Intestinal Absorption of Hexachlorobenzene and Hexachlorocyclohexane Isomers in Rats

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Hexachlorobenzene (C_6Cl_6, HCB) has been implicated in a mass poisoning of humans in Turkey due to the consumption of wheat treated with 2 g of HCB per 100 Kg of seed (CAM and NIGOGOSYAN, 1963). HCB is a widely used fungicide, an important intermediate in organic syntheses, and a by-product of various manufacturing processes (MELNIKOV, 1971). The acute oral toxicity of HCB is relatively low, a minimum lethal dose being about 2 g/Kg for both rats and mice (SAVITSKII, 1964). PARKE and WILLIAMS (1960) have suggested, based on studies in rabbits, that the low toxicity may be due to poor absorption from the gastrointestinal tract. However, coprophagia was not prevented in these experiments.

Hexachlorocyclohexane, commonly misnamed benzenehexachloride ($C_6H_6Cl_6$, BHC) can occur in several isomeric forms of which five, α, β, γ, δ and ε isomers, occur significantly in "technical grade" BHC. Only the γ-isomer, known as Lindane, is used as a pesticide; the other isomers have 50-10,000 fold less toxicity than the γ-isomer (for further discussion see MELNIKOV, 1971). The isomers of BHC other than Lindane are of interest to us in that they are chiefly used as precursors in the synthesis of HCB.

We felt that the absorbability of HCB (and, for comparison BHC) deserved further study under conditions where coprophagia was prevented. The results of our studies in the rat are presented here.

Experimental

Male CD-strain rats (Charles River) weighing approximately 300 g were held in metabolic cages that had coarse screen floors. Feces immediately fell through out of reach, and by a system of baffles were prevented from mixing with urine. The rats were maintained on standard Purina chow and water ad lib. The chlorinated materials were dissolved in acetone, squalane and cottonseed oil were added, and the acetone was evaporated off in a rotary evaporator at 40° under vacuum. The solutions of HCB or BHC and squalane in cottonseed oil prepared in this way were stable for several hours, after which HCB and the highest concentrations of BHC began to slowly crystalize out.

The HCB used here was thrice recrystalized starting from Eastman No. P1552 stock. BHC was technical grade, from the

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Pesticides Repository, Pesticides Research Laboratories, Perrine, Florida. Gas chromatographic analysis (PENNINGTON and MELOAN, 1967) indicated that its isomeric composition was: 70.6% α, 7.2% β, 15.2% γ, 4.8% δ, and 0.8% ε.

Cottonseed oil solutions were administered as a single dose by stomach tube. In addition, 500 g of pulverized rat chow was allowed to soak up a pentane solution of 1 gram of BHC and 0.6 grams of squalane, then air-dried overnight. Rats were allowed to eat this treated chow for a period of two weeks ad lib.

Feces samples were analyzed daily; urine was pooled and analyzed at the end of the experiment. Bile was collected from some animals either three days after feeding the cottonseed oil solutions or immediately at the end of the two-week feeding study. The entire gastrointestinal tract was removed from some of the animals three days after feeding the test compounds. Contents were flushed out and the contents and tissue analyzed separately.

All samples were extracted with chloroform: methanol as described by BLIGH and DYER (1959). The organic phase was evaporated to dryness on a rotary evaporator and the residue taken up in n-hexane: benzene 4:1 (v/v). This solution was chromatographed over 10 g of Florisil, eluting with the same solvent (50 ml). The eluate was concentrated with a rotary evaporator and made up to 1 ml with benzene for gas-liquid chromatography (GLC). Solvents were pesticide grade, from Fisher Scientific Co., Raleigh, North Carolina. Preliminary experiments with spiked samples indicated that the ratios of squalane to the various chlorinated compounds were not changed from those of the preparations fed to the animals by these extraction and clean-up procedures.

GLC was carried out using a Hewlett-Packard Model 5750 instrument with hydrogen flame ionization detectors and with the amplifier output fed to an Autolab System IV computing integrator. Relative molar flame responses were determined (squalane≡1) from standard solutions of the test substances and used to program the computer. In this way, peak area percentages were automatically converted to molar percentages. Injection port and detector temperatures were 250° and 270° respectively; the helium carrier flow was 50 ml/min. at the column outlet.

BHC isomers were resolved on a 2 meter x 1/8" O.D. stainless steel column packed with 4.8% OV-17 on 100-120 mesh Gas Chrom Q. The column was held at 210° for 8 minutes (while BHC eluted), then programmed at 20°/min. to 250° to elute the squalane. Feces samples also contained squalene, requiring that the column be kept at 250° for 16 minutes before recycling. Peak identifications were confirmed by GLC at 210° on 5% OV-225 on 100-120 mesh Gas Chrom Z, 1.35 meter by 1/8"O.D. HCB and squalane were measured using the OV-17 column, programmed from 200° to 250° at 6°/minute without a delay period.

Results and Discussion

We have previously established (ALBRO and FISHBEIN, 1970) that squalane, a saturated C-30 isoprenoid hydrocarbon, is not absorbed by rats. It is excreted quantitatively in the feces, excretion being complete in not over 96 hours. Fecal bacteria do not metabolize squalane, and in the present study we found no loss of HCB or BHC when these compounds plus squalane were incubated with feces in Trypticase Soy Broth at 37° for 24 hours.

Excretion of HCB and BHC in the feces was complete by 96 hours following a single oral dose in the present study. Accordingly, the molar ratio of test compound to squalane in the 96-hour feces is a measure, when compared with the ratio in the sample fed, of the percentage of test substance excreted intact. Using the ratio as a measure obviates any problems from handling losses, variations in sample volumes, and absolute detector sensitivity. This approach has been used successfully to study excretion of hydrocarbons (ALBRO and FISHBEIN, 1970) and polychlorinated biphenyls (ALBRO and FISHBEIN, 1972).

We refer to the percentage of substance fed that is not excreted in the feces as "percentage removed". We assume that the material fed must be either (a) not absorbed and excreted intact, (b) not absorbed but metabolized in the gut and excreted as metabolites (or absorbed only after metabolism has occurred), (c) absorbed but recycled in the bile such that some of what is excreted intact in the feces has been once absorbed, (d) absorbed intact and not excreted in the feces, or (e) some combination of these. What we measure experimentally represents conditions (a) and/or (c). For practical purposes, we assume tentatively that fraction of the material fed appearing in the feces within the time frame of squalane excretion is not likely to have significant physiological effects on the animal. The remaining fraction, whether associated with conditions (b) and/or (d), may have had significant effects and is considered more relevant from an environmental viewpoint than merely the fraction that could be demonstrated to have been absorbed intact.

The less lipid soluble, more polar β and ε isomers of BHC passed through the intestines more slowly than the α and γ isomers. For this reason, only the cumulative data representing total 96-hour feces are given in the first table below. The δ and ε isomers were present at too low levels in the feces for accurate measurement and are omitted from the table.

TABLE 1

Net Excretion of α-, β- and γ-BHC Following a Single Oral Dose of Technical BHC

Dose, mg/Kg	Net Percentage "Removed"[a,b] α	β	γ	Average ± S.D.
30.4	95.3	94.0	96.4	95.2 ± 1.2
62.7	96.7	94.9	97.3	96.3 ± 1.2
125.4	92.4	97.2	98.0	95.9 ± 3.0
Average ± S.D.	94.8	95.4	97.2	95.8 ± 0.57
	± 2.2	± 1.63	± 0.80	

[a]Each Value is the average of 4-6 determinations.
[b]Percentage Removed = 100 minus percentage excreted.

Analysis of variance showed no statistically significant differences in percentage "removal" related to dose or isomer; thus an overall average of 95.8 ± 0.57 percent of a single 30-125 mg/Kg dose of BHC in cottonseed oil (105 mg BHC per ml) was "removed".

We could not detect BHC or squalane in either the intestinal tissue or contents at the end of the 96-hour period. However, the pooled urine contained 0.65% of the α-, 0.98% of the γ- and 0.51% of the δ-BHC fed to the rats. The β-isomer (the least soluble) was not detected.

Results of the continuous feeding study are summarized in Table 2.

TABLE 2

Excretion of BHC Isomers During Continuous Feeding as a Function of Time[a]

BHC Isomer	Percentage Removed, Feces Collected on Day: 3	5	7	10	12	14	Average ± S.D.
α	98.1	97.6	97.4	96.5	96.6	97.9	97.4 ± 0.67
β	90.7	92.0	92.3	89.5	87.1	92.5	90.7 ± 2.1
γ	99.6	99.5	99.6	98.9	99.5	99.0	99.4 ± 0.32
δ	78.6	91.2	98.2	98.7	93.6	90.8	91.9 ± 7.31

[a]Each value is the average of four determinations.

Analysis of variance for the data summarized in the table had $F=7.279$ and $0.001<P<0.01$. Thus the row means differed significantly at the 1% level of probability, and the net excretion/removal of BHC did vary with the particular isomer. The β-isomer was excreted to the greatest extent (nearly 10%) while the most toxic isomer, γ, was barely excreted (intact) at all. The δ-isomer, which is the most lipid-soluble of the four considered, showed considerable time-dependent variation in excretion rate. The grand mean here was 94.9% of the BHC removed on an average, essentially similar to that found following a single dose.

We could not detect BHC in the bile on day 14 of this experiment, suggesting that the value of 5.1% excreted represents that BHC never absorbed.

Hexachlorobenzene was fed at two dose levels, 12 mg/Kg and 30 mg/Kg, limited mainly by the oil solubility of the HCB. The results of this experiment are shown in Table 3.

TABLE 3

Net Excretion of HCB Following a Single Oral Dose

Dose, mg/Kg	Percentage Removed[a] ± S.D.
12	81.8 ± 0.83
30	72.4 ± 2.8[b]

[a]Percentage Removed = 100 minus Percentage Excreted. Each value is the average of 6 determinations.

[b]A 2-tailed t-test gave $p<0.01$ for the means.

The data in this case indicated that HCB was more poorly absorbed and/or metabolized by rats than was BHC, even though HCB was administered in smaller doses than BHC. Percentage removal was less for the higher HCB dose, suggesting that a very small dose might have approached BHC in percentage removal.

We could not detect HCB either in bile or urine collected at the end of the experiment (less than 0.1 ppm). The intestinal tissue contained 6 times as much HCB as the residual intestinal contents, but taken together they accounted for only 2.8% of the HCB fed, 3.4% of the HCB not excreted. At those dose levels, then, it is necessary to conclude that HCB is rather well absorbed or metabolized. These results also suggest, however, that at a lethal dose level of 2 g/Kg, percentage removal might well be quite low. Unfortunately, our experimental approach would not permit the administration of high doses of this rather insoluble compound.

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