

Microbial Metabolism of a Parathion-Xylene Pesticide Formulation

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A mixed bacterial culture was adapted to growth on a mixed carbon substrate consisting of the pesticide parathion and its xylene-based formulation. The environmental growth parameters of temperature, pH, and dissolved oxygen concentration were optimized to obtain complete metabolism of parathion from this mixed carbon substrate. This adapted culture grew rapidly ($\mu = 0.7$ per h) on the pesticide formulation at high parathion suspensions (3,000 mg/liter). Carbon utilization from this mixed substrate was strongly dependent on pH. At slightly acidic pH, xylene was preferentially metabolized, whereas at slightly alkaline pH, parathion was preferentially metabolized. Diethylthiophosphoric acid, a metabolite from parathion, and toluic acid, a metabolite from xylene, also influenced the selection of the primary carbon source.

Parathion (*O,O*-diethyl *O-p*-nitrophenyl phosphorothioate) (PAR) is an extremely toxic organophosphate insecticide which is used extensively as a general insecticide. With the wide usage of parathion and other pesticides comes the subsequent problem of detoxifying pesticide wastes resulting from surplus pesticides, pesticide containers, aircraft spray tanks, and resulting wash waters. Biological oxidation of these wastes presents one method for detoxification of mixed pesticide wastes; however, relatively little is known concerning mixed substrate metabolism of toxic compounds by mixed cultures. Many technical problems must be understood and resolved before biological systems can be utilized.

The metabolism of technical PAR by an adapted, mixed microbial culture in continuous culture has been shown to result in a complete destruction of PAR at rates significantly faster than chemical hydrolysis by strong bases (9). However, the microbial detoxification of PAR, when formulated as a commercial emulsifiable concentrate, would encounter the special problems associated with the metabolism of a mixed carbon substrate by mixed cultures.

This present report deals with the metabolism of PAR in aqueous media in the presence of its commercial pesticide formulation which contained *o*-, *m*-, and *p*-xylene as major constituents. The effects of xylene on the metabolism of PAR were examined, and the parameters crucial for the complete and rapid breakdown of

PAR by a mixed culture in a mixed carbon substrate situation were determined.

MATERIALS AND METHODS

Chemicals. Parathion 4-lb (ca. 18.14 kg) emulsifiable concentrate (PAR EC), 47.5% PAR, the commercial diluent system used to formulate technical PAR, and technical PAR (98.7%) were all obtained from Stauffer Chemical Co. (Richmond, Calif.). The diluent was a 95% commercial mixture of *o*-, *m*-, and *p*-xylene with 5% detergents and emulsifiers. The three isomers of xylene and *p*- and *m*-toluic acid were purchased from Matheson, Coleman and Bell, San Francisco, Calif., all of fine chemical grade.

Microorganisms. Two mixed cultures were used in this study. By use of an aerobic continuous fermentation technique (7, 9), one mixed culture was adapted to growth on PAR EC as a carbon source and the other on technical PAR as a sole carbon source. The first mixed culture was determined to consist of at least 11 isolates, of which eight were identified as fluorescent pseudomonads and one a *Bacillus* sp. (D. M. Munnecke, Ph.D. thesis, Univ. of California, Davis, 1974). The second mixed culture consisted of at least nine isolates, five of which were fluorescent pseudomonads. The remaining four were classified as species of *Brevibacterium*, *Azotomonas*, *Xanthomonas*, and one unknown (9).

Media and culture conditions. Both adapted mixed cultures were maintained in Burk mineral salts 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. The resultant solution, pH 7.2, contained PAR EC or technical PAR as the only added source of carbon. PAR EC was added to the basal salts to create suspensions of 0.1 to 1.0%, or the diluent was added to create 0.04% suspensions. Technical PAR or *o*-, *m*-, or *p*-xylene were added to the mineral salts solution to create 0.04%

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suspensions. For continuous fermentation studies, a New Brunswick Miniform, 600-ml working volume, was used at dilution rates from 0.04 to 0.08 per h using PAR EC as the substrate. A Warburg respirometer, operated at 28 C, with 10 N NaOH in the center well, was used to follow the oxygen uptake of the mixed culture growing on either PAR or on the diluent only. Batch culture experiments with xylene and PAR were conducted in 500-ml Erlenmeyer flasks (200 ml of medium), with cotton stoppers, at 28 C, on a rotary shaker at 160 rpm.

Analytical methods. PAR concentration was determined by a Packard gas chromatograph model 417, equipped with a flame ionization detector and a glass column (1.2 m by 6 mm inner diameter) packed with 6% Apiezon N on Chromosorb Q. The column temperature was maintained at 230 C, and the injector and detector temperatures were both 245 C. Xylene concentrations were determined using a glass column (1.2 m by 6 mm inner diameter) packed with 5% SE-30 on Chromosorb Q. The column temperature was maintained at 70 C and the injector and detector were at 100 and 120 C, respectively.

Thin-layer chromatography studies were conducted using polyamide gel plates (Brinkmann Instruments Inc., Westburg, N.Y.) developed in ethanol-water (3:1). Visualization was by fluorescent quenching under ultraviolet light.

Ultraviolet spectra were obtained with a Beckman DK-2 scanning spectrophotometer. Mass spectra were obtained with a Varian M66 mass spectrometer, equipped with a solid probe. High resolution mass spectroscopy was used to identify metabolite I.

A New Brunswick dissolved oxygen probe was used to monitor the oxygen level in the continuous fermentor during bacterial growth. The change in the rate of oxygen consumption was one method used to determine bacterial growth. This rate was determined by first stopping aeration, but maintaining agitation of the fermenter. Then the time required for the oxygen concentration in the broth to fall from 100% air saturation to 90% air saturation was measured. Corrections were made for solubility changes due to temperature and buffer concentration, and the oxygen uptake rate was then calculated in milliliters per minute at 760 mm of Hg. Cell optical density, determined spectrophotometrically at 600 nm, and plate counts were employed to monitor growth and verify that respiration rates were proportional to optical density or plate count measurements. For optical density measurements, the culture broth was first made acidic (pH 5.0) to avoid interference from the color of *p*-nitrophenol.

The rate of enzymatic hydrolysis of PAR was followed spectrophotometrically at 410 nm with corrections made for the different extinction coefficients of *p*-nitrophenol at various pH values.

RESULTS

Metabolism of PAR EC. (i) Rates. The growth rates of the adapted mixed culture growing on either PAR EC (3,000 mg/liter) or the xylene-based diluent system (3,000 mg/liter) used in the formulation, as measured by the

Warburg respirometer, are shown in Fig. 1. The specific growth rate (μ) for the culture metabolizing only the diluent was 0.13 per h, whereas the growth rate on PAR EC was 0.7 per h, indicating appreciable growth on xylene or PAR. The optimal PAR EC concentration after a 36-day enrichment period was found to be near 5,000 mg/liter, with only a slight decline in the growth rate at 10,000 mg/liter. The metabolism of PAR in the batch cultures appeared to be complete as there was no residual PAR detectable in the spent broth. Under certain continuous fermentation conditions, PAR at influent concentrations of 200 to 700 mg/liters could be degraded at a maximum rate of 50 mg/liter per h, with an effluent PAR concentration that was only 1.5% of the influent concentration (9).

(ii) Temperature. The optimal temperature for the growth of the first mixed culture on PAR EC in a continuous fermenter was determined to be 37 C. This is a fairly high optimal temperature for a predominately pseudomonad culture; however, many pseudomonads do have a temperature optimum in this range (2). Bacterial growth at 23 C was 15% of its maximum, whereas at 44 C it was 55% of its maximum.

(iii) pH. The data describing the optimal pH for the growth of the first mixed culture on PAR EC are shown in Fig. 2. The optimal pH was

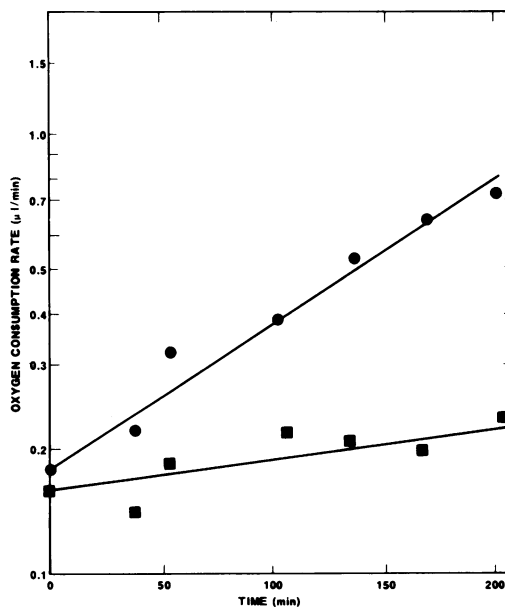


FIG. 1. Growth of a mixed culture on parathion emulsifiable concentrate (●) ($\mu = 0.70$ per h) and on the formulation diluent system without parathion (■) ($\mu = 0.13$ per h) at 28 C in Warburg flasks. Substrate concentration = 3,000 mg/liter.

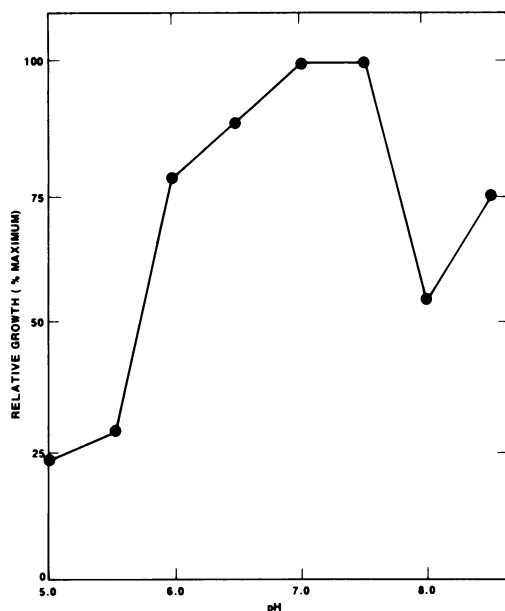


FIG. 2. Effect of pH on the growth rate of a mixed bacterial culture growing on parathion emulsifiable concentrate in batch culture.

determined to lie between pH 7.0 to 7.5, whereas the rate of growth in media below pH 6.0 was drastically reduced. The decline in the growth rate at pH 8.0 and the subsequent increase at pH 8.5 will be discussed under metabolism of xylene.

(iv) Oxygen concentration. Optimal microbial growth on PAR EC in a continuous fermenter occurred when the broth was 100% air saturated (Fig. 3). When the broth was below 20% air saturation, the culture growth rate drastically declined.

(v) Substrate selection. In the laboratory the first adapted mixed culture was continuously maintained in batch or continuous culture on PAR EC in basal salts. During normal growth of the mixed culture on PAR EC in the 7 mM phosphate buffer, the pH of the media repeatedly dropped and, at times, fell below pH 6.0. After 6 months of alternating batch and continuous culture growth, the mixed culture gradually lost the ability to metabolize PAR but was well adapted to xylene metabolism. Therefore, a second mixed culture had to be developed which was able to metabolize PAR as the sole carbon source.

The metabolism of PAR and *o*-, *m*-, and *p*-xylene in batch culture, when all substrates were present simultaneously, by the first mixed culture after it had been maintained in the laboratory for 6 months, is shown in Fig. 4. Both *m*- and *p*-xylene were metabolized rapidly

with *o*-xylene metabolism slightly slower. Once *m*- and *p*-xylene were depleted from the medium, no further metabolism of *o*-xylene was observed, indicating possible co-metabolism of the *o*-isomer by the mixed culture growing on the *m*- and *p*-isomers. During the growth on xylene, PAR metabolism did not occur, but the latter started at a notable rate after xylene metabolism ceased. However, the rate of PAR metabolism in the second phase of the microbial degradation was decreased due to the in-

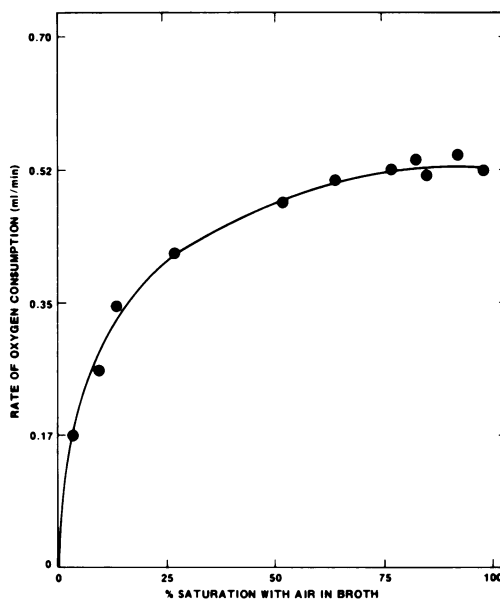


FIG. 3. Effect of oxygen concentration on the rate of respiration of the mixed culture in steady state continuous culture growth on parathion emulsifiable concentrate, 3,000 mg/liter influent concentration.

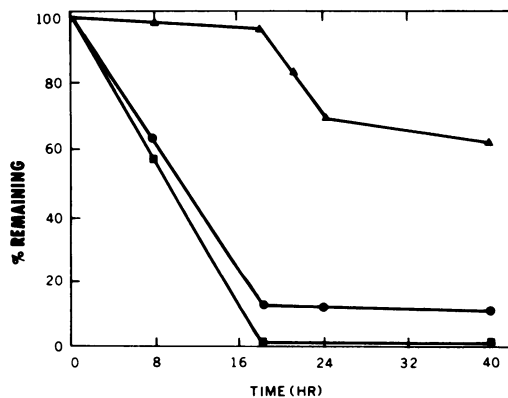


FIG. 4. Batch culture metabolism of parathion and xylene in the insecticide emulsifiable concentrate by acclimated microorganisms. Symbols: parathion, ▲; *o*-xylene, ●; *m*-, *p*-xylene, ■.

creasing acidity in the medium as described in the following section.

Metabolism of xylene. (i) **Effect of pH.** Examination of the microbial metabolism of technical xylene and PAR revealed that selection for xylene metabolizers in the first mixed culture was mainly a result of increasing acidity in the medium during the continuous cultivation of the culture on PAR EC.

The first mixed culture, when grown on xylene, had an optimal growth rate at pH 7.0; however, the culture grew at 68% of its maximum at pH 5.0 and at 75% of its maximum at pH 8.0 (Fig. 5a). When the metabolism of technical PAR by the second mixed culture was examined, the hydrolysis of PAR to *p*-nitrophenol and diethylthiophosphoric acid was found to be rate limiting (9), and parathion hydrolase, an enzyme produced by only one isolate from the mixed culture, a *Brevibacterium* sp. (9), was very dependent on pH (Fig. 5b). PAR hydrolase activity was optimal at pH 9.2 with only 12% of its maximum activity expressed at pH 7.0, the optimum pH for xylene metabolism. Therefore, the different pH optima for xylene metabolism and PAR hydrolysis clarify the diphasic growth patterns as related to pH for cultures growing on PAR EC (Fig. 2).

(ii) **Toluic acid formulation.** When isolates from the first mixed culture were grown on *o*-

m-, or *p*-xylene, good growth occurred on *m*- and *p*-xylene. *o*-Xylene, however, did not support any growth when serving as a sole carbon source, but was metabolized co-metabolically when *m*- and *p*-xylene were added to the media. When the supernatant of cultures grown on *p*- and *m*-xylene were analyzed for metabolites, one metabolite from each isomer (metabolites I and II) was detected. Metabolites I and II had thin-layer chromatography *R_f* values (0.51 and 0.54) similar to the respective *p*- and *m*-toluic acids (0.53 and 0.55). Many previous researchers have demonstrated toluic acid to be an intermediate in xylene metabolism by pseudomonads (5, 10, 11, 14). Based upon ultraviolet and mass spectroscopy (Fig. 6, 7), metabolite I was identified as *p*-toluic acid. During the metabolism of *p*-xylene, *p*-toluic acid accumulated in the medium to levels of 15 mg/liter.

Aromatic ring cleavage. Six isolates from the mixed culture were grown in pure culture on *m*- and *p*-xylene and *p*-nitrophenol as sole carbon sources, then harvested, and tested for mode of aromatic ring fission (16). It was found that the two xylene isomers were cleaved by a meta enzyme system by all six cultures. However, five of the six pseudomonads used an ortho ring fission system for *p*-nitrophenol metabolism. The remaining culture did not grow on *p*-nitrophenol.

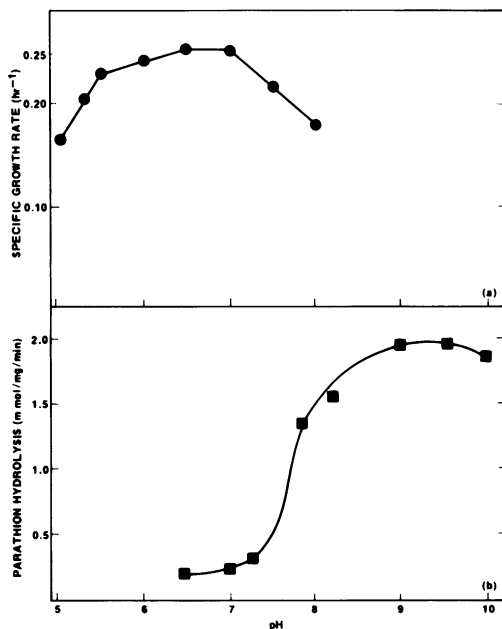


FIG. 5. Effect of pH on the growth rate of the mixed culture on xylene (a) and the rate of enzymatic parathion hydrolysis by a crude extract from the second mixed culture (b).

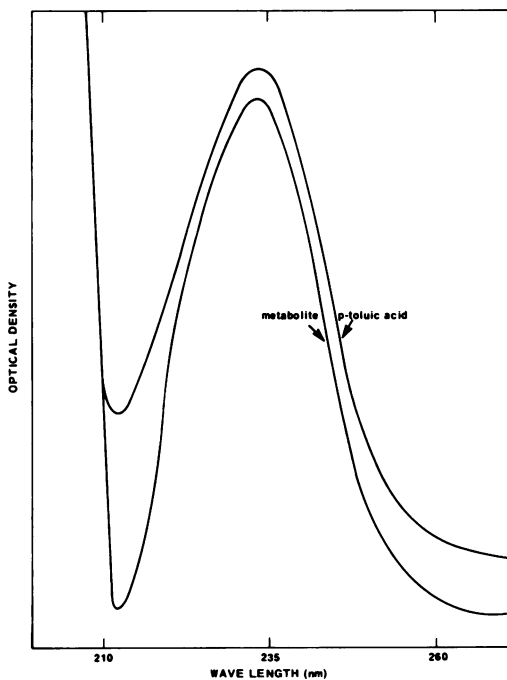


FIG. 6. Scanning ultraviolet spectra of *p*-toluic acid and metabolite I.

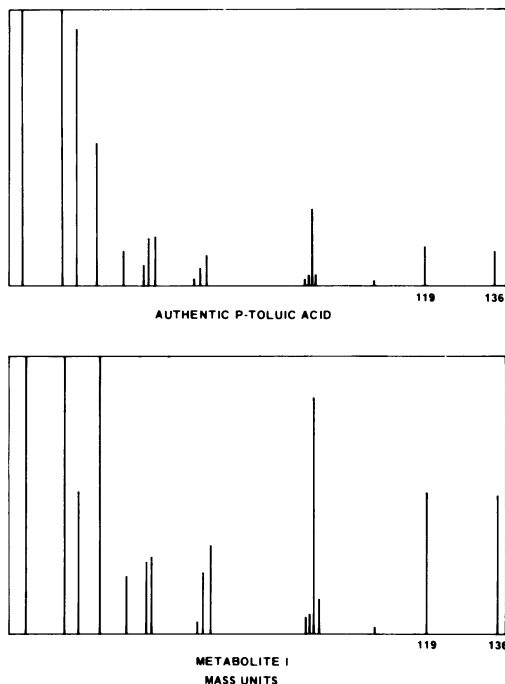


FIG. 7. Comparison of fragmentation patterns of *p*-toluic acid and metabolite I.

DISCUSSION

Under proper environmental conditions an adapted mixed culture in a continuous fermenter was able to degrade 50 mg of PAR/liter per h (9). This rate is 10 times faster than chemical hydrolysis by 1 N sodium hydroxide at 25 C (6) and is more complete since *p*-nitrophenol is not a degradation end product (9). However, the chemical and biological interactions involved in the metabolism of a PAR EC formulation are quite complex. The selection of PAR as the primary carbon source from the mixture of formulation chemicals was dependent on the composition of microflora and their growth environment. Slightly alkaline broth favored the metabolism of PAR over xylene in the PAR EC mixed substrate, whereas slightly acidic broth selected for xylene metabolism.

Metabolites from xylene and PAR metabolism also influenced the metabolism of PAR. Toluic acid, which accumulated from xylene metabolism, lowered the pH of the medium as did a hydrolysis product of PAR, diethylthiophosphoric acid (9), thus indirectly inhibiting PAR hydrolysis and utilization. *p*-Nitrophenol, another hydrolysis product of PAR, was toxic to isolates of the mixed culture at concentrations above 450 mg/liter and growth inhibitory above 30 mg/liter (9). Although *p*-nitro-

phenol did not seem to cause selection for PAR or xylene metabolism, its initial accumulation in the broth strongly affected the lag period and the further metabolism of PAR (9).

The metabolism of the aromatic compounds in the PAR EC formulation proceeds through two different pathways. PAR is hydrolyzed to *p*-nitrophenol which is apparently metabolized by removal of the nitro group followed by ring hydroxylation to yield hydroquinone, and then by a second hydroxylation to yield 1,3,4-benzenetriol (9). The latter is then degraded by an ortho ring cleavage enzyme system (8, 9). The metabolism of *p*- and *m*-xylene by pseudomonads appears to proceed through the respective toluic acids (5, 10, 11, 14). The subsequent metabolism of *m*- and *p*-toluic acid has been shown to proceed through at least five pathways (1, 3, 5, 12, 13, 15), with four of the five pathways employing a meta ring fission enzyme system (3, 4, 5).

In the present study a meta ring fission enzyme system was found to be employed by the pseudomonad cultures for xylene metabolism and an ortho enzyme system for PAR metabolism. The need for the two aromatic ring fission enzyme systems for metabolism of PAR and xylene in the PAR EC formulation represents another possible mechanism for substrate selection. The possibility exists that one ring fission enzyme system may be repressed by the second system in those isolates which possess both systems. This possible control mechanism is consistent with the observation that parathion metabolism by the mixed culture did not occur when there was growth on xylene (Fig. 4).

The findings described above should serve to alert practitioners of pesticide disposal by biological oxidation systems or soil disposal techniques to some of the problems associated with mixed culture metabolism of toxic, mixed substrates. Before developing biological techniques for the disposal of concentrated pesticides and their related wastes, each individual situation must be examined in detail so a viable system can be developed for complete breakdown of the organic compounds in question.

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