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NOTES

Degradation of Lindane by Cell-Free Preparations of *Clostridium sphenoides*

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Cell-free preparations of *Clostridium sphenoides* degraded the insecticide lindane, the γ-isomer of 1,2,3,4,5,6-hexachlorocyclohexane, to the γ-isomer of 3,4,5,6-tetrachloro-1-cyclohexene. The activity appeared to be associated with the membrane fraction and required reduced glutathione. The tetrachlorocyclohexene intermediate was further metabolized by the membrane fraction to unknown substances.

The insecticide lindane, which is the γ-isomer of 1,2,3,4,5,6-hexachlorocyclohexane (γ-HCH), was recently shown to be converted to the γ-isomer of 3,4,5,6-tetrachloro-1-cyclohexene (γ-TCCH) by washed-cell suspensions of *Clostridium sphenoides* (4). This species was the same isolate used in previous studies on γ-HCH degradation (6, 7).

Although there have been a number of reports on the metabolism of γ-HCH by enzyme preparations from insects (1, 2, 5), there have been no successful cell-free studies with bacteria capable of degrading γ-HCH. This paper describes successful attempts to obtain cell-free preparations of *C. sphenoides* that could degrade γ-HCH. The isolate, *C. sphenoides* UQM 780, was grown anaerobically at 30°C for 42 h in 7 liters of a medium described earlier (4). The cells were harvested by centrifugation and washed three times with 0.025 M phosphate buffer (pH 7.2) and finally suspended in the same buffer, modified by the addition of ethylenediaminetetraacetic acid (EDTA) (4 μg/ml) and reduced glutathione (GSH, 7.68 mg/ml). The suspensions were deoxygenated by passing oxygen-free nitrogen through them for 15 min at 200 ml/min before disruption by sonication with an ultrasonic disintegrator (Biosonik III, Bronwill Scientific Inc., Rochester, N.Y.). After sonication and microscopic examination to check the extent of cell disruption, the mixtures were freed of large cell debris by centrifugation at 10,000 × *g* (0°C) for 10 min. Cell-free extracts and membrane fractions were prepared from this supernatant by centrifugation at 30,000 × *g* for 30 min. The supernatant from this centrifugation was used in enzyme assays as the cell-free extract. The membrane fraction represented the pellet, and for the purpose of enzyme assay, it was suspended in 0.025 M phosphate buffer (pH 7.0) containing EDTA and GSH to the equivalent volume of the original cell suspension. The dry weights of these suspensions were between 17 and 19 mg/ml. A portion of the original cell suspension was retained in each experiment and assayed for γ-HCH degradation as a control on whole-cell activity.

The degradation of γ-HCH by cell-free extracts, membrane fractions, and whole cells in the absence of oxygen was followed in glass reaction flasks designed to allow oxygen displacement with oxygen-free nitrogen. Also, frequent sampling was possible via Suba seal plugs by using hypodermic syringes and needles. The assay mixtures had a total volume of 50 ml in all cases and were incubated at 30°C.

Assay mixtures consisted of (i) cell-free extract, GSH and γ-HCH, (ii) cell-free extract and γ-HCH, (iii) membrane fraction, GSH, and γ-HCH, and (iv) buffer, GSH, and γ-HCH. In all assays, three replicates of each mixture were used, and samples were withdrawn periodically for the determination of γ-HCH and γ-TCCH by gas-liquid chromatography. Samples from the assay flasks were extracted into an equal volume of nanograde *n*-hexane, containing δ-HCH (3 μg/ml) as internal standard, in sealed glass vials on a rotary mixer for 10 min. The *n*-hexane layers were withdrawn and dehydrated over anhydrous, granular $MgSO_4$, and 5-μl portions were analyzed by gas chromatography. The gas chromatograph, its operating parame-

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ters, and gas chromatographic identification of substrate and metabolite were exactly as described earlier (4). The concentration of γ-HCH remaining in the reaction mixtures was computed from standard curves for γ-HCH. As the exact amount of γ-TCCH could not be determined, because insufficient authentic γ-TCCH was available for standard curve preparation, the peak heights of γ-TCCH on chromatograms of extracts of assay mixtures have been compared with those of the δ-HCH internal standard in the same extracts and expressed as ratios in Fig. 1. The conversion of γ-HCH to γ-TCCH versus time by cell-free extracts and the membrane fraction of *C. sphenoides* is shown in Fig. 1. Conversion of γ-HCH in the mixtures containing cell-free extract was much greater in the presence of GSH. The activity was low, however, in comparison with whole cells, which converted all of the γ-HCH via γ-TCCH to unknown products within 1 h. No γ-TCCH was produced in mixtures containing buffer, GSH, and γ-HCH. The results from these studies demonstate a requirement for GSH in the degradation of γ-HCH by cell-free extracts of *C. sphenoides*. This requirement for GSH suggests that a glutathione conjugation may be involved in γ-HCH degradation by *C. sphenoides*. Sims and Grover (8) and Ishida and Dahm (5) have reported similar glutathione-dependent, chlorinated hydrocarbon-degrading systems in rat liver and housefly homogenates, respectively.

The membrane preparation of *C. sphenoides* not only showed a more rapid conversion of γ-HCH to γ-TCCH than cell-free extracts, but also conversion of the latter to products as yet unknown (Fig. 1). The enzymes of *C. sphenoides* responsible for the conversion of γ-HCH to γ-TCCH and to other products have, therefore, been shown to be associated largely with the membrane fraction of the cells. Reductive dechlorination of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] has been shown to be associated with the membrane fraction of *Escherichia coli* (3).

The unusually long-term incubation periods used in the present study, particularly for the cell-free extracts, were necessary to achieve an equilibrium with respect to γ-TCCH production. This suggests that other intermediates are formed before γ-TCCH such as γ-2,3,4,5,6-pentachloro-1-cyclohexene or γ-1,2,3,4,5-pentachlorocyclohexane. However, no gas chromatographic evidence based on retention times for the formation of either of these substances was obtained in the present study with either the cell fractions or whole cells.

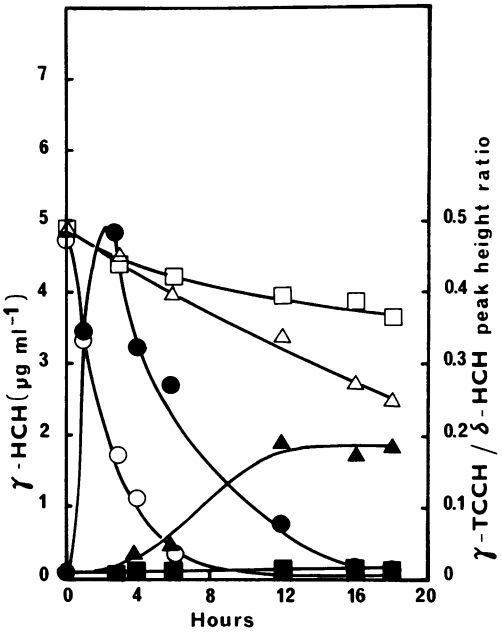

FIG. 1. *Degradation of γ-HCH and γ-TCCH formation by various cell fractions of Clostridium sphenoides. Symbols:* (△, ○, □) *γ-HCH and* (▲, ●, ■) *γ-TCCH in cell-free extract + GSH, membrane fraction + GSH, and cell-free extract alone, respectively.*

The length of the time course of conversion of γ-HCH to γ-TCCH also suggests that the enzymes involved are fairly stable, and this could prove to be a useful property in the purification and characterization of the active protein or proteins.

The results presented here are the first report of the degradation of γ-HCH by bacterial cell-free preparations and localization of the enzymatic activity in the membrane fraction.

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