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EFFECT OF HEXACHLOROBENZENE ON REPRODUCTION IN THE RAT

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This study was designed to determine the effect of hexachlorobenzene (HCB) on rat reproduction. Dietary concentrations of 0, 10, 20, 40, 80, 160, 320, and 640 ppm of HCB were fed to Sprague-Dawley rats in a four generation reproduction study. Pregnancy (number of dams whelping/number of dams mated), viability (pups surviving 5 days/pups born alive), and lactation (pups weaned/live pups on day 5 less culls) indices were measured. Body weight gain of pups was monitored. HCB residues were measured in the 21-day-old pups of the F_1a and F_3a generations. The two highest dietary concentrations were toxic to the F_0 generation females and 50% and 20% respectively, died. Sucking pups were particularly sensitive and many died prior to being weaned. The viability index was zero in the F_1a and F_1b generations for pups from dams fed 320 and 640 ppm of HCB and only 55% for the 160 ppm group. The lactation index decreased from 30% for the F_1a and F_1b generation pups to 0 for the F_2a and F_2b generation pups in the 160 ppm group. Also, the lactation index decreased from 93% in the F_1a generation to 40% in the F_3b generation for the 80 ppm group. Birth, 5-day and 21-day body weights of pups were decreased by treatment. HCB residues accumulated in the pups in a dose-related manner. The relative liver weight and aniline hydroxylase activities were increased in weanlings from dams fed 40 ppm of HCB. No gross abnormalities were observed in the pups.

Hexachlorobenzene (HCB), a persistent fungicide and an industrial contaminant has become ubiquitous in our ecosystem (Koeman *et al.* 1969, Brady and Siyali 1972, Gilbertson and Reynolds 1972).

In Turkey during 1955 to 1959, over 3,000 cases of cutaneous porphyria resulted from the consumption of wheat seed which had been treated with HCB for the control of the fungus *Tillatia tritui* (Cam and Nigogosyan 1963). The main symptoms were skin lesions, porphyrinuria and photosensitization.

A number of papers have been published on the relationship of HCB to porphyria (Ocker and Schmid 1961, De Matteis *et al.* 1961, Vos *et al.* 1971, Grant *et al.* 1974), to enzyme induction (Wada *et al.* 1968, Stonard & Nenov 1974 and Grant *et al.* 1974) and to hepatotoxicity (Vos *et al.* 1971, Medline *et al.* 1973, Szabo *et al.* 1973, and Kimbrough and Linder 1974).

The effect of HCB on reproduction has received very little attention. De Matteis *et al.* (1961) reported that of two pregnant rats given HCB in their food, one died, but the other delivered normally and reared the young until they died with convulsions after 7 to 8 days. Recognizing that HCB residues have been detected in human tissues and the effect of HCB on suckling rats, it was considered essential to undertake a reproduction study in the rat to establish a "no-effect" level for HCB in the diet.

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Methods & procedures

Diet. HCB, organic analytical standard grade, (BDH Chemicals Ltd., England) was suspended in corn oil (purity 99.5%) and mixed with ground stock cubes (Maple Leaf Mills, Master Feeds Division, Toronto). The corn oil constituted 3% by weight of the diet.

Breeding. Sprague-Dawley weanling rats were divided into 8 groups of 20 females and 10 males and fed dietary levels of 0, 10, 20, 40, 80, 160, 320, or 640 ppm of HCB. When the F_0 rats reached 100 days of age, 1 male was caged with 2 females for 1 week, and then the males were rotated for a second one-week mating period. The F_1a pups were weaned after a 21-day lactation period. The F_0 rats were rested for 14 days and then mated to produce the F_1b generation. This schedule was repeated with the F_1b rats to produce the F_2a and F_2b generations, and then with the F_2b rats to produce the F_3a and F_3b generations and finally with the F_3b rats to produce the F_4a and F_4b generations.

Records were made of the number of litters, pups per litter and total litter weight at days 1 and 5. Litter size was reduced to 8 pups per litter on day 5. Litters were examined each day to observe survival of the pups. Individual body weights were recorded on the day of weaning (21 days of age). Five male and 5 female weanlings from each treatment group in the F_1a and F_3a generations were killed by decapitation and blood collected. The weanlings were examined grossly and liver, kidney, heart, and brain removed, weighed and frozen. Approximately 1 g of liver was removed before freezing for aniline hydroxylase analysis (Becking 1973). With the F_1a generation weanlings, the complete gastrointestinal tract was removed and carcass freeze dried, ground to uniform powder, and total fat extracted (A.O.A.C. 1965).

HCB residues. Tissues (plasma, liver, kidney, brain, and body fat), were homogenized with acetonitrile, an equal volume of water was added, and HCB partitioned into hexane and quantitated by GLC-EC. The chromatographic conditions were: Column, 122 cm × 6.3 mm of Supelco Port (80–100 mesh) coated with 4% SE-30 and 6% QF-1; temp., 193°C and N_2 flow rate, 125 ml/min.

Results

Accumulative toxicity. The two highest dietary levels of HCB (320 and 640 ppm) exhibited accumulative toxicity as 2 and 10 females in the F_0 generation died prior to the first whelping and 5 in each group prior to the second whelping. One female, from the 160 ppm group, died prior to the second whelping. The females which died exhibited convulsions prior to death.

Fertility. The fertility indices (Table 1) were decreased by dietary levels of 320 and 640 ppm of HCB in the F_1a and F_1b generations. The average litter size was decreased in the F_1b, F_2a, and F_2b generations, respectively, by dietary levels of 320, 160, and 160 ppm. The two highest dietary HCB levels also increased the number of stillborns. The pups did not exhibit any gross abnormalities.

Table 1. *Fertility indices[a] of rats fed HCB for 4 generations*

Generation	Dietary level (ppm)							
	0	10	20	40	80	160	320	640
F_1 a	70	85	80	60	80	70	28	20
F_1 b	45	75	70	60	55	68	38	40
F_2 a	95	90	85	100	86	78	–	–
F_2 b	85	90	90	90	86	78	–	–
F_3 a	90	85	90	90	75	–	–	–
F_3 b	90	95	85	95	75	–	–	–
F_4 a	80	80	65	85	–	–	–	–
F_4 b	90	70	85	90	–	–	–	–

[a] Fertility index = (Number of litters whelped/number of females exposed to mating) x 100

Survival to day 5. The viability indices (Table 2) were reduced to zero by dietary levels of 320 and 640 ppm of HCB and to less than 60 by 160 ppm in the F_1a and F_1b generations. The viability indices were further reduced to 44 and 35, respectively for the F_2a and F_2b generations fed a dietary level of 160 ppm of HCB.

Survival to day 21. The lactation index (Table 3) was the most sensitive index measured and it was decreased from 30 and 33, respectively, in the F_1a and F_1b generations fed 160 ppm of HCB to 0 in the F_2a and F_2b generations.

Table 2. *Viability indices[a] of rats fed HCB for 4 generations*

Generation	Dietary level (ppm)							
	0	10	20	40	80	160	320	640
F_1 a	92	99	92	93	95	57	0	0
F_1 b	100	98	93	85	93	55	0	0
F_2 a	98	91	96	85	59	44	–	–
F_2 b	93	97	96	92	81	35	–	–
F_3 a	92	76	88	90	70	–	–	–
F_3 b	99	97	95	96	84	–	–	–
F_4 a	97	92	98	94	–	–	–	–
F_4 b	90	96	95	89	–	–	–	–

[a] Viability index = (number of pups surviving 5 days/number pups born alive) x 100

Table 3. *Lactation indices[a] of rats fed HCB for 4 generations*

Generation	Dietary level (ppm)							
	0	10	20	40	80	160	320	640
F_1 a	100	98	100	100	93	30	–	–
F_1 b	95	99	100	90	70	33	–	–
F_2 a	100	100	100	90	74	0	–	–
F_2 b	97	96	95	91	86	0	–	–
F_3 a	100	95	92	84	61	–	–	–
F_3 b	94	94	95	94	39	–	–	–
F_4 a	100	98	97	94	–	–	–	–
F_4 b	95	97	86	82	–	–	–	–

[a] Lactation index = (Number of pups weaned/number of live pups on day 5 less culls) x 100.

Table 4. *Birth weights of rats fed HCB for 4 generations*

Generation	Dietary level (ppm)						
	0	10	20	40	80	160	320
F_1 a	7.2[a]	6.7	6.8	6.8	6.3[c]	6.1[c]	5.4[c]
	0.2[b]	0.3	0.3	0.2	0.1	0.2	0.3
F_1 b	7.0	6.7	7.0	7.1	6.7	6.7	5.9[c]
	0.2	0.2	0.3	0.2	0.1	0.2	0.1
F_2 a	6.5	6.1[c]	6.3	6.1[c]	6.2	5.6[c]	–
	0.1	0.1	0.1	0.1	0.2	0.2	–
F_2 b	6.2	6.2	6.5	6.6	6.2	5.8[c]	–
	0.1	0.1	0.2	0.1	0.2	0.1	–
F_3 a	6.1	6.1	6.5	6.2	6.2	–	–
	0.2	0.2	0.3	0.2	0.2	–	–
F_3 b	6.6	6.4	6.3	6.4	6.1[c]	–	–
	0.1	0.2	0.1	0.1	0.2	–	–
F_4 a	6.3	6.3	6.2	6.3	5.8	–	–
	0.2	0.2	0.1	0.2	0.3	–	–
F_4 b	6.1	6.2	6.4	6.1	–	–	–
	0.1	0.1	0.1	0.1	–	–	–

[a] Mean
[b] SE of Mean
[c] Significantly different from group receiving a dietary level of 0 ppm

Table 5. *Five day weights of rats fed HCB for 4 generations*

Generation	Dietary level (ppm)						
	0	10	20	40	80	160	320
F_1a	11.8[a]	11.1	11.6	11.1	9.4[c]	7.8[c]	–
	0.8[b]	0.5	0.5	0.5	0.3	0.3	–
F_1b	12.1	12.4	12.6	12.2	10.1[c]	8.7[c]	–
	0.6	0.7	0.7	0.8	0.7	0.4	–
F_2a	10.8	10.3	9.5[c]	8.8[c]	8.1[c]	-6.3[c]	–
	0.3	0.4	0.4	0.4	0.7	0.1	–
F_2b	10.0	10.5	10.7	9.9	8.4[c]	6.1[c]	–
	0.3	0.5	0.5	0.5	0.8	0.7	–
F_3a	10.0	10.0	9.9	9.1	7.9[c]	–	–
	0.5	0.6	0.7	0.6	0.7	–	–
F_3b	9.6	9.5	9.0	9.3	7.0[c]	–	–
	0.2	0.4	0.3	0.3	0.5	–	–
F_4a	10.5	10.1	9.4[c]	9.5[c]	–	–	–
	0.3	0.5	0.4	0.4	–	–	–
F_4b	10.5	10.1	10.5	9.8	–	–	–
	0.3	0.4	0.4	0.4	–	–	–

[a] Mean
[b] SE of Mean
[c] Significantly different from group receiving a dietary level of 0 ppm

Body weight. The birth weights were significantly decreased by dietary levels of 80 ppm of HCB and higher in the F_1a generation and by 160 ppm in the F_2a and F_2b generations (Table 4). A dietary level of 80 ppm of HCB consistently decreased the body weight of 5-day-old pups (Table 5) and of 24-day-old pups (Table 6).

Liver weight and aniline hydroxylase activities. In the F_1a and F_3a generations, the liver weights for pups from dams fed 0 ppm of HCB (expressed as percent body weight) were 3.74 and 3.78 respectively, while the liver weights for pups from dams fed 40 ppm of HCB were significantly increased to 4.08 and 4.21. Also, the aniline hydroxylase activities were increased by 40% and 90% respectively, in the F_1a and F_3a generation pups from dams fed 40 ppm. No sex difference was observed, thus the data for males and females were combined. Kidney, heart, and brain weights of pups were not affected by treatment.

HCB residues. Tissue concentrations of HCB are shown in Table 7 for plasma, brain, kidney, liver, and body fat. Unexpected high residues of HCB were found in control F_1a

Table 6. *Weanling weights by sex of rats fed HCB for 4 generations*

	Dietary level (ppm)											
	0		10		20		40		80		160	
Generation	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
F_1 a	51.7[a]	52.1	51.7	50.9	62.0	52.5	52.9	51.1	44.5[c]	43.2[c]	43.1[c]	45.8[c]
	1.4[b]	1.4	0.9	0.8	9.1	0.8	0.9	0.8	0.7	0.7	3.1	1.6
F_1 b	54.1	54.0	57.3	54.6	58.2[c]	57.6[c]	54.3	53.0	51.1	47.2[c]	48.5[c]	46.9[c]
	1.2	1.2	1.3	0.9	0.8	0.8	1.4	1.1	1.5	1.6	0.1	1.2
F_2 a	53.0	52.5	47.7[c]	46.1[c]	47.5[c]	46.4[c]	46.9[c]	46.6[c]	37.1[c]	40.6[c]	–	–
	0.9	0.7	0.7	0.6	0.7	0.9	1.1	0.9	1.4	1.1	–	–
F_2 b	47.3	48.6	49.0	49.0	51.0	50.2	49.6	46.6	42.2[c]	40.8[c]	–	–
	1.0	0.7	0.7	0.7	0.8	0.7	1.2	1.2	1.6	1.7	–	–
F_3 a	52.3	51.4	51.8	50.7	50.7	50.0	46.9[c]	44.5[c]	42.7[c]	43.1[c]	–	–
	0.7	0.8	0.8	0.9	0.8	0.8	1.2	1.1	1.1	1.1	–	–
F_3 b	44.5	41.5	53.5	45.9[c]	41.3[c]	41.9	44.9	45.1[c]	38.9[c]	34.1[c]	–	–
	1.2	1.1	8.0	1.0	1.2	1.0	1.0	0.9	2.1	2.2	–	–
F_4 a	52.1	51.4	49.4[c]	47.6[c]	45.0[c]	46.4[c]	45.5[c]	43.4[c]	–	–	–	–
	0.6	0.5	0.9	0.8	0.9	1.2	1.0	1.2	–	–	–	–
F_4 b	52.2	49.8	55.0[c]	54.8[c]	54.9[c]	53.2[c]	50.0	50.3	–	–	–	–
	0.6	0.6	0.7	0.7	0.9	0.7	1.1	0.9	–	–	–	–

[a] Mean.
[b] SE of Mean.
[c] Significantly different from group receiving a dietary level of 0 ppm.

Table 7. *Tissue HCB levels (ppm) of 21 day old pups from dams fed graded dietary levels of HCB.*

Tissue	Sex	HCB residues at dietary level (ppm) of					
		0	10	20	40	80	160
Plasma[a]	M	0.5	1.7	3.0	4.6	12.8	28.1
	F	0.4	1.4	3.3	5.2	11.7	28.7
Plasma[b]	M	0.005	1.0	2.0	5.1	–	–
	F	0.007	1.1	1.8	4.9	–	–
Brain[a]	M	1.7	4.8	8.7	14.8	30.2	53.0
	F	1.4	4.3	7.9	16.2	32.8	52.7
Kidney[a]	M	1.9	3.8	7.3	12.8	26.1	62.2
	F	1.6	4.3	7.7	14.5	27.7	49.9
Liver[a]	M	3.7	10.8	21.5	38.2	105.0	175.0
	F	3.7	10.6	21.5	49.5	102.0	153.0
Liver[b]	M	0.1	8.7	16.9	49.7	–	–
	F	0.2	8.1	16.9	40.6	–	–
Body fat[a]	M	90.2	260.0	488.0	767.0	1984.0	3059.0
	F	85.1	233.0	527.0	812.0	1887.0	2531.0

[a] Tissues from F_1 a generation pups
[b] Tissues from F_3 a generation pups

rats and will be discussed below. Linear regression analysis showed a highly significant dependence of the residue level on the HCB dietary concentration for each tissue. The slopes of the regression lines were all approximately the same for each tissue when both abcissa and ordinate were plotted on logarithmic scales. The similarity in slope indicates that the ratios of the tissue HCB levels are approximately constant for all dietary levels of the compound. The ratios, calculated at the mean point of the regression line, of the residues in the tissues to plasma for males and females respectively are: body fat 130, 124; liver 7.0, 6.9; kidney 2.3, 2.0; and brain 2.2, 2.3.

Samples of whole blood and plasma were analyzed for HCB residues to determine the distribution of HCB. On a weight basis, whole blood contained approximately 5 times that of plasma.

Discussion

The accumulative toxicity of HCB in female rats observed in this study agrees with studies reported previously (Kimbrough and Linder 1974, Munro *et al.* 1974).

HCB has been shown to cross the placenta of the rat and accumulate in the fetus in a dose-related manner (Villeneuve and Hierlihy 1975). The fact that no gross abnormalities

were observed in the pups agrees with a previous study (Khera 1974) in which HCB administered to pregnant rats at doses up to 120 mg/kg caused no skeletal or visceral abnormalities in the pups.

Dietary HCB adversely affected reproduction in the rat by decreasing the number of litters whelped and the number of pups surviving to weaning. This decrease in survival of pups agrees with the very preliminary findings of De Matteis *et al.* (1961). The decreased body weight of the 5- and 21-day-old pups is thought to be due to decreased milk production by the dams.

In a previous study (Grant *et al.* 1974), dietary levels of HCB were shown to increase liver microsomal enzymes in the rat. This study shows this increase in mixed function oxidases also takes place in suckling pups of dams exposed to HCB.

Residue levels of HCB were determined in various tissues of the weanlings. Surprisingly, significant residues were found in the tissues of F_1a control animals. The problem of residues in the control animals was eliminated in future generations as shown by the results presented for the F_3a generation. Analyses of the control diets did not show significant amounts of HCB that would account for the high residues. Extrapolation from the regression coefficients would indicate an intake by control dams of approximately 3 ppm of HCB, well below the effect levels for any parameter studied. It is possible that an error in feeding was made at some time or there was a general contamination in the animal room during the production of F_1a and F_1b generation rats as only during this time were the 320 and 640 ppm diets fed. As with other organochlorine compounds, the highest levels of HCB were found in the body fat of the weanlings. The ratios of the concentration of HCB in plasma relative to other tissues are considered to be valid for animals only at the time of weaning. The high concentrations observed in the weanlings in relation to the dietary intake of the dam would suggest a high rate of excretion of HCB *via* the milk. Further studies are required to equate levels of HCB in the milk to body burdens in the dam and progeny. The residue data also demonstrate that the weanling rat can tolerate relatively high body burdens without showing any adverse effects on the various parameters measured. Decreased body weight of the weanling was observed only in groups having 1980 ppm or greater in the body fat, with a no-effect level of approximately 800 ppm. Significant induction of a microsomal enzyme (aniline hydroxylase) and increased liver weight were related to approximately 800 and 45 ppm in the body fat and liver, respectively.

Since whole blood residue levels were found to be 5 times those of plasma, it is important to analyze whole blood or to determine the ratio between whole blood and plasma residues when estimating the concentration of HCB circulating in the blood. Burns *et al.* (1974) reported that the cellular constituents of human blood comprised only 13% of the whole blood HCB level. MOSS and HATHWAY (1964) reported that telodrin and dieldrin concentrations in erythrocytes and plasma were equal, while Morgan *et al.* (1972) found less than 18% of the DDT in the erythrocytes of human blood. Thus the distribution of an organochlorine pesticide in blood appears to depend on the compound and the species.

HCB has been found to affect reproduction in the rat, with 20 ppm being the no-effect level. Suckling rats were particularly sensitive. Weanling rats from dams fed HCB contained HCB residues, had enlarged livers with increased hepatic aniline hydroxylase activity. No gross abnormalities were observed in the pups.

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