 7.7	68.0 ± 3.5	72.8 ± 4.8	73.6 ± 6.6	65.4 ± 6.0	66.4 ± 6.0

(continued)

TABLE 1 (continued)

Effects of HCB feeding at different dose levels on lamb weight

Body weight for each group[a] of animals expressed in kilograms as $\bar{x} \pm$ s.d.

Time in days after beginning HCB feeding[b]	HCB fed (ppm)					
	Control	0.01	0.1	0.1 Assoc.	1.0	1.0 Assoc.
222	73.3 ± 8.4	70.0 ± 3.6	73.0 ± 5.0	74.5 ± 6.8	66.4 ± 5.9	66.3 ± 7.9
237	74.8 ± 9.0	71.1 ± 4.4	74.8 ± 5.5	75.2 ± 7.8	66.0 ± 5.4	66.0 ± 7.2
251	77.3 ± 9.4	73.4 ± 4.6	79.2 ± 6.0	79.2 ± 7.6	69.1 ± 5.4	67.9 ± 7.9
264	78.6 ± 10.1	75.6 ± 4.9	80.1 ± 6.9	80.3 ± 8.1	70.4 ± 5.9	69.2 ± 6.0
280	76.3 ± 9.2	73.8 ± 4.6	81.8 ± 6.7	83.0 ± 8.4	72.0 ± 5.3	71.2 ± 8.4
292	82.4 ± 10.2	77.9 ± 4.5	83.0 ± 7.5	84.6 ± 8.7	73.1 ± 6.2	73.6 ± 8.7
306	86.5 ± 10.4	81.9 ± 4.7	85.8 ± 6.5	85.4 ± 8.8	76.8 ± 6.8	74.6 ± 6.8

a. Before day 90: 10 animals in control and each exposed group, 5 in each associate group.
After day 90: 6 animals in control and each exposed group, 3 in each associate group.

b. HCB was fed for 90 days.

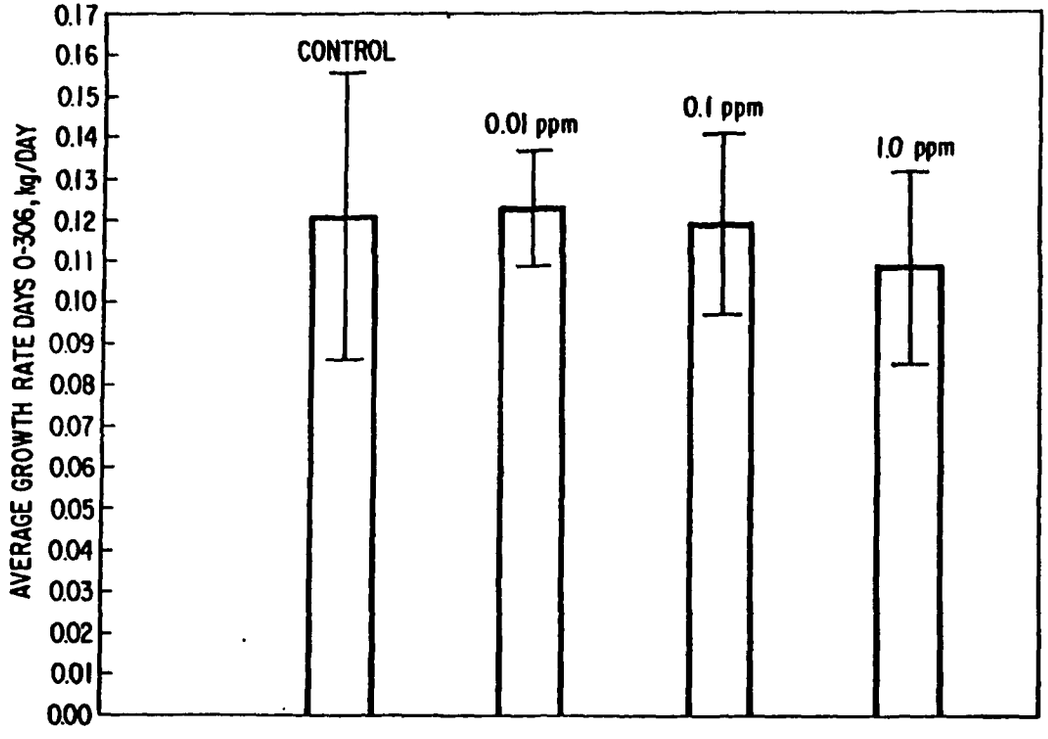


FIGURE 1. Effect of chronic HCB feeding on lamb body weight gain. HCB was fed days 0-90

B. Toxic Effects of HCB Feeding.

1. Specific Materials and Methods

During the course of this experiment the lambs were observed closely for any gross signs of toxicity i.e., listlessness, vomiting, anorexia, etc.

2. Results

No signs of systemic toxicity were observed in lambs fed 0, 0.01, 0.1, and 1.0 ppm HCB for 90 days, and observed for 210 days after cessation of exposure. In the acute feeding of 100 ppm for 19 days there were no apparent signs of toxicity like those described by Sweeny (37) and Nigogosyam (9) and San Martin de Viale, et al. (36).

3. Discussion

No signs of systemic toxicity were noted at any dose level we administered. These observations, coupled with the lack of histopathological abnormalities (see below), led us to conclude the HCB in these doses was not appreciably toxic to these sheep.

C. Determination of Clinical Blood Parameters.

1. Hematocrit

a. Results

The results of the chronic feeding study, expressed as percent packed-cell volume, are shown in Table 2.

There was no significant difference in hematocrit between control sheep and sheep fed HCB at different levels (0.01, 0.1, 1.0 ppm) for 90 days.

Results of a similar nature were obtained after feeding 100 ppm HCB to a group of sheep for 19 days (Table 3).

TABLE 2

Effect of HCB feeding for 90 days
at different dosages on Hematocrit.

Days	Control	0.01 ppm	0.1 ppm	1.0 ppm
0	37.7 ± 0.5	36.8 ± 2.5	37.0 ± 2.7	38.8 ± 2.0
6	36.0 ± 1.3	37.2 ± 1.6	36.0 ± 1.4	36.7 ± 1.2
13	35.2 ± 1.0	35.2 ± 1.8	34.0 ± 2.4	36.2 ± 4.0
20	35.8 ± 2.5	35.5 ± 2.3	34.8 ± 3.6	36.7 ± 1.0
27	36.0 ± 1.7	35.5 ± 1.5	35.5 ± 3.0	36.8 ± 3.2
34	38.0 ± 1.4	37.2 ± 2.4	37.2 ± 2.8	36.5 ± 3.9
41	38.2 ± 2.0	38.0 ± 1.3	36.0 ± 2.0	37.0 ± 5.8
48	38.0 ± 1.4	35.8 ± 3.1	37.0 ± 2.4	36.8 ± 2.6
56	38.0 ± 0.9	35.5 ± 2.2	37.7 ± 1.2	39.5 ± 4.1
64	38.7 ± 1.2	38.2 ± 1.8	34.5 ± 4.9	36.3 ± 3.9
69	38.2 ± 1.8	38.2 ± 1.2	36.2 ± 3.1	37.8 ± 3.8
75	37.5 ± 2.3	38.3 ± 1.4	36.2 ± 3.3	39.0 ± 2.8
82	37.8 ± 1.6	38.2 ± 1.6	36.7 ± 2.6	38.8 ± 3.1

Hematocrit expressed as % red blood cells. Each value represents the mean ± s.d. of 6 sheep.

TABLE 3

Effect of HCB Feeding for 19 days at 100 ppm on Hematocrit.

Days	Control	100 ppm
0	35.5 ± 3.7	36.8 ± 3.0
5	35.6 ± 3.3	35.3 ± 2.5
13	34.2 ± 2.3	35.9 ± 2.0
19	36.5 ± 3.3	36.6 ± 4.3

Hematocrit expressed as % red blood cells. Each value represents the mean ± s.d. of 6 sheep.

b. Discussion

HCB did not influence hematocrit values in any of the dosed groups. A decrease in hematocrit value has been reported in Japanese quail fed HCB (33). In view of this, it is notable that we failed to observe any detrimental effect of HCB on the hematocrit even after 100 ppm. This discrepancy might be explained by species difference.

2. Plasma Protein

a. Results

The effects of HCB feeding on plasma protein are summarized in Table 4. Plasma proteins are expressed as grams per 100 ml plasma.

During the 90 day period of HCB feeding at different dose levels no significant change in the level of plasma protein between control and HCB fed groups was found. A similar result was also obtained when HCB was fed 100 ppm to a group of sheep for 19 days (Table 5).

b. Discussion

The total plasma protein levels are normally maintained at a relatively constant level but may be deranged in some cases due to excessive loss of protein, as in liver disfunction due to the liver's inability to synthesize protein. The protein concentration in plasma also rises when water is lost and is decreased when water is returned to the vascular compartment.

In our study the plasma protein was determined by use of a refractometer. By this means, slight changes in hydration can be detected. During the course of the

TABLE 4

Effect of HCB feeding for 90 days at different dosages on Plasma Protein

Days	Control	0.01 ppm	0.1 ppm	1.0 ppm
0	6.55 ± 0.19	6.88 ± 0.32	6.79 ± 0.22	6.70 ± 0.48
6	6.88 ± 0.48	6.97 ± 0.29	6.48 ± 0.41	6.67 ± 0.43
13	6.82 ± 0.90	6.78 ± 0.23	6.80 ± 0.27	6.45 ± 0.47
20	7.10 ± 1.09	6.98 ± 0.38	6.85 ± 0.48	6.75 ± 0.27
27	6.98 ± 0.69	6.77 ± 0.16	6.90 ± 0.45	6.73 ± 0.20
34	7.15 ± 0.89	6.83 ± 0.22	7.02 ± 0.37	6.88 ± 0.31
41	7.32 ± 0.64	7.02 ± 0.20	7.20 ± 0.43	6.95 ± 0.38
48	7.12 ± 0.63	6.98 ± 0.22	7.05 ± 0.35	6.88 ± 0.45
56	7.17 ± 0.52	6.85 ± 0.35	7.00 ± 0.28	6.88 ± 0.40
69	7.30 ± 0.28	6.95 ± 0.05	7.07 ± 0.58	6.80 ± 0.23
75	7.53 ± 0.31	6.98 ± 0.12	6.97 ± 0.54	6.78 ± 0.27
82	7.40 ± 0.54	6.82 ± 0.04	7.03 ± 0.31	6.95 ± 0.27

Plasma protein expressed as gm per 100 ml. Each value represents the mean ± s.d. of 6 sheep.

TABLE 5

Effect of HCB feeding for 19 days at 100 ppm on Plasma Protein.

Days	Control	100 ppm
0	6.82 ± 0.19	6.65 ± 0.18
5	6.56 ± 0.20	6.48 ± 0.20
13	6.45 ± 0.21	6.41 ± 0.50
19	6.62 ± 0.32	6.52 ± 0.38

Plasma protein expressed as gm per 100 ml. Each value represents the mean ± s.d. of 6 sheep.

experiment we did not find great changes in hydration levels or note clinical signs to indicate alteration in these levels. Our results for plasma protein were consistantly within the normal range of 5.5 to 7.5 grams/ 100 ml (57). Therefore, we can say that HCB does not alter the normal plasma protein level in growing lambs after 90 days of treatment at 1.0 ppm, or after 19 days at 100 ppm.

If Table 4 is examined closely, the sheep seem to increase plasma protein with age. This trend is not felt to be important here; perhaps this reflects a natural maturation process. When again subjected to daily analyses during the last 3 weeks of the study, the percent protein was somewhat less than at 90 days (Table 5).

3. Other Clinical Blood Parameters.

a. Specific Methods

After 90 days of HCB administration blood samples were taken for complete analysis by the Clinical Pathology Laboratory. This was repeated at 300 days. The following parameters were analyzed: total RBC, total WBC, differential WBC, total (Hb) and mean copuscular volume (MCV), erythrocyte sedimentation rate (ESR), and clotting time (CT).

b. Results

No difference were observed between control and experimental groups with regards to any of the measured parameters at either 90 or 300 days.

c. Discussion

The lack of any observable difference between groups would imply that HCB in doses up to 1.0 ppm for

90 days, and 100 ppm for 19 days, has little or no effect on the blood forming tissues.

D. Plasma enzyme analysis after chronic and acute HCB administration.

Examination of the data in tables 6-13 will reveal an apparent fluctuation in the activities of the serum enzymes in the control group from one sampling time to another. These same patterns of fluctuation may be seen in the treated groups as well. These changes were no doubt largely or entirely laboratory induced by unrecognized differences in reaction conditions and by different technicians doing the assays. The reader should therefore compare only the data shown for any particular day of sampling with that day's control values.

1. Alkaline Phosphatase

a. Results

The effects of chronic HCB feeding on the activity of plasma alkaline phosphatase are summarized in Table 6.

No significant difference in alkaline phosphatase activity were found between the control and HCB treated groups. This was true at all levels of exposure for the entire period of the 90 day treatment.

A similar result following 100 ppm HCB feeding for 3 weeks was obtained. The results of this study are summarized in Table 7.

2. Glutamic Oxaloacetic Transaminase

a. Results

The effects of chronic HCB feeding on the plasma GOT activity are summarised in Table 8. No significant difference in plasma GOT activity was found between control sheep and the sheep fed HCB (0.01, 0.1, 1 ppm) for

TABLE 6

Effect of HCB at different dose levels of feeding for 90 days on Plasma Alkaline Phosphatase Activity.*

Days	Control	0.01 ppm	0.1 ppm	1.0 ppm
0	24.03 ± 9.34	28.50 ± 10.83	27.58 ± 11.01	16.23 ± 3.66
6	17.51 ± 4.94	20.79 ± 8.51	25.59 ± 11.68	21.66 ± 2.77
13	21.08 ± 10.21	22.02 ± 9.01	19.45 ± 12.85	15.54 ± 4.81
20	16.87 ± 9.26	14.58 ± 6.77	15.67 ± 7.79	14.77 ± 4.03
27	21.78 ± 11.54	23.05 ± 7.82	20.15 ± 13.45	18.10 ± 4.38
34	14.53 ± 6.46	18.76 ± 9.16	16.08 ± 5.90	14.09 ± 6.58
41	18.10 ± 5.08	25.45 ± 10.63	19.69 ± 13.31	17.24 ± 6.59
48	16.47 ± 6.31	18.47 ± 8.61	15.27 ± 4.25	13.40 ± 4.06
56	24.46 ± 11.25	24.78 ± 7.28	24.03 ± 6.92	20.03 ± 6.22
64	27.99 ± 14.51	34.82 ± 4.98	21.80 ± 5.13	21.81 ± 5.21
69	17.12 ± 5.34	32.16 ± 7.01	23.26 ± 6.14	22.16 ± 4.55
75	5.00 ± 1.87	9.57 ± 2.59	5.98 ± 3.18	5.33 ± 1.54
82	5.66 ± 2.24	10.49 ± 2.39	6.71 ± 4.11	6.34 ± 2.37

*Alkaline phosphatase activity expressed in nmoles of product formed/min/ml plasma. Each value represents the mean ± s.d. of 6 animals.

TABLE 7

Effect on HCB feeding at 100 ppm for 19 days on Plasma Alkaline Phosphatase Activity.

Days	Control	100 ppm
0	28.43 ± 10.98	31.62 ± 5.02
5	31.60 ± 12.04	28.76 ± 4.06
12	29.88 ± 11.38	28.46 ± 6.59
19	24.98 ± 6.42	27.48 ± 8.33

Alkaline phosphatase activity expressed in nmoles of product formed/min/ml plasma. Each value represents the mean ± s.d. of 6 animals.

TABLE 8

Effect of HCB at different dose levels of feeding for 90 days on Plasma GOT Activity.

Days	Control	0.01 ppm	0.1 ppm	1.0 ppm
0	27.01 ± 16.98	31.54 ± 5.45	30.87 ± 7.77	34.15 ± 15.96
6	39.74 ± 18.09	62.07 ± 18.04	43.36 ± 28.41	23.49 ± 8.01
13	72.35 ± 15.48	80.88 ± 14.85	58.02 ± 12.06	85.90 ± 31.64
20	35.59 ± 13.17	46.30 ± 21.32	48.57 ± 8.73	29.08 ± 20.74
27	77.41 ± 17.07	75.38 ± 11.96	70.90 ± 11.86	76.30 ± 13.65
34	58.60 ± 8.20	72.01 ± 16.59	66.56 ± 19.34	60.77 ± 16.16
41	34.77 ± 14.76	57.49 ± 12.68	39.84 ± 10.03	40.66 ± 18.09
48	67.86 ± 55.90	62.84 ± 11.67	44.71 ± 6.90	49.58 ± 21.22
56	56.53 ± 17.41	73.41 ± 39.84	35.11 ± 15.58	42.10 ± 10.95
64	90.19 ± 28.84	123.81 ± 70.27	70.03 ± 5.93	71.24 ± 15.00
69	59.08 ± 55.56	41.38 ± 4.63	57.54 ± 52.81	41.57 ± 15.34
75	46.06 ± 6.95	52.23 ± 7.52	39.93 ± 7.86	48.23 ± 9.79
82	45.72 ± 7.81	55.85 ± 13.84	50.98 ± 16.30	56.91 ± 26.96

GOT activity expressed in nmoles of product formed/min/ml plasma. Each value represents the mean ± s.d. of 6 animals.

TABLE 9

Effect of HCB feeding at 100 ppm
for 19 days on Plasma GOT Activity.

Days	Control	100 ppm
0	67.52 ± 18.81	58.84 ± 7.23
5	41.53 ± 11.24	50.93 ± 11.33
12	104.27 ± 81.75	63.90 ± 14.76
19	90.67 ± 38.01	153.37 ± 77.75

GOT activity expressed in nmoles of product formed/min/ml plasma. Each value represents the mean ± s.d. of 6 animals.

TABLE 10

Effect of HCB at different dose levels of feeding for 90 days on Plasma Glucose-6-Phosphate Dehydrogenase Activity.

Days	Control	0.01 ppm	0.1 ppm	1.0 ppm
0	71.10 ± 28.80	54.90 ± 12.80	50.00 ± 22.90	55.90 ± 29.70
6	57.70 ± 23.50	67.90 ± 15.90	62.10 ± 23.20	43.80 ± 14.00
13	69.70 ± 19.00	54.50 ± 17.90	71.80 ± 24.40	51.70 ± 8.90
20	57.70 ± 19.10	48.00 ± 17.50	74.50 ± 12.70	55.10 ± 11.70
27	69.70 ± 21.50	60.50 ± 10.10	58.50 ± 10.60	64.70 ± 18.70
34	23.30 ± 6.80	18.70 ± 3.70	21.90 ± 8.30	18.67 ± 8.30
41	20.10 ± 9.50	21.10 ± 5.50	23.30 ± 5.30	23.90 ± 11.80
48	28.10 ± 2.70	22.70 ± 2.70	33.20 ± 10.40	19.50 ± 6.10
56	27.70 ± 8.80	32.40 ± 15.70	21.10 ± 4.10	26.00 ± 11.90
64	39.80 ± 11.50	28.40 ± 4.60	40.80 ± 12.30	32.30 ± 7.60
69	43.40 ± 10.10	37.60 ± 11.60	41.80 ± 8.50	33.40 ± 16.20
75	30.00 ± 7.10	22.10 ± 4.30	35.20 ± 5.40	29.60 ± 12.10
82	43.40 ± 11.50	29.10 ± 5.40	34.80 ± 17.20	31.70 ± 8.90

G-6-P.D. activity expressed in nomoles of NADPH formed/min/ml plasma each value represents the mean ± S.D. of 6 animals.

90 days or 100 ppm for 19 days (Table 9).

3. Glucose-6-Phosphate Dehydrogsenase

a. Results

The effects of chronic HCB feeding at 0, 0.01, 0.1, and 1.0 ppm on the plasma glucose-6-phosphate dehydrogenase activity are summarized in Table 10. No significant difference in plasma G-6-PDH activity was found between control sheep and the sheep fed HCB (0.01, 0.1, 1 ppm) for 90 days or 100 ppm for 19 days (Table 11).

4. Succinic Dehydrogenase

a. Results

The effects of chronic HCB feeding on the plasma succinic dehydrogenase activity are summarized in Table 12. No significant difference in plasma succinic dehydrogenase activity was found between control sheep and the sheep fed HCB (0.01, 0.1, 1 ppm) for 90 days or 100 ppm for 19 days (Table 13).

5. General Discussion

Changes in the activities of a number of enzymes in the plasma have been studied in man and animals suffering from various diseases. An increase in the activity of an enzyme in plasma not normally found in high levels is generally thought to be due to the release of intracellular enzymes from damaged tissue (40). Changes in plasma enzyme activity due to liver malfunction can occur in 3 ways:

a. Disruption of hepatic cells resulting from necrosis or altered membrane permeability can cause an elevation of enzyme levels. GOT is a marker enzyme for this type of liver disfunction.

TABLE 11

Effect of HCB feeding at 100 ppm
for 19 days on Plasma G-6-PDH Activity

Days	Control	100 ppm
0	36.58 ± 6.93	23.71 ± 7.88
5	26.13 ± 4.42	25.92 ± 12.48
12	32.56 ± 8.83	34.77 ± 12.19
19	41.40 ± 9.42	34.57 ± 5.04

G-6-PDH activity expressed in nmoles of NADPH formed/min/ml plasma. Each value represents the mean ± s.d. of 6 animals.

TABLE 12

Effect of HCB at different dose levels of feeding for 90 days on Plasma Succinic Dehydrogenase Activity

Days	Control	0.01 ppm	0.1 ppm	1.0 ppm
0	3.45 ± 1.13	2.71 ± 0.62	3.24 ± 1.44	2.83 ± 1.30
6	3.39 ± 0.70	3.15 ± 0.55	4.08 ± 0.89	3.57 ± 0.76
13	4.64 ± 1.60	4.35 ± 1.01	4.05 ± 1.08	4.11 ± 1.18
20	3.10 ± 1.12	3.10 ± 0.40	3.04 ± 0.69	4.05 ± 0.52
27	5.93 ± 1.27	6.49 ± 1.97	5.51 ± 0.51	4.85 ± 0.62
34	8.51 ± 0.68	8.96 ± 1.55	7.32 ± 1.99	6.99 ± 2.07
41	5.48 ± 1.23	6.19 ± 1.27	5.12 ± 0.62	4.91 ± 1.19
48	6.40 ± 1.16	5.42 ± 1.75	5.48 ± 0.65	5.95 ± 1.38
56	7.56 ± 1.28	5.51 ± 1.03	8.42 ± 1.84	9.29 ± 2.99
64	4.67 ± 1.32	5.51 ± 0.83	4.52 ± 0.66	5.80 ± 0.62
69	3.93 ± 0.81	4.85 ± 0.69	3.69 ± 0.57	5.12 ± 1.57
75	6.13 ± 1.29	7.17 ± 1.09	5.86 ± 0.80	6.46 ± 2.14
82	6.01 ± 0.92	5.24 ± 0.66	5.03 ± 0.97	5.92 ± 1.03

Enzyme activity expressed in nmoles of 2,6 dichlorophenol indolphenol reduced/ min/ml of plasma. Each value represents the mean ± s.d. of 6 animals.

TABLE 13

Effect of HCB feeding at 100 ppm for 19 days on Plasma SDH Activity

Days	Control	100 ppm
0	12.95 ± 1.12	13.39 ± 0.67
5	10.71 ± 1.60	10.42 ± 1.08
12	9.97 ± 1.31	8.33 ± 0.87
19	11.01 ± 3.51	11.16 ± 2.09

Enzyme activity expressed in mmoles of 2,6 dichlorophenol indolphenol reduced/min/ml plasma. Each value represents the mean ± s.d. of 6 animals.

b. Elevated enzyme levels due to the lack of biliary excretion as seen in obstructive icterus, exemplified by changes in alkaline phosphatase activity.

c. Lowered plasma enzyme activity can be a result of imparied synthesis by the liver.

As mentioned above, alkaline phosphatase levels in the blood are elevated when tissues rich in this enzyme are damaged. Our findings of no significant changes in the plasma enzyme activity of HCB fed sheep are consistent with no appreciable degree of liver damage as found in pathological studies (see below).

GOT is not a liver specific enzyme, but it can be used diagnostically to measure the level of liver necrosis if no disease exists in other tissues in which this enzyme is found in high concentration. Therefore, as the enzyme appears in extremely high concentration in muscle, both skeletal and cardiac, it is of value in confirming a diagnosis of muscular degeneration was well (41).

No significant difference between GOT activity in the plasma of control and experimental sheep was found, which agrees with pathological studies where no evidence of muscular degeneration was found. This enzyme activity data is also consistant with the absence of hepatic necrosis.

Mountain (67) has mentioned that an elevated glucose-6-phosphate dehydrogenase activity is found in erythrocytes of young infants and also in some adults where abnormal hemoglobins are present in the cells. It is also thought that G-6-PDH could be depressed by lead poisoning. It is theorized that G-6-PDH, as well as other enzymes and cofactors of the

pentose pathway, are probably involved in stabilizing reduced glutathione (GSH) and membrane sulfhydryl in the lung (67). Here again our findings with regard to G-6-PDH did not demonstrate any significant alteration in activity during the experiment at any of the dosage levels of HCB administered. Based on the aforementioned lack of enzyme changes, we can conclude that HCB did not lead to any detectable lung, blood, or liver damage at chronic dosage levels up to 1.0 ppm, and acute (19 days) dosage levels of 100 ppm.

With succinic dehydrogenase, a mitochondrial sulfhydryl enzyme, we did not observe any significant change in activity with respect to the controls. This again is in agreement with no significant histopathological changes; i.e., no observable tissue lesion or damage is produced by HCB at the dose levels administered.

E. In vivo antipyrine metabolism

1. Specific Materials and Methods.

At 90 days, six lambs (3 control, 3 from 1.0 ppm group) were assessed for in vivo antipyrine metabolism. The same experiment was performed after feeding of HCB at 100 ppm for 19 days.

2. Results

The effects of chronic and acute feeding of HCB to lambs at 1.0 and 100 ppm on the plasma half life of antipyrine are summarized in Table 14 and Figure 2. The half life of antipyrine in both cases was shorter than the control group; however, statistical significance ($p<0.01$) was obtained only between control and sheep fed HCB at 100 ppm for 19 days. There was no statistically significant difference ($p>0.05$)

in antipyrine half life between controls and lambs fed HCB 1 ppm for 90 days.

3. Discussion

Soberman et al. (68) have reported that antipyrine is distributed evenly in body water. Antipyrine is completely metabolized via microsomal N-demethylase. This suggests that by estimating changes in plasma half-life of this compound before and after chronic exposure to drugs, information may be obtained regarding the effects of the drug metabolizing enzymes.

Many halogenated compounds are metabolized by the mixed function oxidase system of liver (69, 70). The administration of some drugs that are metabolised by the mixed function oxidase system of liver is known to induce the membrane components of the endoplasmic reticulum involved in the drug metabolism (71, 72). Proliferation of the endoplasmic reticulum can result in the increased metabolism of the drug. During the chronic administration of many drugs this reticular proliferation results in a gradual decline in the plasma concentration of the drug due to the ability of the compound to stimulate its own metabolism by liver microsomes (53). This did not occur in our study (Table 15). The plasma levels of HCB showed a gradual increase up to a few days after the day of cessation of drug administration, then declined (see Table 15). This is undoubtably because of the sequestration of HCB in the fat. Sequestration of a drug in fat has the effect of "smoothing" the fluctuations of plasma level. If one examines the data in Table 15, it can be seen that as time increases towards day 120, the relative (to fat)

TABLE 14

Effect of HCB feeding on
Antipyrine Half-life

Treatment	Minutes	Range
Control	66.00 ± 23.60 (3)	43 - 90
HCB 1.0 ppm[a]	38.60 ± 6.30 (3)[c]	35 - 45
Control	88.50 ± 33.90 (6)	-----
HCB 100 ppm[b]	34.70 ± 7.60 (6)[d]	-----

Antipyrine half-life expressed in minutes.
Each value represents the mean ± s.d. (N).

a. HCB treatment for 90 days.

b. HCB treatment for 19 days.

c. Statistically not significant as compared to its own control ($p>0.05$).

d. Statistically significant at $p<0.01$ level.

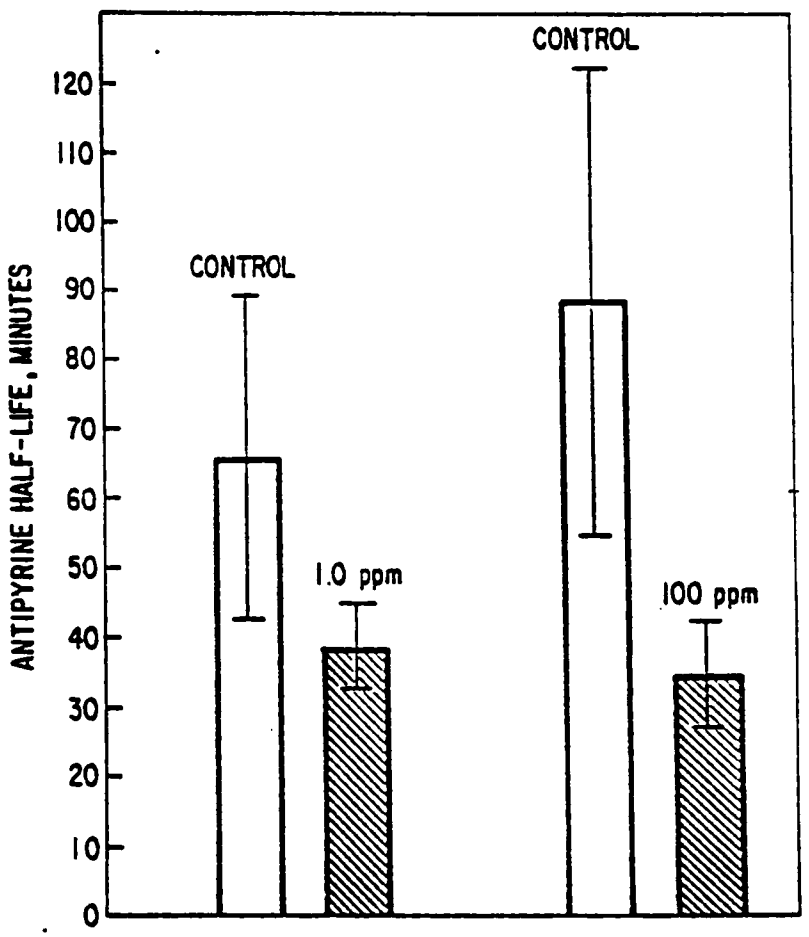


FIGURE 2. Effect of 90 days feeding of HCB at 1.0 ppm, and 19 days feeding of HCB at 100 ppm, on in vivo antipyrine metabolism

TABLE 15

Average plasma levels of HCB in lambs fed HCB at 1.0 ppm during days 0-90

Day	plasma HCB (ppb)	% of fat HCB
0	<4[a]	-
7	<4	-
15	6.7 ± 1.8 (10)[b]	0.3
30	15.9 ± 2.3 (10)	0.4
45	12.2 ± 3.3 (10)	0.2
60	15.7 ± 4.4 (9)[c]	0.2
90	20.8 ± 4.8 (9)[c]	0.2
120	35.8 ± 14.2 (6)	0.4
150	24.3 ± 5.9 (5)[c]	0.3
180	16.9 ± 5.2 (6)	0.3
210	15.3 ± 4.6 (6)	0.4
240	17.0 ± 7.2 (6)	0.5
270	11.3 ± 3.8 (6)	0.4
300	6.6 ± 2.1 (6)	0.3

a. Detection limit 4 ppb

b. $\bar{x} \pm$ s.d. (N)

c. Values below the detection limit were not included in this statistical determination.

level of HCB fluctuates somewhat, but is relatively constant. The continued high blood levels after exposure was stopped no doubt reflects movement of HCB between lipid compartments. This indicates that the blood concentration is a reliable indicator of recent chronic exposure (i.e., greater than 30 days) at the relatively low-level 1.0 ppm exposure.

Our findings in lambs are in agreement with the findings of other investigators that HCB stimulates the activity of the microsomal enzyme system of the rat and the pig (54).

F. In vitro liver enzyme studies

1. Specific materials and methods

Lambs from control and 1.0 ppm groups were sacrificed at day 90, and from control and 100 ppm groups at day 300 after the acute feeding (100 ppm for 19 days) experiment. N- and O-demethylase activities were determined as described. Microsomal protein was determined after the method of Lowry, as modified by Miller (73).

2. Results

The effect of 1.0 ppm and 100 ppm HCB feeding to lambs for 90 and 19 days respectively on the N- and O-demethylase activity and microsomal protein are shown in Table 16 and Figures 3, 4, and 5, respectively. A statistically significant increase in the activity of both enzymes occurred in sheep fed HCB at 1.0 ppm for 90 days as compared to control sheep. At 100 ppm the significant increase was found only in activity of N-demethylase. At this dose, the activity of O-demethylase remained unaffected. A marked increase in the hepatic microsomal protein occurred following 100 ppm HCB feeding; microsomal protein was not measured following the 1.0 ppm regimen.

TABLE 16

Effect of HCB feeding on N- and O-demethylase activity and microsomal protein

------	N-demethylase[a]	O-demethylase[b]	Microsomal Protein[c]
Control group	0.47 ± 0.03 (4)[d]	0.37 ± 0.03 (4)	------
1.0 ppm group[e]	3.34 ± 0.07 (4)	1.17 ± 0.19 (4)	------
p<[f]	0.001	0.002	
Control group	0.71 ± 0.06 (6)	0.29 ± 0.02 (3)	37.60 ± 0.84 (6)
100 ppm group[g]	1.98 ± 0.15 (6)	0.18 ± 0.07 (4)	52.08 ± 1.73 (6)
p<[f]	0.001	N.S.	0.001

a. N-demethylase activity expressed as µm of 4-aminoantipyrine released/hr/gram tissue.

b. O-demethylase activity expressed as µm of HCHO released/hr/gram tissue.

c. Microsomal protein expressed as µg of microsomal protein/mg tissue.

d. $\bar{x} \pm$ s.d. (N).

e. Exposed to HCB for 90 days.

f. Student's t-test.

g. Exposed to HCB for 19 days.

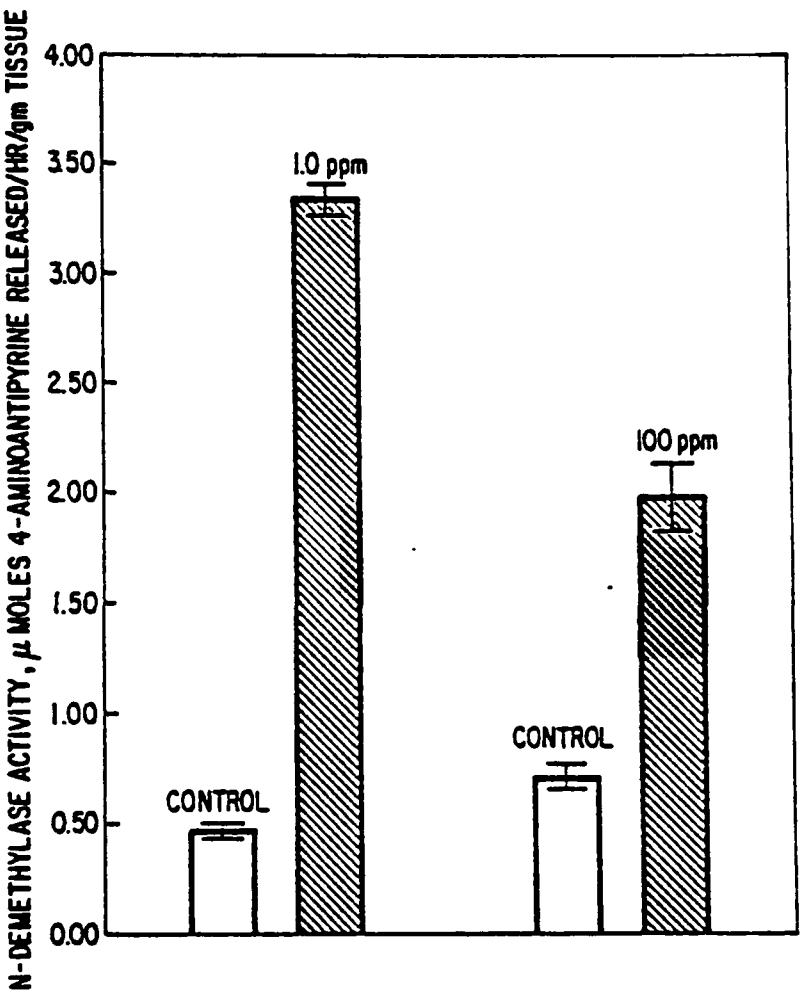


FIGURE 3. Effect of 90 days feeding of HCB at 1.0 ppm, and 19 days feeding of HCB at 100 ppm, on hepatic N-demethylase activity

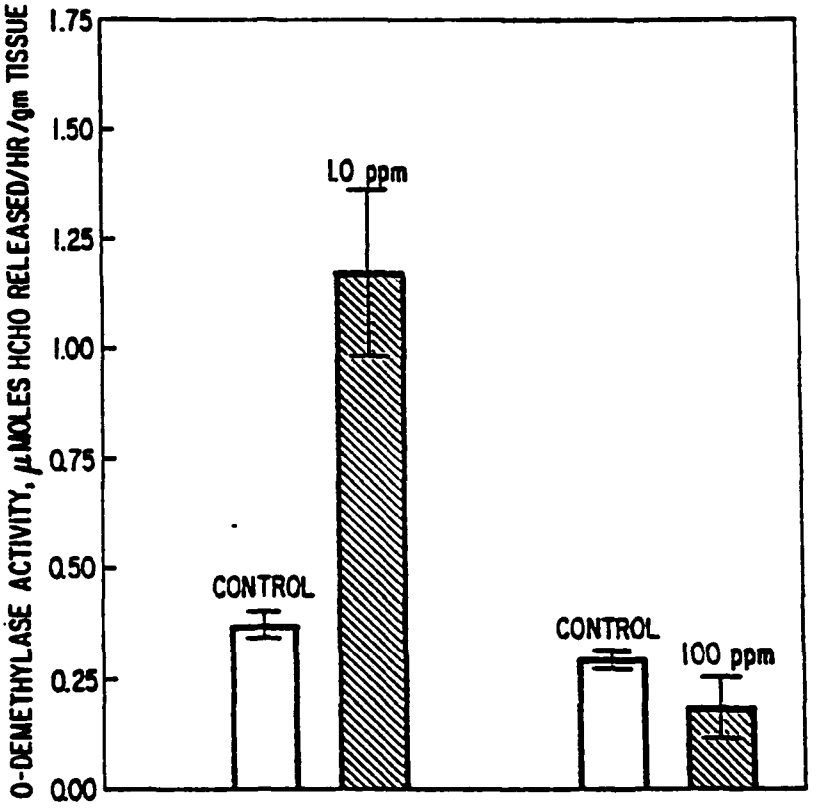


FIGURE 4. Effect of 90 days feeding of HCB at 1.0 ppm, and 19 days feed of HCB at 100 ppm, on hepatic O-demethylase activity

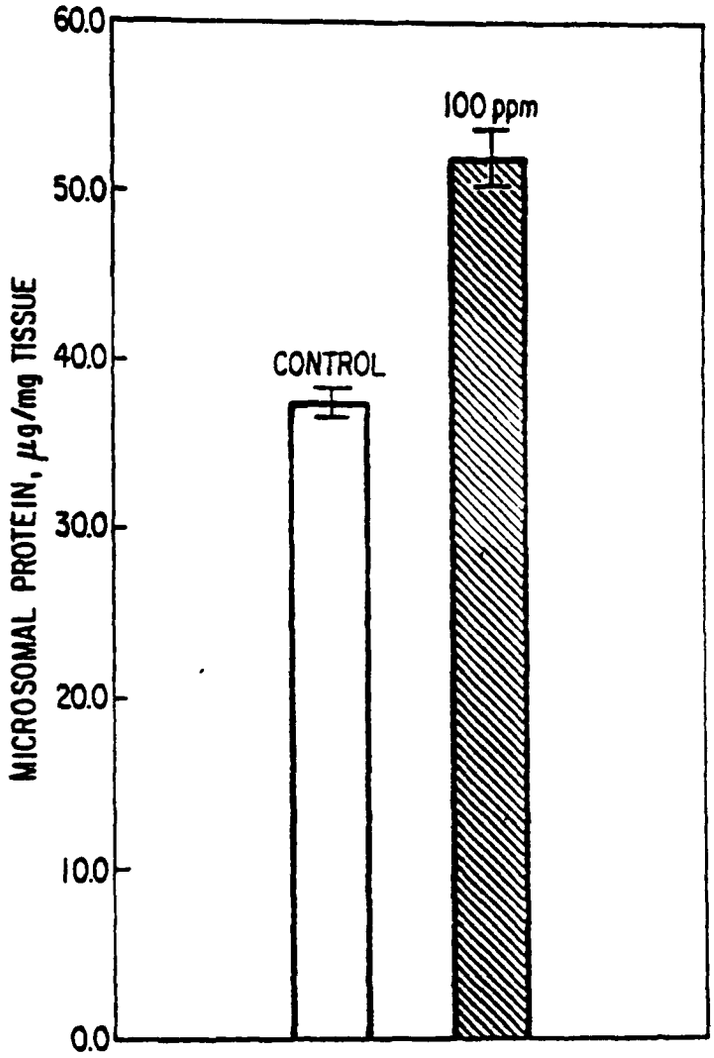


FIGURE 5. Effect of 19 days feeding of HCB at 100 ppm on hepatic microsomal protein concentration

3. Discussion

It has been demonstrated that chronic feeding of HCB to rats causes an increase in microsomal protein (54). An abundance of evidence indicates that the stimulation of microsomal enzyme activity involves new protein synthesis which can be measured *in vitro* using the whole microsomal fraction from liver (65, chapter 14). Many investigators feel that the level of microsomal enzyme activity is a steady-state which is determined by the rate of synthesis and degradation. The increase in microsomal protein found in the present study after 19 days of HCB feeding at 100 ppm could represent a change in this steady-state which led to an increase in the rate of new microsomal protein synthesis and/or a decrease in the rate of degradation. Stodnard and Nenow (54) feel that HCB microsomal induction can be best thought of as qualitatively similar to the induction caused by chronic phenobarbital administration. This, then, would indicate that the induction seen is primarily the result of an increased synthesis of new microsomal protein, as opposed to a decrease in degradation (65, chapter 14).

The chronic feeding of HCB also led to an increase in the hepatic N- and O-demethylase activity. This is consistent with an increased amount of microsomal protein in lambs chronically treated with HCB. It is also consistent with the decreased half-life of antipyrine, metabolised via hepatic N-demethylase, noted *in vivo*.

The effects of HCB at the two dose schedules was not consistent with respect to O-demethylase activity. The enzyme activity was greatly increased following treatment

with 1 ppm for 90 days but no significant increase in the activity of this enzyme was observed following treatment with 100 ppm of HCB for 19 days. This inconsistency is difficult to explain but might be attributed to a basal level of O-demethylase activity lower than that of N-demethylase (74).

G. Uptake and Decay Characteristics of HCB in Omental Fat and Other Tissues

1. Specific Materials and Methods

Biopsies were performed at day 0 and initial HCB fat concentration (detection limit 4 ppb) determined. Feeding was started in the chronic studies at day 0, and continued through day 90 as per the schedule given previously (see section II B, this report). Omental fat samples were taken at days 0, 7, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, and 300. Blood samples were taken at the same times.

a. Samples of brain, liver, kidney, and perirenal fat were taken at day 90, in addition to the omental fat, for HCB residue analysis. Samples of the same five tissues were taken at day 300, 210 days after cessation of HCB feeding.

In the acute feeding experiment, six former associate control sheep (three 0.1 ppm associate control, three 1.0 ppm associate control) were fed 100 ppm HCB for 19 days. Their tissues were analyzed for HCB residues immediately after cessation of feeding; thus, only peak concentration was determined in this group. All determinations of plasma, fat, and tissue HCB residue levels were determined as per the methods of Section II G, this report.

2. Results

The uptake of HCB in omental fat was directly proportional to the dose given. The peak group mean concentration was reached after 90 days of exposure (Table 17). In the 0.01 ppm group the peak concentration was 115.8 ppb. In the 0.1 ppm group it was 1245 ppb. The associate control group showed slight contamination, having an HCB fat concentration of 24.1 ppb at the end of the 90 days. The 1.0 ppm group showed a peak concentration of 10,186 ppb, and its associate group was also slightly contaminated with HCB. The free control group had an initial HCB concentration of 23.7 ppb, which decreased toward the end of the experiment to a final concentration of 12.2 ppb (see Table 17).

After peak concentration was realized at day 90, the levels of HCB residues in the omental fat decayed with half times of 100, 82, and 96 days in the 0.01 ppm, 0.1 ppm, and 1.0 ppm groups, respectively.

Undosed control sheep did not accumulate appreciable amounts of HCB (Table 17), but the associated control groups (0.1 and 1.0 ppm) accumulated significant amounts, probably due to fecal contamination from the treated sheep. Maximum concentrations in their fat were approximately 24.1 ppb and 28.5 ppb, respectively. One lamb in the 1.0 associate control group almost assurredly received an HCB capsule by accident, as his omental fat concentration at day 90 reached 308 ppb. This lamb was omitted from calculations.

Table 18 indicated HCB residue levels immediately after 90 days feeding of HCB at the various levels. It can be seen that the predominant sequestration of the drug occurs in the

TABLE 17

HCB concentration in omental fat after HCB[a] feeding at different dose levels
HCB concentration in omental fat expressed in ppb[a]

Days after feeding	0.00 ppm Control	0.01 ppm HCB	0.1 ppm HCB	0.1 ppm associate	1.0 ppm HCB	1.0 ppm associate
0	23.7 ± 20.8	15.6 ± 2.4	17.5 ± 5.1	18.7 ± 11.2	-------	-------
7	--------	27.9 ± 4.6	177.0 ± 76.7	-------	990 ± 174	-------
15	--------	37.8 ± 7.5	349.0 ± 58.1	-------	2387 ± 448	-------
30	15.5 ± 3.1	49.8 ± 11.7	537.6 ± 99.7	16.5 ± 8.6	4085 ± 418	16.7 ± 4.3
45	--------	59.2 ± 14.8	647.4 ± 126	-------	5801 ± 937	-------
60	16.4 ± 2.5	89.5 ± 13.2	1024.0 ± 213	21.8 ± 10.2	8394 ± 1053	24.9 ± 3.1
90	12.2 ± 1.3	115.8 ± 32.4	1245.0 ± 253	24.1 ± 12.5	10186 ± 1364	28.5 ± 7.0
120	12.6 ± 2.1	80.2 ± 10.1	747.7 ± 141	14.2 ± 1.5	8124 ± 1325	27.1 ± 1.9
150	10.3 ± 1.9	70.1 ± 11.3	716.6 ± 99.5	13.5 ± 1.0	7293 ± 1680	27.7 ± 8.8
180	9.9 ± 0.9	69.0 ± 11.6	596.0 ± 86.0	14.7 ± 2.3	5832 ± 1137	30.1 ± 10.7
210	11.3 ± 0.9	51.2 ± 10.0	449.0 ± 61.7	13.1 ± 3.3	4167 ± 801	-------
240	9.1 ± 0.8	29.5 ± 3.5	289.0 ± 50.3	6.8 ± 1.9	3525 ± 803	26.7 ± 7.4
270	10.3 ± 2.5	36.2 ± 4.0	295.0 ± 46.3	11.5 ± 1.1	2578 ± 711	22.6 ± 9.8
300	11.3 ± 1.7	26.8 ± 6.1	213.0 ± 46.8	-------[c]	2218 ± 331	-------[c]

a. HCB was given everyday for a period of 90 days.

b. Each value represents the mean ± s.d. of ten sheep in each treatment group and five sheep in each associate group for the first 90 days.

After 90 days, each value represents six sheep in each treatment group and three from each associate control.

c. These sheep were used in the acute feeding experiment (100 ppm for 19 days).

TABLE 18

HCB residues in various tissues sampled immediately after 90 days feeding at 0.00, 0.01, 0.1, and 1.0 ppm HCB [results expressed as parts per billion (ppb)]

Tissue	GROUP 0.00 ppm Control	0.01 ppm HCB	0.10 ppm HCB	0.10 ppm Control	1.0 ppm HCB	1.0 ppm Control
Brain	<4(4)[a]	<4(3)	29 ± 1.9(4)[b]	<4(2)	217 ± 53)(4)	<4(2)
Liver	<4(4)	<4(3)	11 ± 0.7(4)	<4(2)	281 ± 41(4)	<4(2)
Kidney	<4(4)	<4(3)	8.0 ± 3.0(4)	<4(2)	112 ± 41(4)	<4(2)
Perirenal fat	-	69 ± 3.8(2)	856 ± 142 (4)	-	7160 ± 1189(4)	-
Omental fat	12 ± 1.7(4)	119 ± 23 (3)	1435 ± 183 (4)	36 ± 11(2)	9503 ± 669(4)	31 ± 0.9(2)

a. Detection limit 4 ppb

b. $\bar{x}$ ± s.d. (N)

fat, particularly omental fat; the levels of HCB residues in brain, liver and kidney were only about 0.01 - 0.02 times the level in the omental fat.

3. Discussion

Hexaclorobenzene administered orally is sequestered in the animal's body fat. This is consistant with known physical characteristics of the compound; i.e., low water solubility, readily oil dissolvable. The difference noted in the levels seen in omental versus perirenal fat does not seem related to anything in particular; perhaps it has something to do with the perfusion characteristics of the two fat loci. In any event, it can be concluded that growing lambs store HCB residues in omental fat at levels 10-12 times the intake level (intake levels 0.01 ppm to 1.0 ppm) after 90 days, and at 50-100 times the levels seen in the other tissues studied.

The decay half times of 100, 82, and 96 days in the 0.01 ppm, 0.1 ppm, and 1.0 ppm groups are all of the same order of magnitude. This is consistant with common pharmacokinetics.

The primary origin of the HCB that contaminated the two associate control groups (Table 17) would presumably have been the feces of the dosed sheep living in the same run. These dosed sheep would presumably have excreted unabsorbed HCB during the 90 day dosing period. This has been previously reported by Avrahami and Steele (30), and confirmed later by Mehendale and Mathews (7), who reported HCB-contaminated fecal material after oral administration of labeled HCB ^{14}C It was also postulated that levels of HCB in the associated

control groups were due to the continued ingestion of bedding containing HCB derived from the dosed sheep during the post-dosing period. We attempted to control this by moving the sheep to clean runs at the end of the exposure period, by bedding only half of the runs, and changing the bedding frequently (every other day).

The residue profile seen (Tables 18 and 19) indicates that HCB might be accumulated in all tissues examined in a dose-dependent manner. In this study the HCB residues were higher in omental fat as compared with the other tissues.

H. Gross and Microscopic Pathological Changes seen after HCB Administration

1. Specific Materials and Methods

Animals were slaughtered at day 90, immediately after the chronic HCB administration. The number of animals from each group was as given in section II B, this report. Gross and microscopic tissue examination was performed as per Section II H, this report. The same procedure was performed at the termination of the 19 day 100 ppm experiment.

2. Results

No grossly observable pathology was noted upon necropsy. Some histopathological changes were observed in lung and intestine of both control and treated groups. These were not felt to be due to the HCB but rather due to parasitic infestation of the animals (i.e., lung worms, coccidia). Similar, parasite-induced changes were occasionally noted in the kidneys of both the control and exposed groups.

Some histopathological changes occurred in the livers of the exposed animals that might be attributable to the HCB.

TABLE 19

HCB residues in various tissues sampled 210 days after termination of 90 days feeding at 0.00, 0.01, 0.1, and 1.0 ppm HCB (i.e., sampled at day 300) [results expressed as parts per billion (ppb)]

	GROUP					
Tissue	0.00 ppm Control	0.01 ppm HCB	0.1 ppm HCB	0.1 ppm Control	1.0 ppm HCB	1.0 ppm Control
Brain	<4(6)	<4(6)[a]	7.0 ± 1.7(5)	-	58 ± 14 (6)	-
Liver	<4(6)	<4(6)	<4(5)	-	36 ± 7.2(6)	-
Kidney	<4(6)	<4(6)	<4(5)	-	21 ± 6.8(6)	-
Perirenal fat	-	27 ± 2.8(6)	200 ± 36 (5)	-	2113 ± 529 (6)	-
Omental fat	11 ± 1.7(6)	27 ± 6.1(6)	213 ± 47 (5)	-	2218 ± 331 (6)	-

a. Detection limit 4 ppb

b. $\bar{x} \pm$ s.d. (N)

TABLE 20

HCB residues in various tissues sampled immediately after 19 days feeding at 100 ppm HCB [results expressed as parts per billion (ppb)]

organ	Control group	HCB-treated group
Brain	<4(6)[a]	2,333 ± 449(6)[b]
Liver	<4(6)	2,197 ± 553(6)
Kidney	<4(6)	522 ± 208(6)
Perirenal fat	-	108,500 ± 23,880(6)
Omental fat	11.3 ± 11.7(6)	100,333 ± 3,098(6)

a. Detection limit 4 ppb

b. $\bar{x} \pm$ s.d. (N)

These changes were minor and not uniformly seen in the exposed animals; they were not noted in the control group. These changes included inflammatory cell infiltration into the portal triad areas, generalized necrosis, and some vaculation of cells.

3. Discussion

As a result of our histopathological findings, we concluded that HCB does not appreciably affect the organs and tissues when it is given in oral doses of 0.01, 0.1, 1.0, and 100 ppm (daily) to growing lambs. This does not conflict necessarily with work of other investigators who found tissue damage (prinicpally liver damage) at a daily dose of 2000 ppm HCB in rats (35), and 100 mg/kg of body weight for 14 consecutive days in rats (34).

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15. SUPPLEMENTARY NOTES

16. ABSTRACT

Growing lambs were exposed to daily doses of hexachlorobenzene (HCB) per os. The daily dose was calculated to provide 0.01, 0.10 or 1.00 ppm of the feed consumed. Exposure was terminated at 90 days and 20 of the 50 lambs were slaughtered. Remaining lambs were monitored for an additional 210 days. Biopsies of omental fat were taken periodically. At slaughter, samples were collected for HCB analysis and histological preparations. Blood samples were periodically collected for determination of HCB, plasma protein, and plasma enzyme activities. At the end of the 90 day exposure, the in vivo metabolism of aminopyrine and the in vitro O and N-demethylation of the liver microsomes were determined. Results of the analyses for HCB in omental fat show that the peak concentration attained was 10.186, 1.024, 0.116 and 0.012 ppm in the 1.00, 0.10, 0.01 ppm and control groups respectively. Following cessation of the exposure, the HCB declined to less than one-half the peak concentration within 100 days. Histological examination of tissues from the slaughtered animals showed no pathological changes attributable to the HCB. Marked changes were noted in the microsomal enzymes but changes were not significant in the plasma enzymes.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
chlorobenzenes pollutants contaminants pathology pathophysiology drug excretion animal nutrition	chlorine aromatic cmpds hexachlorobenzene toxicology drug tissue distribution lamb growth pharmacokinetics	06T/06F

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