First publ. in: Applied and Environmental Microbiology 39 (1980), 2, pp. 463-465

Desulfuration of Dialkyl Thiophosphoric Acids by a Pseudomonad

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A strain of *Pseudomonas acidovorans* used the organophosphorus pesticide breakdown products, ionic *O,O*-diethyl phosphorothioate and ionic *O,O*-diethyl phosphorodithioate, as sulfur sources. The growth yields from the thiophosphates and sulfate were 3.6 to 4.1 kg of protein per mol of sulfur. Elemental sulfur and sulfide also served as sulfur sources but gave lower growth yields.

A key reaction for the metabolic activation of phosphorothionate pesticides in eucaryotes is oxidative desulfuration to the respective oxygen analogs, the highly toxic "oxons." This process requires a monooxygenase and cytochrome P-450 to which the released thiono-sulfur is covalently bound as atomic sulfur (10). The desulfuration reaction has never been demonstrated in procaryotes (6). Phosphorothionate triesters also are subject to hydrolysis to dialkyl thiophosphates (6). In contrast to the neutral phosphorothionate triester, the ionic dialkyl thiophosphate has never been shown to be subject to desulfuration to yield the ionic dialkyl phosphate (6).

In addition to phosphorus pesticides, many other commercially important phosphorus compounds can undergo hydrolysis to yield chemically stable ionic alkyl phosphorus products that can also be toxic (1, 6). Of these products, the dialkyl thiophosphates (e.g., ionic *O,O*-diethyl phosphorodithioate, DEDTP) represent one major class that has no known analog in nature and has shown particular resistance to biodegradation (6). We recently showed that dialkyl thiophosphates could be quantitatively metabolized when present as sole and limiting phosphorus sources for bacteria (1). The potential for the dialkyl thiophosphates to serve also as carbon or sulfur sources for bacteria led us to examine further the degradation of ionic *O,O*-diethyl phosphate (DEP), ionic *O,O*-diethyl phosphorothioate (DETP), and DEDTP.

Growth experiments were done at 30°C in 250-ml Erlenmeyer flasks containing 30 ml of medium. The sulfur-limited growth medium contained 6 mM potassium phosphate buffer (pH 7.4), 0.8 mM $MgCl_2$, 40 mM NH_4Cl, 7.5 mM *p*-hydroxybenzoate, and trace elements (11; at 1/10th the recommended concentration). This solution was autoclaved and amended with the sulfur source (0, 5, 10, 15, or 20 μmol of sulfur per liter). The sulfur sources (Na_2SO_4, DETP, DEDTP, and $Na_2S \cdot 9H_2O$) were dissolved in deionized distilled water, adjusted to pH 7 with NaOH, and sterilized by passage through a membrane filter (0.2-μm pore diameter). Elemental sulfur was dissolved in sulfur-free pyridine and added by a high-pressure syringe to sterile dry flasks. The pyridine was allowed to evaporate, and sterile growth medium was added. The inoculum (3%, vol/vol) was from a culture fully grown with 10 μM DEDTP as sulfur source. In other experiments, the organism was grown with carbon limitation or phosphorus limitation (1, 2). Growth was quantitated as protein (1).

DEP, DETP, and DEDTP in solution were determined by gas-liquid chromatography as their methyl esters by a simultaneous methylation-extraction technique (4a, 5).

Of four bacterial isolates from sewage that were able to use DEDTP as a sole phosphorus source (1), only one could grow with DEDTP or DETP as the sole added sulfur source. This organism, which grew without growth factors near pH 7 (but did not grow below pH 6), was a motile rod. Electron micrographs showed 1 to 3 polar flagella and a wrinkled cell surface characteristic of gram-negative organisms. It accumulated poly-β-hydroxybutyrate, was oxidase positive, and effected a *meta* cleavage of diphenols. It had no methionine requirement, arginine dihydrolase, or capacity for denitrification, and it did not grow at 4 or 41°C. The bacterium grew using adipate, *m*-hydroxybenzoate, norleucine, mannitol, malonate, maleate, acetate, or ethanol but not glucose or testosterone as the sole carbon source. No acid was formed from glucose. Based on these data, the organism was identified as *Pseudomonas acidovorans*. The isolate has

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been deposited in the German Collection of Microorganisms (DSM, Göttingen, West Germany).

Growth of *P. acidovorans* on different sulfur sources was tested in medium buffered with orthophosphate because 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate contained sulfur and tris(hydroxymethyl)methylamine gave slower growth and lower yields. The background of sulfur sources in the growth medium was lowered by reducing the level of trace elements. *P. acidovorans* grew in sulfate-limited medium and yielded 3.6 kg of protein per mol of sulfur (Table 1). This yield compares favorably with 2.4 kg of protein per mol of sulfur calculated from the elemental composition of exponentially growing bacteria (9). The regression line for the protein yield of bacteria as a function of added sulfur indicated a background of about 7 μM of contaminative sulfur in the unamended growth medium.

P. acidovorans grew in a medium containing DETP or DEDTP as limiting sulfur source with yields very similar to those with sulfate as a sulfur source (Table 1). DETP and DEDTP were shown by direct determination to disappear completely during growth. This is the first demonstration of microorganisms using ionic dialkyl thiophosphoric pesticide products as sulfur sources. No intermediate dialkyl phosphates were detected, so the mechanism of desulfuration could not be inferred. Although a claim exists that Diazinon serves as a sulfur source (8), we calculate from the data in that report that the growth yield was 0.001 kg of protein per mol of sulfur (cf., 1) so that presumably the organisms in that study were growing on contaminative sulfur.

P. acidovorans also could grow with elemental sulfur and sulfide as sulfur sources (Table 1), although the apparent yields were not as high as that from sulfate. Although the growth yield from sulfate, when compared with those from DETP and DEDTP (Table 1), suggested that sulfate could be the initial sulfur-containing product, the form in which the thiono-sulfur was removed is unknown. The abilities of bacteria to remove the sulfur from the ionic alkyl phosphorus compounds or to cleave the carbon-phosphorus bond of aliphatic alkyl- or aryl-phosphonates (3, 4, 4a) have apparently never been observed in eucaryotes.

TABLE 1. *Growth yields of P. acidovorans from sulfur sources*

Sulfur source	Growth yield[a] (kg of protein/mol of S)	Correlation coefficient of regression line
Sulfate	3.6	0.997
DETP	4.1	0.997
DEDTP	4.0	0.998
Elemental sulfur	2.3	0.990
Sulfide	1.4	0.986

[a] The least squares regression coefficient from a plot of bacterial growth (kilograms of protein per liter) versus initial sulfur concentration (moles of S consumed per liter).

The metabolism of several forms of inorganic sulfur has been recently reported to occur in a pseudomonad (12). However, even the lowest sulfur concentration (0.001% or 70 μM Na_2SO_4) used in that work was only partially assimilated, giving a yield of 0.7 kg of protein per mol of sulfur (assuming a turbidity of 1.0 equivalent to 85 μg of protein per ml [1]). Care must be exercised in such studies that the sulfur source remains growth limiting.

P. acidovorans grew on ethanol as a sole carbon source with a yield of about 0.003 kg of protein per mol of carbon. This was consistent with our earlier findings that the relative molar requirement of bacteria for carbon and phosphorus is about 300:1 (2) and that growth yields on phosphorus are about 1 kg/mol (1). However, neither DEP nor DEDTP (each at 5 mM) served as a carbon or simultaneous carbon and phosphorus source, although neither compound at 5 mM inhibited growth. Ethanol is the expected product from cleavage of the alkoxy groups of DEP (7) and presumably of DEDTP. This apparent discrepancy possibly results because the amounts of enzyme required to supply the cell with phosphorus or sulfur were insufficient to meet the carbon demand.

We are grateful to W. C. Ghiorse for doing the electron microscopy.

This investigation was supported in part by National Science Foundation grant ENV75-19797 and Public Health Service training grant ES07052 from the Division of Environmental Health Sciences.

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