Biodehalogenation—The Metabolism of the Nematocides *cis*- and *trans*-3-Chloroallyl Alcohol by a Bacterium Isolated from Soil

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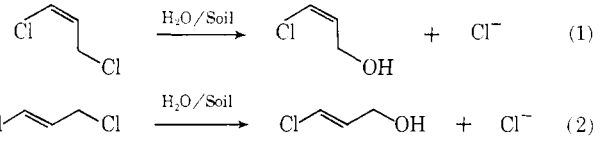
It has been previously demonstrated that *cis*- and *trans*-1,3-dichloropropene chemically hydrolyze in moist soil to the corresponding 3-chloroallyl alcohols which are biocidal. The present work portrays the path of metabolism of each of the isomeric alcohols to carbon dioxide and chloride ion by a *Pseudomonas* species isolated from soil. The dehalogenation step for each of these isomers is a direct hydroxylation of the β-vinylic carbon-chlorine bond of the isomeric 3-chloroacrylic acid intermediates.

Cis- and *trans*-1,3-dichloropropene are widely employed as preplant soil biocides for the control of parasitic nematodes and fungi. No organic halide residues have been detected in crops grown in soil treated with these biocides, and consequently these substances have been exempted from the requirement of a tolerance.

It has been demonstrated (Castro and Belser, 1966) that the dihalopropenes readily hydrolyze in moist soil (Equations 1, 2). Moreover, the resulting *cis*- and *trans*-3-chloroallyl



alcohols are highly nematocidal (Mojé *et al.*, 1957) and they are potent skin irritants.

The residue data coupled with the general lethargy of vinylic chlorides towards chemical reaction suggested that an important biological conversion of the chloroallyl alcohols may occur in soil. As a part of our studies of the fate of nematocides in soil (Castro and Belser, 1968) and the nature of biodehalogenation processes (Bartnicki and Castro, 1969; Castro and Bartnicki, 1965, 1968), we wish to report here the metabolism of *cis*- and *trans*-3-chloroallyl alcohol by a *Pseudomonas* species isolated from soil.

EXPERIMENTAL

Isolation of the Organism. Chloride ion was detected in a sample from our soil screen (Castro and Belser, 1968) that had been incubated with *cis*-3-chloroallyl alcohol. After 15 transfers into a basic salts medium (Castro and Bartnicki, 1965) that contained 0.02% yeast extract and $5 \times 10^{-3}M$ substrate, chloride ion was consistently generated. The organisms were placed on agar plates containing the same levels of nutrients and substrate as the transfer media. A picked colony actively dehalogenated the *cis*-alcohol when placed in the liquid medium. The bacterium was further purified by reisolating it from plates. The gram negative colorless rods obtained in this fashion possessed polar flagella and were identified as a species of *Pseudomonas*. Pure cultures were stored on agar slants that had the constitution noted above, except that 0.2% yeast extract was employed.

Growth and Conversion Conditions. Routinely, 50 to 100 ml of liquid media were inoculated from the slants and allowed to grow for 3 days at room temperature. Twenty-five ml of the inoculum was added to 2.8 l. Fernbach flasks that contained 1 l. of medium. The flasks were incubated at 25° C on a New Brunswick Gyrorotatory shaker for 3 days. Cells were centrifuged at 10,000 rpm for 25 min and washed once with $0.01M$ phosphate buffer at pH 7.4. The cell pack from six flasks, usually ~1 ml, was suspended in 60 ml of a solution of $0.01M$ phosphate buffer at pH 7.4 and $5 \times 10^{-3}M$ substrate. The mixture was stirred slowly at 25° C with a magnetic stirrer. Resting cell suspensions at these concentrations were employed throughout as standard conversion conditions. *cis*-3-Chloroallyl alcohol was wholly consumed within 3 to 6 hr under these conditions (Figure 1). The relative rates of chloride release by resting cells from *cis*-3-chloroallyl alcohol, *trans*-3-chloroallyl alcohol, *cis*-3-chloroacrylic acid, and *trans*-3-chloroacrylic acid were run in similar fashion to the conversion conditions noted above, except that initial substrate concentrations were $3 \times 10^{-3}M$.

Materials. All purchased and prepared substances had physical constants and spectral properties which checked the literature. The infrared spectrum and gas chromatographic properties of radio-labeled substances were identical with the parameters of pure nonlabeled compounds. Pure *cis*- and *trans*-3-chloroallyl alcohol were prepared in the manner previously described (Castro and Belser, 1966). The *cis*- and *trans*-3-chloroacrylic acids were obtained from the reaction of propiolic acid with concentrated HCl at 60° C (Backer and Beute, 1935). Small samples of the isomeric methyl esters were prepared from each of the acids by treatment of an ethereal solution with diazomethane. Dilute aqueous solutions of malonic acid semialdehyde were freshly prepared for gas chromatographic analysis by hydrolysis of the sodium salt of the anion of ethyl formylacetatediethylacetal. The latter was obtained from the condensation of ethyl acetate and ethyl formate with sodium ethoxide in ethanol (Deuschel, 1952). After 24 hr the formation of acetaldehyde from the decarboxylation of formylacetic acid could be detected in these samples.

***cis*- and *trans*-3-Chloroallyl alcohol-1,2,3-^{14}C.** A mixture of 4.2 g of the isomeric alcohols, 70% *cis*-, 30% *trans*-, b.p. 143° to 148° C was obtained from the hydrolysis of 5.5 g of an isomeric mixture of *cis*- and *trans*-1,3-dichloropropene-1,2,3-^{14}C with 60 ml of 10% Na_2CO_3 at 80° C for 25 hr. The procedure was like that described for the nonradioactive alcohols (*vide supra*) except that the product solution was saturated with sodium chloride before repetitive ether extraction. Pure samples of each isomer were trapped by gas chromatography from a carbowax 20M column that contained 3% silver nitrate.

***cis*- and *trans*-1,3-Dichloropropene-1,2,3-^{14}C.** In analogy

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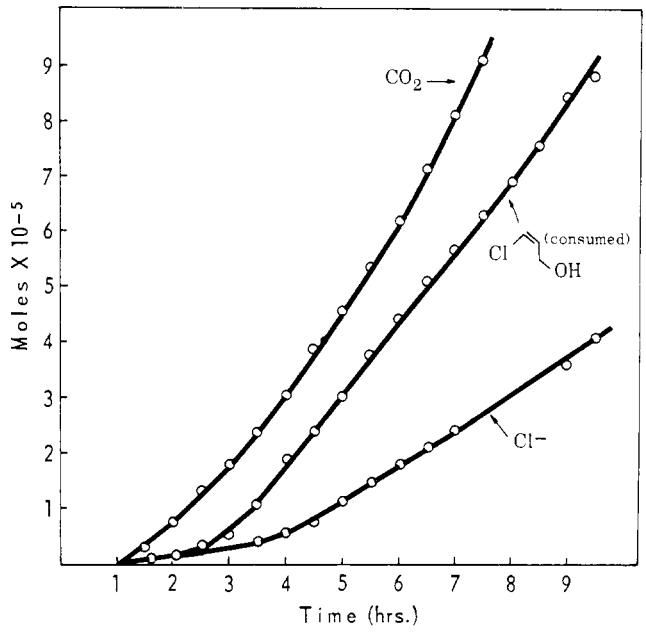


Figure 1. Rates of consumption of 5 × $10^{-3}M$ *cis*-3-chloroallyl alcohol, chloride release, and CO_2 production by resting cells

to the procedure of Hurd and Webb (1936), 1,3-dichloro-2-hydroxypropane-1,2-3-^{14}C, 50 g, and $POCl_3$, 40 g were warmed to reflux from 110° to 165° C over a period of 1 day. The flask was then fitted for distillation with the receiver cooled in an ice water bath. A fraction that had b.p. 103° to 133° C was collected, taken up in ether, and washed thrice with water saturated wtih $NaHCO_3$, and dried over Na_2CO_3. The dried ethereal solution was filtered and distilled through a small Vigreux column. A fraction having a b.p. 104° to 120° C weighed 11.0 g. Upon gas chromatography this fraction was shown to be composed of *cis*-1,3-dichloropropene, 66%, and *trans*-1,3-dichloropropene, 33%. Pure samples of each isomer were obtained by gas chromatographic trapping upon a DC-710 column.

1,3-Dichloro-2-hydroxypropane-1,2,3-^{14}C. The dihalide was prepared in the manner described for the cold compound (Conant and Quayle, 1932). A solution composed of 100 g of 95% glycerine, 2 mc of uniformly labeled glycerine, 3 ml of water, and 2 g of acetic acid was warmed to 115° C whereupon the addition of gaseous hydrogen chloride was begun. After 16.5 hr, workup afforded 56 g of product with b.p. 65–68° C (13 mm).

METHODS

Kinetics. The rates of the whole cell conversion presented in Figure 1 were determined in the following manner. Two identical reactions were run side by side. Each reactor was composed of a 250-ml round bottomed flask fitted with air inlet and outlet ports, a magnetic stirrer, and a serum cap. Each flask was charged with 60 ml of a resting cell suspension. Substrate *cis*-3-chlorallyl alcohol was added at time 0 to the slowly stirred suspension. A slow stream of moist air ~20 ml per min was passed through both flasks, and the exit ports of each were connected in turn to two cold traps followed by a dip tube immersed in a liquid scintillation counting vial that contained 0.2*M* hydroxide of hyamine for CO_2 trapping (Castro and Bartnicki, 1965).

Carbon dioxide was monitored from one of the reactions by employing *cis*-3-chloroallyl alcohol-1,2,3-^{14}C and changing the counting vial at the desired intervals. A blank without cells gave no counts.

Table I. Gas Chromatographic Properties of Substrates and Derivatives

Substance	Temperature (° C)	Emergence Time (min)[a]
cis-3-chloroallyl alcohol	145°	1.6
trans-3-chloroallyl alcohol	145°	2.0
	132°	3.2
cis-3-chloroacrylic acid	132°	6.6
methyl *cis*-3-chloroacrylate	130°	2.6
trans-3-chloroacrylic acid	132°	4.4
methyl *trans*-3-chloroacrylate	130°	2.6
formylacetic acid	80°	2.0
acetaldehyde[b]	130°	1.6

[a] A 1/8 in. diameter 1 ft Porapak P column with a flow rate of 20 ml/min was employed. [b] For this substance a 3 ft length column was used.

The other flask was monitored "simultaneously" for Cl- and *cis*-3-chloroallyl alcohol. Two ml aliquots were removed from the flask with a hypodermic syringe. Chloride ion was determined by direct potentiometry, employing a silver-silver chloride working electrode and a double junction reference electrode. The instrumentation was like that previously employed (Castro and Belser, 1966). Within 10 sec of a chloride reading, 1 μl of the sample was injected into a flame ionization gas chromatograph. A 1 ft × 1/8 in. stainless steel Porapak P column was employed (Table I). The concentration of *cis*-3-chloroallyl alcohol was assessed from peak height.

Organic Products. All products, except formylacetic acid, were visible by direct flame ionization gas chromatography of reaction mixtures. A 60 ml reaction mixture with *cis*-3-chloroallyl alcohol-1,2,3-^{14}C as substrate (*cf.* conversion conditions) was stopped at 6 hr by centrifugation. The supernatant was brought to pH 13 by the addition of 1*N* KOH and extracted three times with ether. The basic ether phase was dried over K_2CO_3, filtered, and concentrated to ~0.1 ml by distillation through a small Vigreux column. Gas chromatography (Table I) upon a machine equipped with an effluent splitter (Castro and Belser, 1968) showed one radioactive peak that coemerged with starting *cis*-3-chloroallyl alcohol. The basic aqueous phase was acidified with cold conc. H_2SO_4 to pH 1, saturated with ammonium sulfate, and extracted with three 100 ml portions of ether. The ether extracts were dried over sodium sulfate and treated in the manner described for the basic fractions. An infrared spectrum of the residue (film) corresponded to *cis*-chloroacrylic acid. Gas chromatography of this fraction afforded a radioactive peak that coemerged with *cis*-3-chloroacrylic acid and a very small peak that coemerged with formyl acetic acid. The peaks vanished in a small fraction treated with base. Another portion, esterified with diazomethane, provided a peak that coemerged with methyl *cis*-3-chloroacrylate. Direct gas chromatographic monitoring of a whole cell conversion of *cis*-3-chloroallyl alcohol at hourly intervals showed the appearance of *cis*-chloroacrylic acid, followed by its decay. After 6 hr no peaks were visible.

With cold *cis*-3-chloroacrylic acid as substrate for the resting cells, after 6 hr repeated ether extraction and concentration afforded formylacetic acid in the condensate subjected to vpc analysis. This condensate was treated with three drops of water and the solution was rechromatographed after 24 hr. At this time acetaldehyde could be seen and only a small amount of formylacetic acid remained. This reaction is characteristic

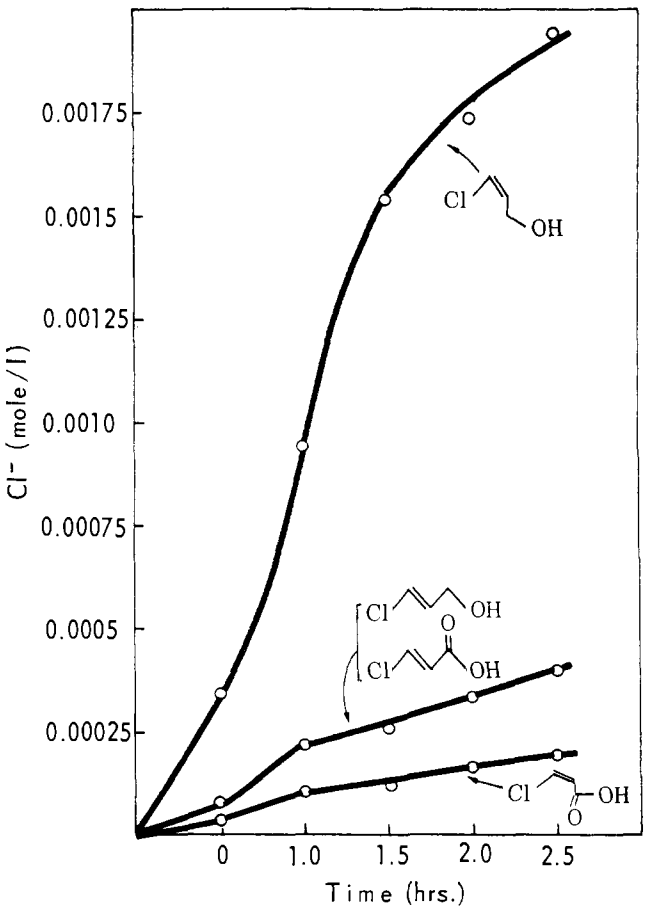


Figure 2. Rates of Cl^- release from *cis*- and *trans*-3-chloroallyl alcohol and *cis*- and *trans*-3-chloroacrylic acid by resting cells. Initial concentration of all substrates was $3 \times 10^{-3} M$

of formylacetic acid, and it was also observed with the synthetic acid under these conditions.

Similar experiments with *trans*-3-chloroallyl alcohol and *trans*-3-chloroacrylic acid demonstrated the *trans*-alcohol was converted to the *trans*-chloro acid and then to formylacetic acid.

The relevant gas chromatographic properties of all products and derivatives are given in Table I.

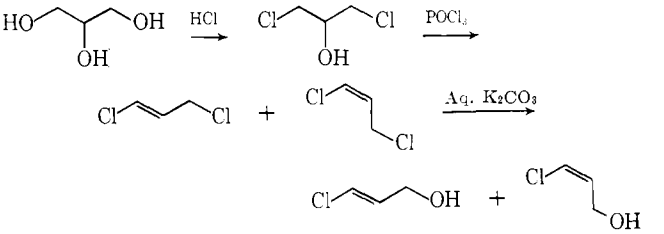
RESULTS AND DISCUSSION

Kinetics. The rates of consumption of *cis*-3-chloroallyl alcohol and the production of chloride ion and carbon dioxide by resting cells are presented in Figure 1.

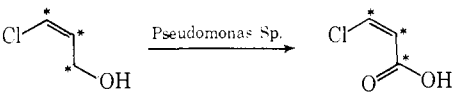
It will be noted that the chloroalcohol is consumed more rapidly than chloride is released. Moreover, from the slopes at 4 hr, the rate of carbon dioxide production is about three times the rate of chloride release, and about equal to the rate of chloroallyl alcohol consumption. These results bespeak a process in which the starting chloroalcohol is converted to a chlorinated intermediate prior to dehalogenation. Moreover, decarboxylation must rapidly ensue after chloride release. The kinetics indicate that only a low steady state concentration of intermediates can be present.

The rate of chloride release from *cis*- and *trans*-3-chloroallyl alcohol and *cis*- and *trans*-3-chloroacrylic acid by whole resting cells is presented in Figure 2. The results serve only to show that significant dehalogenation of all substrates and intermediates does occur. The faster dehalogenation of *cis*-3-chloroallyl alcohol compared to that of *cis*-3-chloroacrylic acid may reflect rates of permeation and/or the toxicity of this acid to the cells at this concentration.

Radiolabeled Substrates. A mixture of 70% *cis*- and 30% *trans*-3-chloroallyl alcohol was prepared from glycerine-1,2,3-^{14}C *via* the route:

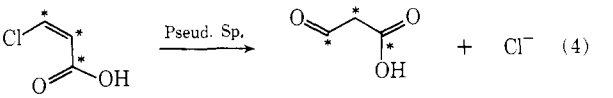


Stoichiometry. In accord with the kinetic findings, the first product from *cis*-3-chloroallyl alcohol 1,2,3-^{14}C is *cis*-3-chloroacrylic acid (Eq. 3). This acid in turn has



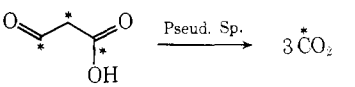
(3)

been found to dehalogenate to formylacetic acid or "malonic acid semialdehyde" (Eq. 4).



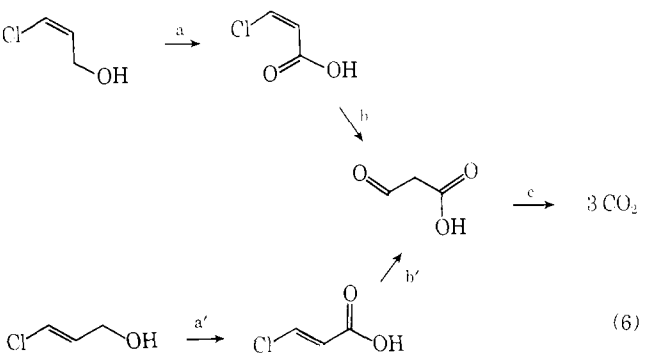
(4)

The latter is rapidly decarboxylated (Eq. 5).



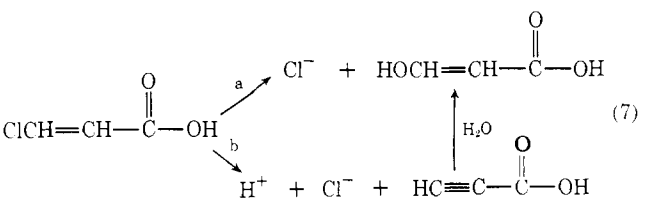
(5)

We have not studied the path of this latter conversion, but it is known to occur in other organisms. An entirely analogous path of metabolism was established for *trans*-3-chloroallyl alcohol. The paths for both isomers are illustrated in Eq. 6. The dehalogenation step for both isomers is the slow step



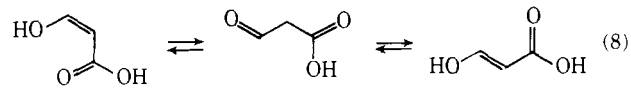
(6)

(6, b, 6, b′). It is the point at which the stereochemistry is lost. There is no prior isomerization of the alcohols or acids. It should be noted that propiolic acid could not be detected in any of the culture media. Hence, we formulate the dehalogenation step as a direct hydroxylation, or water addition at the β-carbon (7, a) rather than an elimination followed by water addition to the acetylene (7, b). Isomeric enols (8)

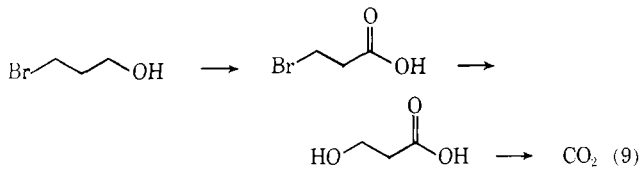


(7)

would not be detected by our methods.


(8)

A direct hydroxylation of the β-carbon-halogen bond and the sequence and timing of the metabolism of the chloroallyl alcohols are consonant with the path established for the metabolism of 3-bromopropanol by another *Pseudomonas* species (Castro and Bartnicki, 1965) (Eq. 9). Common


(9)

features of these processes which may reflect a general path of metabolism of β-haloalcohols are oxidation of the alcohol and hydroxylation of the ensuing β-carbonyl-halide. Coupled with our earlier work (Eq. 1, 2), the present study establishes that the dihalopropene biocides can be completely detoxified in soil.

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