Role of Competition for Inorganic Nutrients in the Biodegradation of Mixtures of Substrates

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A study was conducted to determine whether competition for inorganic nutrients affects the biodegradation of mixtures of substrates. Little benzylamine was mineralized by Pseudomonas putida in solutions with no added P, but the substrate was degraded if the medium contained 100 nM P. The enhancement by P addition did not occur if the medium also contained caprolactam and a caprolactam-utilizing strain of Pseudomonas aeruginosa. The suppression by the second bacterium was overcome by a higher P concentration. The rate of caprolactam utilization by P. aeruginosa was reduced if benzylamine and P. putida were also present in media with 100 nM P, but the suppression was absent if the solution contained a higher P concentration. Glutamate increased and inorganic N plus P decreased the length of the acclimation phase prior to benzylamine mineralization in lake water. We suggest that the effect of one biodegradable substrate on the metabolism of a second often results from a competition for inorganic nutrients.

The biodegradation of organic compounds is often slow because one or more inorganic nutrients needed for microbial growth are in low concentrations in natural environments (3, 9, 17, 18). The addition of N and P may increase the biodegradation of a compound (11), but it can also have no effect or decrease the biodegradation (17). The addition of inorganic nutrients does not always stimulate degradation because other factors may themselves suppress microbial activity or interact with nutrient limitation to slow degradation. Nutrient limitation may be exacerbated by other species using the same elements as those organisms metabolize other organic compounds. The presence of other bacteria (4) or other substrates (6, 8, 13) may reduce the degradation of test compounds. The mechanisms by which other microorganisms and other substrates affect biodegradation are unknown (1, 2).

Inasmuch as biodegradation commonly occurs in environments containing several metabolizable organic substrates and frequently in circumstances in which the supply of one or more inorganic nutrients is less than the demand, a study was undertaken to determine the effect of one species acting on a substrate on the biodegradation of a second substrate by a second species at low concentrations of P. In addition, experiments were conducted with lake water to assess the effects of limiting inorganic nutrients and second carbon sources. Benzylamine and caprolactam were chosen as model substrates.

MATERIALS AND METHODS

Media. Glassware was washed in a P- and N-free detergent (Versa-Clean; Fisher, Springfield, N.J.) and then acid washed in a solution of Nochromix before fall turnover. The samples had a pH of 6.6 and contained 50 mg of organic C, 260 μg of nitrate-N plus nitrite-N, and less than 10 μg of NH₃-N and total P per liter. Solutions of NH₄NO₃ or KH₂PO₄ adjusted to pH 6.6 were purchased from California Bionuclear Corp. (Sun Valley, Calif.) and designated [7-14C]benzylamine (specific activity, 6.9 mCi/mmol) was purchased from California Bionuclear Corp. (Sun Valley, Calif.) and designated [7-14C]benzylamine (specific activity, 6.9 mCi/mmol). The purity of the radiolabeled compounds was 99% or greater. Radiolabeled glutamate was obtained from Sigma Chemical Co. (St. Louis, Mo.). The purity of the unlabeled compounds was 99% or greater.

Organisms. Two bacteria were isolated from samples of Beebe Lake, Ithaca, N.Y., that had been amended with benzylamine or caprolactam. The benzylamine-degrading bacterium is a gram-negative aerobic rod that hydrolyzes arginine, uses citrate as a sole carbon source, produces acetoin from sodium pyruvate, metabolizes glucose, and is oxidase and catalase positive. It was identified as a strain of Pseudomonas putida and designated P. putida Bam2. The caprolactam-degrading bacterium is a gram-negative aerobic rod that is oxidase and catalase positive, hydrolyzes arginine, uses citrate as a sole carbon source, metabolizes gelatin and glucose, and is unable to ferment lactose, glucose, or sucrose. It was identified as a strain of Pseudomonas aeruginosa and designated P. aeruginosa Cap2. At the inoculum sizes used and in solutions with 1 mM P, the degradation of 5 μg of caprolactam per ml by the benzylamine-degrading bacterium P. putida Bam2 and of 5 μg of benzylamine per ml by the caprolactam-degrading bacterium P. aeruginosa Cap2 was detected only after 150 h.

Suspensions of bacteria at the beginning of the stationary phase of growth were diluted in 0.15% NaCl and used as inocula. Unless otherwise stated, the signals were shaken at 30°C at 100 rpm. P. putida and P. aeruginosa were inoculated at approximately 10 cells per ml as determined by plate count. On the basis of the dilution of the inocula, the concentrations of P carried over from the inocula were 1 nM and 1 pM for P. putida and P. aeruginosa, respectively.

Activity of isolates. Radiolabeled benzylamine or caprolactam at 5 or 50 μg/ml and the organism that degraded them were added to the culture flasks. The effects of a second organic compound and P concentration were assessed by comparing degradation in solutions that contained 1 mM, 10 μM, 100 nM, or no added P.

Lake water. Samples were obtained from Moss Lake (Herkimer County, N.Y.), a dimictic oligotrophic lake (14). The samples were collected in September before fall turnover. The samples had a pH of 6.6 and contained 50 mg of organic C, 260 μg of nitrate-N plus nitrite-N, and less than 10 μg of NH₃-N and total P per liter. Solutions of NH₄NO₃ or KH₂PO₄ adjusted to pH 6.6 were added to some of the samples to give 3.5 mg of N and 0.31 mg of P per liter. 14C-benzylamine was added to lake water to give 50 ng/ml, and in some instances, 5 μg of unlabeled glutamate per ml was also added. Each of four replicate flasks was incubated at 30°C on a rotary shaker operating at 100 rpm.

Measurement of biodegradation. Subsamples (1 ml) removed aseptically from cultures were placed in 7-ml glass tubes with 5% NaCl, acidified with 2 drops of 1 M HCl, and aerated with forced air for 2 min to drive off CO₂. Two milliliters of liquid scintillation cocktail (Nuclear Diagnostics, Manville, N.J.) was added, and the radioactivity was determined with a scintillation counter (model LS7500; Beckman, Irvine, Calif.).

Statistical treatment of data. Statistical tests were performed only when
the magnitude of the differences was not large. Degradation rates determined for each treatment were the averages from replicates of that treatment. Rates during the phase of rapid transformation were determined by linear regression, as defined by the line through the points of the active mineralization phase that had the lowest total sum-of-mean-square errors. Prior to linear regression, the data were transformed by the arcsine square-root transformation, which served to normalize the data and stabilize the variances (21). An estimate of acclimation time was obtained by rearranging the common slope-intercept formula (16). To determine the extent of mineralization, an average was taken at time points at which there appeared to be no further degradation that made up a line with a slope not significantly different statistically (P < 0.05) from zero. Differences between rates of degradation, acclimation periods, and extent of degradation were examined by using t tests if two values were being compared or by the use of analyses of variance if more than two values were being compared. The confidence limits were 95 and 90% for tests with axenic cultures and lake water, respectively.

RESULTS AND DISCUSSION

A study was conducted to determine the effect of P concentration on the degradation of 5 μg of benzylamine per ml by P. putida in pure culture and in media that contained 5 μg of caprolactam per ml and the caprolactam-degrading P. aeruginosa. Little of the test compound was mineralized in solutions with no added P (Fig. 1A). However, the addition of P to give 100 nM enhanced the mineralization as shown by the significantly greater (P = 0.05) percentage of benzylamine mineralized at 50 h and thereafter in solutions with 100 nM than in those with no P. Mineralization was rapid and extensive in solutions with 10 μM and 1 mM P.

If the medium also contained caprolactam (5 μg/ml) and a strain of P. aeruginosa that metabolized the second substrate, little degradation again was evident in media with no added P, but mineralization was extensive in media with 10 μM and 1 mM P (Fig. 1B). However, in this instance, the addition of 100 nM P did not cause a statistically significant stimulation of benzylamine degradation. In the presence of the second substrate, P. aeruginosa suppressed the degradation in solutions with no added P or 100 nM P. The rate of degradation of benzylamine in solutions containing 100 nM P was significantly slower in media containing caprolactam and P. aeruginosa than in media without the second bacterium. In solutions with no added P, the rates of transformation in solutions with and without the second substrate were not significantly different, but the percentage of degradation at 8 of 10 time points during the active phase was less in media that contained the second organism than in pure cultures of the benzylamine degrader.

A similar study was conducted to determine the effect of P concentration on the biodegradation of 5 μg of caprolactam per ml by P. aeruginosa. In axenic culture, the rate of degradation was slow in media with no added P and increased with increasing P concentration up to 10 μM (Fig. 2A). The enhancement in rates by P was similar in media containing benzylamine and P. putida (Fig. 2B). The rates were significantly different. Although the rate of degradation of 5 μg of caprolactam was not affected by the presence of benzylamine and P. putida at any P concentration (on the basis of a statistical comparison of the data in Fig. 2A and B), the final extent of caprolactam degradation was statistically greater in pure culture (30% remaining) than in the presence of P. putida (37% remaining); i.e., more of the substrate C remained in the presence of the competitor.

The same experimental design was used in a study in which the test substrate, caprolactam, was supplied at 50 μg/ml, and the inocula contained 10^7 cells of each strain per ml (4 to 8 nM P in the inocula). The cultures were incubated at 20 to 22°C without shaking. When the medium contained 100 nM P, the rate of degradation of caprolactam was markedly reduced by the second bacterium (Fig. 3). Indeed, little mineralization was evident, and approximately 90 and 75% of the C remained at 170 and 360 h, respectively. In this study, the possible inhibition of benzylamine in the absence of a benzylamine-degrading organism was assessed, the data in Fig. 3 showing that the compound was not toxic to P. aeruginosa. In solutions contain-
ing 1 mM P, the degradation of caprolactam at 50 μg/ml was not affected by benzylamine and \( P. \) putida (data not shown).

At a P concentration of 100 nM, the bacteria metabolizing benzylamine and caprolactam in axenic culture had an insufficient supply of the element for maximum activity. This is evident by the greater rate and extent of degradation when the P concentration was higher. At these limiting levels, growth of a second species on a compound not used by the first resulted in a reduction in degradation of the first compound. The decreased degradation was not a result of the toxicity of benzylamine but rather appears to result from a competition between the two \( \text{Pseudomonas} \) species for present in limiting concentrations.

To test whether a second substrate might reduce the degradation of a test compound in lake water because of competition for inorganic nutrients, labeled benzylamine at 50 ng/ml was added to lake water that was either not further amended or amended with inorganic N plus P or glutamate. Glutamate appreciably delayed the onset of detectable biodegradation of benzylamine (Fig. 4). Addition of just the inorganic nutrients significantly shortened the acclimation phase for benzylamine degradation, and the length of that phase was further diminished if glutamate was added together with inorganic N and P. The plot in Fig. 4 designated unamended represents the mean of three of the four replicates, and the curve for degradation in the fourth replicate flask was parallel to the plot in the figure but was delayed for 24 h.

The stimulation in lake water was probably a result of the supplemental P and not N because the water contained 260 μg of nitrate- plus nitrite-N per liter, and glutamate and benzylamine are probably rapidly converted to ammonium. The P concentration was less than the detection limit of 10 μg of P per liter. Phosphate was added in excess amounts since phosphate often becomes biologically unavailable (12).

Competition between the benzylamine- and glutamate-degrading populations could have caused the increase in the acclimation period prior to benzylamine degradation in lake water amended with glutamate alone, but this effect could also have been a diauxic response in which glutamate was more readily utilized than benzylamine. However, diauxie is not known to occur at low substrate concentrations (5). That an inorganic nutrient was likely limiting is suggested by the shortened acclimation prior to rapid benzylamine degradation as a result of adding P plus N.

A suppression of the biodegradation of one compound by a second has been noted frequently (5, 13, 18). This could result from inhibition by the second compound, its conversion to a toxic product, an increased rate of predation by the more numerous protozoa arising because of the biomass growing on the second compound, a deficiency of \( \text{O}_2 \), or competition for limiting inorganic nutrients. Because natural waters frequently contain P in concentrations that limit biodegradation of readily metabolizable substrates (7, 9, 20), competition probably often accounts for the suppression.

Polluted water and soils rarely contain a single organic contaminant, and they contain an array of organic molecules, the degradation of which imposes a need for inorganic nutrients. If two or more of the compounds are readily biodegradable, P, N, or \( \text{O}_2 \) is present at low concentrations, and \( \text{O}_2 \) reentry is impeded, competition for one or more of these elements will likely affect the degradation. In addition, toxic products may be generated during the decomposition (10), or other interactions that lead to a reduced rate or extent of degradation of the organic compounds may occur. Because of the importance of maximizing rates of degradation in the bioremediation of contaminated sites, further research is required to determine the reasons that one organic compound affects the destruction of a second in polluted waters, sediments, and soils.

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**REFERENCES**