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# NOTES

## Mineralization of Diethylthiophosphoric Acid by an Enriched Consortium from Cattle Dip

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**Enrichment cultures were initiated from cattle dip solution with diethylthiophosphoric acid (DETP) as the carbon and energy source. An enriched consortium consisting of at least six distinct bacterial species was subsequently obtained. The consortium mineralized DETP to sulfate and phosphate while utilizing ethyl moieties as a carbon and energy source. These data demonstrate that DETP can be metabolized under environmentally realistic conditions.**

The organophosphate insecticides represent a major class of pesticides that are in widespread use. Although they generally exhibit a very high acute toxicity, they are not considered to be an environmental problem because of their rapid metabolism. The initial step in organophosphate metabolism generally is the hydrolytic cleavage of the organophosphate bond (6) (Fig. 1). It is this reaction that has received the most attention, because it renders the molecule biologically inactive. By comparison, less attention has been paid to the products of this cleavage, particularly the ionic dialkyl moiety. Cook et al. (2) isolated from sewage sludge bacteria that were able to use several dialkylthiophosphoric acids (DETPs) and dialkyldithiophosphoric acids (DEDTPs) as a sole phosphorous source when grown with *p*-hydroxybenzoate as a carbon source. One of these organisms, *Pseudomonas acidovorans*, was also able to use DETP or DEDTP as a sole sulfur source when grown with *p*-hydroxybenzoate as a carbon source (3). Although this organism was capable of growth with ethanol, it did not appear to use DEDTP as a carbon source; results with DETP were not reported.

Although results of these studies demonstrate that specific bacteria are able to metabolize DETP, they do not address the fate of DETP under environmentally realistic conditions, in which carbon substrates rather than sulfate and phosphate are likely to be limiting. As a result of my studies on the biodegradation of coumaphos, an organophosphate insecticide that is used for tick control in cattle dipping vats, I became interested in the fate of the ionic dialkylphosphate hydrolysis product DETP. I report here on an enriched consortium, obtained from cattle dip material, that is able to use DETP as a carbon and energy source in the presence of excess sulfate and phosphate.

The original enrichment culture was initiated from the Laredo City (Laredo, Tex.) vat dip solution, with flowable cattle insecticide (42% coumaphos, 58% inert ingredients; Co-Ral [Mobay Corp.]) used as a carbon and energy source. After I observed that DETP, the hydrolytic cleavage product of coumaphos, was being degraded, an enrichment culture was initiated that consisted of 1,000 μg of DETP per ml and 10 μg of yeast extract per ml in mineral salts (4). Degradation was monitored routinely by measuring DETP disappearance or an increase in biomass. Samples for analysis were obtained by filtering the culture through a 0.2-μm-pore-size disposable filter assembly (Acro LC13; Gelman Sciences, Inc., Ann Arbor, Mich.) and storing the samples at 4°C until they were analyzed. When DETP degradation was complete, a 2% inoculum transfer was made into fresh medium. It was necessary to adjust the pH of the enrichment periodically with 0.5 N KOH because of the production of acidic end products.

Biomass production was determined by filtering 50 ml of culture solution through a 0.45-μm-pore-size filter after growth of the culture on 1,000 μg (50 mg) of DETP per ml–10 μg (0.5 mg) of yeast extract per ml or on 10 μg of yeast extract per ml alone. Filters were allowed to air dry before they were reweighed. Experiments were run in duplicate. Inhibition experiments were conducted by adding 100 μg of either cycloheximide or chloramphenicol per ml to cultures and monitoring the growth. Bacteria were isolated by performing serial dilutions of the culture solution and spread plating the bacteria onto dilute nutrient broth agar (1 g/liter). Bacteria from morphologically distinct colonies were restreaked for purity. The metabolism of DETP was determined by growing isolates to the stationary phase on either 0.1% ethanol or 0.1% yeast extract and adding 1,000 μg of DETP per ml to the culture.

DETP was synthesized by the following procedure. Equal amounts (0.106 mol) of triethylamine (10.7 g in 250 ml of $H_2O$; Aldrich Chemical Co., Inc., Milwaukee, Wis.) and chlorodiethylthiophosphate (20 g in 100 ml of $H_2O$; Aldrich) were combined and heated at 70°C for 6 h (pH 1.0). After the starting materials disappeared, the reaction was cooled, extracted 4 times with 250 ml of methylene chloride, dried over $Na_2SO_4$, filtered, and concentrated in vacuo. The reaction was quantitative. The infrared spectrum indicated the presence of P—OH, P—O—C, and P═S bonds at 2,700 (broad), 1,000 (sharp), and 900 (sharp) $cm^{-1}$, respectively. The mass spectrum showed a parent peak at 170 *m/e*.

DETP was quantified by using a high-pressure liquid chromatographic system (Waters Associates, Inc., Milford, Mass.) with a UV-visible variable wavelength detector (LC-95; The Perkin-Elmer Corp., Norwalk, Conn.) set at 210 nm. Separations were achieved by using a radially compressed



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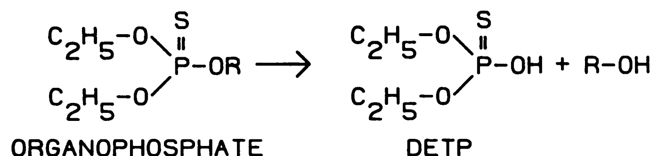


FIG. 1. Hydrolytic cleavage of the organophosphate bond as the initial step in organophosphate metabolism. R, Organic moiety.

cartridge (C-18 Nova-Pak [4 μm]; Waters) with a mobile phase of 50% methanol and 50% 0.75 mM phosphoric acid (pH 2); the flow rate was 2.0 ml/min. Sulfate and phosphate were quantified by using an ion Chromatograph (2000i/SP; Dionex, Sunnyvale, Calif.) equipped with a conductivity detector. Separations were achieved by using a high-efficiency anion column (HPIC-AS4A) with a guard column (NG1). The eluant consisted of 0.75 mM $NaHCO_3$ and 2.2 mM $Na_2CO_3$; the flow rate was 1.8 ml/min. The regenerant consisted of 0.025 N $H_2SO_4$; the flow rate was 3.0 ml/min.

A time course of DETP disappearance and sulfate appearance is shown in Fig. 2. In this experiment, mineral salts were used at one-half strength in order to facilitate quantitation of sulfate and phosphate. DETP was degraded to below the detection limit (10 μg/ml) in 7 days. There was a concomitant increase in the concentration of sulfate; sulfate recovery was 84% of the theoretical value based on the initial concentration of DETP. The uneven rates of degradation in the culture may have been due to daily fluctuations in the pH. Phosphate was also monitored; however, the data are not shown in Fig. 2 because of anomalous results. The phosphate yield was 13.3 mM, which is theoretically impossible based on the initial concentration of DETP (5.8 mM). The majority of the apparent phosphate production (8 mM) occurred between days 1 and 2, when only 1.9 mM DETP was metabolized. This suggests the production of a metabolite with the same retention time as phosphate but with greater conductivity, which interfered with phosphate determination. This experiment was repeated and similar results were obtained, including the anomalous phosphate data. Because of the flocculant nature of growth of the consortium, it was difficult to measure changes in the optical density. However, 6 mg of biomass was produced for every 50 mg of DETP that was consumed, indicating that the ethyl groups were used as a carbon source. Yeast extract alone yielded less than 0.5 mg of biomass.

Microscopic examination of the enriched consortium revealed the presence of several morphologically distinct bacteria as well as several different eucaryotic microorganisms. Incubation with cycloheximide had no effect on growth, while chloramphenicol inhibited growth of the culture, indicating that metabolism of DETP was strictly bacterial. A total of six morphologically distinct bacteria that were present at population densities of greater than $10^6$/ml were isolated from the consortium; two of the strains were able to use ethanol as a sole carbon source. However, none of the strains, either individually or collectively, were capable of metabolizing DETP. This suggests that other bacteria, which were present at low population densities, were responsible for the initial attack on DETP.

The pathway of DETP metabolism is unclear, although there appear to be two possibilities. The ethanol groups could be metabolized preferentially. Previous investigators (1, 5, 7) have demonstrated that certain bacteria are capable of hydrolyzing dialkylphosphates; however, DETP was not included in those studies. Alternatively, the sulfur atom

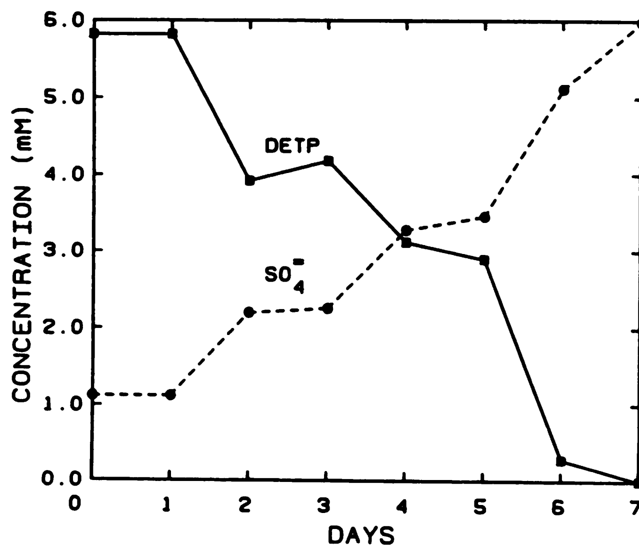


FIG. 2. Time course of DETP disappearance and sulfate appearance.

could be oxidized preferentially. The anomalous phosphate data suggest that there is a possible intermediate with an identical retention time but with a greater conductivity than phosphate.

To my knowledge, this is the first report of the mineralization of DETP as well as its utilization as a carbon and energy source in the presence of excess sulfate and phosphate. Previous investigators (2, 3) have demonstrated that DETP can be used as a sole sulfur or phosphorous source, but only in the presence of an extraneous carbon source. Results of the present study have ramifications for the degradation of many of the more widely used organophosphate pesticides (such as parathion and diazinon), because it demonstrates that DETP can be metabolized under environmentally realistic conditions.

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