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A COMPARATIVE STUDY ON THE EFFECTS OF α-HEXACHLOROCYCLOHEXANE AND ITS METABOLITE β-PENTACHLOROCYCLOHEXENE ON GROWTH AND MONOOXYGENASE ACTIVITIES IN RAT LIVER*

W. PARZEFALL,†‡ J. MÜNSTER and R. SCHULTE-HERMANN
Institut für Toxikologie und Pharmakologie der Philipps-Universität Marburg, Pilgrimstein 2, 355 Marburg, Federal Republic of Germany

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Abstract—The present study adds support to the hypothesis that β-pentachlorocyclohexene (β-PCH) is a primary intermediate in α-hexachlorocyclohexane (α-HCH)§ metabolism in the rat. Degradation of α-HCH to β-PCH was shown to occur *in vitro* and *in vivo*, partially by non-enzymic catalysis. β-PCH accumulated in liver and adipose tissue of α-HCH treated rats, which had received the glutathione-lowering agent diethyl maleate. β-PCH disappears from the body much more rapidly than the parent compound α-HCH: about 50 per cent of a single i.p. dose were degraded within 2.5 hr, while half-life of α-HCH is known to be approximately 130 hr. To maintain equimolar liver concentrations, β-PCH must be given in doses 100-fold higher than α-HCH. β-PCH and α-HCH were fed for a period of ten days at various dose levels to give steady-state liver concentrations. It was found that β-PCH has similar hepatic effects to α-HCH: both agents induced liver growth and a phenobarbital-type pattern of monooxygenase activities, as measured by the following substrates: aminopyrine, ethylmorphine, benzphetamine, 4-nitroanisole, aniline, benzo[*a*]pyrene, ethoxyresorufin and 2,5-diphenyl-oxazole. Threshold doses for these effects were 30–43 μmoles/kg/day for β-PCH and 1.0–1.7 μmoles/kg/day for α-HCH. However, on the basis of molar hepatic concentrations β-PCH was a more potent inducer than α-HCH (2–10 times). Threshold concentrations ranged from 0.4 to 0.6 nmoles β-PCH/g liver and from 0.7 to 1.5 nmoles α-HCH/g liver. β-PCH concentrations in livers of rats treated even with high doses of α-HCH were below the threshold for induction of liver growth and of monooxygenase increases. It is, therefore, highly unlikely that β-PCH is responsible for the effect of α-HCH on rat livers.

The α- and β-isomers of hexachlorocyclohexane (HCH), by-products of commercial γ-HCH (lindane) synthesis, are subject of increasing public concern, mainly as a result of their persistence in biological systems. In rodents, HCH isomers are known to cause liver growth [3–5], induction of drug metabolizing enzymes [6, 7] and resistance towards the effects of convulsants [8–10]. The induction of liver growth, particularly prominent with respect to α-HCH, has recently been shown to be associated with tumor promotion in rodent liver [11, ‖]. The mechanism(s) of action leading to the biological effects described above are largely unknown at present but it would seem feasible that metabolites are involved in this process [12, 13].

Two major pathways of α-HCH metabolism have been established in recent years. The first one depends on cytochrome P-450 [14, 15] and the second includes conjugation reactions with glutathione (GSH) [14, 16–18]. In this latter pathway pentachlorocyclohexenes seem to be primary intermediates [18]. Chemically, PCHs are formed from HCHs by base catalysed monodehydrochlorination. The monodehydrochlorination product of α-HCH was identified by Münster [2, 19] as β-PCH¶. Attempts to demonstrate β-PCH formation in rats exposed to α-HCH have so far been unsuccessful and at best, only traces of this compound were found [15, 19], which could be the result of rapid metabolic clearance of this compound. Indeed, *in vitro* breakdown of β-PCH is a very rapid GSH-dependent process [17, 18]. The experiments reported below show that β-PCH is formed *in vivo* in rats treated with α-HCH. Furthermore, exogenous administration of β-PCH to rats was found to induce liver growth and drug-metabolizing enzymes. Finally we investigated

* A brief account of parts of this work has been published [1]. This paper continues previous studies on the synthesis and chemical properties of β-PCH [2].

† Present address: McArdle Institute for Cancer Research, University of Wisconsin, Madison, WI 53706, U.S.A.

‡ To whom correspondence should be addressed.

§ Abbreviations used: An, aniline; AP, aminopyrine; BP, benzo[*a*]pyrene; BPA, benzphetamine; DCPMA, dichlorophenylmercapturic acids; DEM, diethyl maleate; EM, ethylmorphine; ER, 7-ethoxyresorufin; g.l.c., gas liquid chromatography; GSH, glutathione; α-HCH, α-hexachlorocyclohexane (also known as α-benzene hexachloride); 3-MC, 3-methylcholanthrene; pNA, *p*-nitroanisole; PB, phenobarbital; PC, propylenecarbonate; PCN, pregnenolone-16α-carbonitril; PCH, PCCHE (pentachlorocyclohexene); PPO, 2,5-diphenyloxazole.

‖ R. Schulte-Hermann and W. Parzefall, manuscript in preparation.

¶ This compound was previously synthesized from monochlorobenzene by Kolka *et al.* [20] and termed β-PCH, but the relationship to any HCH isomer remained unclear. Kurihara *et al.* [21] isolated β-PCH by the isomerization of δ-PCH and used a different nomenclature [22] [β-PCH = (*dl*)-346/5-PCCHE).

whether or not β-PCH formed endogenously from α-HCH plays an active role in the induction of changes produced in rat liver by the latter compound.

MATERIALS AND METHODS

Substances

α-HCH (mol. wt = 291) and PCN were gifts of Boehringer, Ingelheim & Schering AG, Berlin; 7-ethoxyphenoxazole (ethoxyresorufin, ER) was synthesized by U. Greeff, Inst. für Pharmakologie und Toxikologie, Marburg. β-PCH (mol. wt = 254) was synthesized as described by Münster *et al.* [2]. It was purified by crystallization and silica gel column chromatography with propylene-carbonate (PC) as the stationary phase (SiO_2:PC 5:2, w/v), and PC-saturated petroleum ether as eluent. Purity of β-PCH: 99.7 per cent, contamination with α-HCH < 0.001%. [^{3}H]Thymidine, sp. act. 6.5 mCi/mmole, was supplied by NEN, Frankfurt, [U-^{14}C]α-HCH, sp. act. 48 mCi/mmole, was supplied by Amersham Buchler and was purified by column chromatography as described above. Bovine serum albumin was obtained from Behringwerke, Marburg. Myrj, a non-ionic dispersing agent, was obtained from Serva, Heidelberg. All other substances were purchased from commercial sources.

Animals and treatment

Female SPF-Wistar rats (5–10 weeks old) weighing 100–170 g, were purchased from the Zentralinstitut für Versuchstiere, Hannover, F.R.G. For most of the experiments both Altromin 1324 feed and tap water were supplied *ad lib*. In experiments designed to study the effects of the inducers on liver DNA synthesis, rats were adapted to an inverted day–light cycle (dark from 9:00 a.m. to 9:00 p.m.), and food was supplied for 5 hr per day only (from 9:00 a.m. to 2:00 p.m.). This regimen is known to synchronize hepatocellular DNA synthesis [5, 23].

The inducers were administered orally by stomach tube unless indicated otherwise. β-PCH, α-HCH and 3-MC were dissolved in corn oil or olive oil; PB was dissolved in 0.9% NaCl; PCN was ground to a fine powder and suspended in 0.9% NaCl with 0.1% Myrj.

Control animals received carrier or were not treated at all. Oil was administered in doses up to 10 ml/kg. Treatment of the animals was carried out between 8:00 and 10:00 a.m. [^{3}H]Thymidine was injected into a tail vein at the time of maximum DNA synthesis, i.e. approximately 10–11 hr after the start of food consumption [5, 23]. The animals were killed 75 min after thymidine injection. Diethylmaleate (DEM) dissolved in corn oil (14% w/v) was administered i.p. at a dose level of 700 mg/kg. The animals were decapitated between 8:00 and 11:00 a.m., unless stated otherwise. The liver was quickly excised, weighed and cooled to 0°, homogenized and processed to obtain the microsomal fractions. Liver DNA samples were stored at −15° for some days prior to analysis.

Continuous exposure experiments. In order to obtain constant inducer levels in the liver, rats received β-PCH and α-HCH continuously via the diet, and the circadian rhythm of food intake was suppressed. Seventeen-day-old rats of both sexes (in which rhythmic feeding is not yet developed) were adapted to and maintained on continuous light until the end of experimentation. They were weaned on day 20. From day 21 onwards the animals (weighing 25–45 g) were fed diets containing either β-PCH or α-HCH *ad lib* for a period of 10 days. Diets were prepared by mixing with solutions of β-PCH and α-HCH in acetone and subsequent drying in air; inducer concentrations were controlled in random samples by g.l.c. and were 1.7, 9.5, 43 and 83 mg α-HCH/kg diet and 150, 190, 420, 840, 1610, 1670, 3150, 3340, 3980 and 6250 mg β-PCH/kg diet. Food intake and body weights were recorded once daily and additionally from day 4 to 7 in 12-hr intervals. No differences could be detected in feeding activities between the different phases of the 24-hr period. The various α-HCH treatments were started by oral administration of initial doses (D_i) of 2, 11, 52 and 106 mg/kg, resp. followed by exposure to dietary maintenance levels (D_m) of α-HCH, which were calculated according to Dost [24]: $D_m = D_i \cdot \ln 2/t_{0.5}$. An initial dose of β-PCH was not given because it has a half-life of less than 3 hr (see Results).

Extraction procedures

Whole body. Rats were killed with ether, minced and frozen in liquid nitrogen as quickly as possible. The mincing procedure was repeated until a fine frozen powder was obtained. The powder was mixed with anhydrous sodium sulfate (1:1, w/w), ground to dryness, and subjected to exhaustive extraction with acidified acetone (1% HCl) in a Soxhlet apparatus. The extract was concentrated under reduced pressure and the remaining residue was extracted into petroleum ether. Chlorinated hydrocarbons were separated from lipid components by repeated extractions into PC. The combined PC-phases were mixed with water (1:2, v/v) and reextracted with petroleum ether. Completeness of each extraction procedure was controlled by counting radioactive residues. β-PCH and α-HCH were completely separated by three consecutive runs on a silica gel column (experimental details were as described above). Recovery for α-HCH and β-PCH was 97.7–103 per cent.

Livers. Since preliminary experiments had shown that liver β-PCH concentrations rapidly decrease after decapitation, liver samples were taken *in situ* under ether/oxygen anesthesia using a pair of tongs, cooled to −196° in liquid nitrogen (freeze stop method [25]).

Frozen liver (0.5–1.0 g) was weighed immediately and homogenized with 5.0 ml ice-cold 2% perchloric acid. The homogenate was extracted with 5.0 or 10.0 ml *n*-hexane under continuous mixing for 1 hr at room temperature. Compound recovery from liver homogenates were 94 ± 6 per cent for β-PCH and 97 ± 1 per cent for α-HCH.

Blood samples. Blood samples (1 g) obtained by cardiac puncture were mixed with 5.0 ml 0.2 M Soerensen buffer and extracted with 10.0 ml *n*-hexane.

Fat. Retroperitoneal adipose tissue (0.1–0.3 g) was mashed and equilibrated with 5.0 ml *n*-hexane.

The mixture was shaken at regular intervals and concentrations were measured 48 hr later.

Muscle tissue. Samples (0.2–0.5 g) were homogenized in 15.0 ml Soerensen buffer. The homogenate was extracted with 30.0 ml *n*-hexane. Residue analyses on chlorinated hydrocarbons were conducted with g.l.c.

G.L.C.—Conditions and radioactivity measurements

A Hewlett–Packard HP 5730A chromatograph with an 3080A integrator and an 7671A automatic sampler was used. Detector: [^{63}Ni]electron capture detector, 300°; column: glass, 1.28 m, i.d.: 2.4 mm, carrier: gaschrom Q (100–120 mesh), stationary phase: 3% OV-1; column temperature: 125 or 140°; carrier gas: argon–methane (9 + 1), 90 or 40 ml/min, resp.; injection port: 200°. Results were quantitated with the use of an external standard calibration curve.

Radioactivity measurements were conducted using Isocap/300 or Tricarb 3380 scintillation counters. Counting efficiency was calculated with internal or external standardization.

Enzyme assays

The activities of drug-metabolizing enzymes were determined in isolated microsomal fractions *in vitro*. The microsomes were prepared through a standard procedure using differential centrifugation [26]. Unless stated otherwise, pooled microsomal fractions were stored for several days at −15° prior to incubation. Incubation mixtures in a final volume of 0.5 ml contained NADP 5×10^{-4} M, isocitrate 5×10^{-3} M, isocitrate dehydrogenase 20 mU, $MgCl_2$ 3×10^{-3}M, microsomes from 20 mg liver tissue, and one of the following substrates: aminopyrine (AP) 10^{-2}M, ethylmorphine (EM) 5×10^{-3}M, benzphetamine (BPA) 1×10^{-3}M, aniline–HCl (An) 2×10^{-3}M or 4-nitroanisole (pNA) 0.5×10^{-3}M. All substances were dissolved in Soerensen phosphate buffer, pH 7.4, M/15. Samples were incubated for 20 min at 37° in a shaking water bath. Enzyme reaction was stopped by transfer to an ice bath and addition of trichloroacetic acid, 1.8 M, 250 μl.

Metabolic conversion of AP, EM and BPA was monitored by measurements of formaldehyde production [24]. *p*-Nitroanisole-*O*-demethylation was assayed by measuring *p*-nitrophenol formation [28] and aniline metabolism by analysis of *p*-aminophenol formation [29]. Ethoxyresorufin-deethylation was assayed by recording the initial velocity of resorufin formation [30, 31], using a Zeiss spectrofluorimeter with two monochromators. Diphenyloxazole (PPO) metabolism was assayed as described by Cantrell *et al.* [32]. Fluorescence intensities of the extracted (still unidentified) phenol were used to compare enzyme activities. Benzo[*a*]pyrene (BP) hydroxylase activity was assayed with a slight modification [33] of the method described by Gielen *et al.* [34].

All enzyme assays were conducted in triplicate. Liver cytochrome P-450 content was assayed with CO-difference spectra. Each cuvette contained 1.3 mg microsomal protein per ml. The concentration of cytochrome P-450 was calculated using the extinction coefficient $\varepsilon_{450\text{-}490} = 91 \times 10^3$ l/mol/cm [35].

DNA, glutathione and protein assays

For DNA assays liver samples (1 g) were homogenized in 10.0 ml 0.2 M ice-cold perchloric acid. After centrifugation the pellet was washed twice with 0.2 M perchloric acid. The pellet was dried with ethanol/ether (3:1) and ether (100%). Ether residues were allowed to evaporate and the dried pellet was subsequently resuspended in 1.0 ml of 0.5 M perchloric acid followed by extraction for 30 min at 80°. After centrifugation DNA was assayed colorimetrically in the supernatant [36].

Reduced glutathione (GSH) was assayed as non-protein-thiol using Ellmans reagent [38]. Protein was determined as described by Lowry *et al.* [39].

Statistical analyses

Means and standard deviations are given. Treatment means were tested on significance using Student's *t*-test.

RESULTS

(1) *Some pharmacokinetic properties of β-PCH.* Administration of β-PCH to rats was well tolerated. Exposure to 700 mg β-PCH/kg body wt daily for 10 days via the diet had no effects on body growth or animal behaviour. However, 930 mg/kg daily resulted in suppressed body growth. A single dose

Table 1. Organ concentrations of β-PCH after application of a single dose of the compound*

	Experiment I: 200 mg/kg p.o.			Experiment II: 300 mg/kg p.o.	
Time (hr)	Blood (μg/g)	Muscle (μg/g)	Fat (μg/g)	Liver (μg/g)	Fat (μg/g)
1	0.6 ± 0.14	1.9 ± 0.6	7.5 ± 3	1.7 ± 0.6	14.9 ± 8.3
2	0.7 ± 0.3	1.4 ± 0.5	27 ± 13	—	—
3	0.9 ± 0.4	2.4 ± 1.3	41 ± 16	—	—
4	—	—	—	1.7 ± 0.2	63 ± 29
5	0.9 ± 0.4	2.4 ± 0.7	64 ± 4	—	—
8	0.8 ± 0.1	2.3 ± 0.8	76 ± 15	6.1 ± 1.5	280 ± 175
12	—	—	—	2.6 ± 0.3	166 ± 44
24	0.1 ± 0.04	0.4 ± 0.06	53 ± 8	—	—
72	0.08	0.2	3.6 ± 1.8	0.04 ± 0.004	0.52 ± 0.6

* Results expressed as mean ± S.D. from three to five animals.

Table 2. Liver growth induced by β-PCH and α-HCH in rats*

Treatment (mg/kg)	No. of animals	Relative liver weight	Liver DNA mg/g liver	%	Liver DNA mg/100 g body wt	%
None	13	4.4 ± 0.4	2.87 ± 0.19	100	12.7 ± 1.01	100
β-PCH						
6 × 150	6	4.4 ± 0.2	—	—	—	—
6 × 450	6	5.7 ± 0.3‡	2.43 ± 0.14‡	85	13.8 ± 0.54†	109
α-HCH						
2 × 200	5	5.9 ± 0.7‡	2.63 ± 0.26†	92	15.6 ± 1.12‡	123

* Animals (120–150 g) were killed 144 hr after beginning of treatment.
† $P < 0.05$.
‡ $P < 0.001$.

of 1020 mg/kg i.p. killed the animals (2/2 rats) within 36 hr.

Absorption of β-PCH administered either i.p. or orally was almost complete, and the rate of absorption increased in the order s.c. < p.o. < i.p. Four hours after i.p. administration of 200 mg/kg (6 rats) or 1020 mg/kg (4 rats) only 1–5 per cent of the β-PCH dose was found in the abdominal cavity. Eight hours after oral administration of β-PCH (200 mg/kg) 25 per cent of the dose was found, and 24 hours after application only traces were found unchanged in the contents of the gastro-intestinal tract. In feces collected during 72 hr after oral administration only 1 per cent of the dose could be detected. After a subcutaneous application (500 mg/kg) β-PCH concentration reached its maximum in retroperitoneal fat tissue not earlier than 24 hr after s.c. application, which indicates that complete absorption of the compound may at least require a similar time interval.

Organ distribution of a single oral dose of β-PCH is shown in Table 1. Highest concentrations were reached in adipose tissue and were, at the time of maximum organ concentrations, almost 100-fold higher than in blood. α-HCH is known to be distributed similarly, the fat:blood concentration ratio being nearly 200 [39]. As the partition coefficients in *n*-heptane:water were 1010 for β-PCH and 1930 for α-HCH, the similarity in organ distribution was not unexpected.

β-PCH elimination from the organs studied was almost complete 72 hr after administration (Table 1). Calculations of elimination rates from these data showed that half-life of β-PCH was in the region of a few hours. In another experiment only 30–35 per cent of a single i.p. dose of 200 mg/kg β-PCH were still present in the bodies of the animals 4 hr after application, suggesting that half of the dose disappeared within 2.5 hr (mean ± S.D. from 4 rats: 2.5 ± 0.6 hr; for experimental conditions: see Table 5). In contrast, the half-life of α-HCH in the liver and fat was calculated to be approximately 130 hr (data from ref. 40). That β-PCH disappears from the body much more rapidly than α-HCH is also shown by findings reported below (section 3).

(2) *Comparative studies on the effects of β-PCH and α-HCH on rat liver.* β-PCH was administered to rats at various dose levels. Six applications of 450 mg β-PCH/kg body wt caused approximately 30 per cent liver enlargement (Table 2). This effect was accompanied by decreases of liver DNA concentration; however, the total liver DNA content was slightly increased (9 per cent). These effects were similar, although less pronounced, than those seen after 2 × 200 mg α-HCH/kg. Liver DNA synthesis in β-PCH treated rats was also enhanced (Table 3); this effect, too, was less pronounced than that produced by α-HCH. These results indicate that β-PCH, like its parent compound α-HCH, is an inducer of liver growth. However, the effects of β-PCH seem to require higher dose levels and this may be

Table 3. Liver DNA synthesis after oral treatment with α-HCH and β-PCH*

Treatment	Dose mmoles/kg	Dose mg/kg	Experiment I (dpm/μg DNA)	Experiment II (dpm/μg DNA)
None	—	—	13.2 ± 5.4 (11)	
β-PCH	0.17	45	29.4 ± 21.2 (5)	8.1 ± 1.9 (5)
	0.52	135	16.7 ± 5.6 (5)	13.9 ± 3.8 (5)
	1.18	300	—	28.8 ± 9.6 (5)†
	1.59	405	51.9 ± 22.6 (5)†	—
α-HCH	0.17	50	37.9 ± 18.9 (5)†	—
	0.52	150	94.1 ± 37.7 (5)†	64 ± 14.3 (4)†

* Body weights: 110–120 g. Number of animals in brackets. In experiment II, β-PCH was administered i.p.
† $P < 0.005$.

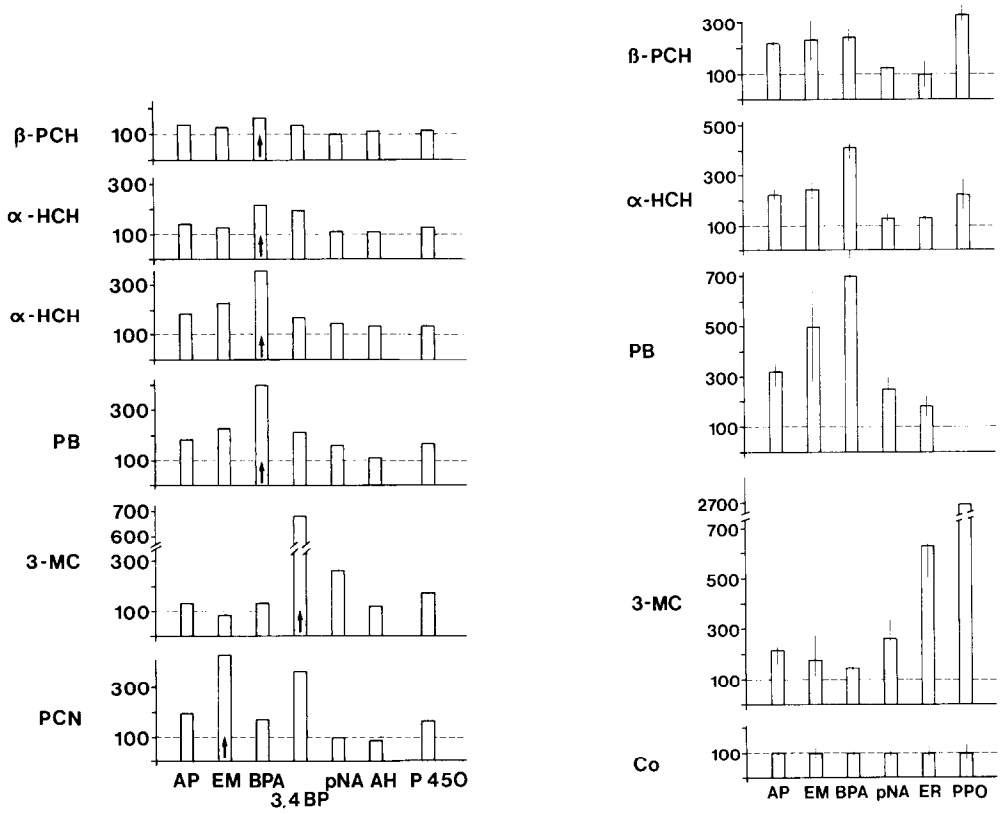

Fig. 1. Induction of rat liver microsomal mono-oxygenases by β-PCH, α-HCH and other stimuli. Panel A: Six rats (150–180 g) per group were treated as follows (doses in mg/kg): β-PCH i.p.: 3 × 300 (1); α-HCH, i.p.: 1 × 50 (3) (upper row); p.o.: 150 + 100 (2) (lower row); PB p.o.: 6 × 100 (1); 3-MC p.o.: 6 × 20 (1); PCN p.o.: 6 × 100 (1). Repeated doses were given on consecutive days. In brackets: days between last treatment and death of animal. Control activities (= 100 per cent) expressed per mg of microsomal protein and 20 min incubation time were as follows: AP = 67, EM = 33, BPA = 47 nmoles formaldehyde; pNA = 10.8 nmoles *p*-nitrophenol; An = 8.3 nmoles *p*-aminophenol; BP = 1.37 nmoles 3-OH-BP/mg protein/10 min; P-450 = 0.67 nmoles/mg microsomal protein. Panel B: Three to four rats (80–90 g) per group were treated as follows (dose in mg/kg): β-PCH s.c.: 1 × 500 + 2 × 300; α-HCH p.o.: 1 × 100; PB p.o.: 3 × 100; 3-MC p.o.: 3 × 20. Repeated doses were given on consecutive days. Decapitation was on the fourth day after the beginning of treatment. Control (CO) activities (= 100 per cent) were: AP = 43.7, EM = 23, BPA = 40 nmoles formaldehyde, pNA = 10.8 nmoles *p*-nitrophenol/mg microsomal protein/20 min; ER = 8.5 pmoles resorufin/mg protein/min.

explained by the shorter half-life of β-PCH (see above).

Both β-PCH and α-HCH enhanced the activities of the hepatic monooxygenase system, as evidenced by increases of cytochrome P-450 levels (+19 per cent) and by enhanced turnover rates of AP, EM and BPA (Fig. 1). Biotransformation of other substrates, i.e. pNA, An, BP, ER, PPO, was increased to a lesser extent or remained unchanged. These different substrates were used to test the specificity of the enzyme(s) induced. β-PCH and α-HCH had no pronounced effects on P-448 specific substrates, such as BP, ER and PPO, and this suggests that the compounds do not belong to the class of '3-MC-type' microsomal enzyme inducers. This confirms results of a previous study in which α-HCH was found to be an inducer of the phenobarbital type [40]. PB pretreatment produces increases in turnover rates of *N*-demethylases in the order AP<EM<BPA (Fig. 1). Relative increases (related to AP = 1) were 1:1.3–1.5:2.2 (AP:EM:BPA). β-PCH tended to induce a similar pattern of enzyme activities. This could be shown most clearly in an experiment with continuous feeding of the inducers for ten days (Fig. 3, see below). Turnover of the substrates AP:EM:BPA increased in a relation of 1:1.3:1.9 as calculated from the slopes of the concentration–response curves. α-HCH produced a similar pattern, as characterized by a 1:1.8:2.3 relation of the slopes (Fig. 3). After pretreatment of rats with PCN, which is known to be a different type of inducer [40, 41, cf. Fig. 1A], this relation was 1:2.3:0.9 (calculated from the date shown in Fig. 1A). These findings strongly suggest that β-PCH and α-HCH induce the same cytochrome P-450 species.

(3) *Inductive potential of β-PCH and α-HCH.* In view of the widely differing half-lives of β-PCH and α-HCH, the inductive potentials could not be compared on the basis of the administered doses; instead, liver concentrations were considered to provide a more useful basis for comparisons. Rats were exposed to various dietary levels of both compounds

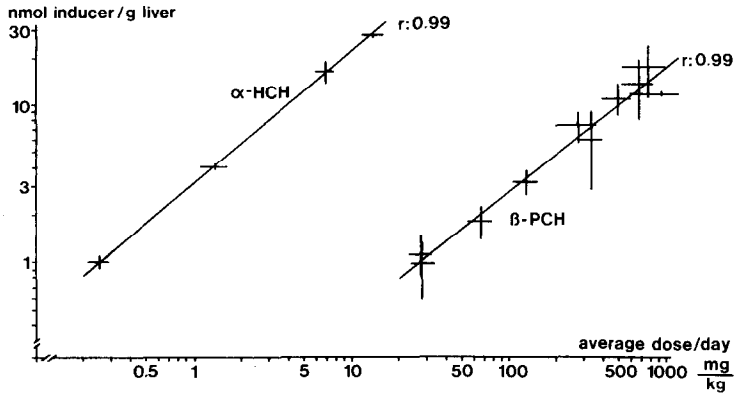

Fig. 2. Relationship between dose levels and liver concentration of β-PCH and α-HCH after application for 10 days. Doses were calculated daily on the basis of food intake and body weights, means ± S.D. are indicated by horizontal bars. Liver concentrations were measured in individual animals by g.l.c., means ± S.D. are shown by vertical bars. Correlation coefficients (r) are indicated. For other details see Materials and Methods.

for a period of 10 days in an attempt to produce steady-state liver concentrations. Daily exposure levels, calculated on the basis of food intake, are shown in Fig. 2 and ranged from 0.2 to 14 mg α-HCH/kg body wt and from 27 to 930 mg β-PCH/kg body wt. Liver concentrations increased linearly with the dose in double logarithmic (Fig. 2) and linear plots. As expected from the different half-lives of the two compounds, maintenance of equimolar liver concentrations required 100-fold-higher doses of β-PCH than of α-HCH. Hepatic concentrations of the two compounds observed after 9 and 9.5 days of feeding were similar to those found at the end of the experimental period (Table 4). This suggests that liver concentrations had reached a steady state and that no major circadian fluctuations were present.

Relative liver weights increased linearly with the logarithm of the respective inducer concentrations (Fig. 3). High doses of β-PCH, i.e. 1000 mg/kg body wt, caused depressed body growth, which resulted in overproportional increases in relative liver weight. These results were not included for estimations of threshold concentrations in liver. On a molar basis, β-PCH appeared to be a more potent inducer of liver enlargement than α-HCH. Three nanomoles β-PCH/g liver was associated with liver enlargement similar to that seen in rats with 30 nmoles α-HCH/g liver. Threshold concentrations (in nmoles/g liver) for the induction of liver growth were calculated to be 0.5 for β-PCH and 1.5 for α-HCH. β-PCH also appeared to be a more potent inducer of microsomal *N*-demethylase activities than α-HCH (Fig. 3). Eight nanomoles β-PCH/g liver produced an increase in aminopyrine *N*-demethylase activity similar to that seen in livers containing 30 nmoles α-HCH/g liver. Threshold concentrations for the induction of liver monooxygenases were 0.4–0.6 nmoles/g liver for β-PCH and 0.7–1.3 nmoles/g liver for α-HCH.*

The results of the experiment shown in Figs. 2 and 3 indicate that a dose of 300 mg β-PCH/kg daily, when administered orally at a constant rate throughout the day, induces increases in activities demethylating AP, EM and BPA by +100, +115 and +180 per cent. In contrast, i.p. application of the same dose once daily (Fig. 1A) produced considerably smaller increases (+37, +29 and +64 per cent). Although the two experiments are not entirely comparable, these observations suggest that continuous exposure to β-PCH is more effective than a single pulse-like i.p. application. This suggestion is supported by the considerable increase of demethylating activity following s.c. treatment with similar doses of β-PCH (Fig. 1B). As noted above, absorption of β-PCH from a s.c. depot is relatively slow.

* It is of interest to note that dieldrin threshold concentrations in rodent liver are similar to those reported in this study for α-HCH and β-PCH ([42], and private communication of A. S. Wright).

Table 4. Liver concentrations of β-PCH and α-HCH after feeding for 9–10 days*

Compound	Intake per day (mg/kg body wt)	9	9.5	10
		nmoles inducer/g liver Duration of treatment (days)		
α-HCH	1.35 ± 0.26‡	4.1	3.6/4.3	4.2/3.9/4.0//4.2†
β-PCH	493 ± 107‡	9.3/11.5	9.6/10.4	5.0/8.2/15//9.8†

* Livers were analysed for β-PCH and α-HCH concentrations 24 and 12 hr before and at the end of experiment. Results shown are from single rats or, where indicated by †, were calculated from Fig. 2.

‡ Means ± S.D. of daily intake.

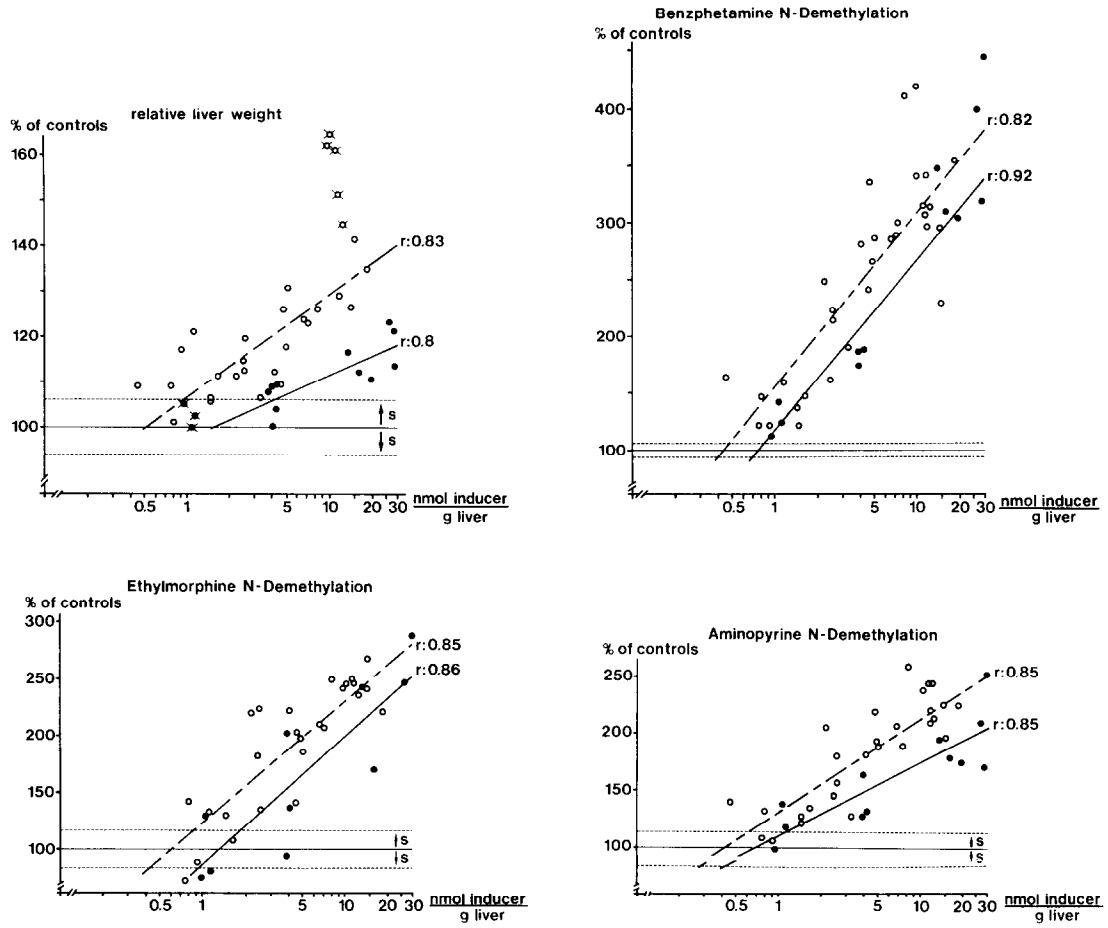

Fig. 3. Relationship between log liver concentration and inductive effects of β-PCH and α-HCH after application for 10 days. ○: β-PCH, ●: α-HCH. Results from individual treated animals are indicated and are expressed as a percentage of the mean of the control group (eight animals). 100 per cent were: 4.08 g liver/100 g body wt; AP = 70, EM = 50, BPA = 46 nmoles HCHO/mg microsomal protein/20 min ± S.D. of controls is indicated by the dotted lines. ⊠, ⊠: values not included in the calculation of regression lines and coefficients. For other details see Materials and Methods and legend to Fig. 2.

It seemed of interest from a practical toxicological point of view to calculate threshold doses for the induction of liver growth and enzyme increases by continuous administration of α-HCH and β-PCH (Figs. 2 and 3). Values found were 0.3–0.5 mg (1.0–1.7 μmoles) α-HCH/kg and day and 8–11 mg (30–43 μmoles) β-PCH/kg and day.

(4) *Formation of β-PCH from α-HCH.* In a first, preliminary attempt to prove the formation of β-PCH from α-HCH in the whole rat, [^{14}C]α-HCH was administered and 'cold' β-PCH was given 12 hr later in order to trap any [^{14}C]β-PCH. At this time (12 hr) liver α-HCH concentration has reached its maximum [39]. The rats were killed 4 hr later, when liver β-PCH concentrations were maximal (data not shown). The whole body was immediately homogenized and, after separation of β-PCH from α-HCH, radioactivity found in the β-PCH fraction was calculated as a percentage of that in the α-HCH fraction. Results varied considerably and an average of 1.45 per cent was found (Table 5). A control rat received radioactive α-HCH i.p. after being killed and was processed immediately. Here 1.7 per cent of the radioactive α-HCH was degraded to β-PCH. Only a little β-PCH was formed during the analytical procedure (Table 5). These findings suggest that α-HCH is degraded to β-PCH in the rat, but it is not clear whether degradation occurs by enzymic or non-enzymic catalytic effects of biological material.

In vitro, β-PCH is formed slowly from α-HCH in aqueous solution at physiological pH (Table 6); β-PCH formation was enhanced several-fold by addition of denatured liver homogenate and even more by addition of native serum albumin. These results show that β-PCH can be formed from α-HCH in the presence of unspecific protein. Formation of β-PCH by similar mechanisms should be possible also *in vivo*.

Small amounts of β-PCH (less than 0.1 per cent of the α-HCH present) were consistently found in livers and adipose tissue of α-HCH treated rats (Table 7). *In vitro*, β-PCH has been shown to accumulate during incubation of α-HCH with liver cytosol protein prepared by acetone precipitation and thus being free of GSH [18]. Therefore, we depleted α-HCH treated rats of most (90 per cent) of their

Table 5. Formation of [^{14}C]β-PCH in rats treated with [^{14}C]α-HCH*

Experiments	Recovery of radioactivity (% of applied dose)	α-HCH specific activity: Prior to treatment (dpm/mmole)	α-HCH specific activity: After termination of experiment (dpm/mmole)	β-PCH/α-HCH-ratio in the purified fractions (%)
Rat 1	70	2054	1921	2.8
Rat 2	72	1092	1020	0.6
Rat 3	73	1092	1081	0.95
				Mean 1.45 ± 1.18
Rat 4 (control)	78	1092	1072	1.72
Procedure without rat	97.7	1962	2051	0.16

* Rats were treated with [^{14}C]α-HCH p.o. (160 mg/kg). Twelve hours later they received cold β-PCH i.p. (200 mg/kg), and were killed 4 hr after β-PCH treatment. The control rat was killed by ether and received 200 mg/kg [^{14}C]α-HCH i.p.

liver GSH. This procedure resulted in small but reproducible (three experiments) increases of β-PCH concentration in liver and adipose tissue (Table 7).

Even under conditions of GSH-depletion, liver β-PCH concentrations in α-HCH treated rats were 0.38 nmoles/g at maximum (Table 7), which is below the observed threshold concentration for induction of liver growth and drug metabolizing enzymes (0.4–0.7 nmoles/g liver, Fig. 3). These results strongly suggest that the rate of β-PCH formation from α-HCH *in vivo* is not sufficient to induce the changes produced in rat liver by the latter compound.

DISCUSSION

The present investigation adds support to previous suggestions [18] that β-PCH is an intermediate in α-HCH metabolism *in vivo*. It is shown that β-PCH is formed *in vivo* from α-HCH. These results are in agreement with previous studies in which trace amounts of β-PCH were demonstrated in α-HCH treated rats [15, 19]. That the amounts of β-PCH found in α-HCH treated rats are very small is easily explained by the short half-life of β-PCH. The formation of a common metabolite, i.e. an isomeric pentachlorocyclohexene (356/4-PCH), in the livers of rats treated with either α-HCH or β-PCH provides additional support for the intermediary role of β-PCH in α-HCH metabolism (present study, data not shown).* Other metabolites common to α-HCH and β-PCH, i.e. dichlorophenylmercapturic acids (DCPMA), have been obtained *in vitro* after incubation with cytosol or with an isolated GSH-*S*-transferase (E.C. 2.5.1.18) in the presence of GSH [18]. The relative proportions of the 4 DCPMA isomers formed from α-HCH or β-PCH were almost identical, but were different from those obtained with other HCH's or PCH's.

In vitro formation of β-PCH from α-HCH is shown to occur by unspecific catalysis of proteins. The turnover rates of α-HCH in the presence of denatured liver protein (10 pmoles/hr/mg protein) or serum albumin (25 pmoles/hr/mg protein) would be sufficiently high to account for β-PCH accumulation in the liver *in vivo*, as observed in the experiment shown in Table 7 (approximately 0.5 pmoles/hr/mg protein). However, enzymic catalysis also seems to participate in α-HCH degradation [18, 43, 44]. The isolated enzyme was shown to catalyze α-HCH dechlorination *in vitro* much more effectively (V_{max} = 300 nmoles/hr/mg protein [43]) than did denatured liver protein or serum albumin. Therefore, degradation of α-HCH to β-PCH *in vivo* may occur by both enzymic and non-enzymic catalysis.

Since α-HCH elimination follows almost first order kinetics with a half-life of about 130 hr (see above and ref. 40), it may be calculated that 0.5 per cent of the dose is degraded per hour. From 10 to 20 per cent of a single dose of α-HCH is excreted in the form of DCPMA (J. Portig, personal communication). Hence, approximately 0.05–0.1 per cent per hour is metabolized via GSH-conjugation. It is of interest that the rate of β-PCH accumulation in liver and adipose tissue of α-HCH treated, GSH-depleted rats appears to be of the same order of magnitude (0.02–0.04 mole% per hr, as estimated from the data shown in Table 7). These findings are in agreement with the assumption that β-PCH is an intermediate of GSH-dependent α-HCH metabolism.

The concentration of 0.2–0.4 nmole β-PCH/g of tissue, as found in the liver of α-HCH treated rats, is below the observed threshold concentration for induction of liver growth and hepatic monooxygenase(s). Since β-PCH concentration was measured at the time of maximum α-HCH levels in the liver [39], it may be concluded that the amount of β-PCH formed will at no time suffice to make a significant contribution to the effects of α-HCH. The findings make it also unlikely that metabolites of β-PCH mediate the α-HCH effects. One might speculate that intermediates of the P-450-dependent pathway of α-HCH metabolism could be involved. In this pathway the only intermediary products sufficiently stable appeared to be tri- and tetrachlorophenols [14, 45]. However, several chlorophenols, namely

* Identification of 356/4-PCH rested on combined g.l.c.–mass-spectrometry and g.l.c. on two different stationary phases (OV1 3% and DEGS 15%). Isomerization of β-PCH to 356/4-PCH can be explained by hydrogen–chlorine exchange at the double bond.

Table 6. β-PCH formation from α-HCH *in vitro**

Incubation time (hr)	Soerensen buffer pH 6 pmoles/ml	%	Soerensen buffer pH 7.4 pmoles/ml	%	Denatured liver homogenate pmoles/mg protein	%	Serum albumin pmoles/mg protein	%
0	0	0	1.7	0.02	2.6	0.07	1.4	0.06
3	1.9	0.022	4.7	0.055	29	0.7	78.2	3.4
6	1.9	0.022	12	0.14	53	1.4	128	5.5
24	4.6	0.053	39	0.45	173	4.4	220	9.5

* The incubation mixture (2.0 ml) contained 5 μg α-HCH (17.2 nmoles) in Soerensen buffer and 2.2 mg/ml denatured liver protein or 3.7 mg/ml bovine serum albumin as indicated. pH was 7.4 unless stated otherwise. The reaction was studied at 37° for the periods shown. Liver protein was denatured by $HClO_4$, followed by boiling for 5 min.

trichlorophenols [46, 47] and pentachlorophenol [47–49], were shown to be essentially ineffective as inducers of liver growth and of phenobarbital-inducible monooxygenases. In conclusion, there is no evidence to indicate that any metabolite of α-HCH is responsible for the changes induced in rat liver by this agent. This suggests that induction of liver growth and of hepatic monooxygenase activities is essentially due to direct action of the parent compound α-HCH.

In most studies on induction of liver growth and drug-metabolizing enzymes reported so far, inducers with relatively long biological half-lives have been used. Substances with short half-lives were frequently found to be weak inducers, e.g. short-lived barbiturates [50–52]. However, the present study shows that β-PCH as a short-lived inducer, when administered at a sufficiently high level and at a constant rate rather than as a single i.p. 'pulse', causes quantitatively similar effects to long-lived agents such as α-HCH or phenobarbital. Based on steady state concentrations in the target organ, β-PCH was even more potent than α-HCH. These findings provide experimental support for the contention that an inducer must be present at its target for a certain critical period of time in order to induce the eventual initiation of DNA replication and of cytochrome P-450 synthesis. β-PCH may be a useful tool to study the nature of early inducer-dependent steps in the induction process.

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Table 7. β-PCH in liver and fat tissue of rats treated with α-HCH*

Treatment	μmoles GSH/g liver	nmoles/g organ α-HCH	nmoles/g organ β-PCH	$\frac{\text{nmoles } \beta\text{-PCH}}{\text{nmoles } \alpha\text{-HCH}} \times 100$
Liver				
None	6.6	—	—	—
α-HCH†	4.1 ± 0.9	327 ± 25	0.22 ± 0.046	0.067 ± 0.016
α-HCH + DEM‡	0.57 ± 0.03	312 ± 45	0.27 ± 0.02	0.086 ± 0.009‖
α-HCH + DEM§	0.4 ± 0.09	408 ± 85	0.38 ± 0.08	0.094 ± 0.021¶
Fat				
None		—	—	—
α-HCH†		5403 ± 1300	2.75 ± 0.76	0.052 ± 0.016
α-HCH + DEM‡		4553 ± 740	3.34 ± 0.75	0.075 ± 0.022‖
α-HCH + DEM§		5703 ± 931	4.63 ± 1.06	0.085 ± 0.014¶

* The β-PCH fraction included 10–20 per cent 356/4-PCH (see Discussion).
† α-HCH (200 mg/kg p.o.) was administered 13.5 hr prior to kill.
‡ 700 mg DEM/kg i.p., 30 min prior to kill.
§ Three i.p. applications: an initial dose of 700 mg DEM/kg, 1.5 hr prior to kill, and two subsequent treatments with 300 mg DEM/kg at half hourly intervals.
‖ $P < 0.05$. Five animals per treatment group.
¶ $P < 0.01$. Five animals per treatment group.

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