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In Vivo Levels of Chlorinated Hydroquinones in a Pentachlorophenol-Degrading Bacterium

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Sphingomonas chlorophenolica **RA-2 is a soil microorganism that can grow on pentachlorophenol (PCP) as a sole carbon source. In this microorganism, PCP is converted to tetrachlorohydroquinone (TCHQ), trichlorohydroquinone, and 2,6-dichlorohydroquinone. The remainder of the pathway has not yet been defined. The ability to grow on PCP as a sole carbon source is remarkable because of the toxicity of PCP and its chlorinated hydroquinone metabolites. Experiments in which the levels of PCP and chlorinated hydroquinones were measured in cells metabolizing [U-^{14}C]PCP revealed that the levels of chlorinated hydroquinones in the cytoplasm are in the low micromolar range. The toxicity of chlorinated hydroquinones was evaluated by exposure of *Escherichia coli* cells that had been treated with EDTA (to remove the outer membrane) to TCHQ. Significant toxicity due to TCHQ was not apparent until concentrations of 500 μM and higher. Thus, an important part of the explanation for why *S. chlorophenolica* RA-2 is able to grow on PCP as a sole carbon source is undoubtedly that it can process sufficient carbon for growth without accumulating high levels of toxic intermediates.**

Pentachlorophenol (PCP), a widely used wood preservative, is the only highly chlorinated aromatic compound still used in large quantities (approximately 23 million kg per year) in the United States (5, 12). PCP is listed as a Priority Pollutant by the Environmental Protection Agency because of its toxicity and widespread distribution in the environment. PCP is found at parts-per-million levels in 80% of drinking water supplies sampled in the United States. Accumulation of PCP in the food chain, including fruits, vegetables, grains, dairy products, and meats, results in an average daily intake of PCP of approximately 16 μg per person (12).

Several microorganisms that can degrade PCP have been isolated from contaminated sites. We are studying the biodegradation of PCP by *Sphingomonas chlorophenolica* RA-2 (29, 30). We have identified the initial steps in the pathway for degradation of PCP in this microorganism (Fig. 1). PCP is converted first to protonated tetrachlorohydroquinone (TCHQ), and then to trichlorohydroquinone (TriCHQ) and 2,6-dichlorohydroquinone (DCHQ). The pathway determined thus far is identical to that reported for *Flavobacterium* sp. strain ATCC 39723 (42, 43), which has now been reclassified as *S. chlorophenolica* (25). Several other microorganisms (including *Mycobacterium chlorophenolicus* [1, 38], *Rhodococcus* sp. strain CP-2 [10], and *Mycobacterium fortuitum* CG-2 [11]) also metabolize PCP through chlorinated hydroquinone intermediates.

The ability to grow on PCP as a sole source of carbon is remarkable because it requires not only the metabolic enzymes to degrade this unusual carbon source but also adaptations to counteract the toxic effects of PCP and its metabolites. The toxicity of PCP is attributed to its ability to uncouple oxidative phosphorylation (14, 22, 31, 33, 36) and to alter membrane properties (32, 34, 37). Some soil microorganisms avoid the toxicity of PCP either by excluding it from the cell (an ability bestowed by the outer membrane of gram-negative bacteria [14, 15]) or by converting it to a nontoxic, dead-end metabolite by methylating the hydroxyl group (9). Neither of these strategies is available to a microorganism that must use PCP as a sole source of carbon. Thus, *S. chlorophenolica* RA-2 must somehow strike a balance between the toxicity of PCP and the need for carbon for cell growth.

Consideration of the pathway for PCP degradation reveals additional problems beyond the toxicity of PCP. The intermediates in the degradation pathway are chlorinated hydroquinones, which are toxic for a variety of reasons. While they would be expected to have effects similar to those of PCP on oxidative phosphorylation and membrane properties, there is an additional route for toxicity due to the facile oxidation of hydroquinones to semiquinones and benzoquinones (Fig. 2). This process produces superoxide and possibly other reactive oxygen species which can lead to oxidative damage to DNA (6, 16, 18). Both TCHQ (6, 8, 41) and DCHQ (16) have been shown to cause single-stranded breaks in DNA, predominantly through the formation of reactive oxygen species. Furthermore, benzoquinones are quite electrophilic and can react with a variety of cellular nucleophiles, including glutathione (40), proteins (28, 39), and DNA (41). For example, TCHQ has been shown to bind covalently to both DNA (41) and proteins (39), presumably after oxidation to tetrachlorobenzoquinone (TCBQ). Finally, the redox cycling caused by repeated oxidation of hydroquinones, followed by reduction by glutathione, can deplete the level of this important intracellular reductant (17).

Here we describe experiments designed to evaluate the levels of chlorinated hydroquinones accumulated in the cytoplasm during metabolism of PCP and to determine whether these levels are sufficient to cause toxicity to the cells.

MATERIALS AND METHODS

Materials. PCP was obtained from Aldrich. TCHQ was obtained from Kodak. [U-^{14}C]PCP was obtained from Sigma. [U-^{14}C]TCHQ was prepared from [U-^{14}C]PCP by using an extract from *Escherichia coli* CCL3, which overexpresses PCP monooxygenase from *S. chlorophenolica* ATCC 39723. [U-^{14}C]DCHQ was prepared from [U-^{14}C]TCHQ by using TCHQ dehalogenase purified from *E. coli* JDO1 (21), which overexpresses TCHQ dehalogenase from *S. chlorophe-*

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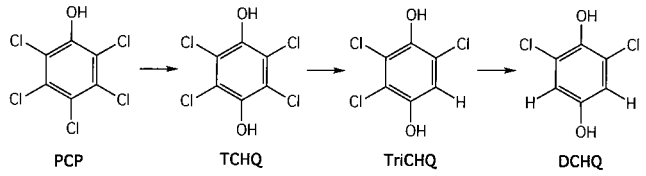

FIG. 1. Initial pathway for degradation of PCP in *S. chlorophenolica* RA-2.

nolica ATCC 39723. *E. coli* CCL3 and JDO1 were generous gifts from Cindy Orser.

Microorganisms and growth conditions. *S. chlorophenolica* RA-2 was obtained from Steve Schmidt of the University of Colorado at Boulder. Growth medium contained (per liter) 2.4 g of Na_2HPO_4, 0.3 g of KH_2PO_4, 0.1 g of NH_4NO_3, 0.02 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 1 ml of trace element solution (27), and 250 to 300 mg of PCP. Cells were grown at 25°C with shaking. *E. coli* JM105 was grown at 37°C in the same medium except that PCP was replaced with glucose (0.2%) and thiamine was added (0.005%).

Preparation of extracts for enzyme assays. Bacteria were harvested from liquid cultures by centrifugation at 5,900 × *g* for 20 min at 4°C. The cells were suspended in lysis buffer (50 mM Tris-HCl [pH 8.0], containing 10% [vol/vol] glycerol, 1 mM EDTA, 100 mM NaCl, 0.2% [vol/vol] Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 μg each of leupeptin, antipain, and pepstatin per ml). Lysozyme was added to a final concentration of 0.5 mg/ml, and the mixture was incubated at 4°C for 1 h with gentle stirring. DNase I was added to a final concentration of 0.1 mg/ml, and the incubation was continued for 2 h more. The extraction mixture was then centrifuged at 17,000 × *g* for 20 min at 4°C to remove cellular debris and dialyzed to remove Triton X-100.

Enzyme assays. PCP monooxygenase activity was measured by monitoring the conversion of [U-^{14}C]PCP into [U-^{14}C]TCHQ. Reaction mixtures contained 50 mM Tris-HCl (pH 7.3), 10 μM [U-^{14}C]PCP, 1 mM NADPH, 1 mM ascorbate (to protect TCHQ from oxidation), 1 mM $MgCl_2$, 2 mM dithiothreitol, 50 μg of bovine serum albumin per ml, and crude extract of *S. chlorophenolica* RA-2 cells (60% of the final volume). At intervals, aliquots of the reaction mixture were taken. PCP and TCHQ were separated by thin-layer chromatography using toluene saturated with acetic acid and water as the mobile phase. Spots corresponding to PCP and TCHQ were cut out, and the amount of radioactivity present was determined by scintillation counting.

TCHQ dehalogenase activity was measured by monitoring the disappearance of TCHQ. Reaction mixtures included 100 μM TCHQ, 1 mM glutathione, 5 mM ascorbate (to protect hydroquinone substrates and products from oxidation), 1 mM EDTA, and crude extract of *S. chlorophenolica* RA-2 cells (10% of final volume) in 25 mM potassium phosphate buffer, pH 7.2. At intervals, aliquots were taken, acidified with HCl, and analyzed by reverse-phase high-pressure liquid chromatography on a Rainin Microsorb C_{18} column (4.6-mm inside diameter [i.d.] by 5 cm) using 25% acetonitrile in 0.1% acetic acid as the mobile phase.

Gas chromatography-mass spectrometry (GC-MS) analysis of products of enzymatic reactions. Reaction mixtures from assays of either PCP monooxygenase or TCHQ dehalogenase were acidified by the addition of 0.1 volume of 6 N HCl and extracted three times with equal volumes of ether. The ether extract was dried with anhydrous magnesium sulfate, and the ether was removed with a stream of nitrogen. The extracted compounds were methylated by treatment with ethereal diazomethane prior to analysis by GC-MS with a Hewlett-Packard 5988A GC-mass spectrometer.

Extraction of total cellular metabolites of [U-^{14}C]PCP. Six liters of cell cultures was harvested, yielding 610 mg of cells. (The yield of cells is low because 67% of the mass of PCP is due to chlorine, so the supply of carbon for cell growth is small.) The cells were resuspended in 20 ml of growth medium containing [U-^{14}C]PCP (250 mg/liter, 0.94 mM; specific radioactivity = 9.05×10^5 dpm/μmol). After 1 h of incubation at 25°C, the cells were harvested by centrifugation at 8,300 × *g* at 4°C for 15 min. (At this point, the medium contained 1.8×10^5 cpm/ml, and the cell pellet contained 5.3×10^5 cpm/ml. Thus, the presence of a small amount of extracellular medium in the cell pellet would not significantly alter the amounts of metabolites detected. This is particularly true for the chlorinated hydroquinones, which would be prone to oxidation and would therefore be present at extremely low concentrations in the extracellular medium.) The cell pellet was resuspended in 1.5 ml of 0.1 N HCl and extracted multiple times with chloroform. The insoluble layer at the interface of the aqueous and chloroform layers was collected and also extracted multiple times with chloroform. The chloroform layers were pooled and evaporated to dryness. The residue was dissolved in 0.2 ml of ethanol, and a portion was analyzed by reverse-phase high-pressure liquid chromatography on a Rainin Microsorb C_{18} column (4.6-mm i.d. by 25 cm) using a gradient of acetonitrile in 0.1% acetic acid. Fractions were collected, and radioactivity was determined by scintillation counting.

Measurement of octanol-buffer partition coefficients. Radiolabelled compounds (PCP, TCHQ, and DCHQ) were added to tubes containing equal volumes of octanol and 20 mM sodium phosphate (pH 7.2). After vigorous vortexing, the organic and aqueous phases were separated by centrifugation. Aliquots of each phase were taken, and the concentration of the test compound was determined by scintillation counting. Octanol-buffer partition coefficients were determined from the ratio of the concentrations of each compound in the octanol and aqueous phases.

Assessment of toxicity of TCHQ to *E. coli*. *E. coli* JM105 was used to assess the toxicity of TCHQ to gram-negative bacteria. In order to ensure that the cells were fully exposed to the test compounds, aliquots of cells were exposed to EDTA (30 nmol per 10^9 cells) in 120 mM Tris-HCl buffer (pH 8.0) for 2 min at 37°C in order to remove the outer membrane (19). The EDTA treatment was stopped by addition of a 10-fold excess of $MgSO_4$. The cells were harvested by centrifugation and resuspended in minimal medium. Tests of the toxicity of TCHQ and TCBQ were conducted by exposing the cells to the test compounds at various concentrations for approximately one doubling time and then spreading diluted aliquots onto plates containing glucose for assessment of cell viability. (Five plates were used for each concentration tested.) In some cases, ascorbate, catalase, and superoxide dismutase were added to final concentrations of 0.1%, 1.6 U/ml, and 4.4 U/ml, respectively.

RESULTS

Identification of PCP monooxygenase and TCHQ dehalogenase activities in crude extracts. Crude extracts from *S. chlorophenolica* RA-2 contain an enzyme that converts PCP to TCHQ in the presence of NADPH (data not shown). The identity of the product was confirmed by extracting the reaction mixture with ether, treating the extracted compounds with ethereal diazomethane, and analyzing the composition of the mixture by GC-MS. Peaks due to 1,2,4,5-tetrachloro-3,6-dimethoxybenzene (formed by methylation of TCHQ; molecular ion at $m/z = 274$) and to pentachloroanisole (formed by methylation of residual PCP; molecular ion at $m/z = 278$) were found (data not shown).

Partially purified extracts from *S. chlorophenolica* RA-2 catalyzed the disappearance of TCHQ in a reaction requiring glutathione (data not shown). The products of this reaction were TriCHQ and an unidentified isomer of DCHQ. The identities of the products were confirmed by GC-MS as described above. Peaks due to 1,3,4-trichloro-2,5-dimethoxybenzene (formed by methylation of TriCHQ; molecular ion at $m/z = 240$) and dichloro-1,4-dimethoxybenzene (formed by methylation of an unknown isomer of DCHQ; molecular ion at $m/z = 206$) were found (data not shown). Although the existence of glutathione in *S. chlorophenolica* has not been addressed, we expect that glutathione is the physiological thiol substrate for this enzyme because high levels of glutathione are found in various gram-negative bacteria such as *Flavobacterium* sp. strain 12.154 (24), *E. coli*, and *Pseudomonas fluorescens* (23) and because the enzyme from a related strain (*S. chlorophenolica* ATCC 39723) shows some striking resemblances to proteins in the glutathione *S*-transferase superfamily (20, 21, 26).

Quantitation of metabolites of PCP in cellular extracts. *S. chlorophenolica* cells from an actively metabolizing culture were harvested and resuspended in medium containing [U-^{14}C]PCP at a final concentration of 0.94 mM. The cells were harvested after 70 min when the PCP level had fallen to 0.67 mM. The cell pellet was resuspended in 0.1 N HCl and extracted extensively with chloroform. The pooled chloroform layers were evaporated to dryness, and the residue was redissolved in eth-

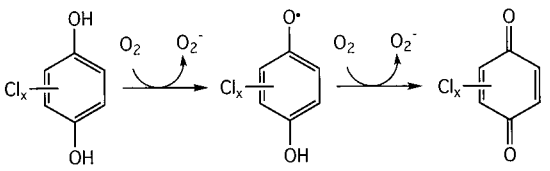

FIG. 2. Oxidation of chlorinated hydroquinones to chlorinated benzoquinones.

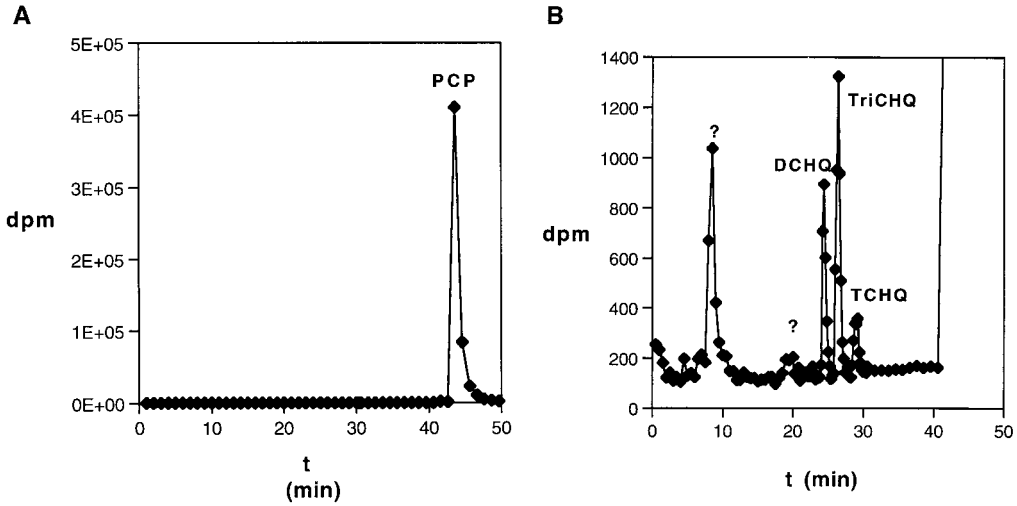

FIG. 3. (A) Chromatogram of chloroform extract of *S. chlorophenolica* RA-2 cells grown on [^{14}C]PCP. (B) Expansion of early region of chromatogram shown in panel A. Chromatography was carried out on a Rainin Microsorb C_{18} column (4.6-mm i.d. by 25 cm) with a gradient of acetonitrile in 0.1% acetic acid. Fractions were collected, and radioactivity was determined by scintillation counting.

anol. A portion of this solution was analyzed by reverse-phase chromatography (Fig. 3). The chromatogram showed a large peak of radioactivity corresponding to PCP and much smaller peaks of radioactivity corresponding to TCHQ, TriCHQ, DCHQ, and two unidentified metabolites. Concentrations of these metabolites in the cell pellet were determined by using the known specific radioactivity of the [U-^{14}C]PCP (9.05 dpm/μmol) and the measured volume of the cell pellet (Table 1).

Quantitation of the identified metabolites of PCP leads to a striking conclusion. The concentrations of the various chlorinated hydroquinone metabolites in the cell pellet are quite low (Table 1). The concentration of these metabolites in the cytoplasm will be even lower, since chlorinated hydroquinones are hydrophobic and tend to partition into the lipid bilayer. While the exact concentrations in the cytoplasm cannot be determined accurately, the average cellular concentration can be roughly partitioned into contributions from the membranes and the cytoplasm by using (i) the octanol-buffer partition coefficients for each compound as estimates for the corresponding membrane-cytoplasm partition coefficients, (ii) the estimate that the lipid bilayers of the inner and outer membranes occupy about 10% of the cellular volume (based upon electron micrographs of *S. chlorophenolica* RA-2 cells [29] and the known dimensions of phospholipid bilayers [4]), and (iii) the assumption that the membrane and cytoplasmic compartments are at equilibrium.

Approximate cytoplasmic concentrations (concn) of metabolites were determined by using the following relationships:

$$K_{\mathrm{mc}} = C_m/C_c \approx K_{\mathrm{ob}} \quad (1)$$

$$C_{cp} = C_m V_m + C_c V_c \quad (2)$$

In equation 1, K_{mc} is the membrane-cytoplasm partition coefficient for the metabolite of interest, which was approximated by the octanol-buffer partition coefficient (K_{ob}), and C_m and C_c are the concentrations of metabolite in the membrane and cytoplasm, respectively. The octanol-buffer partition coefficients measured at pH 7.2 for PCP, TCHQ, and DCHQ were 377, 38, and 75, respectively. The octanol-buffer partition coefficient for TriCHQ was assumed to be midway between those of TCHQ and DCHQ. Equation 2 expresses the average concentration of the metabolite in the cell pellet (C_{cp}) as a weighted average of its concentrations in the cytoplasm and the membrane. (For this purpose, the periplasm and the cytoplasm are considered together.) V_m and V_c refer to the membrane and cytoplasmic fractional volumes, respectively. Equations 1 and 2 contain only two unknowns and can be readily solved to give the concentrations of the metabolite in the membrane and in the cytoplasm. Estimates for the cytoplasmic concentrations calculated with equations 1 and 2 are given in Table 1. Note that because of uncertainties in parameters such as the intracellular pH and the actual volume of cells in the cell pellet, and the necessary assumption that the cytoplasmic and membrane compartments are in equilibrium, these calculations of cytoplasmic concentrations of metabolites are only estimates.

Toxicity of TCHQ to *E. coli* JM105. In order to measure the toxicity of TCHQ, we used a strain of *E. coli* that is unable to degrade TCHQ. The outer membrane, which provides a permeability barrier to uptake of toxic compounds, was removed by treatment of the cells with EDTA in 120 mM Tris-HCl (pH 8.0) at 37°C for 2 min (19). The cells were then harvested by centrifugation and resuspended in minimal medium. The toxicity of TCHQ was investigated by exposing cells to TCHQ at varying concentrations in minimal medium at 37°C for approximately one doubling time. (The control cells were treated with EDTA as described above, but were not exposed to TCHQ.) In

TABLE 1. Concentrations of PCP and metabolites in cells harvested from medium containing 670 μM PCP

Metabolite	Concn in cell pellet (μM)	Approx cytoplasmic concn (μM)
PCP	4,600	0–120[a]
TCHQ	9	2
TriCHQ	33	5[b]
DCHQ	19	2

[a] Since the assumption that the membrane and cytoplasmic compartments are in equilibrium is not likely to be valid for PCP, only a range of possible values can be determined.

[b] The octanol-buffer partition coefficient for TriCHQ is assumed to be between those of TCHQ and DCHQ.

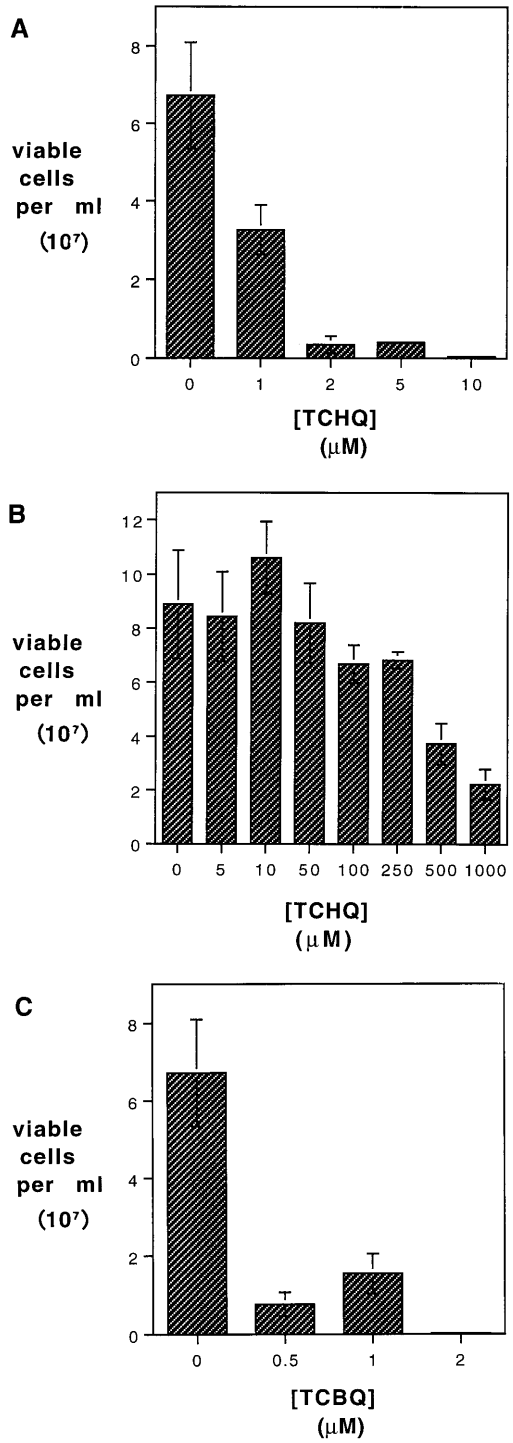

FIG. 4. Effect of exposure of EDTA-treated *E. coli* cells to chlorinated hydroquinones and benzoquinones. (A) TCHQ alone; (B) TCHQ in the presence of 0.1% ascorbate; (C) TCBQ.

some cases, ascorbate, catalase, and superoxide dismutase were also added. Aliquots were then spread on agar plates containing glucose to assess cell survival. The toxicity of TCBQ was also assessed in this manner. The results (Fig. 4) show that treatment with as little as 1 μM TCHQ causes significant toxicity. However, inclusion of ascorbate in the medium obviates this toxicity, and significant toxic effects are not seen until the concentration of TCHQ reaches 500 μM. Notably, TCBQ (the oxidation product of TCHQ) is quite toxic at even 0.5 μM.

DISCUSSION

The identification of PCP monooxygenase and TCHQ dehalogenase activities in crude extracts of *S. chlorophenolica* RA-2 suggests that the pathway for degradation of PCP in this microorganism (Fig. 1) is identical to that previously found in *S. chlorophenolica* ATCC 39723 (42, 43). The isomer of DCHQ formed by the *S. chlorophenolica* RA-2 TCHQ dehalogenase was not determined. However, recent studies of the ATCC 39723 TCHQ dehalogenase have shown that the enzyme produces 94% 2,6-DCHQ and 6% 2,3-DCHQ (3). Since the gene sequences of the enzymes from RA-2 and ATCC 39723 are nearly identical (7), the distribution of isomers produced is certain to be identical.

Quantitation of the identified metabolites of PCP leads to a striking conclusion. The concentration of the various chlorinated hydroquinone metabolites in the cells is very low (Table 1). The concentration of these metabolites in the cytoplasm will be even lower, since chlorinated hydroquinones are hydrophobic and tend to partition into the lipid bilayer. While the exact concentrations in the cytoplasm cannot be determined accurately, the average cellular concentration can be roughly partitioned into contributions from the inner and outer membranes and the cytoplasm as described in the previous section. Estimates for the cytoplasmic concentrations are given in Table 1. These results must be evaluated in the context of data on the toxicity of these compounds in order to determine whether simply maintaining these low levels of chlorinated hydroquinones is sufficient to protect the cells from toxicity.

In order to determine whether the levels of TCHQ found in *S. chlorophenolica* RA-2 cells are likely to have significant toxic effects, we have examined the toxicity of TCHQ to *E. coli* JM105. Since this microorganism cannot degrade TCHQ, complications resulting from the removal of the test compound by metabolism can be avoided. Furthermore, methods for removal of the outer membrane from *E. coli* cells are well established (19). (The outer membrane of gram-negative bacteria acts as a permeability barrier that protects the cells against toxic compounds.) Thus, we were able to ensure that the *E. coli* cells were exposed to cytoplasmic levels of TCHQ comparable to those observed in *S. chlorophenolica* cells growing on PCP.

E. coli cells whose outer membrane had been removed by treatment with EDTA were exposed to various concentrations of TCHQ for 2 h. The time required for repair of the outer membrane is generally two-thirds of the doubling time (19), which in this case (doubling time = 1 1/4 h) corresponds to 50 min. In the presence of TCHQ, the repair time will probably be longer, since uncouplers of oxidative phosphorylation have been shown to impede repair and TCHQ is structurally similar to PCP, a known uncoupler. Thus, under these conditions, the cells should have been exposed to TCHQ for a time at least equivalent to one generation time and possibly longer. Following exposure, the cells were diluted and spread on plates containing glucose to allow an assessment of cell viability.

The results shown in Fig. 4 demonstrate that exposure of EDTA-treated *E. coli* cells to TCHQ causes significant toxicity at levels as low as 1 μM. However, this toxicity is due to an oxidation product of TCHQ, rather than to TCHQ itself. Oxidation of TCHQ produces TCBQ and superoxide. Superoxide dismutates in solution to hydrogen peroxide and O_2. In the presence of transition metal ions, hydrogen peroxide can be cleaved to hydroxyl radicals. TCBQ and any or all of the

reactive oxygen species produced could contribute to the toxic effect. TCBQ is clearly a major culprit, since treatment of the cells with TCBQ causes major toxicity at even 0.5 μM, and addition of ascorbate (which reduces semiquinones and benzoquinones back to hydroquinones) to the reaction mixture causes a dramatic diminution of the toxicity. Under these conditions, the cells can tolerate up to 250 μM TCHQ without significant adverse effects. Furthermore, superoxide dismutase and catalase did not provide any additional protection over ascorbate alone (data not shown), suggesting that reactive oxygen species were not an important cause of the toxic effects. These results show that TCHQ is not highly toxic if it can be maintained in its reduced form. The cytoplasm of *E. coli* is a reducing environment (the intracellular redox potential is −0.28 to −0.42 V with respect to the Ag/AgCl electrode, and therefore −0.08 to −0.22 V with respect to the normal hydrogen electrode (NHE), as measured by the equilibrium between intracellular reduced and oxidized flavodoxin [35], while the redox potential of an aqueous solution [pH 7.0] equilibrated with air is +0.81 V with respect to NHE [13]). The Nernst equation can be used to approximate the concentration of TCBQ that would exist at equilibrium in a reducing environment with a redox potential of −0.10 V. For the two-electron reduction shown in equation 3, the Nernst equation is as shown in equation 4.

$$\mathrm{Ox} + 2e^- + 2\mathrm{H}^+ \rightarrow \mathrm{Red\text{-}H_2} \quad (3)$$

$$\mathrm{E} = \mathrm{E_0} + 0.059 \log (\mathrm{H}^+) - \frac{0.059}{2} \log \frac{(\mathrm{Red\text{-}H_2})}{(\mathrm{Ox})} \quad (4)$$

This equation must be modified as shown in equation 5 to take into account the significant ionization of TCHQ at physiological pH. (The pK_a for TCHQ is 7.8 [20].)

$$\mathrm{E} = \mathrm{E_0} + 0.059 \log (\mathrm{H}^+) - \frac{0.059}{2} \log \frac{(\mathrm{TCHQ}^-)\,(\mathrm{H}^+)}{(\mathrm{TCHQ})\,(\mathrm{K_a})} \quad (5)$$

To use equation 5, we must know E_0 for the TCBQ-TCHQ couple. It would be difficult to measure a value of E_0 for this redox couple because of the extremely low solubility of TCBQ in aqueous solutions. However, a value of 0.6 V can be estimated from the value of 0.7 V reported for alcoholic solutions containing 0.5% HCl (2) by taking into account the difference in proton concentration. When $E = -0.10$ V and $E_0 = 0.6$ V, then $(TCHQ^-)/(TCBQ)$ is equal to 5×10^8. Since $(TCHQ^-)$ represents 20% of the total tetrachlorohydroquinone species (from the Henderson-Hasselbach equation), the concentration of TCBQ in the cytoplasm would be expected to be approximately 1 fM. The total cellular concentration would be higher, since TCBQ is very hydrophobic and should exist predominantly in the membranes, but would probably still be less than 1 pM. Such concentrations are below our limit of detection, and in fact, we do not observe TCBQ in cellular extracts. (TCBQ elutes after TCHQ under the conditions shown in Fig. 3.)

Our data are consistent with previous in vitro experiments described by Witte et al. (41) and Juhl et al. (16), who have shown that induction of single-strand breaks in DNA by TCHQ (41) and DCHQ (16) is just detectable at concentrations of 5 μM. However, single-strand breaks caused by much higher levels of DCHQ (30 μM) can be prevented by inclusion of catalase (50 μg/ml) in the reaction mixture (16). Thus, it is likely that the normal cellular mechanisms that protect against oxidative damage in *S. chlorophenolica* cells can prevent damage to DNA from the low levels of chlorinated hydroquinone metabolites.

An additional interesting finding is that the concentration of PCP in the cells is approximately 4.6 mM. This level is several times that in the medium at the time the cells were harvested (0.67 mM) but not as high as the maximal level expected if the cells were in equilibrium with the medium. Assuming that the octanol-buffer partition coefficient accurately reflects the partitioning of PCP between the lipid bilayers and the aqueous medium and cytoplasm and that the lipid bilayers of the outer and inner membrane occupy approximately 10% of the cell volume, the total concentration of PCP in the cells should be about 25 mM if PCP were completely equilibrated between the medium, lipid bilayers, and cytoplasm. Since the total concentration is significantly lower than this level, the first enzyme in the metabolic pathway, PCP monooxygenase, must be doing an effective job of converting PCP into the next metabolite (TCHQ), thus preventing the accumulation of PCP to equilibrium levels.

The cytoplasmic concentration of PCP cannot be estimated by using the assumptions used to calculate the cytoplasmic concentrations of the chlorinated hydroquinone intermediates. The cytoplasmic and membrane compartments are unlikely to be at equilibrium, since the net flux of PCP is into the cell from the medium. However, bounds on the possible concentrations can be established. At one extreme, if PCP monooxygenase were extremely efficient, all of the PCP might be found in the membrane, with only very low levels in the cytoplasm. At the other extreme, if conversion of PCP to TCHQ by PCP monooxygenase were very slow, PCP would equilibrate between the membrane and the cytoplasm. Under these circumstances, the concentration of PCP in the cytoplasm would be about 120 μM. Thus, PCP monooxygenase is able to maintain cytoplasmic PCP levels that are at least severalfold (and perhaps more) below those in the medium, despite the necessity of admitting this toxic molecule into the cell and the unavoidable consequence that significant levels accumulate in the membrane. The strategies used by *S. chlorophenolica* for overcoming the toxic effects of PCP are currently under investigation.

Conclusions. The development of novel pathways for the catabolism of toxic xenobiotics imposes serious difficulties upon bacteria in terms of avoiding and/or mitigating the toxic effects of the xenobiotic and its metabolites. The optimal solution to this problem is to keep the levels of all toxic intermediates very low. This requires that the levels and activities of the metabolic enzymes in the pathway be carefully orchestrated. The rate of each step in the pathway, which is dependent on the k_{cat} of the enzyme, the concentration of the enzyme, and the concentration of the substrate, must be equal to or greater than that of the preceding step in order to avoid the accumulation of intermediates. This ideal situation may not be achieved early in the evolution of a new catabolic pathway. However, the microorganism may still be able to survive if it is able to protect itself from the toxic effects of the new metabolites. In the case considered here, the toxic effects are due to the products of oxidation of chlorinated hydroquinones. Cells have a multitude of defenses against the types of oxidative damage that can be caused by superoxide, hydrogen peroxide, hydroxyl radicals, and chlorinated benzoquinones. These include enzymes such as catalase, superoxide dismutase, diaphorase, glutathione reductase, and glutathione peroxidase and intracellular reductants such as glutathione. These protective capabilities may be crucial in the early stages of development of a new catabolic pathway. Ultimately, they may become less important as the relative activities of the metabolic enzymes are adjusted to maintain low levels of toxic intermediates.

The ability of *S. chlorophenolica* to degrade PCP is probably a relatively recent development. PCP has been used as a pes-

ticide for only about 50 years, and it is unlikely that such highly chlorinated aromatic compounds are present in the natural environment. Some aspects of the enzymology of PCP degradation are also consistent with this interpretation. In *S. chlorophenolica* ATCC 39723 (which is closely related to *S. chlorophenolica* RA-2), the first enzyme in the pathway is a monooxygenase which can convert PCP to TCHQ but is much more effective with a variety of other substrates (44). The second enzyme in the pathway (TCHQ dehalogenase) is expressed constitutively (26), suggesting either that it has some other essential function in the cell and has been recruited to serve as a dehalogenase or that a mechanism for its regulation has not yet evolved. However, despite the evidence suggesting that these first two enzymes are not yet ideally suited for their functions in the degradation of PCP, the pathway for degradation in this microorganism has reached a level of sophistication in which the rates of the initial catabolic steps are appropriately controlled so as to maintain low levels of chlorinated hydroquinone intermediates and thus to avoid oxidative damage to the cell.

REFERENCES

1. **Apajalahti, J. H. A., and M. S. Salkinoja-Salonen.** 1987. Complete dechlorination of tetrachlorohydroquinone by cell extracts of pentachlorophenol-induced *Rhodococcus chlorophenolicus*. J. Bacteriol. **169:**5125–5130.
2. **Conant, J. B., and L. F. Fieser.** 1923. Reduction potentials of quinones. I. The effect of the solvent on the potentials of certain benzoquinones. J. Am. Chem. Soc. **45:**2194–2218.
3. **Cort, T., and S. D. Copley.** Unpublished results.
4. **Cronan, J. E., Jr., R. B. Gennis, and S. R. Maloy.** 1987. Cytoplasmic membrane, p. 31–55. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
5. **Crosby, C. G.** 1981. Environmental chemistry of pentachlorophenol. Pure Appl. Chem. **53:**1052–1080.
6. **Dahlhaus, M., E. Almstadt, P. Henschke, S. Luttgert, and K. E. Appel.** 1995. Induction of 8-hydroxy-2-deoxyguanosine and single-strand breaks in DNA of V79 cells by tetrachloro-p-hydroquinone. Mutat. Res. **329:**29–36.
7. **Ederer, M. M., R. L. Crawford, R. P. Herwig, and C. S. Orser.** 1997. PCP degradation is mediated by closely related strains of the genus *Sphingomonas.* Mol. Ecol. **6:**39–49.
8. **Ehrlich, W.** 1990. The effect of pentachlorophenol and its metabolite tetrachlorohydroquinone on cell growth and the induction of DNA damage in Chinese hamster ovary cells. Mutat. Res. **244:**299–302.
9. **Häggblom, M. M., D. Janke, P. J. M. Middeldorp, and M. S. Salkinoja-Salonen.** 1989. O-methylation of chlorinated phenols in the genus *Rhodococcus*. Arch. Microbiol. **152:**6–9.
10. **Häggblom, M. M., D. Janke, and M. Salkinoja-Salonen.** 1989. Hydroxylation and dechlorination of tetrachlorohydroquinone by *Rhodococcus* sp. strain CP-2 cell extracts. Appl. Environ. Microbiol. **55:**516–519.
11. **Häggblom, M. M., L. J. Nohynek, and M. S. Salkinoja-Salonen.** 1988. Degradation and O-methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains. Appl. Environ. Microbiol. **54:**3043–3052.
12. **Hattemer-Frey, H. A., and C. C. Travis.** 1989. Pentachlorophenol: environmental partitioning and human exposure. Arch. Environ. Contam. Toxicol. **18:**482–489.
13. **Hungate, R. E.** 1969. A roll tube method for cultivation of strict anaerobes. Methods Microbiol. **3B:**118.
14. **Ito, M., and Y. Ohnishi.** 1982. *Escherichia coli* mutants resistant to uncouplers of oxidative phosphorylation. Microbiol. Immunol. **26:**1079–1084.
15. **Izaki, K., M. Takahashi, Y. Sato, Y. Sasagawa, K. Sato, and C. Furusaka.** 1981. Some properties of pentachlorophenol-resistant Gram-negative bacteria. Agric. Biol. Chem. **45:**765–767.
16. **Juhl, U., J. K. Blum, W. Butte, and I. Witte.** 1991. The induction of DNA strand breaks and formation of semiquinone radicals by metabolites of 2,4,5-trichlorophenol. Free Radical Res. Commun. **11:**295–305.
17. **Kappus, H., and H. Sies.** 1981. Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. Experientia **37:**1233–1241.
18. **Kolachana, P., V. V. Subrahmanyam, K. B. Meyer, L. Zhang, and M. T. Smith.** 1993. Benzene and its phenolic metabolites produce oxidative DNA damage in HL60 cells *in vitro* and in the bone marrow *in vivo*. Cancer Res. **53:**1023–1026.
19. **Leive, L.** 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. **243:**2373–2380.
20. **McCarthy, D. L., and S. D. Copley.** Unpublished results.
21. **McCarthy, D. L., S. Navarrete, W. S. Willett, P. C. Babbitt, and S. D. Copley.** 1996. Exploration of the relationship between tetrachlorohydroquinone dehalogenase and the glutathione S-transferase superfamily. Biochemistry **35:** 14634–14642.
22. **Mitchell, P.** 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol. Rev. **41:**445–502.
23. **Newton, G. L., and R. C. Fahey.** 1990. Glutathione in prokaryotes, p. 69–77. *In* J. Vina (ed.), Glutathione: metabolism and physiological functions. CRC Press, Inc., Boca Raton, Fla.
24. **Newton, G. L., R. C. Fahey, G. Cohen, and Y. Aharonowitz.** 1993. Low-molecular-weight thiols in streptomycetes and their potential role as antioxidants. J. Bacteriol. **175:**2734–2742.
25. **Nohynek, L. J., E. L. Suhonen, E.-L. Nurmiaho-Lassila, J. Hantula, and M. Salkinoja-Salonen.** 1995. Description of four pentachlorophenol-degrading bacterial strains as *Sphingomonas chlorophenolica* sp. nov. Syst. Appl. Microbiol. **18:**527–538.
26. **Orser, C. S., J. Dutton, C. Lange, P. Jablonski, L. Xun, and M. Hargis.** 1993. Characterization of a *Flavobacterium* glutathione-*S*-transferase gene involved in reductive dechlorination. J. Bacteriol. **175:**2640–2644.
27. **Pfennig, N., and K. D. Lippert.** 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Mikrobiol. **55:**245–256.
28. **Ploemen, J. H. T. M., B. van Ommen, and P. J. van Bladeren.** 1991. Irreversible inhibition of human glutathione *S*-transferase isoenzymes by tetrachloro-1-4-benzoquinone and its glutathione conjugate. Biochem. Pharmacol. **41:**1665–1669.
29. **Radehaus, P. M.** 1992. Ph.D. thesis. University of Colorado at Boulder, Boulder.
30. **Radehaus, P. M., and S. K. Schmidt.** 1992. Characterization of a novel *Pseudomonas* sp. that mineralizes high concentrations of pentachlorophenol. Appl. Environ. Microbiol. **58:**2879–2885.
31. **Ravanel, P., and M. Tissut.** 1986. Toxicity of pentachlorophenol on isolated plant mitochondria. Phytochemistry **25:**577–583.
32. **Smejtek, P., A. W. Barstad, and S. Wang.** 1989. Pentachlorophenol-induced change ζ-potential and gel-to-fluid transition temperature in model lecithin membranes. Chem.-Biol. Interact. **71:**37–61.
33. **Stockdale, M., and M. J. Selwyn.** 1971. Effects of ring substituents on the activity of phenols as inhibitors and uncouplers of mitochondrial respiration. Eur. J. Biochem. **21:**565–574.
34. **Suwalsky, M., M. A. Espinoza, M. Bagnara, and C. P. Sotomayor.** 1990. X-ray and fluorescence studies on phospholipid bilayers. IX. Interactions with pentachlorophenol. Z. Naturforsch. **45c:**265–272.
35. **Taylor, M. F., M. H. Boylan, and D. E. Edmondson.** 1990. *Azotobacter vinelandii* flavodoxin: purification and properties of the recombinant, dephospho form expressed in *Escherichia coli*. Biochemistry **29:**6911–6918.
36. **Ting, H. P., D. F. Wilson, and B. Chance.** 1970. Effects of uncouplers of oxidative phosphorylation on the specific conductance of bimolecular lipid membranes. Arch. Biochem. Biophys. **141:**141–146.
37. **Trevors, J. T.** 1983. Effect of pentachlorophenol on the membrane fluidity of *Pseudomonas fluorescens*. FEMS Microbiol. Lett. **16:**331–334.
38. **Uotila, J. S., M. S. Salkinoja-Salonen, and J. H. A. Apajalahti.** 1991. Dechlorination of pentachlorophenol by membrane bound enzymes of *Rhodococcus chlorophenolicus* PCP-1. Biodegradation **2:**25–31.
39. **van Ommen, B., A. Adang, F. Müller, and P. J. van Bladeren.** 1986. The microsomal metabolism of pentachlorophenol and its covalent binding to protein and DNA. Chem.-Biol. Interact. **60:**1–11.
40. **van Ommen, B., A. Koster, H. Verhagen, and P. J. van Bladeren.** 1992. The glutathione conjugates of *tert*-butyl hydroquinone as potent redox cycling agents and possible reactive agents underlying the toxicity of butylated hydroxyanisole. Biochem. Biophys. Res. Commun. **189:**309–314.
41. **Witte, I., U. Juhl, and W. Butte.** 1985. DNA-damaging properties and cytotoxicity in human fibroblasts of tetrachlorohydroquinone, a pentachlorophenol metabolite. Mutat. Res. **145:**71–75.
42. **Xun, L., and C. S. Orser.** 1991. Purification and properties of pentachlorophenol hydroxylase, a flavoprotein from *Flavobacterium* sp. strain ATCC 39723. J. Bacteriol. **173:**4447–4453.
43. **Xun, L., E. Topp, and C. S. Orser.** 1992. Purification and characterization of a tetrachloro-*p*-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. J. Bacteriol. **174:**8003–8007.
44. **Xun, L., E. Topp, and C. Orser.** 1992. Diverse substrate range of a *Flavobacterium* pentachlorophenol hydroxylase and reaction stoichiometries. J. Bacteriol. **174:**2898–2902.