Biodehalogenation. The Metabolism of Chloropicrin by *Pseudomonas* sp.

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Four *Pseudomonas* sp. have been isolated from soil that dehalogenate chloropicrin. The path of reaction of chloropicrin with *Pseudomonas putida* PpG-786 (ATCC culture 29607) has been determined. A small portion of the halide (~4%) is converted to carbon dioxide. The major path of metabolism entails three successive reductive dehalogenations to nitromethane:

$$Cl_3CNO_2 \rightarrow Cl_2CHNO_2 \rightarrow ClCH_2NO_2 \rightarrow CH_3NO_2$$

In addition, a highly water soluble substance, presumably a small peptide, is produced by a nonenzymatic reaction of chloropicrin with live or dead cells.

Chloropicrin is a highly toxic and noxious substance (Merck & Co., 1976). It was employed as a trench gas during World War I. Subsequently it found use in agriculture as an efficient preplant soil fumigant. It is used widely today, usually in combination with methyl bromide, as a soil sterilant. Moreover, it is an effective agent for protecting forest products from microbial decay and it is used as a disinfectant for cereals and grains. The broad-spectrum biocidal properties of this molecule suggest that it would be difficultly detoxified in the biosphere. The substance contains no carbon–hydrogen bonds. This characteristic itself could confer upon the substance a lethargy to microbial attack, for there are no sites for an initial oxidative metabolism.

As a part of our effort to discern the fate of such substances in the environment, we report here that chloropicrin is dehalogenated by several species of *Pseudomonas* isolated from soil. The path of metabolism by *Pseudomonas putida* is reported in detail. In this initial work we have employed the organism PpG-786 because it contains a high concentration of the enzyme P-450 cam. In theory, this kind of hemeprotein should be capable of cleaving carbon–halogen bonds (Castro, 1982; Bartnicki et al., 1978).

EXPERIMENTAL SECTION

Materials. *^{14}C-Labeled chloropicrin* was synthesized from UL phenol via the sequence

$$\text{phenol} \xrightarrow{H_2SO_4} p\text{-hydroxyphenylsulfonic acid} \xrightarrow{HNO_3} \text{picric acid} \xrightarrow{Ca(OCl)_2} \text{chloropicrin}$$

The method follows essentially that reported for the nonlabeled substance (Reed, 1924; Frahm, 1951). The physical properties of the final product matched those in the literature. It had a specific activity of 1.88×10^{-3} mCi/mmol. The mass spectrum of chloropicrin was typical of the family of chloronitromethanes studied in this work. The high mass peak corresponded to the CCl_3 fragment (parent – NO_2) at 123, 121, 119, and 117. **Caution**: This preparation should be conducted in a good fume hood. All transfers of pure chloropicrin were performed in a hood.

Dichloronitromethane was obtained in poor yield by the borohydride reduction of chloropicrin. A stirred solution of 20.2 mL (0.2 mol) of trichloronitromethane in 150 mL of dry dimethylformamide (DMF) was cooled to 5 °C. A solution of 7.1 g (0.18 mol) of sodium borohydride in DMF, prepared by grinding the tablets under DMF, was added slowly over 3 h. The reaction is vigorous and sodium chloride begins to precipitate immediately. during borohydride addition the mixture was kept below 20 °C. After standing overnight the reaction mixture was steam distilled. The steam distillate was separated into two phases, and the aqueous phase was extracted twice with methylene chloride. The organic fractions were combined, dried over sodium sulfate, concentrated, and distilled twice under argon through a small vigreux column. The fraction boiling at 116–118 °C was collected as dichloronitromethane: MS 87, 85, 83 (P – NO_2); NMR δ 7.1 (s, 1 H); IR 3020 and 2910 (C–H), 1545 cm^{-1} (C–NO_2). Gas chromatographic analysis indicated the substance contained ~3% chloropicrin but no chloronitromethane. Reactions run at higher temperature or with "wet" solvent yielded only trace amounts of the steam distillable product.

Monochloronitromethane was obtained by the *tert*-butyl hypochlorite chlorination of nitromethane in the presence of 1-hexene (Heasley et al., 1971): bp 119–120 °C; MS 51, 49 (P – NO_2); NMR δ 5.45 (s, 2 H). Nonradioactive chloropicrin and nitromethne were obtained from Eastman Kodak and used without purification.

The Soil Screen. Incubation of chloropicrin with a wide range of soil samples in shake culture was conducted in the manner previously described (Castro and Belser, 1968). At least four organisms were isolated that would continually produce chloride ion upon incubation with chloropicrin (10^{-3} M) in a minimal salt medium. All of these were species of *Pseudomonas* as defined by the characteristics in Bergey's Manual. However, *Pseudomonas putida* [PpG-786 (ATCC 29607)] was selected for this initial metabolic work because the organism has a high content of the enzyme P-450 cam. A slant of the organism was obtained from Professor I. C. Gunsalus.

Growth of *P. putida*. *P. putida* was grown on camphor in a minimal salt medium according to the procedures outlined by Peterson (O'Keefe et al., 1978).

Reaction of Chloropicrin with *P. putida* G-786. Whole cells were harvested, washed twice with buffer, and resuspended in 0.1 M phosphate buffer at pH 7.4. A relatively thick slurry of cells, 0.5–1.0 g wet weight/10 mL of buffer was employed and maintined on shake at 30 °C. A 40-mL solution of $(0.7–2.0) \times 10^{-3}$ M [^{14}C]chloropicrin in buffer was prepared in the 250-mL reaction flask by shaking the glass-stoppered flask at 30 °C for 2 h. Reaction was begun by adding an equal volume of the cell suspension to the chloropicrin solution. The single-neck flask was fitted with a glass-stoppered joint equipped with a serum-capped stopcock.

Overall Material Balance. Reaction mixtures were shaken at 30 °C for 2–3 days. With the use of hypodermic tubing the gas phase of the reaction was swept with argon into a barium hydroxide solution that had been purged with argon for $^1/_2$ h. The $BaCO_3$ produced was vacuum filtered, washed with water, dried, and weighed, and

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Table I. Distribution of Counts[a] and Cl^- Yields

	counts				
% Cl^- [b]	whole mix	CO_2	cell paste	aqueous	% CH_2Cl_2
80		2.5	6.4	32	33
100		2.0	12.7	32	
94	93	3.8			
92	75		4.3	34	35
100	97	3.4			
95	93	3.9	7.4	39	40
21	(dead cells)			18	[c]

[a] Percent of original counts in $Cl_3{}^{14}CNO_2$. [b] $(Cl^-)(100)/(Cl_3CNO_2)(3)$. [c] Only starting (Cl_3CNO_2).

counted for ^{14}C. Carbon dioxide, determined gravimetrically or by ^{14}C counting, was in good agreement and represented only a small portion of the initial carbon, 2–4%. Chloropicrin solutions without cells yielded no $BaCO_3$ from the gas or aqueous phase during this time period. An aliquot of the whole cell reaction mixture was removed via hypodermic syringe for liquid scintillation counting and Cl^- analysis. The latter was determined potentiometrically by the method previously described (Castro and Belser, 1966). At this point the flask was opened and the contents were centrifuged for 20 min at 10^4g. The water washed cell paste contained 4–12% of the counts while the bulk, 60–80%, remained in the aqueous supernatant solution. This solution was extracted twice with methylene chloride and separated. Somewhat more than half of the counts were consistently extracted into the organic phase. The distribution of the counts between the aqueous and organic phase was not altered by employing other extractants, e.g., ether, or varying the pH of the aqueous solution from 1 to 11 before extraction. The overall distribution of counts for several runs is given in Table I.

Gas chromatography of the entire reaction mixture proved too complex for analytical purposes. The organic fraction itself did show up to six peaks. In these overall bulk runs, however, only one section of emergents was radioactive. The best chromatograms of the CH_2Cl_2 extract were obtained with a $^1/_8$ in., 2.5 ft Poropak Q column at 140 °C and a thermal conductivity detector. In the runs outlined above, vapor-phase chromatography of the organic product solution exhibited a peak at 1.5 min that was readioactive and coemergent with nitromethane. In contrast, the aqueous phase showed no radioactive fractions upon gas chromatography. A radioactive aqueous fraction was, however, held on G-10 Sephadex, indicating a relatively low molecular weight. Attempts to characterize the Sephadex G-10 chromatographed radioactive fraction as a potential one-carbon metabolite failed. Thus, formic acid was added to the highly aqueous soluble "metabolite". A *p*-bromophenacyl ester was prepared and recrystallized to purity. It was not radioactive. Similarly, acid hydrolysis (formamide) and derivatization yielded no radioactivity in the ester. Methylamine was eliminated by diluting and isolating the corresponding methylphenylthiourea, devoid of radioactivity. Carbon dioxide was eliminated as the product by adding the aqueous phase to barium hydroxide. Only a trace of ^{14}C was found in the barium carbonate. Thin-layer chromatography of the radioactive G-10 Sephadex fraction on silica gel G and elution with *n*-butyl alcohol, acetic acid, and water afforded a spot that was consistently ninhydrin positive and radioactive. Repeated thin-layer chromatography of the isolated area under a range of conditions and development procedures indicated the substance(s) to be a low molecular weight peptide(s). Full characterization of this fraction was abandoned when it was learned that a substance possessing the same solubility and chromatographic behavior was obtained upon exposing dead cells to chloropicrin.

Dead cells were prepared by exposing the cell pack to a 10% glutaraldehyde solution for 3 h. These cells, when subjected to the regimen outlined above, showed an 18% release of Cl^- from chloropicrin after 3 days. The organic phase contained only chloropicrin, and the aqueous phase was the same as that described above.

Time-Course Studies. Reactions were conducted in the fashion described for the material balance runs outlined above. A typical run consisted of 10 g (wet weight) of cells in 100 mL of phosphate buffer that was 10^{-3} M in chloropicrin. After the cells and radioactive substrate were mixed, 5-mL aliquots of the reaction solution were removed at intervals by hypodermic syringe and quenched in serum-capped vessels that contained 0.25 mL of concentrated HNO_3 and 2 mL of CH_2Cl_2. The quenched aliquot was shaken and centrifuged. The aqueous phase was analyzed for chloride ion by direct potentiometry. An aliquot of the CH_2Cl_2 phase was counted for total reactivity. A 5-μL sample was analyzed by gas chromatography under the conditions described above. In addition to solvent, these samples showed numerous peaks. Depending upon timing, however, four were consistently radioactive. These corresponded to nitromethane 2.2 min, chloronitromethane 6.0 min, dichloronitromethane 12 min, and chloropicrin 18 min. Qualitative identification of these substances was accomplished with nonradioactive starting material by trapping the vapor-phase chromatographic peaks in a CO_2-cooled cold finger and subjecting them to mass spectral analysis. Each substance coemerged with a standard and had the correct mass spectrum (vide infra). Best estimates of the relative amounts of these substances proved to be obtained by directly trapping the radioactive fractions (1 min on either side of the emergence time above) into "Insta-Gel" (Packard) counting fluid in toluene and determining the ^{14}C by liquid scintillation counting. The results of two of these time-course studies are presented in Figures 1 and 2.

Liquid scintillation counting was accomplished with a Packard Tri-carb Model B-3255 with automatic external standard. All counts were quench corrected and background was subtracted from them.

RESULTS

The overall material balance upon exposure of cells to chloropicrin for 2–3 days in a closed system is illustrated in Table I. It should be emphasized that the volatility of the starting substrates and the organic products plagued our attempts at a tight quantitattion. Thus, with ^{14}C-labeled chloropicrin, counts were always lost upon manipulation of the reaction solution. In addition, dead cells did cause some consumption of chloropicrin (bottom line of Table I). The counts in the aqueous fraction following dead cell exposure correspond in kind and amount to those remaining in the aqueous product from live cell runs. In both cases counts were vested in what appears to be a low molecular weight peptide or other ninhydrin-positive entity. This assignment is based upon chromatography on G-10 Sephadex and subsequent repeated TLC analysis. Attempts to derivatize this (these) substance(s) as a carboxylic acid (*p*-bromophenacyl ester) or amine (thiourea) failed. Specific checks for water-soluble one-carbon fragments that might be obtained by hydrolysis and/or reduction of chloropicrin (carbon dioxide, formic acid, formamide, methylamine) failed. Our attempts to characterize this (these) substance(s) further were abandoned

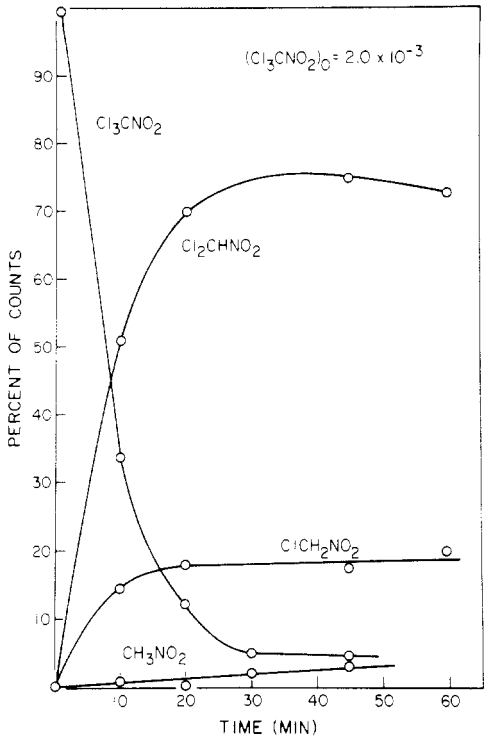

Figure 1. Time course of the conversion of trichloronitromethane to nitromethane, $(CP)_0 = 2 \times 10^{-3}$ M.

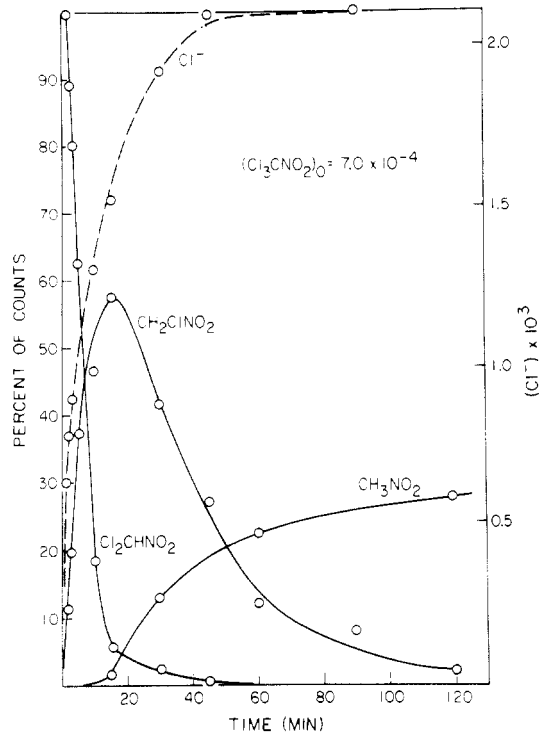

Figure 2. Time course of the conversion of trichloronitromethane to nitromethane, $(CP_0) = 0.7 \times 10^{-3}$ M.

when it was realized the same entity is obtained from dead cells. We presume this product represents a chemical alkylation of cell proteins by chloropicrin.

The results of the table demonstrate that chloropicrin is metabolized and that carbon dioxide is a minor product. Also, gas chromatographic analysis of the organic extract showed nitromethane to be the major organic product. These results are summarized in eq 1. The path of conversion of chloropicrin to nitromethane was determined primarily by a combination of gas chromatography and

$$Cl_3C\text{-}NO_2 \xrightarrow[\text{nonenzymatic}]{p.\ putida} 3Cl^- + CO_2 + CH_3NO_2 \quad (1)$$

low molecular weight peptides

mass spectrometry along with liquid scintillation counting of trapped vapor-phase chromatographic fractions.

Time-course studies of the metabolism of chloropicrin by *P. putida* are illustrated in Figures 1 and 2. In Figure 1, the disappearance of chloropicrin is accompanied by a rise in concentration of dichloronitromethane. At 60 min the latter is beginning to fall off while chloronitromethane and more slowly nitromethane are being produced. The slower rates of reaction in this figure as compared to those in Figure 2 were chosen to show the initial phases of the reaction. They also illustrate the deleterious effect of the higher concentration of starting toxicant. The data in Figure 2 were obtained with about one-third the concentration of chloropicrin. In this case chloropicrin consumption was so fast that none was apparent at the first time point. Indeed, the data show the rapid decline of dichloronitromethane, the first metabolite, followed by the rise and fall of chloronitromethane. This in turn, after a slight lag, is followed by the steady build up of nitromethane, the final organic product. The rate of Cl^- production is also plotted on the same graph at one-third scale. The general pathway established by these kinetics is sketched in eq 2. At each step Cl^- is released. Qualitatively, the relative rates of the processes are $k_{2a} > k_{2b} > k_{2c}$. An average approximate ratio of rates from several runs is 40:4:1. It should be emphasized that chloropicrin blanks in this buffer do not produce detectable amounts of Cl^- in this time period (Castro and Belser, 1981).

$$Cl_3CNO_2 \xrightarrow[a]{P.\ putida} HCCl_2NO_2 \xrightarrow[b]{} H_2CClNO_2 \xrightarrow[c]{} CH_3NO_2 \quad (2)$$

DISCUSSION

The reductive dehalogenations observed here are typical of the reaction of iron(II) porphyrins and heme proteins (Wade and Castro, 1973; Bartnicki et al., 1978) with this class of substrate. The bacterium used contains a large amount of the enzyme P-450 cam. The results suggest that the enzyme P-450 cam in intact *P. putida* is capable of catalyzing the observed transformations. Studies of the isolated enzyme and active sites models are under way.

The fact that such a highly toxic substance can be metabolized by soil microbes in a tribute to their resilience and chemical capabilities. The results constitute an important biological complement to the degradation of this subtance in the environment by photohydrolysis (Castro and Belser, 1981) or other physical means.

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Registry No. Chloropicrin, 76-06-2; dichloronitromethane, 7119-89-3; monochloronitromethane, 1794-84-9; carbon dioxide, 124-38-9; nitromethane, 75-52-5.

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Interactions of the Herbicides EPTC and EPTC plus R-25788 with Ozone and Antioxidants in Corn

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In greenhouse studies, the potential interactions of the herbicide EPTC (*S*-ethyl dipropylthiocarbamate) with ozone (O_3) or the antioxidants piperonyl butoxide and *n*-propyl gallate on corn (*Zea mays* L., "Pioneer 3780") were investigated in the presence or absence of the herbicide antidote R-25788 (*N,N*-diallyl-2,2-dichloroacetamide). Commercial formulations of EPTC (EPTAM) or EPTC plus R-25788 (ERADICANE) were incorporated into the soil at 4.5, 5.6, and 6.7 kg/ha, and they were evaluated against 0.2 and 0.3 ppm of O_3 or against 4.5, 6.7, and 9.0 kg/ha of soil applications of the two antioxidants. The interactive effects between selected treatment combinations of EPTC plus R-25788 and ozone or the two antioxidants were highly synergistic. In the absence of R-25788, the interactive effects of EPTC with O_3 or the two antioxidants were additive, although EPTC at 6.7 kg/ha combined with some rates of piperonyl butoxide interacted synergistically. The implications of these findings as to the potential mode of action of the antidote R-25788 are discussed.

Preplant incorporated applications of the herbicide EPTC (*S*-ethyl dipropylthiocarbamate) at rates of 3.4–4.5 kg/ha are effective in controlling many annual grass weeds in corn and in other crops (Mullison, 1979). In addition, EPTC at higher rates (6.7 kg/ha) is very effective in managing certain tough-to-control perennial weeds such as quack grass [*Agropyron repens* (L.) Beauv.] and yellow nut sedge (*Cyperus esculentus* L.) or in suppressing Johnson grass [*Sorghum halepense* (L.) Pers.] (Mullison, 1979). However, at these rates, EPTC is commonly injurious to corn (Burnside et al., 1971; Burt and Akinsorotan, 1976; Burt and Buzio, 1979; Chang et al., 1972; Martin and Burnside, 1982; Meggitt et al., 1972; Rains and Fletchall, 1971). The phytotoxicity of EPTC to corn is greatly reduced with the use of selected herbicide antidotes such as R-25788 (*N,N*-diallyl-2,2-dichloroacetamide) (Chang et al., 1972; Martin and Burnside, 1982; Meggitt et al., 1972; Pallos et al., 1975; Rains and Fletchall, 1971). Currently, in the United States, all EPTC used for weed control in corn is exclusively formulated as a prepackaged mixture of the herbicide EPTC and the antidote R-25788 in a 12:1 ratio. This formulation is marketed under the trade name ERADICANE. Use of the antidote R-25788 does not reduce the toxicity of EPTC to other crop or weed species (Stephenson and Chang, 1978).

The exact mechanism(s) by which R-25788 counteracts the toxicity of EPTC to corn are not very well understood at the present time. Extensive research on the mode of the antidotal action of R-25788 has resulted in a plethora of proposed mechanisms, none of which is unequivocally accepted. Among the mechanisms proposed, two that have attracted considerable attention are those proposing either a counteraction of EPTC phytotoxicity by R-25788 through a competitive inhibition at some common site within the protected plant (Ezra and Gressel, 1982; Ezra et al., 1982; Görög et al., 1982; Leavitt and Penner, 1979a; Stephenson et al., 1978, 1979; Wilkinson and Smith, 1975) or an increase in the rate of metabolic detoxication of EPTC caused by the antidote R-25788 (Carringer et al., 1978; Kömives and Dutka, 1980; Lay and Casida, 1976; Leavitt and Penner, 1979b; Rennenberg et al., 1982).

Air pollutants such as ozone (O_3) have been reported to intereact with several agricultural practices including chemical weed control (Rich, 1975). During the past decade, studies have shown that O_3 may interact with selected herbicides on certain plant species, thereby modifying either the overall plant response to these herbicides (Carney et al., 1973; Reilly and Moore, 1982; Sung and Moore, 1979) or the metabolism of herbicides in fumigated plants (Hodgson et al., 1973, 1974; Hodgson and Hoffer, 1977a,b). In addition to air pollutants, insecticide synergists such as piperonyl butoxide [α-[2-(2-butoxyethoxy)ethoxy]-4,5-(methylenedioxy)-2-propyltoluene] or other antioxidant compounds have also been reported to interact synergistically with selected herbicides on crop plants (Kömives and Dutka, 1980; Rubin et al., 1980).

The objectives of the present study were to evaluate any potential interactions between the herbicide EPTC and the air pollutant ozone (O_3) or the antioxidants piperonyl butoxide and propyl gallate on corn in the presence or absence of the herbicide antidote R-25788 and then to explain possible relationships between these interactions and the responses they evoke.

MATERIALS AND METHODS

Chemicals. The herbicides and antioxidant compounds used in the present study were obtained from the following sources: EPTC (formulated EPTAM herbicide) and EPTC plus R-25788 (formulated ERADICANE herbicide) were kindly provided by Stauffer Chemical Co., Westport, CT. Piperonyl butoxide was purchased from Pflatz and

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