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Anaerobic Formation of α-Hexachlorocyclohexane from γ-Hexachlorocyclohexane in Soil and by *Escherichia coli*

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In two soil types small amounts of α-hexachlorocyclohexane were formed from γ-hexachlorocyclohexane under submerged conditions, but not under aerobic conditions. In the submerged soils γ-tetrachlorocyclohexene was detected as a major metabolite of γ-hexachlorocyclohexane, whereas aerobic soil incubation yielded γ-pentachlorocyclohexene. Growing cultures of *Escherichia coli* produced α-hexachlorocyclohexane from the γ-isomer only upon anaerobic incubation. Moreover, heavy, washed suspensions of *E. coli* incubated anaerobically with γ-hexachlorocyclohexane (7.5 mg/liter) in a glucose-phosphate buffer medium converted 1.6% of the γ-hexachlorocyclohexane into the α-isomer in 10 days. Growing mycelia of *Aspergillus niger* incubated with γ-hexachlorocyclohexane produced no α-isomer.

INTRODUCTION

In many countries the use of the insecticide hexachlorocyclohexane (HCH)[1] has been restricted to its γ-isomer (lindane). Yet, analysis of certain food commodities in these countries reveals the presence of α-HCH. This may be partly attributed to the presence of α-HCH in animal feed imported from countries where the crude HCH mixture is still used. However, as another possible source of α-HCH its formation from γ-HCH by microorganisms in the environment was indicated by several authors. Newland *et al.* (1) observed formation of α- and δ-HCH from γ-HCH in aquatic sediments. More recently it has been shown (2, 3) that cultures of *Pseudomonas putida* formed small amounts of α-HCH from γ-HCH in the presence of NAD. However, in the reports of these studies it has not been indicated whether aerobic or anaerobic incubation conditions had been applied. Kohnen *et al.* (4) found formation of α-HCH from γ-HCH in submerged soil, but not in aerobic soil. No further study was made of this phenomenon. The formation of α-HCH in mixed anaerobic bacterial cultures obtained from soil or rumen has also been mentioned (5, 6).

Since the role of oxygen in the formation of α-HCH had never been investigated, we compared the formation of α-HCH from γ-HCH in soil and by pure cultures of microorganisms under aerobic and anaerobic conditions. This paper describes the results of these investigations.

MATERIALS AND METHODS

Soils. Two soil types were used, viz. a sandy loam and a silt loam. Characteristics of the soils are given in Table 1. The sandy loam was obtained from Tollebeek, North East Polder, The Netherlands, and the silt loam was taken from Schinnen, Limburg, The Netherlands.

Incubation of the soils. The soils were air dried and screened through a 2-mm sieve. Samples of soil (50 g wet wt) were placed in conical flasks (300 ml) and treated with a solution of 0.375 mg of γ-HCH in 0.5 ml of acetone. After treatment the flasks were closed with a cotton plug. Subsequently the soils were mixed thoroughly

[1] Abbreviations used: γ-HCH, 1α,2α,3β,4α,5α,6β-hexachlorocyclohexane; α-HCH, 1α,2α,3β,4β,5α,6β-hexachlorocyclohexane; γ-PCCH, 2,3α,4β,5β,6α-pentachlorocyclohex-1-ene; γ-TCCH, 3α,4α,5β,6α-tetrachlorocyclohex-1-ene.

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TABLE 1

Chemical and Physical Characteristics of the Soils Used

	Silt loam	Sandy loam
Organic matter content (g/100 g)[a]	2.3	4.3
Moisture content (%)[b]	35	41
pH (KCl)	6.0	7.3
Granulation (%)		
Clay (<2 μm)	15.6	12.7
Silt (2–50 μm)	77.5	33.3
Sand (>50 μm)	7.1	54.0

[a] Data refer to dry soil.

[b] Of maximum water-holding capacity.

for 1 hr by mechanical shaking of the flasks. After adjusting the soil moisture content to the original value by the addition of distilled water, the flasks were shaken again for 1 hr. The final concentration of γ-HCH in the soil was 7.5 mg/kg.

Aerobic soils were incubated at 24°C in a moist box. To obtain submerged conditions 50 ml of H_2O was added to the soil samples. This was enough to keep a ca. 1-cm layer of water over them. Incubation was carried out at 24°C in the dark. Submerged soils become largely anaerobic after a short period.

Sterilization of the soils was carried out by Gammaster (Ede, The Netherlands) with 2.4 Mrad of γ-rays.

Microorganisms. Aerobic incubation of *Escherichia coli* B with γ-HCH was performed in shake cultures in conical flasks (3 l) containing 500 ml of peptone–glucose medium, containing 1% Bactopeptone, 1% glucose, 0.4% K_2HPO_4, and 0.1% KH_2PO_4 in tap water, final pH 7. A solution of γ-HCH in acetone (0.5 ml) was added to the autoclaved, still warm medium, to give a final γ-HCH concentration of 7.5 mg/liter. After cooling, the flasks were inoculated with a bacterial suspension from agar slants. Anaerobic incubation was carried out in round-bottom flasks (1 l) containing 500 ml of peptone–glucose medium. After autoclaving and adding γ-HCH (final concentration 7.5 mg/l) and the inoculum, the flasks were provided with a goose-neck shaped water seal. Subsequently the air above the culture medium was removed by evacuation and replaced by nitrogen. Incubation was carried out at 37°C.

To obtain cells for heavy, washed suspensions, *E. coli* was grown overnight under aerobic conditions in a LKB 1601 fermentor containing 3 liters of peptone–glucose medium. Thereafter 200 ml of a concentrated glucose solution (15%) sterilized by filtration was added and further growth was allowed for 2 hr. The bacteria were then harvested by sterile centrifuging, washed twice with 0.5% phosphate buffer (pH 7), and resuspended (13 mg dry weight/ml) in 0.5% phosphate buffer or in 0.5% phosphate buffer containing 1% glucose. Incubation with γ-HCH was carried out under nitrogen in 150 ml round-bottom flasks containing 60 ml of heavy suspension. The flasks were provided with a water seal.

To obtain spent medium, *E. coli* was grown in peptone–glucose medium for 14 days under anaerobic conditions. Bacteria were harvested by centrifuging and the spent medium was filter-sterilized. Five hundred milliliters of the spent medium was incubated anaerobically with γ-HCH (7.5 mg/l) for 14 days.

Experiments with *A. niger* were carried out in glucose–mineral salts medium, pH 7 (7). γ-HCH, final concentration 7.5 mg/liter, was added as a solution in acetone (0.5 ml) to the autoclaved medium.

Chemicals. γ-HCH was crystallized repeatedly from ethanol to obtain an α-HCH content <7.5 mg/kg.

γ-PCCH was prepared according to Reed

and Forgash (8) by dehydrohalogenation of γ-HCH with a solution of NaOH in acetone. The crude preparation was chromatographed on a silicagel column and distilled at 90°C/1.5 mm Hg (Lit.: 115–6°C/4 mm Hg).

γ-TCCH was prepared from γ-HCH by treatment with zinc in ethanol according to Beland et al. (9). The compound was purified by fractioned crystallization from hexane, followed by preparative tlc on Al_2O_3 with cyclohexane. After elution with $CHCl_3$ the compound was crystallized from hexane to give white crystals, mp 89°C. Orloff *et al.* (10) reported a mp of 89°C.

All solvents were analytical grade.

Extraction and gas–liquid chromatography (glc). All samples were analyzed immediately after termination of the incubation.

Both submerged and aerobic soils were analyzed in the same way, except that to aerobic soils 50 ml of water was added prior to the extraction. Samples were extracted with three 50-ml portions of acetone by mechanical stirring. The combined extracts were diluted with 250 ml of a 2% aqueous sodium sulfate solution and extracted with two 25-ml portions of hexane. The combined hexane fractions were washed with 50 ml of sodium sulfate solution and analyzed by gas–liquid chromatography.

Of the cultures usually 50 ml was shaken with 50 ml of hexane. Emulsions were broken by centrifugation. The hexane fraction was analyzed by gas–liquid chromatography.

Gas liquid chromatography with electron capture detection was performed on a Carlo-Erba Fractovap GB gas chromatograph, fitted with two 1.80 × 4-mm i.d. glass columns, packed with 3% OV-210 and 1.8% OV-1/2.7% OV-210 on Gas Chrom Q 80/100 mesh, respectively. Isothermal GLC was conducted at 170°C and the carrier gas used was nitrogen at a flow rate of about 30 ml/min.

The effectiveness of the analytical method was checked by addition of suitable amounts of the compounds involved to samples incubated without the presence of γ-HCH. Recoveries were better than 90% and the results reported were not corrected for these percentages.

The present analytical method was not designed for the determination of β-hexachlorocyclohexane.

Chromatograms obtained upon γ-HCH injections showed also peaks of γ-PCCH and γ-TCCH, caused by on-column decomposition of γ-HCH. This decomposition ranged from 0.05–0.2% of the amount of γ-HCH injected, depending on the condition of the columns. Besides the presence of sample background peaks, especially this phenomenon caused higher detection limits for γ-PCCH and γ-TCCH compared to that of α-HCH although the response factors of these compounds are of the same order of magnitude.

Gas–liquid chromatography–mass spectrometry. The presence of γ-PCCH, γ-TCCH, and α-HCH was confirmed in some samples by 70 eV electron impact mass spectrometry using a Varian MAT 112 mass spectrometer equipped with a Varian 2700 gas chromatograph and coupled to a Varian SS 100 computer system. A 50 m × 0.5 mm-i.d. SE-30 coated glass capillary column was used at 150°C with helium as the carrier gas at a flow rate of about 3 ml/min.

RESULTS

Soil

Upon submerged incubation, detectable amounts of α-HCH were present after a 4-weeks incubation period (Table 2). Within this period most of the oxygen in the soils had been consumed and largely anaerobic conditions had been established. This was evident by formation of black deposits of iron(II)sulfide in the soil samples. After 8 weeks the concentrations of α-HCH were 14 and 4 μg/kg in the sandy loam and the silt loam, respectively. γ-PCCH was detected as a major conversion product under aerobic conditions, whereas γ-TCCH was a major product under submerged conditions. The identity of α-HCH, γ-TCCH, and γ-PCCH was confirmed by gas chromatogra-

TABLE 2

Formation of α-HCH, γ-PCCH, and γ-TCCH upon Incubation of Two Soil Types with γ-HCH (7.5 mg/kg) under Aerobic and Anaerobic Conditions

Soil	Condition	Incubation period (weeks)	Percentage γ-HCH recovered	Compounds formed (μg/kg)		
				α-HCH	γ-PCCH	γ-TCCH
Sandy loam	Anaerobic	4	61(59)[a]	5.4(6.2)	—[b]	40(40)
		8	27(27)	14.0(13.4)	—	Trace(trace)[c]
Silt loam	Anaerobic	4	56(45)	Trace(trace)	—	100(80)
		8	29(29)	4.0(3.6)	—	20(20)
Sandy loam	Aerobic	8	85(85)	—	120(100)	—
		16	80(80)	—	180(180)	—
Silt loam	Aerobic	8	83(83)	—	80(80)	—
		16	80(80)	—	120(100)	—
Sandy loam	Anaerobic, sterile	8	91	—	120	—
Silt loam	Anaerobic, sterile	8	83	—	60	—
Sandy loam	Aerobic, sterile	16	83	—	160	—
Silt loam	Aerobic, sterile	16	83	—	60	—

[a] Duplicate values indicated in parentheses.
[b] Not detected, i.e., less than 1, 10, and 10 μg/kg for α-HCH, γ-PCCH, and γ-TCCH, respectively.
[c] Trace: between 1 and 2 μg/kg for α-HCH; between 10 and 20 μg/kg for γ-TCCH.

phy–mass spectrometry. Additional peaks were observed on gas chromatograms of soil extracts, but no further attempts were made to identify them.

No α-HCH was formed upon incubation of sterile soils with γ-HCH, indicating the role of microorganisms in its formation. γ-PCCH was not only detected in the unsterile, aerobic soils, but also after sterile submerged or aerobic soil incubation.

Pure Cultures

The facultative anaerobe *E. coli* was used to compare the microbial formation of α-HCH from γ-HCH under aerobic and anaerobic conditions. Growing cultures of *E. coli* formed α-HCH from γ-HCH under anaerobic conditions, but not under aerobic conditions (Table 3). γ-TCCH was detected predominantly in the anaerobic cultures, smaller amounts being formed aerobically. γ-PCCH formation was not observed in this experiment.

No α-HCH was formed in control experiments in which sterile nutrient medium was incubated with γ-HCH for 28 days. Neither was α-HCH formed upon anaerobic incubation for 14 days of a spent medium of *E. coli* with γ-HCH.

Figure 1 shows the formation of α-HCH upon anaerobic incubation of *E. coli* with γ-HCH during a 28-day period. Since the stationary phase of the culture is reached already after 2–3 days, α-HCH formation obviously proceeds also after cessation of growth. In view of this result, experiments

TABLE 3

Formation of α-HCH and γ-TCCH upon Aerobic and Anaerobic Incubation of E. coli with γ-HCH (7.5 mg/liter) in a Peptone–Glucose Medium (pH 7) for 14 days at 37°C

Incubation condition	Percentage γ-HCH recovered	α-HCH found (μg/liter)	γ-TCCH found (μg/liter)
Aerobic	80(80)[a]	—[b]	6(Trace)[c]
Anaerobic	88(88)	2.2(3.4)	14(16)

[a] Duplicate values in parentheses.
[b] Not detected, less than 0.20 μg/liter.
[c] Trace: between 2 and 4 μg/liter.

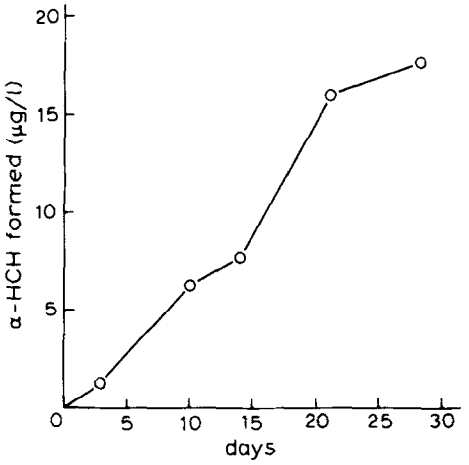

FIG. 1. Formation of α-HCH from γ-HCH (7.5 mg/l) by *E. coli* in peptone–glucose medium, pH 7, at 37°C, under anaerobic conditions.

with heavy, washed suspensions of *E. coli* were carried out. Results of anaerobic incubation with γ-HCH for 3 and 10 days are given in Table 4. These results show that more α-HCH was produced by washed suspensions than by growing cultures, probably due to the high cell density (13 mg dry wt/ml) in the former. Addition of glucose stimulated the formation of α-HCH, although the rate of degradation of γ-HCH was not influenced. A substantial amount of γ-HCH (1.6%) was converted into α-HCH in 10 days by the heavy suspension to which glucose had been added, at a concentration of 7.5 mg of γ-HCH per liter. At a γ-HCH concentration of 75 mg/l, 0.7% was converted into α-HCH.

To investigate whether the metabolite γ-PCCH might be converted into α-HCH a heavy, washed suspension of *E. coli* was incubated for 10 days with γ-PCCH (7.5 mg/liter) under anaerobic conditions in the presence of glucose (1%). Only 2.4 µg/liter of α-HCH was found, although the γ-PCCH initially added had been completely degraded.

A. niger, a common soil fungus, cultivated either as mycelial pads or in shake cultures in a glucose–mineral salts medium with γ-HCH (7.5 mg/liter) for 2 or 4 weeks did not yield α-HCH or γ-TCCH, but γ-PCCH (6-10 µg/liter) was detected.

DISCUSSION

Our experiments with *E. coli* clearly show that α-HCH accumulates only under anaerobic and not under aerobic conditions. Previous reports mentioned formation of α-HCH by anaerobic mixed cultures (5, 6) or by *Pseudomonas putida* under undefined conditions, which were in our view restricted in oxygen supply (2, 3). The results of our experiments as well as those of earlier investigations indicate that α-HCH formation can occur in the environment. The results obtained strongly suggest that this formation will prevail under anaerobic or microaerophilic conditions.

In the present experiments *E. coli* was chosen as a model representative of facultative anaerobes. However, this microorganism does not normally occur in the environment. Further experiments on the for-

TABLE 4

Formation of α-HCH and γ-TCCH by Heavy, Washed Suspensions of E. coli during Anaerobic Incubation at 37°C with γ-HCH (7.5 and 75 mg/liter) in Phosphate Buffer (pH 7)

Initial concentration of γ-HCH (mg/liter)	Glucose (1%) added	Incubation period (days)	Percentage γ-HCH recovered	α-HCH found (µg/liter)	γ-TCCH found (µg/liter)
7.5	+	3	69	61	20
7.5	+	10	53	117	10
7.5	–	3	67	27	29
7.5	–	10	53	53	14
75	+	10	28	543	53

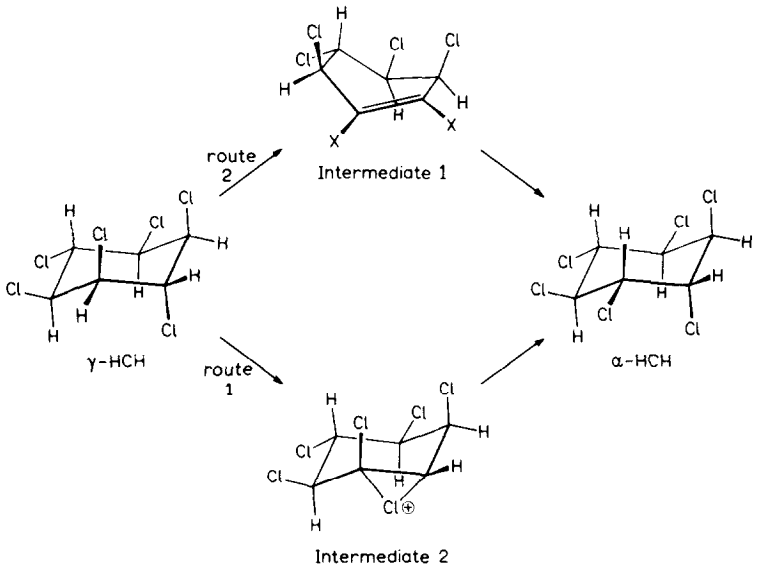

FIG. 2. Configuration of γ- and α-HCH and proposed isomerization mechanisms. Only one possible configuration of the intermediate 1 is shown. X = H or Cl.

mation of α-HCH from γ-HCH by facultative and strictly anaerobic bacteria occurring in soil and mud should be carried out.

Nothing is known about the mechanism of the microbial isomerization of γ-HCH into α-HCH. This isomerization requires the inversion of the configuration at one of the C atoms of γ-HCH, since the position of the chlorine atoms in γ-HCH is *eeeaaa* (*e* = equatorial, *a* = axial), whereas that in α-HCH is *eeeeaa* (10). It may be speculated that an intermediate containing a double bond (Fig. 2, route 2) is formed. Formation of α-HCH from the potential intermediates γ-TCCH and γ-PCCH requires addition of two chlorine atoms or HCl, respectively, to the latter compounds, which does not seem very probable. Another potential intermediate could be hexachlorocyclohexene (HCCH). HCCH has been detected as a metabolite of γ-HCH in rats and by rat liver microsomes by Chadwick *et al.* (12). It is not known whether this compound is an intermediate in the degradation of γ-HCH by microorganisms.

Formation of an intermediate containing a bridged chloronium ion (Fig. 2, route 1) also offers an attractive explanation for the observed isomerization of γ-HCH. The required abstraction and addition of hydride (H^-) is a well-known process in nicotinamide-nucleotide coenzyme-mediated enzymatic hydrogen-transfer reactions. reactions.

In our experiments production of α-HCH was always accompanied by that of γ-TCCH. Matsumura *et al.* (3) observed that addition of NAD stimulated both α-HCH and γ-TCCH formation by *P. putida*, whereas rotenone inhibited this effect. However, based on the considerations discussed above γ-TCCH does not seem a probable precursor of α-HCH. A similar enzyme system might be responsible for formation of both compounds, or they might have a common precursor. The simultaneous decrease of γ-TCCH concentration and increase of α-HCH concentration from Days 3 to 10 upon incubation of γ-HCH with *E. coli* (Table 4) may rather be ascribed to relatively fast degradation of γ-TCCH (11) than to conversion into α-HCH.

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