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Molecular Probe Techniques for the Identification of Reductants in Sediments: Evidence for Reduction of 2-Chloroacetophenone by Hydride Transfer

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The reduction of 2-chloroacetophenone (2-CAP) was examined in anoxic sediment slurries from both freshwater and marine sources. The reduction of 2-CAP produces acetophenone via electron transfer and 2-chloro-1-phenylethanol (2-CPE) via hydride transfer. Experimental results demonstrate that 2-CAP is an effective probe molecule for distinguishing and quantifying reductive transformations occurring by electron transfer and hydride transfer in anaerobic sediments. These results varied among the sediments examined, with freshwater sediments generating more 2-CPE (hydride transfer product) than the marine sediments. Enantiomeric excess of (*R*)-2-CPE (over the (*S*)-enantiomer) demonstrated that reduction by hydride transfer is enantioselective, providing direct evidence that the source of hydride is a chiral reductant. Temperature studies demonstrate that increasing temperature eliminates the production of 2-CPE, further evidence for an enzyme-mediated pathway.

Introduction

Many agrochemicals, textile dyes, pharmaceuticals, and solvents contain functional groups that are susceptible to facile reduction in anaerobic environments. Because reducing environments abound in nature (subsurface waters and soils, aquatic sediments, hypolimnia of stratified lakes), there is interest in understanding the reduction of organic molecules in such environments and in identifying the natural reducing agents responsible for the degradation of reducible anthropogenic pollutants. The latter has proven to be particularly challenging due to the complex nature of natural sediments. Complications that are of particular concern include organic carbon, solid mineral phases, and dissolved inorganic ions.

To develop a better understanding of reductive transformations in natural sediments, the reduction of organic molecules has been studied in many natural and model media: anaerobic soils/sediments (*1*−*4*); biomimetic model systems (*5*−*12*); ferrous iron/mineral oxide model systems (*13*−*14*); and other model systems that contain sulfur nucleophiles, quinones, and zerovalent metals (*15*−*18*). These studies have provided mechanistic information about reduction reactions, but none provide direct identification of the primary reductants responsible for the reaction of reducible organic compounds in sediments.

The use of organic analytes as probe molecules to acquire information about reactivity in environmental systems has been an important part of many studies. Analytes that have been used as probes include alkyl halides (*19*); resofurin ethers (*20*); nitroxide radicals (*21*−*24*); meso- and D,L-2,3-dibromopentane (*25*); [1,2-^{13}C]chloroacetic acid, -acetamide, and -acetonitrile (*26*, *27*); and nitroaromatic compounds (*7*, *16*, *28*−*30*). This study examines the reactivity and potential utility of a different probe molecule, 2-chloroacetophenone (2-CAP), which reacts by at least two characterized pathways (Figure 1). Previous studies have shown that 2-CAP is reduced to 2-CPE via hydride transfer (reaction 1) and to acetophenone via electron transfer (reaction 2) in simple laboratory systems (*31*). Acetophenone can be further reduced to *sec*-phenethyl alcohol via hydride transfer (reaction 3).

α-Haloacetophenones, including 2-CAP, have been used as mechanistic probes for the enzyme-mediated (horse liver alcohol dehydrogenase, HLADH) reduction by nicotinamide adenine dinucleotide (NADH). The reduction of the α-haloacetophenones by NADH alone was found to be limited, yielding only traces of the acetophenone product. In the presence of NADH/HLADH, however, both α-chloro- and α-fluoroacetophenones gave the hydride reduction product, optically active 1-phenyl-2-haloethanol. Other studies have demonstrated the use of these compounds as probes for the study of reduction by organometal hydrides (*32*−*35*) and NADH model compounds (*36*, *37*).

2-CAP can also be used to probe the stereoselectivity of a reduction pathway. Because the two faces of the carbonyl group in 2-CAP are enantiotopic, chiral reductants have the potential to distinguish between the enantiotopic faces of the carbonyl group (Figure 2). Attack at the enantiotopic faces of the carbonyl carbon by a chiral hydride reagent (e.g., NADH coupled with HLADH) will give diastereomeric transition states of different energies that are formed in unequal amounts. Conversely, attack by an achiral hydride reagent (e.g., $NaBH_4$) at the enantiotopic faces of the carbonyl group of 2-CAP will give enantiomeric transition states of equal energy. In this situation, a racemic mixture (50%(*R*)/50%(*S*)) of products will result. Because there are no known achiral sources of hydride in environmental systems, enantioselective reduction of 2-CAP would almost certainly indicate reaction with a biological reductant (such as NADH, mediated by dehydrogenase enzymes).

The objective of this research was to demonstrate the use of 2-CAP to characterize naturally occurring reductants and reduction pathways in four different anoxic sediments. This was accomplished by measuring reduction rates and product yields of 2-CAP in anoxic sediments from both freshwater and marine sources. The products of both electron transfer (acetophenone) and hydride transfer (2-CPE) were quantified using liquid chromatography. In addition, the enantiomeric ratio of 2-CPE was measured using gas chromatography. Future work will attempt to characterize other possible reduction pathways of 2-CAP so that it can be used to estimate reductant concentrations, which are necessary to begin developing quantitative kinetic expressions describing the reductive transformations of organic chemicals.

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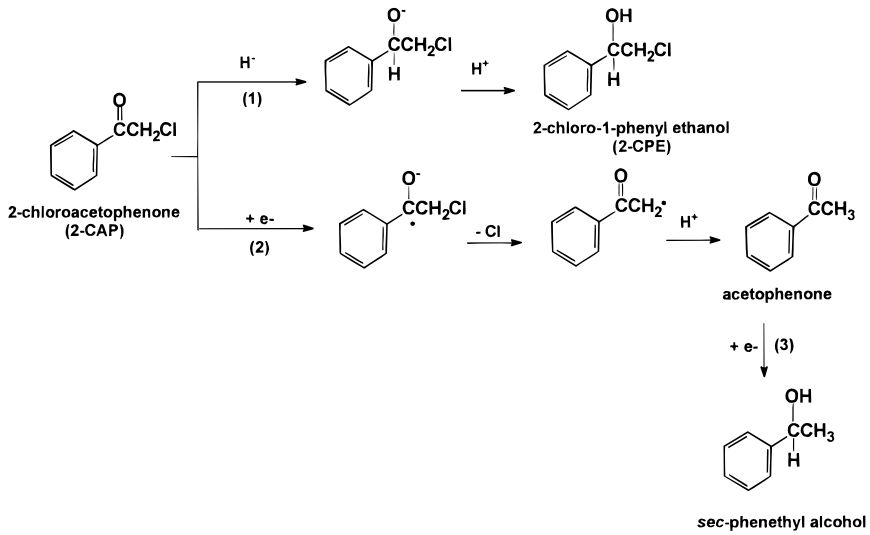

FIGURE 1. Reduction pathways of 2-CAP: (1) hydride transfer to produce 2-CPE; (2) electron transfer to produce acetophenone; and (3) hydride transfer to produce *sec*-phenethyl alcohol.

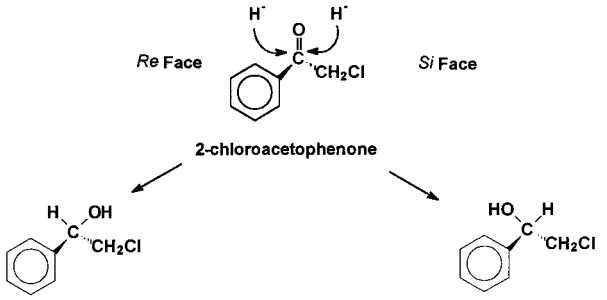

FIGURE 2. The two faces of the carbonyl group in 2-CAP are enantiotopic. Attack by achiral and chiral hydride will produce different ratios of enantiomers.

Materials and Methods

Chemical Reagents. 2-Chloroacetophenone [532-27-4], acetophenone [98-86-2], *sec*-phenethyl alcohol [98-85-1], (*R*)-(−)-2-chloro-1-phenylethanol [56751-12-3], (*S*)-(+)-2-chloro-1-phenylethanol [70111-05-6], acetonitrile (HPLC grade), sodium borohydride, and horse liver alcohol dehydrogenase (HLADH, conversion of 1 μmol of ethanol to acetaldehyde per min at pH 8.8, 25 °C) were all obtained from Aldrich Chemical Co. at purities greater than 97% and used as received. The reduced form of nicotinamide adenine dinucleotide (NADH) was obtained from Sigma Chemical Company with purity greater than 98% and used as received.

Sediment Sources. Freshwater sediments were collected from Rock Creek, a tributary of the Tualatin River in northeast Oregon, and Cherokee Park, a freshwater pond located in a residential area of Athens, GA. Institute Marsh sediments were collected from a pristine salt marsh at Sapelo Island, GA. Young's Bay sediments were collected from part of the Columbia River estuary at the confluence of two small tributaries. Young's Bay is influenced by tides of moderate range, but water chemistry is dominated by freshwater input so the mean salinity intrusion is only 10−15 ppt.

Sediments were collected in wide-mouthed Mason jars from the first 5 cm below the sediment/water interface. In the laboratory, each sample was sieved (1 mm) to remove large particles, resealed, and stored at 4 °C until used. Sediment slurries for batch experiments were prepared in the anaerobic chamber by diluting the sample with either site pore water or Nanopure water (Barnstead/Thermolyne) to the desired solids concentration. This material was allowed to equilibrate for 2 days in the anaerobic chamber (95% N_2 (g); 5% H_2 (g)) prior to use in batch experiments. All subsequent manipulations of the sediment slurry were conducted in the anaerobic chamber.

TABLE 1. Sediment and Control System Characteristics: pH, % Solids, and Fe(II) Concentration

batch system	pH	% solids	Fe^{II} (mol/L)
Cherokee Park	7.5	2	3.4×10^{-5}
Rock Creek	7.4	7−10	1.3×10^{-4}
Young's Bay	7.6	2	3.3×10^{-5}
Institute Marsh	8.8	2	4.4×10^{-7}
Goethite/Fe(II)	7.3	3	1.0×10^{-3}
NADH	7.0		
HLADH/NADH	7.0		

Experimental Setup. Forty milliliters of the equilibrated sediment slurry (% solids listed in Table 1) were transferred to amber-glass serum bottles using an automatic pipettor and a 10-mL glass pipet. The serum bottles were fitted with Teflon-lined gray rubber septa. At the start of each experiment ($t = 0$), 1.0 mL of a 2-CAP stock solution (2.15 mM, 50/50 (v/v) acetonitrile/H_2O) was added to the serum bottles to produce a final substrate concentration of approximately 60 μM. The bottles were crimp-sealed, removed from the anaerobic chamber, and placed on a temperature-controlled shaker table (250 rpm, 25 °C). For sampling, bottles were returned to the anaerobic chamber, and a 1-mL aliquot of the sediment-water slurry was transferred to a 1.5-mL polypropylene microcentrifuge tube. The sample was then centrifuged at 13 000 rpm for 10 min. The supernatant then was transferred to a clean tube and centrifuged for 5 min at 13 000 rpm. Concentrations of 2-CAP in the supernatant were determined by HPLC. Each experiment was performed in duplicate.

Control Studies. Four control studies without sediments were performed to verify that the two reaction pathways of 2-CAP were distinguishable. The four batch systems consisted of 2-CAP (prepared as above) and (i) a slurry consisting of 0.5 g/L FeOOH (goethite) and 1.0 mM Fe(II) (added as $FeCl_2 \cdot 4H_2O$); (ii) a solution consisting of only NADH and 0.05 M potassium phosphate buffer (pH 7.0); (iii) a solution consisting of NADH, HLADH, and 0.05 M potassium phosphate buffer (pH 7.0); or (iv) a methanol solution containing 0.04 M sodium borohydride. The remainder of the experimental

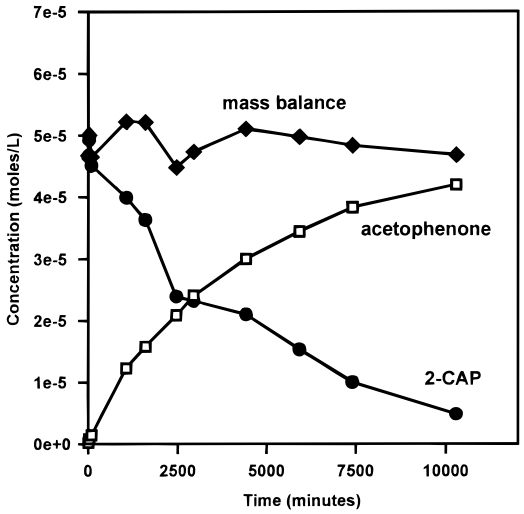

FIGURE 3. Reduction of 2-CAP to acetophenone. Reaction conditions: 1 mM Fe^{II} in 0.5 g/L FeOOH(s), pH 7 phosphate buffer.

procedure was identical to that used in the batch experiments performed with sediment slurries.

Control experiments were also performed to determine if any loss of the reduction products was due to sorption by the sediments. Rock Creek sediment was spiked with each of the reduction products: acetophenone, *sec*-phenethyl alcohol, and 2-CPE. Their concentrations were measured over a period of 10 days by liquid chromatography as described below.

Analytical Methods. The extent of 2-CAP reduction was determined by following loss of the parent compound using reversed-phase HPLC: Alltech column (RSIL, C18, 5 micron, 250 × 4.6 mm); Adsorbosphere guard column (C18, 5 micron); and Applied Biosystems (Model 783A) programmable UV absorbance detector (λ = 210 nm). The isocratic eluents consisted of 35% acetonitrile and 65% Nanopure water, with a flow rate of 1.0 mL/min. The three reduction products (acetophenone, *sec*-phenethyl alcohol, and 2-CPE) were identified and quantified using authenticated standards. The products of the reactions were further characterized using a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 5972 Series Mass Selective Detector.

Plots of ln [2-CAP] versus time were linear, indicating that the reduction reactions in sediment slurries were first order with respect to 2-CAP. 2-CAP reduction rate constants (k_{obs}, min^{-1}) correspond to the slope of these plots. Kinetic experiments were monitored over two to three half-lives of the 2-CAP (1−5 days).

Separation and analysis of the (*R*)- and (*S*)-enantiomers of 2-CPE was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a flame ionization detector and a capillary chiral column (Chiraldex, B-cyclodextrin dimethyl, 30 m × 0.25 mm, Advanced Separation Technologies, Inc., Whippany, NJ). Samples were prepared for analysis by extraction from the aqueous phase with methylene chloride. Baseline separation was achieved with an isothermal column temperature of 170 °C, injector temperature of 210 °C, and detector temperature of 220 °C. Helium was the carrier gas with a flow of 1.0 mL/min.

Results and Discussion

Control Experiments. Control experiments were conducted to demonstrate the two reaction pathways of 2-CAP: electron transfer and hydride transfer. 2-CAP was reduced in a slurry of goethite containing 1.0 mM ferrous iron (Figure 3). This system was chosen because Fe(II) sorbed onto metal oxides is known to be an effective reductant of nitroaromatics (*13*), chromate (*38*), pertechnetate (*39*, *40*), and nitrite (*41*) via an electron-transfer pathway. In addition, structural Fe(II) and Fe(II) adsorbed to the surface of iron oxides have been found to reduce carbon tetrachloride (*42*−*45*) and trichloroethene (*46*). The results of the control experiments show that all of the 2-CAP reacted in the Fe(II)/goethite batch system can be accounted for by the production of acetophenone, the reduction product that is expected to form via electron transfer.

Control systems including only NADH and phosphate buffer did not result in detectable transformation of 2-CAP. Under similar conditions, Tanner and Stein found that only trace amounts of acetophenone were produced (*31*). However, the addition of HLADH to batch systems containing NADH resulted in complete transformation of 2-CAP. The only product detected in this control experiment was 2-CPE, the reduction product resulting from hydride transfer. These results are in agreement with previous reports (*31*). Further analysis using gas chromatography demonstrated that the (*R*)-enantiomer of 2-CPE was produced in 97% enantiomeric excess (Table 2). This enantiomeric excess is direct evidence of enantioselective reduction of 2-CAP by a chiral reagent. The final control experiment employed an achiral hydride reducing agent, sodium borohydride, and it produced the expected result: a 50%/50% mixture of the (*R*)- and (*S*)-enantiomers of 2-CPE.

Sediment-Mediated Reductions: Kinetics and Mass Balance. 2-CAP underwent reductive transformation in all four natural sediments. In the Rock Creek sediment (Figure 4A), 35% of the 2-CAP lost from the sediment suspension was reduced to 2-CPE, while 20% of the 2-CAP was reduced to acetophenone. We believe that this is the first direct evidence for the reduction of dissolved organic species by hydride in natural sediment. 25% of the initial 2-CAP concentration was further transformed via acetophenone to *sec*-phenethyl alcohol (Figure 1; reaction 3).

Loss of 2-CAP in the Cherokee Park sediment (Figure 4B) resulted in 50% conversion to acetophenone with only 10% of the 2-CAP transformed to 2-CPE, implying that the primary reduction pathway is electron transfer. The Institute Marsh and Young's Bay sediments (Figure 4 (parts C and D)) reduced 2-CAP primarily to acetophenone, which accounted for 20% of the 2-CAP lost. Formation of 2-CPE was 5% or less in both of these sediments. Recovery of the starting compound and the reaction products was especially difficult in the marine sediments. To assess whether the reduction reaction in these systems required the presence of a surface, Rock Creek and Institute Marsh pore waters (separation by decanting) were spiked with 2-CAP. No reaction was observed in these sediment-free systems.

Sorption Experiments. The sorption of 2-CAP and its reduction products was investigated in an attempt to account for the variable and incomplete mass balance that was observed in the marine sediments. Rock Creek sediment was spiked with each of the known reduction products (acetophenone, *sec*-phenethyl alcohol, and 2-CPE) at concentrations typical of those used in kinetic batch experiments. The measured concentrations of all three of the standards were constant over time, indicating that losses due to sorption were insignificant. Extraction of 2 mL of slurry with 1 mL of acetonitrile did not significantly change the recovery relative to aqueous samples, which is consistent with the conclusion that sorption is not responsible for the missing mass. The time dependence in the mass balance data varies with the sediment, suggesting that it reflects some other transformation that is sediment-associated.

One possibility is that sulfide is reacting with 2-CAP by nucleophilic displacement of the chloride ion to form 2-thioacetophenone. Later, under oxidizing conditions, the

TABLE 2. Kinetic Data for Sediment and Control Systems[a]

batch system	k_{obs} (min^{-1})	acetophenone (%)	2-CPE (%)	(%*R*)/(%*S*)
Cherokee Park	$(2.40 \pm 0.09) \times 10^{-4}$	50	10	84/16
Rock Creek	$(2.75 \pm 0.50) \times 10^{-3}$	20 (+25)	35	62/38
Young's Bay	$(3.06 \pm 0.53) \times 10^{-3}$	20	5	63/37
Institute Marsh	$(7.01 \pm 0.68) \times 10^{-3}$	20	3	–
Goethite/Fe(II)	$(2.10 \pm 0.36) \times 10^{-4}$	100	–	–
NADH	no rxn	–	–	–
HLADH/NADH	3.33×10^{-5}	<1	99	97/3
$NaBH_4$	not measured	<1	99	50/50

[a] k_{obs} (first-order rate constant), % of 2-CAP converted to acetophenone, % of 2-CAP converted to 2-CPE, ratio of (*R*)- and (*S*)-enantiomers of 2-CPE.

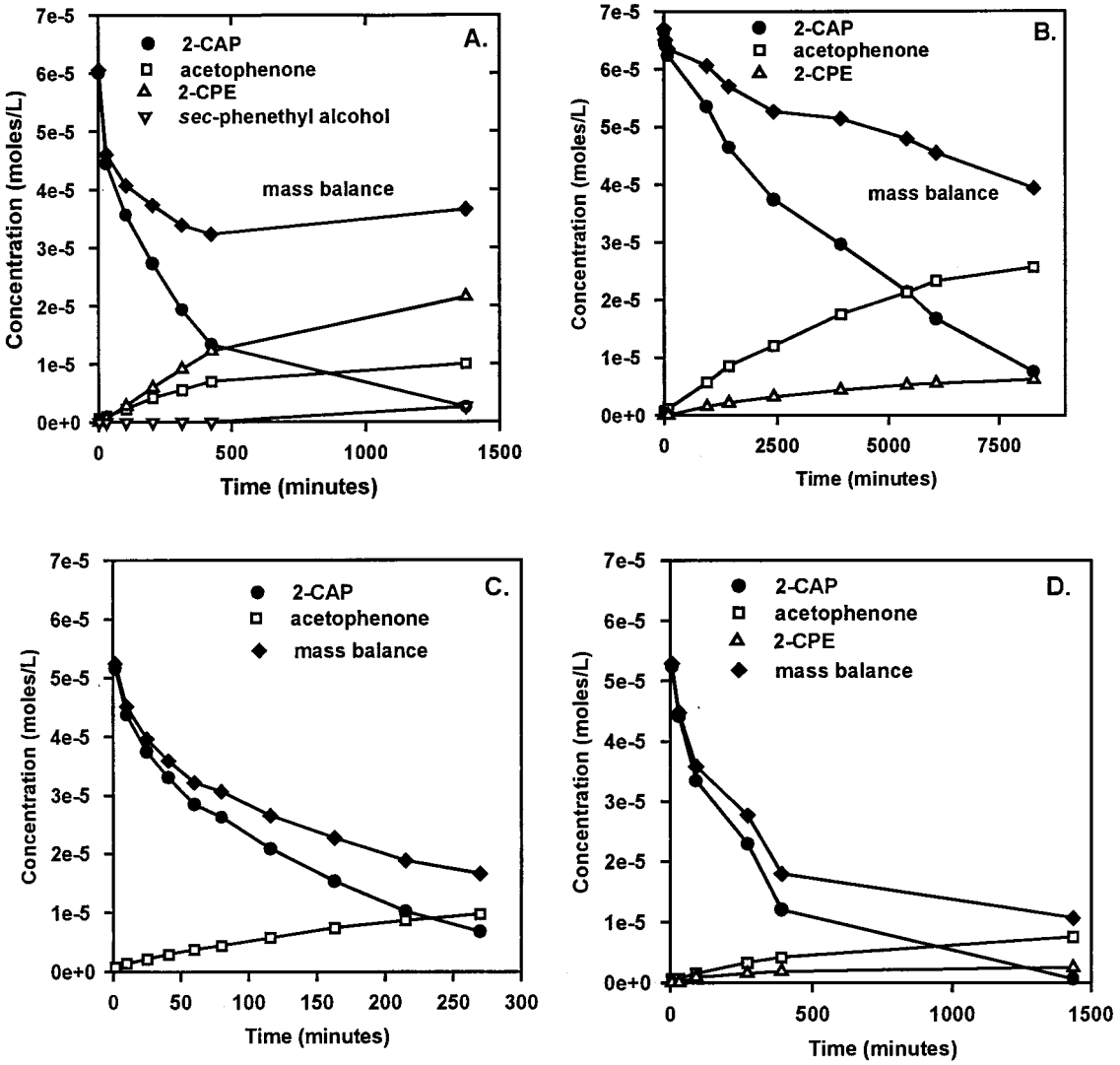

FIGURE 4. Reduction of 2-CAP as a function of time in four sediment slurries: A. Rock Creek, B. Cherokee Park, C. Institute Marsh, and D. Young's Bay.

thiol might be oxidized to the disulfide. The reaction of sulfide and other sulfur-based nucleophiles with chloroacetanilides has been well-documented (*47*, *48*). Preliminary studies demonstrated that treatment of 2-CAP with 1.0 mM bisulfide ion resulted in the facile disappearance of 2-CAP. Although, detailed product analyses have not yet been completed, we were able to conclude that neither acetophenone or 2-CPE were formed in the reaction of HS^- with 2-CAP.

Enantioselective Formation of 2-CPE. In all three sediment/water systems in which an adequate amount of 2-CPE was formed for analysis by gas chromatography (Cherokee Park, Rock Creek, and Young's Bay), the (*R*)-enantiomer was produced in enantiomeric excess from 62% to 84% (Table 2). Baseline separation of the enantiomers of 2-CPE detected in Rock Creek sediment was achieved using gas chromatography equipped with a chiral column (Figure 5). The observed enantioselectivity of the hydride transfer reaction provided direct evidence that the reductant was chiral.

The most likely explanation for chiral reduction by hydride is enzyme-catalyzed reduction with a coenzyme such as NADH, a metabolic process exhibited by microorganisms. Dehydrogenase enzymes are known to catalyze hydride transfer by NADH, and extracellular dehydrogenase activity has been reported in soils (*49*, *50*). NADH-dependent horse

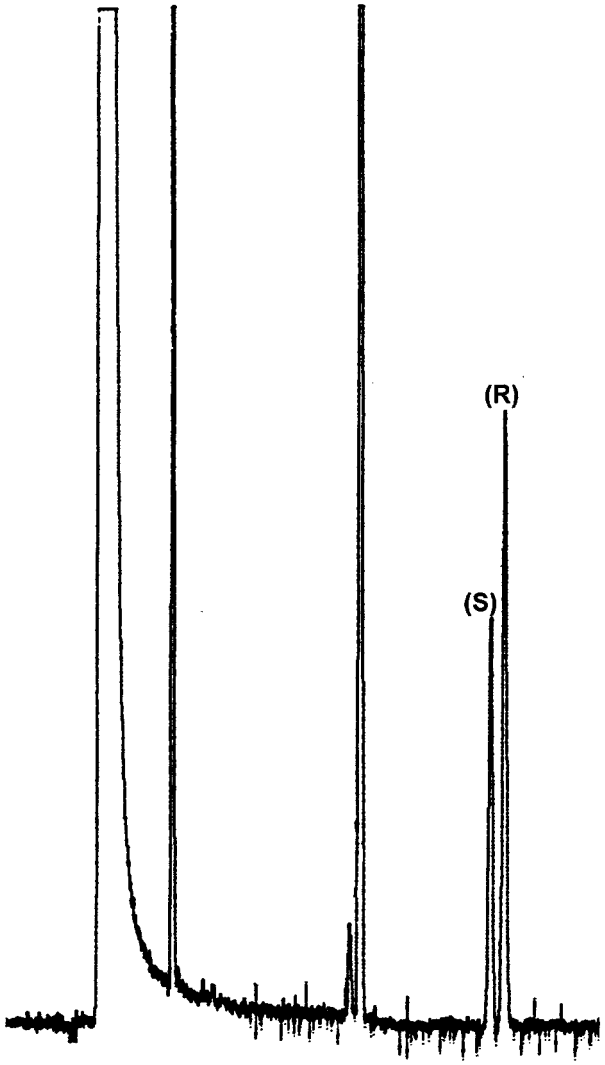

FIGURE 5. Demonstration of enantioselective reduction of 2-CAP in Rock Creek sediment: GC chromatogram of the (*R*)- and (*S*)-enantiomers of 2-CPE.

liver alcohol dehydrogenase catalyzes the reduction of aldehydes and ketones with broad specificity (*51*). Other coenzymes such as NADPH (nicotinamide adenine dinucleotide phosphate), FADH (flavin adenine dinucleotide), and dihydroflavin could also be serving as the hydride donor. Although these considerations complicate the interpretation of results with the 2-CAP probe, the implication that reduction involves hydride is still a more specific identification of a natural reducing agent in sediments than has been achieved by other methods.

Temperature Studies. The kinetics of 2-CAP reduction were studied in Rock Creek sediment as a function of temperature to further assess the role that enzymes might play in the reduction process. Biological reactions typically demonstrate a temperature optimum, whereas the rate of abiotic reactions will increase in proportion to the reaction temperature increase (*52*). Over the range of 25−65 °C, k_{obs} (min^{-1}) increased monotonically with increasing temperature (Figure 6). A plot of ln k_{obs} vs $1/T$ is linear, demonstrating Arrhenius behavior. Looking specifically at the rate of product formation, we observed that the kinetics of the formation of the hydride reduction product (2-CPE) resulted in a temperature optimum (Figure 7), while the rate of formation of acetophenone increased with temperature much like the overall reduction rate of 2-CAP increased with temperature. The observed temperature optimum for the production of

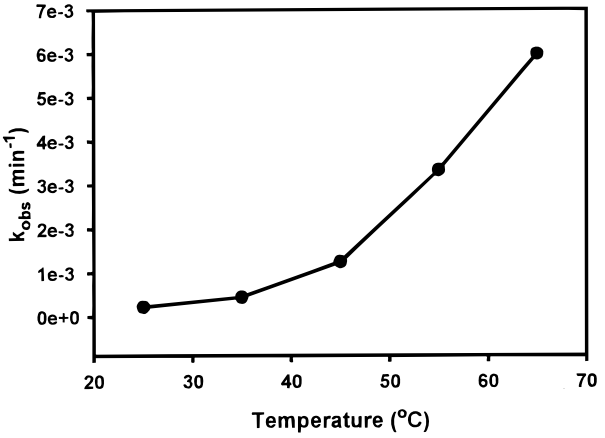

FIGURE 6. Effect of temperature on 2-CAP reduction in Rock Creek sediment.

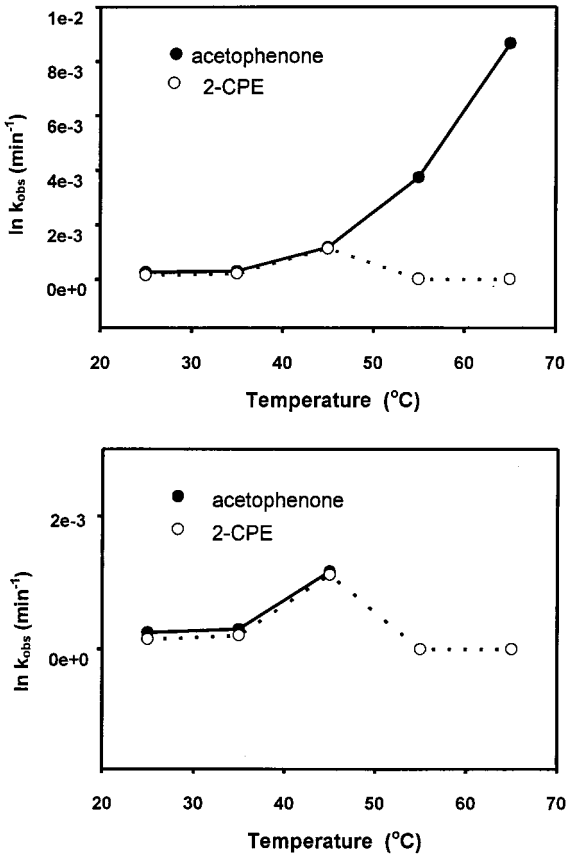

FIGURE 7. Effect of temperature on acetophenone and 2-CPE production in Rock Creek sediment. The lower graph represents the same data with a smaller range on the *y*-axis.

2-CPE suggests that an enzymatic reaction is responsible for the hydride transfer reaction pathway.

The increase in the rate of formation of acetophenone with temperature suggests that enzyme-mediated reduction of 2-CAP to acetophenone was not significant in the sediments we investigated. Bacterial enzymes containing transition-metal coenzymes, such as heme (an iron porphyrin complex), cobalamins (vitamin B_{12} derivatives), and F-430 (nickel-centered porphinoid) have been proposed as important catalysts for reductions in biological systems (*53, 54*). The generalized mechanism for the reaction of metallocoenzymes and halogenated hydrocarbons involves transfer of the halogen atom directly from carbon to the metal, resulting in the formation of a carbon-based free radical and

the metal-halide ion adduct (*55*). Consequently, the reduction of 2-CAP by metallocoenzymes would be expected to result in the formation of acetophenone. The reduction of acetyl chloride by nickel(I) octaethylisobacteriochlorin, a good structural model for Factor F-430, gives only the acetaldehyde (*55*). Reduction of the carbonyl group was not observed. The reduction of 2-CAP by metallocoenzymes will be addressed in future studies.

Although further work is necessary to characterize the enzymes that apparently mediate reduction of 2-CAP to 2-CPE in sediments, the data strongly indicate that hydride is a reductant that is contributing to the transformation of 2-CAP. Thus, it would seem that hydride must be considered along with Fe(II), sulfide, and NOM among the electron donors that contribute to contaminant reduction in reducing sediments. The reaction pathways observed in this study expand the list of compounds that are thought to undergo reductive transformation in natural sediments as well as the list of potential transformation products. Considering reduction by hydride could provide additional pathways for pollutants already susceptible to reductive transformations.

Acknowledgments

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