Degradation and Metabolization of Lindane and Other Hexachlorocyclohexane Isomers by Anaerobic and Aerobic Soil Microorganisms

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Z. Naturforsch. **34 c**, 1066–1069 (1979); received June 1, 1979

Microbial Metabolization of Hexachlorocyclohexane, Anaerobic and Aerobic Microorganisms, Dechlorination of Hexachlorocyclohexane

Microbial degradation of lindane or other hexachlorocyclohexane isomers has been observed to occur more rapidly in flooded than in upland soils. Experiments with a mixed bacterial flora enriched anaerobically from soils showed a rapid dechlorination and degradation of lindane. Screening studies with pure bacteria indicated that *Clostridium* spp. and several representatives of *Bacillus* spp. and *Enterobacteriaceae* effectively degraded lindane, while representatives of *Lactobacillaceae* and *Propionibacterium* were inactive. During degradation organic bound chlorine was released as chloride and nearly chlorine-free, partly volatile metabolites were formed. The chloride formation was promoted by the addition of glucose, pyruvate or formate.

Lindane was the most easily degraded isomer, while α- and especially β- and δ-hexachlorocyclohexane was also, but more slowly, dechlorinated. These isomers are relatively frequently observed as environmental contaminants, their occurrence might be caused by their greater persistence or/and by an isomerization of the γ- into α- and other isomers during microbial incubation as it was discussed by several authors. Experiments, however, whether or not this isomerization can be carried out by microbes, were negative or showed that it only can occur to a very minor extent.

Aerobic degradation of lindane proceeded only slowly and chiefly by formation of chlorinated aromatic compounds. One of the main intermediate metabolites seemed to be γ-pentachlorocyclohexene which was further transformed by the release of HCl or hydrogen and by addition of water. During anaerobic incubation, γ-tetrachlorocyclohexene intermediately occurred which was further dechlorinated to nearly chlorine-free compounds.

Introduction

Lindane, the γ-isomer of hexachlorocyclohexane (γ-HCH), belongs to the less persistent organochlorine insecticides [1]. Especially in flooded or organic nutrient rich soils it disappears rapidly while in well aerated soils it is more stable [2–4]. Besides γ-HCH also other HCH-isomers are commonly found in the environment. Especially α- and β-HCH occur sometimes more than γ-HCH [5, 6]. They are probably released by the application of technical HCH-mixtures or of insufficiently purified lindane. Some authors, however, also report about an isomerization of γ- into α-HCH or into other isomers [7–9].

Several microorganisms are reported to degrade lindane. A mixed bacterial flora enriched anaerobically from arable soil showed a rapid dechlorination and the formation of nearly chlorine-free volatile compounds [10]. A similar rapid dechlorination and formation of volatile compounds was also observed by incubation with bovine rumen fluid or in anaerobic culture media inocculated with rumen fluid [11]. Microorganisms from silage, however, did not or much less degrade γ-HCH. A rapid dechlorination was also observed with several Clostridia or similarly with *Enterobacteriaceae* and *Bacillus* spp. [12, 13]. Mixed aerobic cultures from soil or aerobic bacteria and fungi degraded lindane much less and metabolized it partly into highly chlorinated phenols and benzenes [14, 15, 19]. The following report deals with the anaerobic and aerobic degradation of γ-HCH and other isomers. Furthermore some studies are reported whether microbes can transform γ-HCH into other isomers.

Abbreviations: HCH, hexachlorocyclohexane; PCH, pentachlorocyclohexene; TCH, tetrachlorocyclohexene.

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0341-0382 / 79 /1100-1066 $ 01.00/0

Materials and Methods

Experiments as far as they are reported from the author's laboratory were generally made with ^{36}Cl- or/and ^{14}C and ^{3}H-labelled HCH-isomers. These were prepared by chlorination of benzene with $^{36}Cl_2$ or of ^{14}C- or ^{3}H-labelled benzene with $^{35}Cl_2$. The mixture of isomers was separated by column and preparative thin layer chromatography into γ-, α-, β-, and δ-HCH [10, 13]. Incubation studies were made with

either mixed or pure bacterial cultures under anaerobic or aerobic conditions. The pure bacteria were either isolated from soil or obtained from different other sources.

Results and Discussion

Former investigations showed a more than 90 per cent dechlorination of γ-HCH with anaerobic mixed cultures of soils or rumen fluid within a few days [10, 11]. In such anaerobic systems strict, facultative and aerotolerant anaerobes can proliferate. Selected representatives of these groups were tested for their capability to dechlorinate γ-HCH and some results are shown in Table I. Included were experiments with several organisms which under anaerobic conditions use nitrate or sulphate as an electron acceptor.

This table shows obviously that *Clostridia* as strict anaerobes but also *Enterobacteriaceae* and *Bacillaceae* as facultative anaerobes are effectively dechlorinating γ-HCH. The ineffective species belonged to the aerotolerant anaerobes such as *Lactobacillus* and *Leuconostoc* spp. or *Propionibacterium*. Also ineffective was *Paracoccus denitrificans* which under anaerobic conditions uses nitrate as an electron acceptor. These results indicate that only organisms which are known to evolve hydrogen during fermentation are effective. Organisms which ferment substrates only by internal oxidoreduction reactions are ineffective. These are *e. g.* the homo- or heterofermentative lactic acid bacteria or *Propionibacterium*. Also ineffective seem to be organisms like *Paracoccus denitrificans*. The facultative anaerobic bacteria grow in the presence or absence of oxygen. Several experiments indicated that the aerobically grown organisms actively degraded γ-HCH during a subsequent anaerobic incubation. This degradation, however, was significantly promoted if an additional energy source, such as glucose, pyruvate or formate was added [13]. Since even arable soils have always microenvironments with changing redox potentials this observation seems to be important. Water logged pores or the surroundings of rapidly decomposing plant particles are anaerobic and favour the degradation of γ-HCH or other chlorinated hydrocarbons.

Table I. Dechlorination of ^{36}Cl-γ-HCH upon anaerobic incubation in a complex glucose medium by several bacteria during 4—6 days (per cent of the organichlorine released as chloride).

Organisms	% rel. as Cl^-	Organisms	% rel. as Cl^-
Clostridium butyricum	93	*Citrobacter freundii*	80
C. pasteurianum	80	*Escherichia coli*	21
Bacillus polymyxa	45	*Enterobacter aerogenes*	45
B. circulans	10	*E. cloacae*	20

Inactive Organisms: *Lactobacillus* spp., *Leuconostoc* spp., *Propionibacterium shermanii*, *Paracoccus denitrificans*.

Further investigations about the anaerobic γ-HCH degradation were made with ^{36}Cl-, ^{14}C- and ^{3}H-labelled preparates. Table II shows an example where *Citrobacter freundii* was anaerobically incubated in liquid cultures supplied with 20 ppm γ-HCH. At distinct time intervals aliquots of the culture solution were extracted with benzene, and the distribution of the radioactivity was measured in the unextracted culture solution, in the benzene extracts, in the extracted solution or in absorbents of the supernatant gas atmosphere. Table II indicates that the ^{36}Cl-activity remained nearly completely in the culture solution while the ^{14}C- and ^{3}H-activity decreased with time and was partly

Table II. Degradation of ^{14}C-, ^{3}H- and ^{36}Cl-labelled γ-HCH during anaerobic incubation with *Citrobacter freundii*. Distribution of the radioactivity in the culture medium, benzene extract, extracted medium and in absorbed volatiles from the gas above the culture liquid (figures indicate % of the added activity).

Fractions	Incubation time								
	2			3			5 days		
	% ^{14}C	^{3}H	^{36}Cl	% ^{14}C	^{3}H	^{36}Cl	% ^{14}C	^{3}H	^{36}Cl
Culture medium	61	66	101	47	58	102	20	37	94
Benzene extract	52	50	26	32	34	16	11	12	1
Extracted medium	8	13	72	12	23	83	8	24	90
Evolved as gas	25	22	0	28	26	0	41	43	4

released as volatiles which could be collected in absorbents. After 5 days only little of the ^{36}Cl-activity could be extracted any more with benzene but remained as chloride in the extracted solution. The ^{14}C- and ^{3}H-activity in the culture solution could be still extracted with benzene, however, the extractability of the ^{3}H was somewhat less than that of ^{14}C. These results indicate that the organic bound chlorine atoms are released as chloride but they are not stoichiometrically released together with the hydrogen substituents in the form of HCl. It is therefore plausible that the release of the organic bound chlorine as chloride needs suitable external substrates to provide the necessary reduction equivalents. The formation of highly chlorinated aromatic compounds from γ-HCH appears to be inessential since very little of the ^{36}Cl could be any more extracted along with ^{14}C.

A comparison of the dechlorination rate of several HCH-isomers by anaerobic mixed or pure cultures is shown in Table III. It is obvious that γ-HCH is the most easily degraded isomer, also α-HCH is considerably but less dechlorinated. More stable appeared to be the β- and especially the δ-isomer. A similar sequence ($\gamma > \alpha > \beta \geqq \delta$-HCH) in the degradation of the isomers in soil was found by MacRae *et al.* [16]. The slower degradation of α- and β-HCH probably contributes towards their accumulation in the environment. Another contribution to the frequent occurrence of α- and β-HCH, however, should be an isomerization of γ- into α- or β-HCH [7–9]. This isomerization, however, is difficult to prove, since even purified γ-HCH preparations always contain small quantities of the other isomers which are rather difficult exactly to be determined. We therefore tried to solve these problems by the use of the reverse isotope dilution method and incubated therefore distinct amounts of ^{14}C-labelled γ-HCH together with smaller amounts of non labelled α-HCH and measured at distinct times whether radioactivity in the α-HCH was increased. These incubation studies were made with several bacteria which have been described to cause isomerization [8] or from which we presumed they should cause it. Three representative organisms are shown in Table IV. They were incubated with about 10 ppm of ^{14}C-labelled γ-HCH together with 2 or 1.5 ppm of added non-labelled α-HCH under either anaerobic or aerobic conditions. The culture media were thereafter exhaustively extracted with benzene and γ- and α-HCH were separated by preparative gas chromatography and their amounts and specific radioactivities were determined.

Table III. Dechlorination of ^{36}Cl α-, β-, γ- or δ-HCH by an anaerobic bacterial flora enriched from soil, by *Citrobacter freundii*, *Clostridium butyricum* or by *C. pasteurianum* within a six-days-incubation period.

Isomer	Mixed soil flora	*C. freundii*	*C. butyricum*	*C. pasteurianum*
	% of organic ^{36}Cl released as $^{36}Cl^-$			
α-HCH	6.5	13.9	97.4	53.2
β-HCH	7.4	15.3	23.8	10.1
γ-HCH	78.0	91.5	98.3	91.0
δ-HCH	1.6	2.8	38.5	5.0

Table IV. Screening experiments for a possible isomerization of γ- into α-HCH by bacteria during incubation. Mixtures of 2 mg of ^{14}C-labelled γ-HCH with 0.5 or 0.3 mg of unlabelled α-HCH were incubated in 200 ml complex glucose medium. At distinct times the two isomers were separated and their quantitiy and specific activity was determined. The standard deviation s was calculated from 3 replicates.

Organism	Incub. time [d]	μg γ-HCH	dpm/μg $\pm s$	μg α-HCH	dpm/μg $\pm s$
Citrobacter freundii	0	1910	1740 ± 4	553	14 ± 3
anaerobe	2	1450	1795 ± 20	543	15 ± 6
	3	833	1756 ± 15	529	13 ± 4
	5	0	—	153	16 ± 2
Serratia marcescens	0	2005	1822 ± 17	305	22 ± 2
aerobe (resting cultures)	13	1913	1906 ± 11	298	25 ± 6
	27	1826	1856 ± 15	254	21 ± 3
	34	1755	1721 ± 26	266	26 ± 4
Pseudomonas putida	0	1985	2005 ± 21	351	27 ± 6
aerobe (shake cultures)	13	1950	2020 ± 16	327	23 ± 3
	27	1870	1953 ± 14	303	19 ± 4
	34	1753	1856 ± 19	297	25 ± 8

C. freundii degraded γ-HCH rapidly and α-HCH less, while *S. marcescens* and *P. putida* degraded γ- and α-HCH only slowly. The little radioactivity in the α-HCH at zero time was caused by a small impurity of α-HCH in the labelled γ-HCH. Within the limits of the experimental error (<0.1%) none of the three organisms, however, caused an increase of the specific radioactivity in the α-HCH. Similar tests with several *Clostridia, Escherichia coli, Klebsiella oxytoca* or *Pseudomonas testosteroni* were also negative. It therefore seems that an isomerization of γ- into α-HCH does not occur or occurs only to a very small extent.

During the anaerobic degradation of lindane the main intermediate metabolite was characterized as γ-tetrachlorocyclohexene (γ-TCH) [13, 17]. This is further dechlorinated into benzene, monochlorobenzene besides very small amounts of tri- and tetrachlorobenzenes. A formation of δ-TCH from α-HCH has also been reported [17]. During the aerobic incubation of lindane in soil small amounts of γ- and α-TCH seem also to occur [15, 18]. However, γ-pentachlorocyclohexene (γ-PCH) was here the much more prominent metabolite [2, 15]. Chlorinated benzenes and phenols have been observed as additional prominent metabolites beside γ-PCH during degradation with fungi and aerobic bacteria [14]. Engst *et al.* [14, 19] summarized schematically the metabolites observed during aerobic degradation of γ-HCH by several bacteria and fungi. They proposed that the γ-PCH is slowly transformed into several chlorinated benzenes and phenols by the elimination of HCl or through chemical or biochemical dehydrogenation and by the addition of water. During this dehydrogenation very small amounts of hexa- and pentachlorobenzenes are possibly formed. More prominent, however, are tetra- and trichlorobenzenes and corresponding phenols. Obviously the fast anaerobic and the slow aerobic degradation pathways differ already in the first steps. During the anaerobic degradation organic chlorine substituents become rapidly eliminated by external electron donors while during aerobic incubation HCl is removed and still chlorinated aromatic compounds are progressively formed. These aromatic compounds are only found in negligible amounts during the anaerobic incubation. The observation that only those bacteria rapidly degrade hexachlorocyclohexane during anaerobic incubation from which iron sulfur protein dependant fermentative hydrogen evolution is known to occur, indicates that these co-enzymes may play a role during the dechlorination process.

Acknowledgements

Several bacteria were kindly provided by Prof. W. Koransky, Marburg. The experiments were partly subsidized by the European Community under the Contract No. 174-77-4 ENVD.

[1] C. A. Edwards, Insecticides (C. A. I. Goring and J. W. Hamaker eds., pp. 513–568, M. Dekker Inc., New York 1972.
[2] T. Yoshida and T. F. Castro, Soil Sci. Soc. Amer. Proc. **34,** 440–448 (1970).
[3] K. Raghu and I. C. MacRae, Science **160,** 263–265 (1966).
[4] R. Kohnen, K. Haider, and G. Jagnow, Environm. Qual. Safety, **Vol. III** (F. Coulston and F. Korte eds.), pp. 222–225,, G. Thieme, Stuttgart 1975.
[5] G. Bodenstein, Toxicology of lindane, II Suppl. (E. Ulmann ed., "Lindane"), pp. 11–73, K. Schillinger, Freiburg 1976.
[6] A. Szokolay, A. Madaric, and J. Uhnak, J. Environ. Sci. Health **B 12,** 193–212 (1977).
[7] L. W. Newland, G. Chesters, and G. B. Lee, J. Water Poll. Cont. Fed. **41,** R 174–183 (1969).
[8] J. H. Benezet and F. Matsumura, Nature **243,** 480–481 (1973).
[9] H. Steinwandter and U. Schlüter, Bull. Environm. Contam. Toxicol. **20,** 174–179 (1978).
[10] K. Haider und G. Jagnow, Arch. Microbiol. **104,** 113–121 (1975).
[11] K. Haider, G. Jagnow, und R. Rohr, Landw. Forsch. **32/II,** 147–152 (1976).
[12] I. C. MacRae, K. Raghu, and E. M. Bautista, Nature **221,** 859–860 (1969).
[13] G. Jagnow, K. Haider, and P.-Chr. Ellwardt, Arch. Microbiol. **115,** 285–292 (1977).
[14] R. Engst, R. M. Macholz, and M. Kujawa, Res. Rev., **Vol. 68** (F. A. Gunther and J. D. Gunther eds.), pp. 59–90, Springer, New York, Heidelberg, Berlin 1977.
[15] C. M. Tu, Arch. Microbiol. **105,** 131–134 (1975), Arch. Microbiol. **108,** 259–263 (1976).
[16] I. C. MacRae, K. Raghu, and T. F. Castro, J. Agr. Food Chem. **15,** 911–914 (1967).
[17] A. D. Heritage and I. C. MacRae, Appl. Environm. Microbiol. **34,** 222–224 (1977).
[18] Y. Tsukano and A. Kobayashi, Agr. Biol. Chem. **36,** 166–167 (1972).
[19] R. Engst, R. M. Macholz, and M. Kuyawa, Chemosphere **7,** 401–418 (1977).