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Mechanism of aerobic transformation of carbon tetrachloride by poplar cells

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Abstract

The biochemical mechanism of carbon tetrachloride transformation by poplar cells was investigated using an axenic poplar cell culture. After one-day incubations of poplar cells under aerobic conditions, about 1.5% of dosed carbon tetrachloride was transformed to carbon dioxide, about 0.001% to chloroform and about 3% of the carbon was bound to insoluble poplar cellular materials. The production of carbon dioxide increased under aerobic conditions while the formation of chloroform and cell binding of carbon tetrachloride-carbon was enhanced under anaerobic conditions. Both carbon dioxide production and cell binding were significantly inhibited by a general inhibitor of cytochrome P-450 activity (carbon monoxide) and by specific P-450 2E1 inhibitors (chlorzoxazone, isoniazid, 4-methylpyrazole and 1-phenylimidazole). However, no inhibitory effects were observed when the cells were incubated in the presence of lignin peroxidase inhibitors ($NaVO_3$ and 3-amino-1,2,4-triazole). These results suggest that an enzyme similar to mammalian cytochrome P450-2E1 is involved in the metabolism of carbon tetrachloride by poplar cells. This study demonstrates an environmental biodegradative process for carbon tetrachloride that operates under aerobic conditions.

Introduction

Carbon tetrachloride (CT) is one of the major contaminants on the Hazardous Waste list of the U.S. Environmental Protection Agency. Traditional pump-and-treat remediation of groundwater contaminated with CT is very expensive and often does not achieve desirable final treatment levels (Semprini 1995). *In situ* microbial degradation of CT occurs under anaerobic conditions, but often results in the accumulation of hazardous metabolites such as chloroform (CF). In addition, microbial degradation of CT can be inhibited when CT is present in the milligram per liter range (Hashsham et al. 1995).

In recent years, phytoremediation – the use of plants to clean up groundwater and soil contaminated with hazardous organics and toxic metals – has become recognized as a cost-effective environmental technology. Because of their fast-growing and deep-rooting characteristics, poplar trees have been studied for the remediation of groundwater and soil contaminated with trichloroethylene (TCE), trinitrotoluene (TNT) and atrazine (Newman et al. 1999; Thompson et al. 1998; Burken & Schnoor 1997). The results from the pilot-scale field experiments of Newman et al. (1999) suggested that TCE uptake and degradation by poplar trees may play a role in the removal and dechlorination of TCE by tree plantations in contact with polluted groundwater. Our on-going field experiments have indicated that plantings of poplar trees could remove more than 90% of applied CT with about 60% dechlorination of the lost CT (Wang et al. 1999). These losses may have been due to microbial activity in the rhizosphere or to uptake and transformation within the poplar trees themselves. This study was undertaken to determine the extent and mechanism of biochemical transformation of CT by poplar.

It is well known that higher organisms have a general mechanism of oxidative transformation and detoxification of xenobiotic compounds. Cytochrome P-450 monooxygenases, heme proteins of the endoplasmic reticulum, play a critical role in the degradative processes of xenobiotic compounds in higher organisms (Estabrook & Werringloer 1977). The P-450 enzymes are involved in the transformation of CT in mammalian systems (Henschler 1985), and CO_2 and CF were found to be the major metabolites, in addition to covalent binding of some reactive intermediates to cell proteins and lipids. We have found no studies of the mechanism of CT transformation by plants, but P-450-mediated reactions have been reported to be involved in the detoxification of herbicides and pesticides by various plants (Schuler 1996). The involvement of a P-450 enzyme in the degradation of TCE by poplar trees has been suggested, based on the nature of TCE metabolites in plant tissues (Newman et al. 1997). Therefore, it seems reasonable to suggest that P-450 enzyme may be responsible for the transformation of CT by poplar.

In addition to the P-450 mediated oxidative transformation of CT in mammalian systems, CT was also found to be mineralized by the lignin peroxidase activity of white rot fungus (Phanerochaete Chrysosporium) under aerobic conditions. The resulting products included CO_2 and trichloromethyl radical (Shah et al. 1993; Khindaria et al. 1995). Lignin peroxidases are also present in woody plants where they are involved in the biosynthesis of lignin. Therefore, it is also important to determine if lignin peroxidase is involved in the oxidative transformation of CT in the plant system. The objectives of this study were to identify the metabolites of CT transformation in poplar cells and to determine whether inhibitions of P-450 and lignin peroxidase would affect CT transformation by poplar cells. The final goal was to determine whether P-450 or lignin peroxidase was involved in CT metabolism by poplars. Because the growth and chemical conditions of cell cultures are easier to control compared with those of whole plants, the mechanism of CT transformation by poplar was investigated using suspended cell culture experiments. This approach also simplified the system by eliminating plant-soil-microbe interactions with CT metabolism.

Materials and methods

Cell culture

Axenic H11-11 tumor cells produced by transforming shoots of the *Populus trichocarpa* × *P. deltoides* clone [H11-11] with *Agrobacterium tumefaciens* A281 were used for the experiment, and the cells were grown in the Murashige and Skoog basal salt medium (MS medium) without hormones (Newman et al. 1997).

Reactor

A special reactor system, described below, was used in all cell culture experiments to determine the mechanism of CT transformation by poplar cells. Twenty mL of cell culture suspension was placed in a 125-mL, two-necked flask with a screw cap containing a lined Teflon septum on the side neck and a septum valve mounted in a screw cap (Mininert) on the top neck. Radiolabelled or non-radiolabelled CT was added to the culture through the septum valve. The flask was sealed to prevent losses of CT and any volatile metabolites, and the cells were incubated for 24 hours with shaking at 100 rpm. Under these conditions the cells could be exposed to as much as 300 mg/L CT in the aqueous phase without producing any decrease in cell mass or viability when compared with non-dosed cells. Cell mass was determined by weighing the cells after separation from the MS medium by filtration. Cell viability was determined using the method of trypan blue exclusion (Moldeus et al. 1978). At the end of the cell incubation, the dissolved oxygen concentration in the cell suspension was measured using a dissolved oxygen electrode meter. Dissolved oxygen levels were generally above 4 mg/L and always above 2 mg/L in the experiments under aerobic conditions.

After 24 hours of incubation, the cell suspension was purged with air to remove volatiles. A tube containing granular activated carbon (Coconut Charcoal, SKC, Cat. No. 226-09), divided into two sections by a glass fiber plug, was attached to the septum valve of the flask. The outflow from the activated carbon tube was attached to an impinger, containing 20 mL of 1 N NaOH, which was connected to a vacuum pump. Glass and Teflon shrink tubes were used to make all connections in the system. A needle was inserted through the Teflon-lined septum on the side neck of the flask to allow air to enter the flask, while the vacuum pump was used to pull air through the system for 2 h. For the non-radiolabelled cell culture experiments described below, the air entering the flask also flowed

through a tube containing activated carbon to remove impurities. Air flowed from the flask, through the activated carbon tube, and then through the impinger. No ^{14}C radioactivity above background was found in the second section of the activated carbon tubes, indicating that all non-transformed CT and volatile chlorinated hydrocarbon metabolites of CT (such as ^{14}C-CF) were completely trapped by the activated carbon in the first section of the carbon tube and did not reach the downstream NaOH solution. Carbon dioxide was trapped in the NaOH solution. Radiolabelled carbon bound to cellular materials remained in the cells and radiolabelled, non-volatile soluble metabolites of CT remained in solution. After the cell cultures were purged with air, the cells were separated from the MS medium by filtration using Ahlstrom filter paper. The total recovery of radiolabelled carbon was calculated from the sum of the ^{14}C radioactivity in the NaOH, MS medium, filtered cells and activated carbon (determined as described below). This sum was compared to the total radioactivity in applied CT and the ratio reported as percent recovery.

Fractionation of CT metabolites

To determine the extent of CT oxidation to CO_2 and of ^{14}C binding to cells, a radiolabelled cell culture experiment was carried out using the flask reactor system and procedures described above. Two μl of radiolabelled CT (dissolved in methanol with a specific activity of 4.0 mCi/mmole) was injected into 20 mL of cell suspension in the two-necked reactor flasks, producing a final concentration of 0.24 mg/L. Controls consisted of MS medium without cells and cell suspensions with cells killed by autoclaving (20 mL each). Both controls were dosed with 2 μl ^{14}C-CT and incubated under the same conditions as the live cell cultures. After 24 hours of incubation the reactor flasks were purged with air in the same way as described above. The cells, MS medium, activated carbon and NaOH solution were sampled and analyzed. The amount of radioactivity trapped as $^{14}CO_2$ in the NaOH solution was determined by mixing 0.2 mL NaOH with 7 mL liquid scintillation cocktail (Ultima Gold) and counting in a Beckman LS 7000 liquid scintillation counter (LSC). The amount of ^{14}C associated with the cells was determined by combusting cell samples using a sample oxidizer (Packard), collecting the resulting $^{14}CO_2$ in scintillation cocktail (15 mL), and quantifying ^{14}C radioactivity on the LSC.

To assure that the ^{14}C radioactivity found in the cells represented the binding of CT-carbon to the cells and was not due to ^{14}C-CT adsorbed to the cells, 3 ml of 99.5% TCE was added to the cells for 3 days to extract CT, then the TCE was removed and the cells were combusted using the oxidizer and counted for ^{14}C radioactivity.

In order to calculate the recovery of ^{14}C from the reactors, the ^{14}C radioactivity associated with activated carbon and MS medium was also determined. An aliquot of MS medium (0.2 mL) was mixed with 7 mL liquid scintillation cocktail and counted by LSC. The ^{14}C adsorbed on the activated carbon was extracted with 3 mL of 99.5% TCE, then 0.2 mL of the extraction solvent was mixed with 7 mL liquid scintillation cocktail and counted by LSC.

In order to investigate the formation of CF and other possible metabolites during the transformation of CT by poplar cells, a non-radiolabelled cell culture experiment was conducted. Ten μl of non-radiolabelled CT was added to 160 mL cell suspension in 1-L three-necked flasks, which yielded a CT concentration of about 100 mg/L. Controls consisted of MS media alone and cell suspension with cells killed by autoclaving. After 24 h cell incubation, the system was purged with air as described above and activated carbon, cells and MS medium were sampled and analyzed for chlorinated metabolites. To analyze chlorinated metabolites in cells and MS medium, 2 g frozen cells or 8 mL MS medium were added to cold centrifuge tubes and the tubes were closed with septum caps. Acid (2 mL of 1 N H_2SO_4/10% NaCl) was added to the tubes and the tubes were shaken for 2 min, after which 10 mL of hexane was added. After shaking for 1 min, the sample was centrifuged for 10 min at 8,000 rpm. Seven mL of hexane was taken from the tube and placed in a septum-capped vial containing 2 g Na_2SO_4. After 1 hour, 1 mL of hexane extract was added to an autosampler vial and analyzed for chlorinated metabolites by injecting 2 μl onto a Perkin Elmer gas chromatograph with electron capture detector (GC-ECD) with a RTx-1 column, a helium gas flow set at 0.86 mL/min, oven temperature of 100 °C, detector temperature of 380 °C. To determine chlorinated metabolites associated with activated carbon, the activated carbon from the trap was placed in a 3-mL vial under a septum cap. Three mL of hexane was added to the vial, which was shaken for 30 min. After extraction, about 0.5 mL of the hexane was added to an autosampler vial and analyzed by GC-ECD as described above.

Inhibitor study

The chemicals selected as P-450 inhibitors for this experiment included carbon monoxide (CO), SKF-525 (TRC), isoniazid (Sigma), piperonyl butoxide (Aldrich) (Mico et al. 1983), 4-methylpyrazole (Sigma) (Feierman and Cederbaum 1985), 1-phenylimidazole (Aldrich), chlorzoxazone (Aldrich) (Anari et al. 1996) and piperonylic acid (Aldrich) (Schalk et al. 1998). Inhibitors of lignin peroxidase were $NaVO_3$ (Aldrich) (Ralph & Catcheside 1999) and 3-amino-1,2,4-triazole (Aldrich) (Tuisel et al. 1992). All inhibitors except CO and piperonylic acid were first dissolved in water or methanol to make stock solutions. The effects of different levels of methanol on both cell viability and CT metabolism were tested, and within the range of the amount of methanol used in our experiment, no effect was observed. Different amounts of stock inhibitor solutions were added to 20 mL cell suspensions in reactor flasks to produce the desired inhibitor concentrations (shown in the results section). Piperonylic acid was first added in phosphate buffer (pH = 6.6) and then 1N NaOH solution was added until piperonylic acid was dissolved. The final pH of the piperonylic acid stock solution was 7.1. In experiments using CO, the air in the flasks was replaced with a mixture of N_2, O_2, and CO. Only the ratio of N_2 to CO varied among different treatments. The oxygen concentration in the gas mixtures of each treatment was initially 20%, a level sufficient to maintain aerobic conditions during the 24 hour incubations. Cell suspensions without inhibitor were used as controls for each experiment. After addition of the inhibitors, 2 μl of radiolabelled CT was added to each flask as described previously. Cell incubation, system purging, and ^{14}C measurement followed the procedures described above.

All reported results were for inhibitor concentrations that did not inhibit cell growth and cell viability. To determine if the inhibitors were toxic to cells, both cell mass increases (growth) and cell viability (trypan blue exclusion) were determined for a range of inhibitor concentrations. Only the inhibitor concentrations that caused no toxic effects on cells are reported.

Effect of oxygen on CT transformation

To determine the effects of oxygen on CT transformation, the headspaces of the cell suspension reactor flasks were replaced by flushing with various mixtures of O_2 and N_2. The percentage of O_2 in the headspaces ranged from 0 to 50%. To study the effect of O_2 level on CO_2 production and carbon binding, 2 μL of ^{14}C-CT was added to each of the 125-mL, two-necked flasks, which contained 20 mL of cell suspension. To study the effect of O_2 levels on CF formation, 10 μL non-radiolabelled CT was added to 1-L, three-necked flasks, which contained 160 mL of cell suspension. The rest of the procedures were as previously described. Cell mass and cell viability were determined after one day incubations and no decreases in growth or viability were observed for any oxygen level.

Statistical methods

A two-factor ANOVA analysis was used to determine differences between treatments. P-value of 0.05 was used to evaluate significance.

Results

Biotransformation of CT by poplar H11-11 cells

Table 1 shows that $^{14}CO_2$ production, ^{14}C associated with the cells and CF formation were significantly higher in living cells than in MS medium and dead cell controls. Radioactivity of the cells was not extractable by TCE, indicating that ^{14}C was strongly bound to the cells. Thus the excess of the ^{14}C bound to living cells over the ^{14}C bound to killed cells, shown in Table 1, was unlikely to be due to CT sorbed to cell material. Excess ^{14}C bound to the living cells may have been due to covalent binding of reactive intermediates of CT metabolism to cellular materials.

Effect of P-450 inhibitors on CT metabolism by poplar cells

Among the inhibitors tested, carbon monoxide is a general P-450 inhibitor, piperonylic acid is a specific P-450 C4H inhibitor, chlorzoxazone, isoniazid, 4-methylpyrazole and 1-phenylimidazole are specific P-450 2E1 inhibitors, and the rest of the inhibitors have shown inhibitory effects on P-450-mediated CT transformation in mammalian systems (Kubic & Anders 1980; Mico et al. 1983; Lindros et al. 1990; Sipes et al. 1977). The effects of these inhibitors on CT metabolism by poplar cells are shown in Table 2. Carbon monoxide, isoniazid, 4-methylpyrazole, 1-phenylimidazole, chlorzoxazone significantly inhibited both CO_2 production and ^{14}C binding. In most cases, the inhibitory effects became stronger as the inhibitor concentrations increased, and the differences

Table 1. Transformation of carbon tetrachloride by poplar cells in suspension culture

Incubation Condition	^{14}C as CO_2 (dpm* per bottle)	^{14}C in cell pellet (dpm per bottle)	Chloroform production (μg per bottle)
Living cells	4450 ± 201	12700 ± 2400	0.93 ± 0.23
MS medium	2490 ± 155	0	0.09 ± 0.02
Dead cells	1730 ± 86	3370 ± 138	0.07 ± 0.07

Mean $\pm$ standard deviation of the mean.
*Disintegrations per minute.

between control and the other treatments were significant at the 5% level or less. CO_2 production decreased significantly in the presence of piperonyl butoxide, but there was no effect on ^{14}C binding. CO_2 production from the cells with piperonylic acid was lower than the control cells, however, the difference was not statistically significant. Interestingly, ^{14}C binding increased in the presence of piperonylic acid, although this incremental effect was significant only at 0.25 mM piperonylic acid. SKF-525 had no significant inhibitory effect on either CO_2 production or ^{14}C binding. Variations in the activity of uninhibited controls were due to variations in cell mass and culture age.

Effect of lignin peroxidase inhibitors on CT metabolism by poplar cells

Two lignin peroxidase inhibitors, $NaVO_3$ and 3-amino-1,2,4-triazole, were tested for their effects on CO_2 production and ^{14}C binding (Table 3). The results indicated that there were no inhibitor effects on either CO_2 production or ^{14}C binding.

Effect of oxygen levels on CT metabolism by poplar cells

Table 4 shows that CO_2 production from CT by poplar cells appeared to increase as the oxygen concentration was increased from 0 to 20%, but CO_2 production was less at an oxygen concentration of 50%. Statistical analysis indicated that CO_2 production in 20% O_2 was higher ($p = 0.05$) than at either the 0% or 50% O_2 treatments. Table 4 also shows that the ^{14}C bound to cell tissue significantly decreased as oxygen level increased ($p = 0.05$).

The effect of oxygen level on CF production by poplar cells is presented in Table 5. Samples of the cells, the MS medium, and the activated carbon were analyzed for the presence of CF. CF was detected only in the activated carbon. Higher amounts of CF were formed under anaerobic conditions (Nitrogen

Table 2. Effects of P-450 inhibitors on carbon tetrachloride transformation

Inhibitors	Doses	CT transformation products measured as ^{14}C (dpm per bottle)	
		CO_2	Cell-bound ^{14}C
CO	0%	4480 ± 158	12400 ± 2210
	10%	3310 ± 132^a	9260 ± 117
	40%	2570 ± 155^a	7080 ± 358^a
	70%	1660 ± 166^a	6100 ± 331^a
Isoniazid[b]	0 mM	3810 ± 231	8130 ± 190
	1 mM	2890 ± 221^a	6170 ± 163^a
	5 mM	2160 ± 145^a	5910 ± 62^a
	10 mM	1630 ± 127^a	5320 ± 77^a
Chlorzoxazone[b]	0 mM	1240 ± 115	3140 ± 57
	0.1 mM	1140 ± 16	2740 ± 132^a
	0.2 mM	871 ± 170^a	1840 ± 200^a
	0.3 mM	969 ± 52^a	1730 ± 112^a
4-Methylpyrazole[b]	0 mM	1230 ± 61	2550 ± 96
	0.1 mM	1080 ± 34^a	1990 ± 48^a
	0.2 mM	977 ± 35^a	1450 ± 68^a
	0.4 mM	776 ± 15^a	1390 ± 80^a
1-Phenylimidazole[b]	0 mM	1700 ± 33	6980 ± 180
	0.1 mM	1270 ± 90^a	4760 ± 226^a
	0.2 mM	1020 ± 35^a	2500 ± 418^a
	0.3 mM	1070 ± 35^a	2220 ± 139^a
SKF-525	0 mM	3730 ± 175	7650 ± 769
	0.1 mM	3530 ± 630	8380 ± 1250
	0.2 mM	3770 ± 276	6820 ± 778
Piperonyl butoxide	0 mM	3190 ± 196	7740 ± 778
	0.01 mM	2250 ± 332^a	9040 ± 1160
	0.05 mM	1840 ± 123^a	7350 ± 668
Piperonylic acid[c]	0 mM	2767 ± 255	2871 ± 313
	0.25 mM	2066 ± 62	4207 ± 206^a
	0.5 mM	2295 ± 201	4574 ± 639

Mean $\pm$ standard deviation of the mean.
[a] Significantly different from response with no inhibitor ($p = 0.05$).
[b] Specific inhibitors of mammalian cytochrome P-450 2E1.
[c] Specific inhibitor of plant cytochrome P-450 C4H.

Table 3. Effects of lignin peroxidase inhibitors on carbon tetrachloride transformation

Inhibitors	Doses	CT transformation products measured as ^{14}C (dpm per bottle)	
		CO_2	Cell-bound ^{14}C
$NaVO_3$	0 mM	4913 ± 162	2496 ± 389
	0.25 mM	5300 ± 476	2152 ± 247
	0.5 mM	5534 ± 163[a]	2548 ± 873
3-Amino 1,2,4-triazole	0 mM	3550 ± 176	3609 ± 375
	10 mM	3957 ± 566	3622 ± 156

Mean ± standard deviation of the mean.
[a] Significantly different from response with no inhibitor ($p = 0.05$).

Table 4. Effect of oxygen levels on carbon tetrachloride transformation by poplar cells

O_2 levels*	CO_2 production (dpm per bottle)	Cell binding of ^{14}C (dpm per bottle)
0%	2360 ± 468[a]	19700 ± 2290
5%	2550 ± 505	14880 ± 4420
20%	2930 ± 441	11080 ± 504
50%	2410 ± 389[a]	8480 ± 1080

Mean ± standard deviation of the mean.
* Remainder of headspace gas was N_2.
[a] Significantly different from response at atmospheric oxygen level of 20% ($p = 0.05$).
All cell binding data were significantly different between all oxygen levels ($p = 0.05$).

Table 5. Effect of oxygen level on chloroform formation

Headspace composition	Total CF formed (μg per bottle)
Air	0.17 ± 0.03
Nitrogen gas	2.79 ± 0.11

Mean ± standard deviation of the mean.

gas) than in the aerobic environment (Air). Both CF formation and cell binding of ^{14}C were promoted under low oxygen conditions, while CO_2 production was enhanced under standard atmospheric oxygen levels.

^{14}C distribution and recovery in cell culture experiments

The distribution of ^{14}C after poplar cells were exposed to radiolabelled CT for 24 hours is shown in Table 6. About 1.5% of the radiolabelled CT carbon was recovered as CO_2 from living cultures. About 3% of dosed ^{14}C was bound to living cells and about 7% of the ^{14}C applied was recovered in the MS medium. About 85% of the dosed ^{14}C was found in the activated carbon trap as non-transformed CT, as confirmed by GC-ECD analysis. CF was not detectable in these trap samples since the CT dose used in radiolabelled cell culture experiments yielded a level of CF below the detection limit (about 0.01 mg/L). When the cells were dosed with 100 mg/L non-radiolabelled CT in a separate experiment, about 0.001% of the CT was transformed to an amount of CF that could be detected by GC-ECD.

Table 6. Distribution of ^{14}C from applied ^{14}C-carbon tetrachloride after 24 hours of cell incubation

Fraction	Dead cells (%)	Living cells (%)
Recovered as CO_2	0.3 ± 0.3	1.5 ± 0.2
Bound to cell tissue	1.1 ± 0.5	3.0 ± 0.1
Remaining in medium	13 ± 5.5	7.1 ± 2.8
Trapped on carbon tube	88 ± 33	84 ± 10
Total recovery	102 ± 33	96 ± 13

Mean ± standard deviation of the mean.

Table 6 shows that total recovery of dosed ^{14}C in a typical radiolabel experiment was approximately 100%. The percentage of metabolites recovered in the various fractions varied with experimental factors such as cell mass and cell age; but the general distribution of the recovered label was consistent.

To ensure that the detected metabolites were not due to contaminants in the CT or to transformations of such contaminants, we analyzed the purity of both radiolabelled and non-radiolabelled CT. No chlorinated hydrocarbons other than CT were detected by GC-ECD analysis of the non-radiolabelled and radiolabelled CT. The non-volatile residue of the radiolabelled CT accounted for about 0.04% of the total radioactivity, much less than fraction of applied ^{14}C recovered as CO_2 and bound to cells (about 1.5%

and 3%, respectively). Therefore, the transformation products detected in our experiments were produced from the metabolism of CT by poplar cells.

Discussion

Aerobic CT transformation has been observed in both mammalian and fungal systems. While P-450 enzymes are responsible for CT transformation in mammals, lignin peroxidases are involved in CT transformation by fungi. Although lignin peroxidases are also present in plant systems, lignin peroxidase inhibitors had no inhibitory effects on CT transformation in our experiments with poplar cells (Table 3). These results suggest that lignin peroxidase was not involved in the biotransformation of CT by poplar cells. It is also possible that lignin peroxidase activity was not expressed in the non-differentiated cell cultures.

The transformation of CT in mammalian systems has been extensively investigated. The first step of the reaction is the cytochrome P-450-dependent reductive dechlorination of CT to the trichloromethyl radical ($\cdot CCl_3$) (Poyer et al. 1980). The trichloromethyl radical may undergo three different reactions. Under sufficient oxygen concentrations, trichloromethyl radical can be oxidized by oxygen to form phosgene ($COCl_2$), which then reacts with water to produce carbon dioxide (Harris & Anders 1981; Shah et al. 1979). At low oxygen condition, the trichloromethyl radical may abstract a hydrogen atom from its environment to form chloroform ($CHCl_3$) (Ahr et al. 1980; Kubic & Anders 1981). Alternatively, the trichloromethyl radical may bind covalently to the lipids and proteins of cells (Trudell et al. 1982; Diaz Gomez 1973).

The results of our cell culture experiments are consistent with the conclusion that the transformation system for CT in poplar cells is similar to that found in mammalian systems. Both CO_2 and CF were identified as products of CT transformation by poplar cells, and significant binding of CT-carbon to poplar cells was observed (Table 1). The identification of CF as a CT-derived product is particularly important because CF is an expected metabolite of P450-mediated CT metabolism. The production of CF suggests that the first step in the metabolism of CT by poplar cells is a one-electron reductive dechlorination to trichloromethyl radical, which then abstracts a hydrogen atom from some component of the medium to form CF. This explanation was further supported by the effects of oxygen on metabolite production by poplar cells (Table 4 and Table 5). Under oxygen limiting conditions, more CF or more binding of CT-carbon to poplar cells would be produced from trichloromethyl radical, because less oxygen would be available to compete for trichloromethyl radicals to produce CO_2. With greater oxygen availability, more trichloromethyl radicals could be oxidized to CO_2; therefore, less trichloromethyl radicals would be available to form CF or to bind to cells. When the oxygen level was increased to 50%, CO_2 production from CT was also reduced (Table 4). This effect may have been due to inhibition of the dechlorination of CT to trichloromethyl radical at higher oxygen concentrations. As a result, both CO_2 production and cell binding would be reduced at the highest oxygen level.

The results from the P-450 inhibitor experiments further support involvement of a P-450 enzyme in the CT transformation process in poplar cells (Table 2). Carbon monoxide, which is a general inhibitor of P-450 enzymes and is also an inhibitor of many other cellular enzymes, significantly inhibited both CO_2 production and cell binding of ^{14}C from radiolabelled CT. Chlorzoxazone, isoniazid, 4-methylpyrazole and 1-phenylimidazole, which are specific inhibitors of the P-450 2E1 enzyme in mammalian systmes, also significantly inhibited both CO_2 production and ^{14}C binding. Piperonyl butoxide inhibited only CO_2 production. The significant inhibitory effects of CO, isoniazid and piperonyl butoxide on CT metabolism have also been observed in mammalian systems (Kubic & Anders 1980; Mico et al. 1983; Lindros et al. 1990). SKF-525 produced no inhibitory effect on the CT metabolism by poplar cells. SKF-525 has also been shown to be a weak inhibitor of covalent binding of CT-carbon to protein (Sipes et al. 1977) and of formation of phosgene and electrophilic chlorine from CT metabolism in mammalian systems (Kubic & Anders 1980; Mico et al. 1983). P-450 C4H is a member of the superfamily of P-450 enzymes in plant systems. The lack of inhibitory effect of piperonylic acid, a specific P-450 C4H inhibitor, on both CO_2 production and ^{14}C binding suggested that the biodegradation of CT by poplar cells was not associated with P-450 C4H (Table 2). Of the multiple forms of P-450 present in mammalian systems, P-450 2E1 has been identified as the primary catalyst of CT metabolism (Raucy et al. 1993). Significant inhibitory effects of P-450 2E1-specific inhibitors on CT metabolism have been observed in our cell culture experiments. These results suggest involvement of a P450-2E1-like enzyme in the CT metabolism in plant systems.

While CT removal rate was about 50 μg CT/d/g dry weight of root and leaf mass when poplar trees were tested in field trials (Wang et al., unpublished), the percentage of CT mineralization and fixation by poplar cell cultures was low (Table 6), amounting to about 2 μg CT/d/g dry weight cells. A similarly low level of TCE transformation was also observed in experiments with the H11-11 cell culture (Newman et al. 1997). Two explanations may be advanced to account for these discrepancies. First, degradation of CT in the field may be due primarily to microbial activity in the rhizosphere. Second, the undifferentiated cell line used in our tissue culture experiments may have had limited detoxifying activity compared to full-grown plants. It has been demonstrated that differentiated and transformed plant cell cultures degrade PCBs with much higher efficiency than amorphous and nontransformed ones of the same species (Mackova et al. 1997).

Cell culture work is a useful approach for the study of the mechanism of chlorinated hydrocarbon degradation in plants. However, the results cannot be quantitatively extrapolated to field applications because of the inherent methodological limitations described above. These limitations prevent conclusions based on laboratory experiments with cell cultures regarding the relative importance of plant versus microbial activity in phytoremediation applications.

In summary, the results of this investigation demonstrate that poplar cells were able to mineralize CT and suggest that a P450-2E1-like enzyme was involved in an oxidative pathway of CT degradation in plant tissue.

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