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Degradation of Hexachlorocyclohexanes and Structurally Related Substances by *Clostridium sphenoides*

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Abstract

Washed cell suspensions of *C. sphenoides* adapted to the γ-isomer of 1,2,3,4,5,6-hexachlorocyclohexane (γ-HCH), metabolized α- and γ-HCH but not β- and δ-HCH. Temperature and pH optima for the metabolism of γ-HCH were found to be 40°C and pH 8·0. The γ-HCH-adapted suspensions also metabolized the β-, γ- and δ-isomers of 3,4,5,6-tetrachloro-1-cyclohexene, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol, γ-1,2,3,4,5,6-pentachlorocyclohex-1-ene, and 1,2,3,4,5-pentachlorocyclohexane. The suspensions did not metabolize a selection of chlorinated benzenes, dichlorocyclohexane and chlorocyclohexane.

Introduction

The conversion of the α- and γ-isomers of 1,2,3,4,5,6-hexachlorocyclohexane (α- and γ-HCH) to δ- and γ-3,4,5,6-tetrachloro-1-cyclohexene (TCCH) respectively by *Clostridium sphenoides* has been demonstrated (Heritage and MacRae 1977*a*). Also, cell-free preparations of the same bacterium were found to convert γ-HCH to γ-TCCH (Heritage and MacRae 1977*b*). As the γ-TCCH that was formed in mixtures of whole cells or cell-free extract and γ-HCH was noted to disappear from the mixtures, it was assumed to be metabolized further. However, no information was obtained regarding the identity of its degradation products.

Studies involving mammalian cells (Freal and Chadwick 1973) and insect preparations (Reed and Forgash 1968, 1969, 1970) have yielded results which indicate that enzymatic degradation of γ-HCH involves the formation of chlorinated benzene derivatives. Also, there is evidence that several bacteria and fungi isolated from soil could convert γ-HCH to γ-2,3,4,5,6-pentachloro-1-cyclohexene (γ-PCCH), α-3,4,5,6-tetrachloro-1-cyclohexene (α-TCCH), β-TCCH, γ-TCCH and pentachlorobenzene (PCB) (Tu 1976). One of the cultures, *Pseudomonas* sp. No. 62, when grown on γ-HCH, was simultaneously adapted to γ-PCCH, α-TCCH, β-TCCH, γ-TCCH, PCB, 1,2,3,4-tetrachlorobenzene (1,2,3,4-TCB) and 1,2,4,5-tetrachlorobenzene (1,2,4,5-TCB). The formation of γ-PCCH during the metabolism of γ-HCH has been indicated for other bacteria (Yule *et al.* 1967; Benezet and Matsumura 1973; Francis *et al.* 1975) as well as soya bean seeds (Nash and Harris 1973) and houseflies (Ishida and Dahm 1965).

While the pH optimum for γ-HCH metabolism by housefly preparations has been recorded as pH 7·6 (Ishida and Dahm 1965) nothing seems to have been recorded for pure cultures of γ-HCH-metabolizing microbes.

In the present study, the pH and temperature optima for γ-HCH degradation by *C. sphenoides* were determined. In addition, the metabolism of the four common isomers of HCH and structurally related substances by *C. sphenoides* was studied to gain more information concerning the pathway of degradation of γ-HCH by this bacterium.

Materials and Methods

Materials

The α- and γ-HCH were procured from Fluka AG (Switzerland); PCB, 1,2,3,4-TCB, 1,2,3-trichlorobenzene (1,2,3-TCB), 1,3,5-trichlorobenzene (1,3,5-TCB) from K and K Laboratories Inc. (U.S.A.); 1,2,4,5-tetrachlorobenzene (1,2,4,5-TCB) from Eastman Organic Chemicals (U.S.A.); *trans*-1,2-dichlorocyclohexane (DCCH) and chlorocyclohexane (CCH) from Aldrich Chemical Co. Inc. (U.S.A.); chlorobenzene (CB) from Merck (West Germany); 1,2,4-trichlorobenzene (1,2,4-TCB) from Chemicals Procurement Laboratories Inc. (U.S.A.). The following were obtained as gifts: β- and δ-HCH from the International Rice Research Institute (Philippines); pentachlorocyclohexane (PCCHa), β-, γ- and δ-TCCH from Professor N. Kurihara (Japan); γ-TCCH from Dr H. J. Benezet (U.S.A.); and 2,3,4,5,6-pentachloro-2-cyclohexane-1-ol (PCCOL) from Dr R. W. Chadwick (U.S.A.). The γ-PCCH was prepared by the method of Nakajima *et al.* (1949).

Organism and Growth Conditions

The organism used in this study was the *C. sphenoides* UQM 780 described earlier (Heritage and MacRae 1977*a*). Cell suspensions were prepared from liquid cultures after anaerobic growth for 42 h at 30°C in a medium having the following composition (in g/l): K_2HPO_4, 0·1; $(NH_4)_2HPO_4$, 0·5; $MgSO_4.7H_2O$, 0·2; $Ca(NO_3)_2.4H_2O$, 0·01; $FeSO_4.7H_2O$, 0·001; Difco yeast extract, 20·0; γ-HCH, 0·006; distilled water, pH 7·1. Cells were harvested by centrifugation, washed in the appropriate buffer, depending on the nature of the experiment, and finally suspended in the same buffer to give a dense suspension having a dry weight (100°C) value between 5 and 35 mg/ml when diluted 1 in 10. For studies on the effect of pH and temperature on the degradation of γ-HCH, 0·025 M phosphate buffer solutions in the range pH 5–8 were used to prepare the cell suspensions, while in the range pH 8–10, 0·05 M 2-amino-2-methyl-1,3-propanediol adjusted with 0·05 M HCl was used. All other cell suspensions were made up in 0·025 M phosphate buffer, pH 7·2.

The anaerobic degradation of the various substances by the washed cell suspensions was followed in 100 ml glass reaction flasks designed so that oxygen could be displaced with high purity oxygen-free nitrogen. Forty-five ml of the appropriate buffer was added to the flasks and substrates added as concentrated solutions in acetone to give the desired concentration without the addition of more than 0·5 ml acetone. To aid dispersion of the substrates into the buffer solutions, the flasks containing the mixtures were subjected to ultrasonic treatment (55 kHz) for 5 min. The flasks were sealed and placed in a thermostat-controlled waterbath. Oxygen-free nitrogen was passed through the contents of the flask at 200 ml/min for 15 min, after which 5 ml of the concentrated cell suspension was injected into the flask via a Suba seal plug near the base of the flask. In the case of controls, 5 ml of the same cell suspension that had been heat-killed by autoclaving at 121°C for 5 min was used. The contents were again deoxygenated by passing oxygen free nitrogen through the flasks for 5 min. Frequent sampling of the mixtures in the flasks for gas chromatographic analysis was made via the Suba seal plugs.

Extraction of Chlorinated Substrates

Samples taken from the reaction flasks were extracted for 10 min with nanograde n-hexane to which had been added a known amount of a suitable internal standard reference compound. In addition to making allowance for day by day variation in response of the electron capture detector, the internal standard method proved useful in studies with substances that were not available in sufficient amounts to allow the preparation of standard curves. The internal standards used were as follows: γ-HCH as internal standard for δ-TCCH, δ-HCH, β-HCH and α-HCH; α-HCH for

1,2,4,5-TCB, 1,2,3,4-TCB, β-TCCH and PCCHa; 1,3,5-TCB for CB, CCH and DCCH; 1,2,3,4-TCB for 1,3,5-TCB, 1,2,4-TCB, 1,2,3-TCB and PCB; δ-HCH for γ-TCCH and γ-PCCH.

The ratio of sample volume to solvent volume proved to be critical and depended to a large extent upon the number of chlorine atoms per molecule of chlorinated substrate. The following ratios of sample volume to n-hexane volume were adopted: 6 Cl atoms/molecule, 1:3; 3–5 Cl atoms/molecule, 1:1; 2 Cl atoms/molecule, 12·5:1; and 1 Cl atom/molecule, 50:1. When the phases had separated after extraction, the hexane layer was dehydrated over anhydrous $MgSO_4$ and analysed by gas chromatography.

Analysis by Gas Chromatography

Gas chromatographic analyses were performed with a dual-column Shimadzu model GC-IC instrument having dual tritium electron capture detectors. All columns used were of stainless steel (3 mm int. diam.) and the column support was Gas-Chrom Q 80/100 mesh (Applied Science Laboratories, U.S.A.). The carrier gas was oxygen-free nitrogen. The following stationary phases and chromatographic conditions were used: for the HCH isomers, 1% silicone GE XE60, column (1·5 m) temperature 170°C, carrier gas 150 ml/min; for trichlorobenzenes and PCCOL, 15% silicone DC QFI, column (1·8 m) temperature 170°C (trichlorobenzenes) and 160°C (PCCOL), carrier gas 150 ml/min; for (*a*) pentachlorobenzene, tetrachlorobenzenes, γ-PCCH, (*b*) TCCH isomers, (*c*) dichloro- and chloro- compounds, 7·5% silicone GE Versilube F-50 modified with 0·75% epikote, column (2·7 m) temperature (*a*) 170°C, (*b*) 150°C and (*c*) 120°C, carrier gas (*a*) 62·5 (*b*) 70 and (*c*) 31·5 ml/min.

Detector and injection port temperatures were 195 and 220°C respectively.

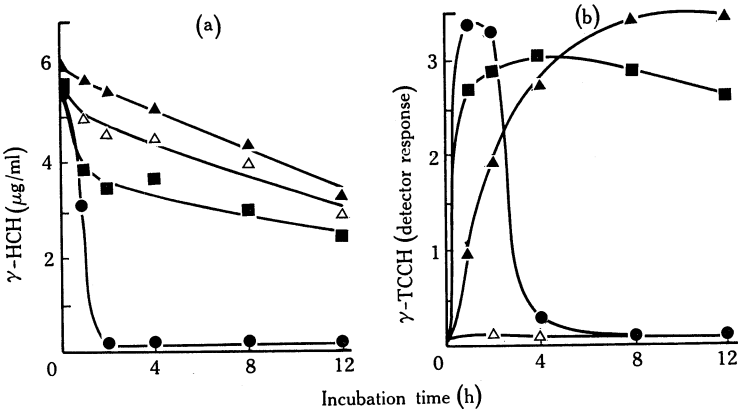

Fig. 1. (*a*) γ-HCH degradation and (*b*) γ-TCCH accumulation in washed cell suspensions (5·9 mg/ml dry wt) of *C. sphenoides* incubated anaerobically at pH 7·0 in the presence of γ-HCH at 30°C (▲), 40°C (●), 50°C (■) and 60°C (△).

Results

In washed cell suspensions of *C. sphenoides* (pH 7·0), degradation of γ-HCH was most rapid at 40°C (Fig. 1*a*). Almost all of the γ-HCH had been degraded within about 2 h. Formation of the intermediate γ-TCCH from γ-HCH was also fastest at 40°C (Fig. 1*b*). Subsequent loss of this intermediate from the washed cell suspensions was most rapid at 40°C. While approximately the same concentration of γ-TCCH was reached in those mixtures incubated at 30 and 50°C, the intermediate in γ-HCH degradation persisted for much longer. Very small amounts of γ-TCCH were detected in suspensions incubated at 60°C.

Studies on the effect of pH and temperature on γ-HCH degradation and γ-TCCH formation revealed that the fastest rate of γ-HCH degradation by washed cell suspensions of *C. sphenoides*, compared with autoclaved cell controls, occurred at pH 8 and 40°C (Fig. 2). Losses of γ-HCH from reaction mixtures at pH 9·0 and 10·0

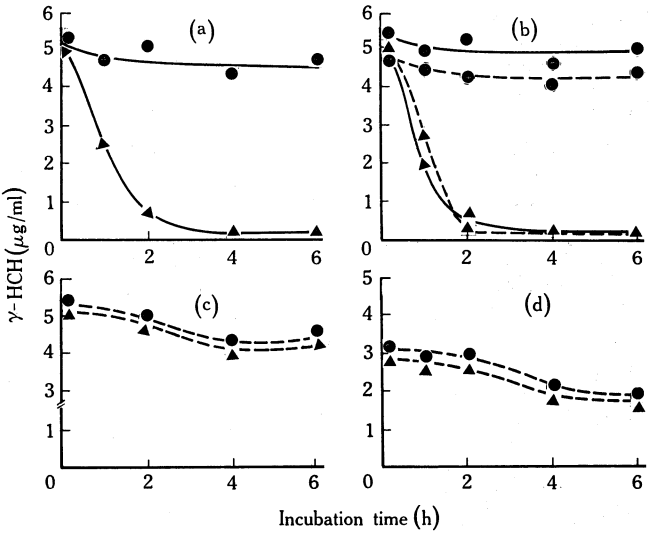

Fig. 2. γ-HCH degradation by washed cell suspensions (7·8 mg/ml dry wt) of *C. sphenoides* incubated anaerobically at pH values 7 (*a*), 8 (*b*), 9 (*c*) and 10 (*d*) at 40°C; live cell suspensions (▲), autoclaved cells (●), 0·025 M phosphate buffer (—) and 0·05 M ammediol buffer (– – –).

were similar to those found in control flasks containing heat-killed cells. At 50°C, the greatest degradation of γ-HCH occurred at pH 7·0. The optimum pH of γ-HCH degradation by washed cell suspensions of *C. sphenoides* decreased from pH 9·0 to pH 7·0 as the temperature increased from 30 to 50°C. Maximum accumulation of the intermediate γ-TCCH was found under conditions of poor γ-HCH degradation at pH 6·0 and 30°C.

Table 1. Degradation of chlorinated cyclic compounds by washed suspensions of *C. sphenoides*
Dry weight (100°C) of cells 15–25 mg/ml

Compound	Concn (μg/ml)	Extent of degradation (%)	Time (h)	Compound	Concn (μg/ml)	Extent of degradation (%)	Time (h)
γ-HCH	5	100	2	γ-PCCH	5	100	1
α-HCH	5	100	4	PCCOL	2·5	100	1
β-HCH	10	0	24	β-TCCH	5	100	1
δ-HCH	10	0	24	δ-TCCH	5	76	8
PCCHa	5	62	18	γ-TCCH	5	100	4

Complete degradation of α-HCH by *C. sphenoides* occurred within 4 h (Table 1). No degradation of either the β- or the δ-isomer of HCH was evident after 24 h as opposed to the complete disappearance of γ-HCH in 2 h with the same cell suspension.

Washed cell suspensions of *C. sphenoides* derived from cultures grown in the presence of γ-HCH were able to degrade γ-PCCH, PCCOL, β-TCCH and γ-TCCH completely within 4 h and without any evidence of a lag period (Table 1). Degradation of PCCHa and δ-TCCH was much slower but also proceeded without a lag period. No evidence was obtained to suggest that the bacterium could metabolize any of the chlorinated benzenes or the dichloro- and chlorocyclohexane tested. Washed cell suspensions of *C. sphenoides* degraded α-HCH to δ-TCCH. Apart from α- and γ-HCH no degradation products were detected from any of the other substances listed in Table 1. However, the formation of a substance from δ-HCH was indicated by the presence of a second peak on gas chromatograms of extracts from the control flasks containing heat killed cells. No such peak appeared on chromatograms from flasks containing live suspensions of the bacterium and δ-HCH.

Discussion

Results of studies into the effect of varying temperature and pH on the degradation of γ-HCH by washed cell suspensions of *C. sphenoides* have indicated that maximum activity occurs at 40°C and pH 8. This result is similar to the pH 7·6 optimum found by Ishida and Dahm (1965) for the attack on γ-HCH by housefly enzyme preparations. Similarly, degradation of γ-HCH in mud was found to be most rapid at pH 7 and 9 with some persistence at pH 5 (Lichtenstein *et al.* 1966). However, a common feature of that study and the present one is that at pH 9 and 10 some alkaline dehydrochlorination of the γ-HCH to γ-PCCH (Yule *et al.* 1967) or other undetected products may have accounted for the loss of some of the γ-HCH.

While the degradation of γ-HCH in soil and sewage sludge is known to be stimulated by a rise in temperature from 20 to 35°C (Hill and McCarty 1967; Yoshida and Castro 1970) no account of effect of temperatures above 35°C has been recorded. If similar organisms to the one used in the present study are active in γ-HCH degradation in soil and sewage sludge, then peak activity could occur at 40°C.

The rapid disappearance of γ-TCCH from mixtures of cells and γ-HCH, particularly at pH 8 and 40°C indicates that this intermediate is further metabolized. However, the nature of the products is not known.

There was a marked difference in the degradation of the four stereoisomers of HCH tested. The α- and γ-isomers were degraded rapidly via δ- and γ-TCCH respectively. In attempts to promote degradation of the β- and δ-isomers, heavier cell suspensions of the organism and the conditions most conducive to γ-HCH degradation, pH 8·0 and 40°C, were used. In spite of this, no stimulation of β- or δ-HCH breakdown occurred. While it seems that the bacterium is unable to degrade these two isomers, it is possible that conditions of pH and temperature different from those chosen might be required for degradation of the β- and δ-isomers.

The accumulation of low levels of an intermediate compound in control flasks containing buffered solutions of δ-HCH and heat-killed cells could have arisen from the chemical monodehydrochlorination of δ-HCH to δ-pentachlorocyclohex-1-ene at pH 8·0. The absence of a similar peak on gas chromatograms of extracts from mixtures containing live cells probably indicates that the compound is being degraded by the cells.

Both the α- and γ-isomers of HCH have adjacent carbon atoms with axial chlorine substituents on the side of the molecule opposite three equatorial chlorines (Fig. 3).

If this diaxial chlorine arrangement, which is not found in β- or δ-HCH, is the conformation enabling the enzyme present in *C. sphenoides* to initiate breakdown of the molecule, then the η-isomer of HCH may be predicted to be degraded readily by this bacterium. This isomer was not available.

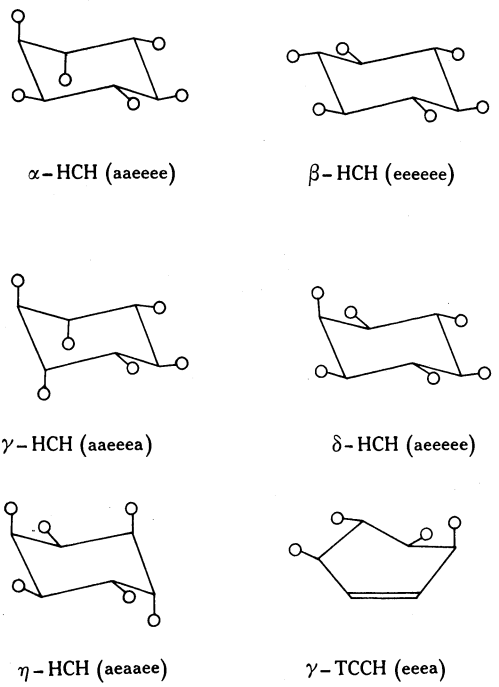

Fig. 3. Common isomers of hexachlorocyclohexane (HCH) after Hornstein (1955) and tetrachlorocyclohexene (TCCH) after Orloff *et al.* (1953). ○ Chlorine; *a*, axial bond; *e*, equatorial bond.

Disappearance of both γ-PCCH, the product of monodehydrochlorination of α- and γ-HCH, and PCCOL was very rapid compared with α- and γ-HCH. The ability of certain pseudomonads to oxidize γ-PCCH has been described by Haider *et al.* (1974). None of the intermediates that have been described as being formed during the metabolism of γ-PCCH by insects were observed during degradation of this compound by *C. sphenoides*. These intermediates include the tri-, tetra-, and pentachlorobenzenes (Reed and Forgash 1969). Also, no evidence has been found in this study for the formation of the 2,4,5- and 2,3,5-trichlorophenols. These substances have been found in the urine of rats fed γ-PCCH (Grover and Sims 1965; Freal and Chadwick 1973).

Chadwick and Freal (1972) found it difficult to account for the formation of PCCOL, which has double chlorine substitutions of the double bond, during γ-HCH metabolism. They proposed that a different mechanism from that leading to the formation in rat urine of tetra-, tri- and dichlorophenols from the corresponding chlorobenzenes must have been responsible. Although γ-PCCH has not been detected as a γ-HCH breakdown product in mammals, the possibility exists that if it is formed, a proportion of it is changed to PCCOL. This double chlorine saturation of the

double bond of PCCOL has not inhibited its degradation by *C. sphenoides*. Rather than being an intermediate product of γ-HCH degradation by this bacterium, this compound is possibly a structural analogue of an intermediate.

Pentachlorocyclohexane (PCCHa) is the product speculated by Sethunathan *et al.* (1969) as the compound expected after a single dechlorination of γ-HCH. Like the other pentachloro- alicyclic compounds studied, PCCHa disappeared faster from test flasks than from autoclaved cell controls. Disappearance of PCCHa was slow, however, when compared with that of α- and γ-HCH, γ-PCCH and PCCOL. This is the first report of the degradation of PCCOL and PCCHa by bacteria.

Very rapid breakdown of β-TCCH was recorded for test flasks, although significant losses of substrate occurred from control flasks, presumably by volatilization. Volatilization losses from reaction mixtures were common, particularly with the chlorinated benzenes. This appears to be the first report of microbial degradation of δ-TCCH.

The inactivity of *C. sphenoides* suspensions towards the penta- and tetrachlorobenzenes was similar to the results of Reed and Forgash (1969, 1970).

While the formation of γ-PCCH during γ-HCH breakdown by microbes and insects has been widely reported, no evidence for its formation was obtained in the present study. Also, the formation of γ-TCCH was not observed during the rapid degradation of either γ-PCCH or PCCHa. If a pentachloro- compound is an intermediate of γ-HCH degradation, its failure to accumulate in solution may be because it is degraded at a faster rate than γ-HCH. However, in the absence of TCCH formation during γ-PCCH or PCCHa degradation, it is difficult to account for one of these pentachloro- compounds as a primary intermediate of HCH degradation. Since the evidence points to the removal of two chlorine atoms from γ-HCH to form γ-TCCH, it is possible that later steps proceed in this fashion. A dichlorocyclohexadiene and finally benzene would be expected to be the products of such dechlorinations. Benzene formation during the breakdown of γ-HCH in cattle dip baths was described by Allan (1955). Hydrogen and other gases evolved during the anaerobic growth of bacteria in the baths was proposed as the agents of dechlorination.

Acknowledgments

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