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Microbial Decontamination of Parathion and *p*-Nitrophenol in Aqueous Media

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A mixed microbial culture was adapted to growth on parathion to determine the feasibility of using microorganisms to detoxify concentrated parathion in agricultural wastes. In a 600-ml chemostat, the culture was able to degrade 50 mg of parathion per liter per h. Para-nitrophenol, produced by enzymatic hydrolysis of parathion, caused delays in exponential growth which were directly proportional to its concentration. A pseudomonad, isolated from the mixed culture, exhibited optimal growth at 0.21 mM *p*-nitrophenol and grew in concentrations up to 3.5 mM. In metabolic studies using [^{14}C]*p*-nitrophenol, the nitro group was removed in stoichiometric quantities as nitrite and hydroquinone was tentatively identified as a metabolite.

Parathion (*O*,*O*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) (PAR) is an extremely toxic organophosphate insecticide which has been used extensively as a general insecticide. With this wide usage comes the subsequent problem of detoxifying PAR containing wastes from containers, aircraft spray tanks, accidental spill sites, and other such areas. The usual chemical detoxification procedure (15) utilizes a strong alkaline solution to hydrolyze PAR to *p*-nitrophenol (PNP) and diethylthiophosphoric acid. However, this hydrolysis is slow (4, 6), and the products, as well as the strong alkaline solution, are environmental pollutants. A more complete degradation under milder conditions was therefore needed.

Microorganisms are known to metabolize PAR both in soil (9, 13) and in water (W. L. Gibson, Ph.D. thesis, Mississippi State University Starkville, 1972; 10). Thus, it seemed feasible to adapt a mixed microbial culture to growth on concentrated PAR suspensions and to effect complete degradation of the molecule. An enrichment technique was used to select for PAR degraders, which were in turn adapted to utilize high levels of PAR (7). Preliminary chemostat experiments showed that this culture could metabolize PAR quite efficiently (50 mg per liter per h); however, under certain conditions, PNP accumulated initially in the broth to levels which were inhibitory or even toxic to various microorganisms (1, 5).

To design a proper start-up of the chemostat, metabolism of PAR, PNP was studied as an initial substrate to determine its inhibitory and toxic levels, the maximal specific growth rate of the culture on this substrate, and its catabolic pathway.

MATERIALS AND METHODS

Chemicals. Parathion (4 lb; ca. 1.8 kg) emulsifiable concentrate and technical PAR (98.7%) were obtained from Stauffer Chemical Co., Richmond, Calif. PNP was obtained from Matheson, Coleman and Bell, Los Angeles, Calif. [2,6-^{14}C]PNP (20 to 40 μCi/μmol) was obtained from ICN Isotope and Nuclear Division, Irvine, Calif.

Microorganisms. Samples of sewage and soil microorganisms were adapted to grow on PAR in a continuous fermentor by initially adding glucose (0.1%) to the broth and then gradually increasing PAR influent concentration from 10 μg/ml to 3 mg/ml over a 30-day period (7). The adapted mixed culture was maintained in Burk mineral salt solution: K_2HPO_4, 0.2 g; KH_2PO_4, 0.8 g; $MgSO_4$, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.1 g; $Na_2MoO_4 \cdot 2H_2O$, 0.0033 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $(NH_4)_2SO_4$, 1.0 g in 1,000 ml of distilled water; pH 7.2, with PAR as the only carbon source.

Media and culture conditions. Burk mineral solution was used with either PAR (98.7%) at concentrations from 0.01 to 1.0% or with PNP at 0.01 to 0.05% as the sole source of carbon. Resting cell experiments were conducted in 6 mM phosphate buffer at pH 7.2. For chemostat studies, a New Brunswick Miniferm chemostat, 600-ml working volume, was used at dilution rates of 0.04 to 0.08 h^{-1} for PAR studies and 0.3 h^{-1} for PNP. In chemostat experiments with ^{14}C-PNP, metabolites from cold PNP were removed from the effluent by absorption on a XAD-2 resin column. After the cold metabolites in 10 liters of effluent had been collected, ^{14}C-PNP (1×10^6 dpm) was added to the chemostat, and another 2-liter volume of effluent collected before the metabolites

were eluted from the resin. The resin was air-dried and washed with acetone. The resulting acetone-water mixture was acidified, and the metabolites were extracted into chloroform. For experiments using resting cells, the culture was grown on PNP for 48 h, centrifuged at 12,000 × *g* for 10 min, washed with buffered medium, resuspended, and again centrifuged at 12,000 × *g*. The suspended cells were then added to flasks containing 10 ml of phosphate buffer. ^{14}C-PNP was added (1 × 10^6 dpm) to each flask, and the catabolism was stopped at various times by addition of 40 ml of acetone. The broth was then sonicated for 1 min and acidified, and the metabolites were extracted into chloroform.

Analytical methods. PAR concentration was determined by a Packard Gas Chromatograph Model 417 flame ionization detector with a 4-ft (ca. 121 cm), ¼ in. (ca. 0.64 cm) outer diameter glass column packed with 6% Apiezon N on Chromosorb Q. The column temperature was maintained at 230 C, whereas the injector and detector temperatures were 245 C. PNP was determined spectrophotometrically at 410 nm at pH 10. Cell density was determined spectrophotometrically at 600 nm and pH 5.0 to avoid any yellow interference from PNP. Nitrite was determined colorimetrically at 550 nm by a Griese-Saltzman reaction (12). Nitrate and ammonia concentrations were determined with specific electrodes manufactured by Orion and Corning Glass Works, respectively (Scientific Products, Menlo Park, Calif.). The ^{14}C studies were conducted by using a Packard liquid scintillation counter for quantitating labeled metabolites and Kodak x-ray film was utilized for thin-layer chromatography (TLC) visualization.

RESULTS

Isolation of bacteria from the adapted mixed culture. Nine pure culture isolates were obtained from the PAR-adapted culture by plating on agar plates containing mineral salts and either PAR, PNP, or glucose as a sole source of carbon. The isolates were classified according to various biochemical and morphological observations (2), with their genera given in Table 1. A *Brevibacterium* was the only gram-positive organism isolated; all the others were gram negative, predominantly of the genus *Pseudomonas*. Isolates 3P and 8P were used in studies concerning the toxicity and metabolic pathway of PNP catabolism.

Chemostat metabolism of parathion emulsifiable concentrate. Once the adapted mixed culture was in steady-state growth on PAR emulsifiable concentrate in the chemostat, it was possible to vary the influent feed rate and PAR concentration over a select range with no subsequent increase in effluent PAR concentration (Fig. 1). The chemostat operated successfully at dilution rates from 0.04 to 0.08 h^{-1} and at PAR influent concentrations from 1.38 to 4.8 mmol/liter, with a maximal metabolic rate of 0.36 mmol (50 mg) per liter per h. However, at higher feed rates, the PAR concentration in the effluent increased from less than 1.5% of influent value to over 25%. Further increase in the feed rate caused a higher rate of PAR metabolism (115 mg per liter per h), but the PAR level in the effluent rose to 50% of the influent concentration. Thus, the chemostat could be optimized for either maximal rate of PAR metabolism or for minimal effluent PAR concentration.

Parathion catabolism and p-nitrophenol production. When the dormant, adapted culture, which had been grown in batch culture on technical PAR as a sole carbon source, was activated by addition of PAR, there was an appreciable lag period (8 h) before growth, during which time the disappearance of PAR and the accumulation of PNP was linear (Fig. 2). Depending on the length of time the culture had been dormant, the lag period varied from a few minutes to over 40 h, and the PAR hydrolysis was sometimes completed before the growth began. This PAR hydrolysis by the lag-phase culture was due to an enzyme produced during the previous growth cycle that

TABLE 1. *Tentative identification and characteristics of microorganisms isolated from a mixed culture growing on technical parathion in Burk mineral solution*[a]

Isolate	Genus	Gs	M	F	PNP	PAR	MN	G	Gel
1P	*Pseudomonas*	−	+	+	+	−	−	+	−
2P	*Pseudomonas*	−	+	+	+	+[1]	−	+	−
3P	Unidentified	−	+	−	−	+[1]	−	+	−
4P	*Xanthomonas*	−	+	−	−	−	−	−	−
5P	*Azotomonas*	−	+	−	−	+[1]	+	+	−
5Pa	*Brevibacterium*	+	+	−	−	+[1]	−	−	+
6P	*Pseudomonas*	−	+	+	+	+[1]	−	+	−
8P	*Pseudomonas*	−	+	+	+	+[1]	−	+	−
9P	*Pseudomonas*	−	+	+	+	+[1]	−	−	−

[a] Gs, Gram stain; M, motility; F, fluorescence; MN, mannitol, nitrogen-free media; G, glycerol; Gel, gelatin liquidification; +[1] denotes growth on PAR after initial isolation only, growth stopped after second transfer.

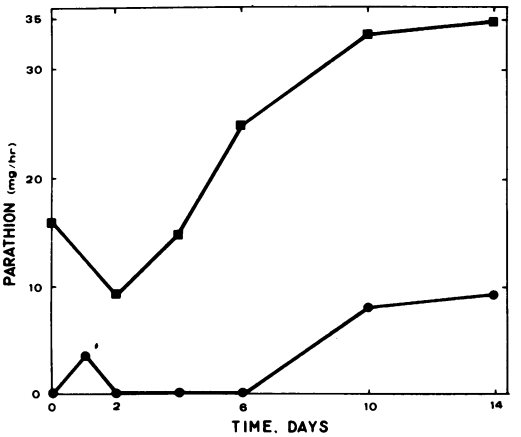

FIG. 1. *Removal of parathion in a continuous fermentor. Total parathion entering (■) and leaving (●) a 600-ml working volume chemostat apparatus.*

retained its activity long after growth ended. When PAR was again added, this activity in the broth produced PNP up to 2.2 mM. The lag period was governed by how much PNP was produced before the cells could be induced to metabolize it. This hydrolysis of PAR by a crude cell extract was optimum at pH 9.2 (19.5 mmol per mg of protein per min) with only 10% of its maximal activity at pH 7.2 (unpublished data).

p-Nitrophenol-induced lag period. When the isolates from the mixed culture were grown on PNP as the initial substrate, a lag period occurred which was directly proportional to increasing PNP concentration (Fig. 3). Cells were prepared for this experiment by growth in batch culture at PNP concentrations never exceeding 0.65 mM. This culture was used to inoculate fresh media containing 0.07 to 3.24 mM PNP. Thus, in a step-down experiment to 0.07, 0.21, and 0.35 mM, the lag period was absent; however, in step-up experiments to 0.72, 1.08, 2.50, 2.88, and 3.24 mM the lag period was appreciable. After a culture inoculated into 2.16 mM PNP medium had entered exponential growth subsequent to a 30-h lag period, the cells were centrifuged and transferred to fresh 2.16 and 2.88 mM media. Exponential growth continued in the 2.16 mM medium, but there was a 12-h lag period before growth resumed in the 2.88 mM medium.

p-Nitrophenol toxicity. The toxic concentration of PNP and its concentration for maximal rate of bacterial growth were determined for the pseudomonad isolate 8P by growing the cells in batch cultures at various PNP concentrations and then measuring the growth rate during early exponential growth. The optimal concentration for bacterial growth was determined to be 0.21 mM, and the toxic level was 3.6 mM (Fig. 4).

Nitrite release from p-nitrophenol. When the eight isolates of the mixed culture were grown on PNP in Burk mineral solutions, they released stoichiometric quantities of nitrite from PNP (Fig. 5). The fate of this nitrite was further studied to determine whether the culture could utilize this nitrite as a source of nitrogen if ammonium sulfate was not provided. In the absence of ammonium sulfate, the released nitrite was reduced and assimilated into cellular material, thus removing nitrite as a waste product. To test if the aromatic nitro group is removed before ring cleavage, the following radiolabeled PNP study was conducted.

[^{14}C]p-nitrophenol studies. [^{14}C] PNP was added to a resting cell suspension of culture 8P.

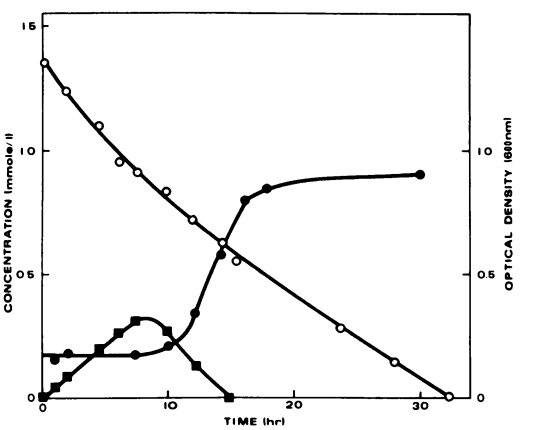

FIG. 2. *Parathion catabolism and p-nitrophenol production in batch culture. Symbols: parathion* (○), *p-nitrophenol* (■), *and cell density* (●).

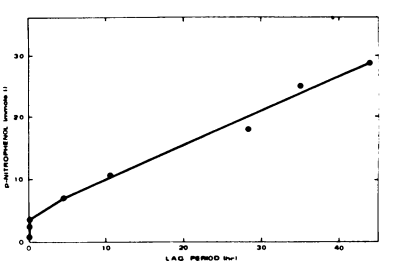

FIG. 3. *p-Nitrophenol-induced lag period in the growth of pseudomonad 8P on p-nitrophenol. Cells were prepared for this experiment by growth in batch culture at p-nitrophenol concentrations never exceeding 0.65 m/liter.*

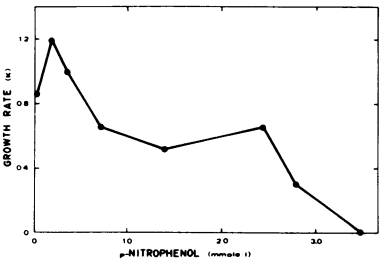

FIG. 4. *Growth rate of pseudomonad 8P at various p-nitrophenol concentrations.*

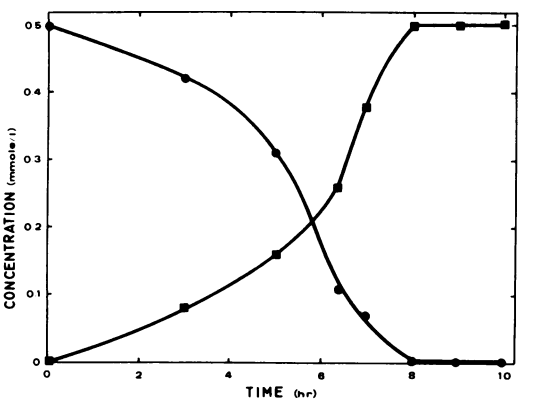

FIG. 5. *Stoichiometric release of nitrite* (■) *during growth on p-nitrophenol* (●) *by pseudomonad 8P in batch culture.*

Samples taken at various times were extracted and analyzed for metabolites by TLC. Three metabolites appeared in two TLC systems, and one, based on similar R_f values on two TLC systems and a single R_f value on one TLC system when co-chromatographed with standard, was tentatively identified as hydroquinone. The other two metabolites were not similar to any of the possible metabolites listed in Table 2, thus suggesting the possibility that they were metabolites formed after ring fission. The very low accumulation of suspected hydroquinone (30 μg/liter) and the other two metabolites precluded any further physical or chemical characterization. This tentative identification of hydroquinone as an intermediate in PNP metabolism supports the hypothesis that the nitro group is removed before aromatic ring fission. The production of the hydroquinone-like metabolite from PNP over the time course of the radio-labeled experiment is indicated in Fig. 6.

To determine if any primary metabolites were present in the chemostat effluent, labeled PNP was added to a chemostat culture growing on PNP, and the effluent was then checked for the primary metabolites listed in Table 2. None were found in the chemostat effluent. When batch cultures were grown with ^{14}C-PNP, 20% of the added label remained as cell mass, and only 3% remained in the supernatant after it was gassed with nitrogen to remove residual, labeled carbon dioxide.

TABLE 2. *Thin-layer chromatography of nitrophenol and possible microbial nitrophenol metabolites*

Compound	R_f values[a]	
	System A	System B
Quinone	.00	.68
4-Nitrocatechol	.39	.17
p-Nitrophenol	.45	.36
Catechol	.61	.23
1,3,4-Benzenetriol	.69	.01
p-Aminophenol	.77	.14
Hydroquinone	.68	.11
Unknown	.68	.10

[a] System A was ethanol-water (3:1) on polyamide gel plates. System B was chloroform-ethanol-0.1 N NaOH (100:5:1) on Silica Gel G plates. Visualization was by fluorescent quenching under ultraviolet light.

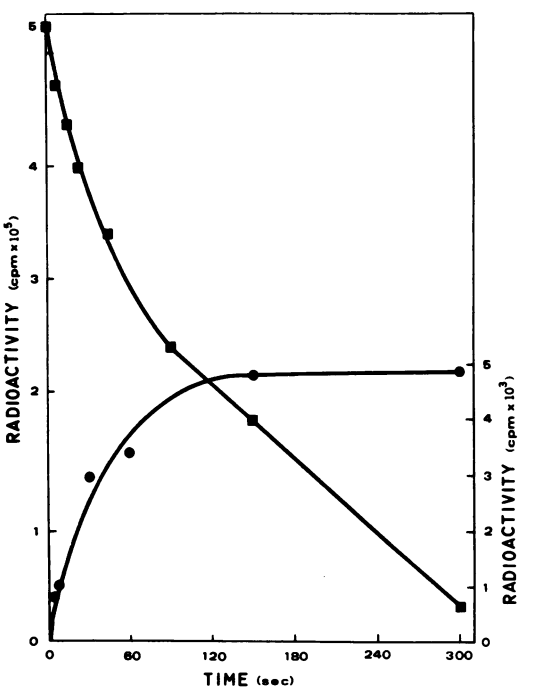

FIG. 6. *Radiolabeled hydroquinone production* (●) *by resting cell culture 8P metabolizing labeled p-nitrophenol* (■).

DISCUSSION

Although it has been reported that a single pseudomonad was capable of growing on PAR as the sole source of carbon (14), no isolate from our mixed culture could continue to grow on PAR. All isolates from the mixed culture lost their ability to grow on PAR after a second transfer on PAR agar plates. Symbiosis may have occurred in the metabolism of PAR by the mixed culture. One isolate (5 Pa) could hydrolyze PAR but could not grow on it or utilize PNP for growth, whereas others could metabolize PNP but could not hydrolyze PAR. It is also possible that a nutrient provided by an organism's growth on PNP was essential for growth or metabolism of PAR by PAR-degrading organisms. Still others metabolized neither PAR nor PNP. The complete degradation of PAR seems to be effected by a concerted action of the component microorganisms in the mixed culture. An improper start-up of the mixed culture could result in a lag period until the metabolic activity of different organisms was adjusted so that exponential growth of the mixed culture as a whole could occur. The effect of recombinations of strains on PAR metabolism is presently under investigation.

The detected lag can also be explained on the basis of metabolite inhibition. For exponential growth to begin, the monooxygenase and dioxygenase essential for aromatic hydroxylation and ring fission must be induced. In contrast, PAR hydrolase activity in the broth of the dormant culture remains significant; thus, when PAR is added, high levels of PNP are produced which can inhibit cell growth and either directly or indirectly inhibit the induction of the two oxygenases. The mixed cultures' unregulated hydrolysis of PAR in the supernatant demands a counter mechanism to overcome the inhibition resulting from high concentrations of PNP. The evidence that the lag period for growth on PNP is directly proportional to its concentration in the medium implies that the cells must synthesize some moiety or enzyme which removes PNP from its inhibitory sites, thus protecting the metabolic activities of the cell. How this is done is conjectural at this time.

Several groups have studied the catabolism of aromatic phenols. On the basis of manometric experiments with a pseudomonad, Simpson and Evans (Biochem. J., 1953, **55**:XXIV) proposed that the catabolism of PNP proceeded through the formation of hydroquinone. Chamberlain and Dagley (3), also working with a pseudomonad, found that 1,4-dihydroxy aromatic compounds had to be hydroxylated at the three position before ring fission could occur. Larvay and Evans (8), working on resorcinol metabolism by a pseudomonad, found 1,3,4-benzenetriol as an intermediate before ortho ring fission. Raymond and Alexander (11), in studying the metabolism of PNP by a *Flavobacterium*, found stoichiometric release of nitrite from PNP; however, when the cells were treated with 0.1% chloroform, 4-nitrocatechol accumulated in the broth, supporting the postulation of an aromatic hydroxylation before nitro removal. Our studies with a pseudomonad support the pathway postulated by Simpson and Evans and expanded upon by Chamberlain and Dagley and include to our knowledge, for the first time, the tentative isolation of hydroquinone as an intermediate in PNP metabolism. The tentative pathway for PNP metabolism by a pseudomonad would then include hydroquinone and 1,3,4-benzenetriol as intermediates before ortho ring cleavage.

Recently, Siddaramappa et al. (14) have reported the metabolism of PAR by a *Pseudomonas* sp. at low PAR levels (<4 mg/liter). They found PNP to initially accumulate slightly (1 mg/liter), with the nitro group of PNP released in stoichiometric amounts as nitrite once parathion was hydrolyzed. In neither our study nor the study by Siddaramappa has the fate of diethylthiophosphoric acid been determined.

The chemical hydrolysis of PAR by strong alkaline solution is dependent on the solubility of PAR in water (6). In 1 N potassium hydroxide at 25 C, hydrolysis proceeds at approximately 5 mg per liter per h regardless of initial PAR concentration. Microorganisms, in a chemostat operation, metabolized 50 mg per liter per h, a rate ten times greater than chemical hydrolysis. This microbial hydrolysis was then followed by a complete catabolism of the PNP moiety. Our study, therefore, shows that microbial degradation does offer an effective alternative for the detoxification of parathion in an aqueous system if certain precautions are taken to insure that PNP production does not interfere with this decontamination process.

ACKNOWLEDGMENT

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