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Parathion Utilization by Bacterial Symbionts in a Chemostat

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A continuous-culture device was used to select and enrich for microorganisms, from sewage and agricultural runoff, that were capable of using the organophosphorus insecticide parathion as a sole growth substrate. Parathion was dissimilated by the highly acclimated symbiotic activities of *Pseudomonas stutzeri*, which non-oxidatively and cometabolically hydrolyzed the parathion to ionic diethyl thiophosphate and *p*-nitrophenol, and *P. aeruginosa*, which utilized the *p*-nitrophenol as a sole carbon and energy source. Ionic diethyl thiophosphate was found to be inert to any transformations. Methyl parathion was dissimilated in an analogous way. The device functioned as a chemostat with parathion as the growth-limiting nutrient, and extraordinarily high dissimilation rates were attained for parathion (8 g/liter per day) and for *p*-nitrophenol (7 g/liter per day). This is the first report of parathion utilization by a defined microbial culture and by symbiotic microbial attack and of dissimilation of an organophosphorus pesticide in a chemostat.

Continuous-cultivation techniques have rarely been used for studying the interactions of microorganisms and xenobiotics, e.g., microbial degradation of pesticides and toxic wastes. Ware and Evans (69) and Evans and Kite (14) gave perhaps the first reports of continuous culture applied to detoxifying aqueous wastes (i.e., phenols). Chemostats have been used to investigate the microbial utilization of phenol (26, 31, 70, 73), *p*-hydroxybenzoate (29), and 2,4-dichlorophenol (68), three possible products of pesticide breakdown. There are only two reports of pesticides (both water-soluble herbicides) serving as growth-limiting nutrients in a chemostat: 2,4,-dichlorophenoxyacetic acid (68) and dalapon (61, 62). Chu and Kirsch (7) reported the continuous cultivation of a bacterium on pentachlorophenol. McClure (38) constructed a membrane filter device that kept the influent and effluent separate from the bacteria for use in the enhanced biodegradation of the herbicide propham. This device operated in a manner similar to that of a continuously fed batch system with constant removal of soluble wastes. Pesticides and environmental pollutants of limited water solubility have experienced even fewer investigations. Hsieh and Munnecke (28, 46) mentioned the growth of a bacterial community on parathion in continuous culture, but it was not able to maintain a steady state because the parathion was fed to the growth vessel as an emulsion of uncontrolled and variable concentration. Pritchard and Starr (57) described a novel device for the continuous cultivation of bacteria on oil.

We have previously reported the utilization of parathion by symbiotic bacterial activity in a chemostat device (12) and the use of these bacteria for accelerated degradation of parathion in soil (C. G. Daughton and D. P. H. Hsieh, Bull. Environ. Contam. Toxicol., in press). Parathion is an unusual substrate for microbial utilization. Although it has poor water solubility (24 μg/ml, 82 μM), parathion can be hydrolyzed to two potential substrates that contain carbon, nitrogen, phosphorus, and sulfur: (i) fairly water-soluble and moderately toxic *p*-nitrophenol and (ii) extremely water-soluble potentially toxic ionic diethyl thiophosphate. We report here the descriptions of the parathion-acclimated bacteria and the data obtained from their growth in a two-stage chemostat; the second stage was used to study possible diauxic growth on metabolites that were not utilized in the first stage.

MATERIALS AND METHODS

Substrates. Technical-grade 98% parathion (*O,O*-diethyl *O*-*p*-nitrophenyl phosphorothionate) (PTN) was kindly supplied by Stauffer Chemical Co., Richmond, Calif. Technical-grade 96% methyl parathion (*O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothionate) (MPTN) was kindly supplied by Monsanto Chemical Co., Anniston, Ala. *p*-Nitrophenol (mp 113 to 114°C) (*p*-NP) was obtained from Matheson, Coleman and

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Bell, San Francisco, Calif. All other chemicals were of analytical reagent grade.

Continuous culture. The continuous-culture device was of a two-stage, single-stream design, without feedback. The first stage (St-1) was a BioFlo model C30 (New Brunswick Scientific Co., New Brunswick, N.J.), 240 to 300 ml, maintained at 26°C and aerated at 0.8 liter/min; foam control was not needed. The effluent overflowed into the second stage (St-2) (690 ml). All tubing was either silicone rubber or glass. A concentrated salts solution (25-fold) contained (grams per liter of deionized water): $(NH_4)_2SO_4$, 25.0; $MgSO_4 \cdot 7H_2O$, 5.0; $CaCl_2$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.175; $MnCl_2 \cdot 4H_2O$, 0.05; $NaMoO_4 \cdot 2H_2O$, 0.025; $ZnCl_2$, 0.0125; and $CuCO_3$, 0.0125. This solution, in a reservoir separate from that of the phosphate buffer ($K_2HPO_4 \cdot 3H_2O$, 2 to 3 g/liter), was pumped to St-1 at 1/25 the rate of the buffer by a multichannel precision variable-speed peristaltic pump (Sage model 375A, Orion Instruments). This obviated the need for sterilizing the buffered minimum salts solution to prevent ammonia oxidizer growth in the reservoir and for organic chelating agents, which are sometimes toxic to pseudomonads and can serve as additional substrates and as catalysts for the hydrolysis of PTN (34). Since technical PTN has only limited water solubility (24 μg/ml, 82 μM), it was metered into St-1 at 4 to 67 μl/h through a Teflon needle from a 1.0- or 2.5-ml gas-tight syringe powered by a variable-speed precision syringe pump (Sage model 335, Orion). When MPTN (mp = 38°C) was metered into St-1 as the sole substrate, the syringe and Teflon inlet line were heated to 40°C by wrapping them with Nichrome wire and applying sufficient voltage. The theoretical or "effective" PTN concentration in the influent buffered salts solution (S_r-PTN, micrograms per milliliter) was calculated from the flow rate (F, milliliters per hour), the PTN flow rate (P, microliters per hour), and the specific gravity of the PTN (1.25 mg/μl):

$$S_r\text{-PTN} = 1{,}000 \times (P \times 1.25)/F$$

The pH of each stage was maintained between 6.5 and 7.2. When *p*-NP was fed as the sole substrate, it was dissolved in the buffer reservoir.

The two stages were checked for efficient homogeneous mixing by following the washout of *p*-NP from each of the reactors (cell free) over time at a constant dilution rate. The concentrations of *p*-NP in St-1 ($\bar{s}_1$-*p*-NP) and St-2($\bar{s}_2$-*p*-NP) were predicted very well by the following equations, indicating nearly perfect mixing:

$$\bar{s}_1 = s_0 e^{-D_1 t} \quad \text{and}$$

$$\bar{s}_2 = D_2/(D_2 - D_1) \cdot s_0 e^{-D_1 t} + Ce^{-D_2 t}$$

where s_0 is the initial *p*-NP concentration in both St-1 and St-2, D_1 and D_2 are dilution rates for St-1 and St-2, respectively, and C is a boundary condition at time (t) zero.

Acclimated microorganisms. The microbial inoculum was obtained from the University of California, Davis, Sewage Treatment Plant aeration tank, which also receives agricultural runoff. Cells in the sample were selected, enriched, and acclimated for growth on PTN as the sole carbon and energy source and maintained in continuous culture for over 2 years. Sterile techniques were not used while these organisms were maintained in continuous culture.

Assays. Steady-state conditions in St-1 and St-2 were determined by monitoring turbidity with a Klett-Summerson colorimeter with a blue filter (400 to 450 nm). Generally, for each step-change imposed on the system, more than five residence times for each stage elapsed before measurements were made. For all analyses, samples were withdrawn from the reactors and immediately processed. Dry mass was determined by filtering measured volumes through tared 0.2-μm Sartorius membrane filters, in quadruplicate, followed by rinsing with 10 ml of deionized water and drying at 90°C for at least 1 h. The same procedure was followed for blanks of equivalent volumes of water. Dry mass was calculated after duplicate weighings on a Mettler model H20T semimicro balance as: average mass (sample) − average mass (blank).

PTN was quantitated by extraction with ethyl acetate or ethyl ether at pH 3 (65), dried over anhydrous Na_2SO_4, and injected on a Becker model 417 gas chromatograph equipped with a phosphorus-thermionic detector and a 1-m glass column, 2 mm ID packed with 10% Apiezon N on 60/80 Gas-Chrom Q. Ionic diethyl thiophosphate (DETP) and ionic dimethyl thiophosphate were obtained as the potassium salts from American Cyanamid Co., Princeton, N.J. Dimethyl and diethyl phosphates were obtained as the free acids from American Cyanamid Co., Wayne, N.J., and Eastman Chemical Co., Rochester, N.Y., respectively. These were quantitated as their methyl esters by using the procedure of Daughton et al. (11) without the resin preconcentration step. Methylation of DETP and ionic dimethyl thiophosphate yields both the *O*- and *S*-methyl esters, the latter of which can be formed in 90% yield if a protic solvent, such as methanol or water, is present during methylation with ethereal-ethanolic diazomethane; this agrees with published data (39). The methylated samples were injected on a gas chromatograph (phosphorus-thermionic) with a 1.8-m glass column, 2-mm ID, packed with equal mixtures of 15% QF-1 and 10% DC 200 on 80/100 Gas-Chrom Q. Identification was confirmed by gas chromatography-mass spectrometry.

When present at less than 0.5 μg/ml, *p*-NP was quantitatively extracted at pH 3 with 2 equal volumes of ethyl acetate (65), which was then dried over anhydrous Na_2SO_4; the volume was decreased by evaporation in vacuo, and the mixture was dried under nitrogen gas. The residue was suspended in 1% Na_2CO_3 and membrane-filtered to remove suspended solids; the absorbance at 410 nm was read in a Spectronic 100 (Bausch & Lomb, Inc., Rochester, N.Y.), with 1% Na_2CO_3 used as the blank. Samples containing higher concentrations of *p*-NP were quickly membrane-filtered, and the filtrate was made alkaline with concentrated aqueous NaOH.

Qualitative identification of possible mono- and dihydric phenolic metabolites was achieved by extracting an acidified sample with either ethyl acetate, ethyl ether, or methyl-isobutyl ketone (10);

forming the trimethylsilyl derivatives by adding *N,O*-bis(trimethylsilyl)acetamide (Aldrich Chemical Co., Milwaukee, Wis.) to the dry residue; diluting with carbon disulfide; and comparing the retention times with those of standards on a Becker gas chromatograph equipped with a flame ionization detector and with a QF-1, DC 200 Gas-Chrom Q column.

Qualitative estimation of "total" phenols was accomplished by the method of Chrastil (6), which gave responses to all the phenols of interest when present at 5 μg or more per ml.

RESULTS

Acclimated microorganisms. After 2 years of continuous growth on PTN or *p*-NP as the sole carbon and energy source, the continuous culture apparently consisted only of three morphologically distinct bacterial forms: two motile rods and one immotile coryneform. Of the initial isolates, one could grow on *p*-NP as the sole substrate, but none could hydrolyze PTN. Therefore, many late-growing and slowly developing colonies on nutrient agar were screened for PTN-hydrolytic activity. Finally, a colony was isolated that possessed extremely active PTN-hydrolysis ability. Together with the isolate that grew on *p*-NP, these two isolates symbiotically utilized PTN as the sole carbon and energy source in the chemostat and in batch culture. In batch culture, PTN hydrolysis was so rapid that *p*-NP accumulated in toxic quantities, resulting in delayed growth by the *p*-NP-utilizing species.

By using the procedures and data of Stanier et al. (63) and Palleroni et al. (53), except for the test for arginine dihydrolase (66), the isolates were identified as follows.

(i) ***Pseudomonas aeruginosa.*** Gram-negative, motile rod, with yellow-green pigmentation, highly fluorescent; *ortho* cleavage of catechol; no lytic response to *P. putida* phage; abundant growth at 40 and 26°C, no growth at 4°C; oxidase positive; denitrification without gas production; arginine dihydrolase rapidly positive; starch hydrolysis negative; no methionine requirement; very rapid growth; no PTN-hydrolysis activity. Growth on the following compounds as sole carbon sources: *p*-NP (with much release of nitrite), *p*-hydroxybenzoate, acetamide, DL-valine, arginine, D-glucose, betaine, sucrose (without levan formation), β-hydroxybutyrate, lactate, glycollate, 2-noneic acid, succinate, β-alanine, ethylene glycol, propylene glycol, mucate, D-fructose, and L-histidine. No growth on: mannitol, cellobiose, starch, DL-norleucine, DL-tryptophan, *myo*-inositol, *m*-hydroxybenzoate, fucose, adipate, maltose, trehalose, maleate, lactose, L-threonine, and D-arabinose. Variable growth on D-galactose.

(ii) ***P. stutzeri.*** Rapidly hydrolyzes PTN; gram-negative, motile rod; no pigmentation or fluorescence; *ortho* cleavage of catechol; colonies often dry and wrinkled; no growth at 40 or 4°C; variable growth at 38°C; growth at 26°C; oxidase positive; vigorous denitrification without gas production; arginine dihydrolase weakly positive; starch hydrolysis positive; no methionine requirement; no accumulation of polyhydroxybutyrate. Growth on the following compounds as sole carbon sources: mannitol, cellobiose, DL-valine, *p*-hydroxybenzoate, D-glucose, sucrose (without levan formation), β-hydroxybutyrate, lactate, fucose, maltose, trehalose, lactose, D-galactose, D-fructose, and propylene glycol. No growth on: starch, acetamide, DL-norleucine, DL-tryptophan, *myo*-inositol, *m*-hydroxybenzoate, arginine, betaine, glycollate, adipate, 2-noneic acid, maleate, β-alanine, *p*-NP, mucate, L-threonine, and D-arabinose. Variable growth on: ethylene glycol and L-histidine.

The isolate of *P. stutzeri* did not possess several characteristics of the species (e.g., utilization of starch and glycollate), but since it had been growing in mixed continuous culture, exclusively on PTN for over 2 years, these properties could have been lost through mutation. With all utilizable substrates, *P. stutzeri* grew much slower than did *P. aeruginosa*. All of the studies discussed here involve this highly acclimated culture containing the two defined bacterial species.

Parathion-limited chemostat. Continuous cultivation with parathion supplied as the sole carbon source revealed that steady-state dry cell mass in St-1 ($\bar{x}_1$) increased linearly as the theoretical influent concentration of PTN (S_r-PTN) increased (Fig. 1). Steady-state PTN concentrations in St-1 ($\bar{s}_1$-PTN) were less than 2% of S_r-PTN. From the relationship $\bar{x} = Y_{ob}(S_r - \bar{s})$ (56), the observed yield (Y_{ob}), calculated on the basis of the benzene nucleus carbon of PTN, was 36%. The yield was calculated only on the basis of the carbon in the *p*-nitrophenyl moiety since the diethyl phosphoryl carbon was not utilized, as shown below.

Steady-state analyses for ionic alkyl phosphates and thiophosphates revealed the exclusive presence of DETP in both St-1 and St-2. More importantly, the steady-state concentration of DETP in St-1 and St-2 was always equimolar to the theoretical S_r-PTN (150 to 2,274 μg/ml; 0.52 to 7.8 mM) (Fig. 2). The DETP concentration was the same for a given steady state whether determined by extraction of

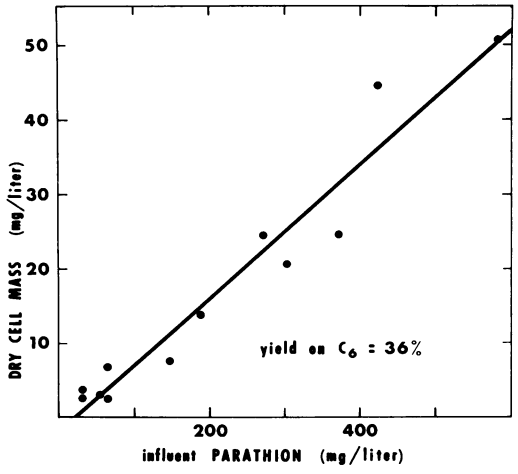

FIG. 1. *Parathion chemostat: $\dot{x}_1$ versus S_r-PTN. Steady-state dry-cell mass in St-1 (milligrams per liter) versus the concentration of parathion in the influent (milligrams per liter). Model equation $\dot{x} = (Y_{ob}/100)(S_r - \bar{s})$. Least-squares line: $\bar{x}_1 = 0.088 S_r - 1.7$, $r^2 = 0.94$. $s_{y \cdot x} = 4.04$, $s_b = 1.71$, $s_m = 0.007$ ($n = 13$).*

whole-cell suspensions or by using the 0.2-μm membrane filtrate from cell suspensions. When the supply of phosphate or sulfur was withdrawn from the chemostat, $\dot{x}_1$ decreased to very low values, the cells clumped, and DETP remained unaltered. These results indicate the following. (i) Since DETP was present exclusively in the extracellular fluid, hydrolysis of PTN was probably mediated exocellularly. Intracellular hydrolysis of PTN would have required the active transport of DETP from the cell, since DETP is an ionic phosphate. In addition, suspended, stationary-phase cells of *P. stutzeri* from nutrient agar, which had been washed and membrane filtered, could immediately hydrolyze PTN, whereas the filtrate possessed no activity; hydrolytic activity was shown by the extracellular appearance of *p*-NP. (ii) Since DETP reached steady-state concentrations equimolar to S_r-PTN, this fortuitously confirmed that the theoretical calculation of S_r-PTN was valid and that the infusion of PTN to the reactor was constant. (iii) There was no significant loss of PTN by volatilization, since all of the PTN entering the system could be accounted for as DETP. (iv) DETP was recalcitrant to microbial and chemical transformations, even when supplied as a sole phosphorus and sulfur source.

When MPTN was infused as the sole substrate, dissimilation was analogous to that of PTN. Hydrolysis resulted in accumulation of nearly equimolar quantities of ionic dimethyl thiophosphate. High concentrations of MPTN were perhaps less readily hydrolyzed since MPTN is a solid below 38°C; this resulted in a lessening of MPTN emulsification, due to solid material floating on the culture surface or adhering to the reactor walls, and a decrease in its accessibility to hydrolytic enzymes.

When S_r-PTN was gradually increased to 2,274 μg/ml, Y_{ob} decreased by nearly 50%, but $\bar{s}_1$-PTN still remained relatively low (52 μg/ml). These results were achieved for a dilution rate (D) of 0.15/h and represent a dissimilation rate of 8 g of PTN per liter per day. The steady-state concentration of phenols, including *p*-NP, remained <0.5 μg/ml. The decrease in Y_{ob} was probably due to the high concentrations of DETP.

p-NP-limited chemostat. Since DETP was not utilized, PTN utilization was directly related to that of *p*-NP. Initiation of the continuous cultivation of the acclimated bacteria on *p*-NP, as a sole carbon and energy source, required low values for influent concentration of *p*-NP (250 μg/ml) and dilution rate (0.1/h). To achieve a higher S_r or D, the incremental increases needed to be small (e.g., 150 μg/ml or 0.02/h); otherwise, what would have been a transient increase in the amount of *p*-NP present in the reactor could have become growth inhibitory. Evans and Kite (14) pointed out an important characteristic of a continuous fermentor used for degradation of toxic substrates: when it operates at dilution rates close to maxi-

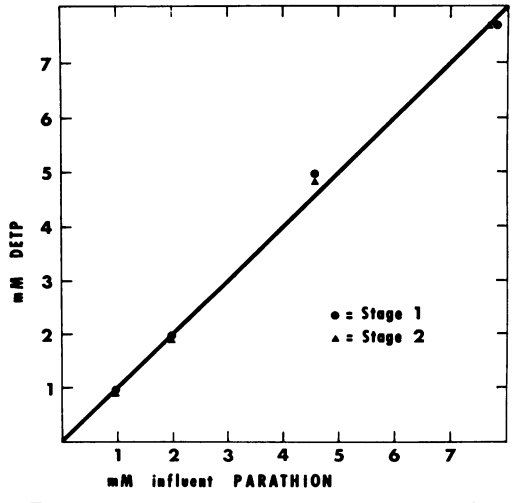

FIG. 2. *Parathion chemostat: $\bar{s}_1$-DETP and $\bar{s}_2$-DETP versus S_r-PTN. Steady-state concentration of DETP in St-1 (●) and St-2 (▲) (mM) versus the concentration of parathion in the influent to St-1 (mM).*

mum, fluctuations (e.g., step increases) in influent concentrations or flow rate can lead to effluent concentrations that inhibit growth.

Steady-state dry-cell mass ($\bar{x}$) versus S_r-*p*-NP (D = 0.13/h) is plotted (Fig. 3). As S_r was increased, $\bar{x}$ increased, but in proportionately smaller amounts. Dry-cell mass yields were calculated on the basis of the milligram equivalents of the benzene-nucleus carbon of consumed *p*-NP. With S_r-*p*-NP increasing from 240 to 960 μg/ml, Y_{ob} was 30 and 18%, respectively. The steady-state dry-cell mass versus S_r-PTN is shown in Fig. 4. The values for St-2 are noticeably lower than those for St-1, compared to the values resulting from the run when *p*-NP was the substrate (Fig. 3). Since liberation of DETP is the only distinguishing feature of PTN metabolism compared to *p*-NP metabolism, the lower values for $\bar{x}$-PTN, probably due to cell lysis, indicate that DETP is toxic.

Presumably, *p*-NP undergoes a nitro-group removal as nitrite by a nitro-reductase prior to its utilization (59). To be sure that this was the rate-limiting step, so that *p*-NP disappearance could be used as a direct measure of its utilization, samples were analyzed for steady-state concentrations of possible *p*-NP phenolic metabolites in the extracellular fluid and in the organic extracts of the acidified cell suspension. No phenols other than *p*-NP were detectable by either the total phenol or the gas chromatography method.

Further verification that the chemostat was *p*-NP limited involved following turbidity (in Klett units as related to dry mass) and the steady-state *p*-NP concentration in St-1, while increasing D over a period of 3 weeks at a constant S_r-*p*-NP (720.5 μg/ml). Figure 5 shows a plot of $1/D$ versus $1/\bar{s}_1$-*p*-NP. The equation

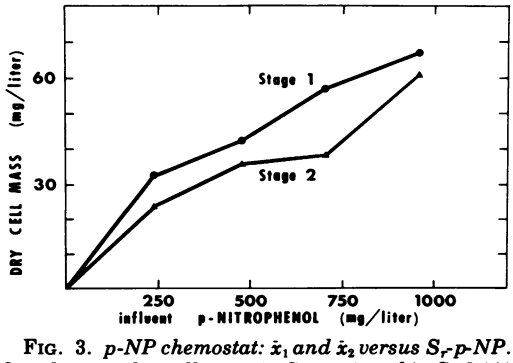

FIG. 3. *p-NP chemostat: $\bar{x}_1$ and $\bar{x}_2$ versus S_r-p-NP. Steady-state dry-cell mass in St-1 (●) and in St-2 (▲) versus concentration of p-NP (milligrams per liter) in the influent. D = 0.13/h.*

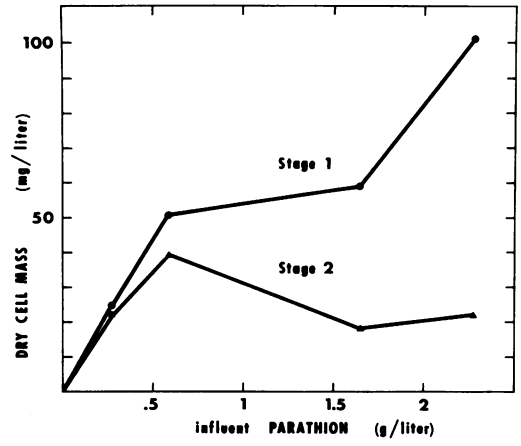

FIG. 4. *Parathion chemostat: Effect of large concentrations of parathion; $\bar{x}_1$ and $\bar{x}_2$ versus S_r-PTN. Steady-state dry-cell mass (milligrams per liter) in St-1 (●) and St-2 (▲) versus the concentration of parathion in the influent to St-1.*

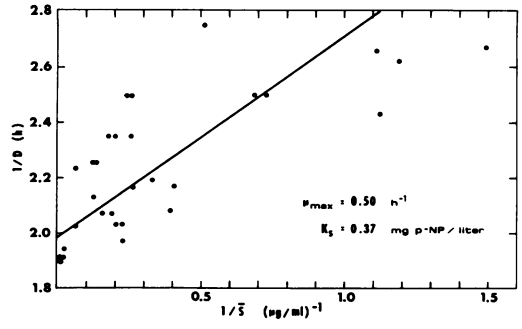

FIG. 5. *p-NP chemostat: $1/D$ versus $1/\bar{s}_1$. Double-reciprocal plot of dilution rate (per hour) versus steady-state concentration of p-NP (micrograms per milliliter). S_r-p-NP = 720.5 mg of p-NP per liter. Model equation: $1/D = [(K_s/\mu_{max})(1/\bar{s})] + [1/\mu_{max}]$. Least-squares line: $1/D = [0.731(1/\bar{s})] + 1.979$, $r^2 = 0.56$, $\mu_{max} = 0.50/h$, $K_s = 0.37$ mg of p-NP per liter. $s_{y \cdot x} = 0.170$, $s_b = 0.042$, $s_m = 0.081$ (n = 31).*

model for this line is $1/D = [K_s/\mu_{max}(1/\bar{s})] + [1/\mu_{max}]$, where K_s is the Monod saturation constant and μ_{max} is the maximum specific growth rate (56). The least-squares regression line gives a theoretical μ_{max} = 0.50/h and K_s = 0.37 mg of *p*-NP per liter.

Figure 6 shows a plot of $1/Y_{ob}$ versus $1/D$ (= $1/\mu$). The equation for the line, as given by Pirt (56), is $1/Y_{ob} = (m/\mu) + (1/Y_g)$, where m is the maintenance coefficient and Y_g is the "true" growth yield or that which would be observed in the absence of any maintenance requirement. From the least-squares regression line, the following values result: m = 0.21 mg of *p*-

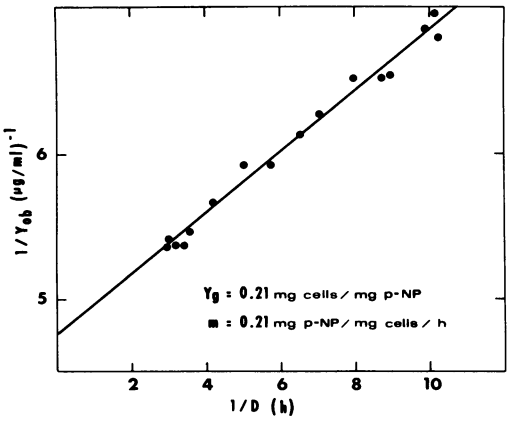

FIG. 6. *p-NP chemostat: $1/Y_{ob}$ versus $1/D$. Double-reciprocal plot of milligrams of dry-cell mass yield per milligram of p-NP in St-1 (Y_{ob}) versus dilution rate (per hour). S_r-p-NP = 720.5 mg of p-NP per liter. Model equation: $1/Y_{ob} = (m/\mu) + (1/Y_g)$ ($D = \mu$, at steady state). Least-squares line: $1/Y_{ob} = 0.21(1/D) + 4.755$, $r^2 = 0.987$, $m = 0.21$ mg of p-NP per milligram of dry-cell mass per hour, $Y_g = 0.21$ mg of dry-cell mass per milligram of p-NP consumed. $s_{y \cdot x} = 0.070$, $s_b = 0.095$, $s_m = 0.014$ ($n = 16$).*

NP per mg of dry cell mass per hour; $Y_g = 0.21$ mg of cells per mg of *p*-NP.

Figure 7 shows a plot of $\bar{x}$ and $\bar{s}$-*p*-NP versus D. The theoretical curve through the data points of $\bar{s}$ versus D was generated from the Monod equation: $\bar{s} = DK_s/(\mu_{max} - D)$. The curve through the points of $\bar{x}_1$ verus D was generated from the Pirt modification of the Monod equation (56): $\bar{x} = DY_g[(S_r - \bar{s})/(mY_g + D)]$; this equation takes the effects of maintenance energy at lower dilution rates into consideration. The experimental washout point (critical dilution rate) was $D_c = 0.53$/h. Notably, $\bar{x}$-*p*-NP dropped to a lower plateau when D reached 0.36/h; at this dilution rate, the steady-state concentration of *p*-NP exceeded 1 µg/ml. *p*-NP is a potent "uncoupler" of oxidative phosphorylation (15). This perhaps accounts for the step decrease in yield at the higher D values.

To determine the maximum *p*-NP dissimilation rate, S_r-*p*-NP was increased in 150-µg/ml increments starting from 250 µg/ml at the dilution rate that gave a maximum $\bar{x}$ (i.e., D = 0.36/h). When S_r-*p*-NP reached 940 µg/ml, the chemostat became unstable; but at D = 0.13/h this same concentration resulted in a steady-state level, suggesting that *p*-NP was still the limiting nutrient. The Monod model for the chemostat predicts that $\bar{s}$ should be independent of S_r for a given D, but, when S_r-*p*-NP was increased, $\bar{s}_1$-*p*-NP increased from <0.25 to 3.5 µg/ml. Grady and Williams (20) reported similar results, in contradiction to the Monod model. The maximum experimental dissimilation rate achieved for *p*-NP (D = 0.13/h and S_r-*p*-NP = 940 µg/ml) was 7 g/liter per day. This rate, compared to the maximum achieved rate for PTN dissimilation (8 g/liter per day), is equivalent to 15 g of PTN per liter per day and indicates that the rate-limiting step in PTN utilization was its hydrolysis.

To determine if the nitro-group of *p*-NP could be used as a sole nitrogen source, the *p*-NP-limited chemostat was operated at D = 0.29/h and S_r-*p*-NP = 674 µg/ml, and the sole exogenous nitrogen source, $(NH_4)_2SO_4$, was withdrawn from the influent salts solution. The cell mass concentration reached a steady-state level 25% lower, indicating that the nitro-group of *p*-NP was utilizable but that Y_{ob} was reduced by 25%. Reinitiation of the nitrogen source resulted in $\bar{x}$ increasing to its original level (Fig. 8).

DISCUSSION

Although the biological treatment of organophosphorus manufacturing wastes was first reported by Coley and Stutz (9) and Lue-Hing and Brady (36), we have presented the first account of an organophosphorus insecticide serving as the growth-limiting substrate in a chemostat, allowing for a quantitative description of its utilization, and the first documented report of PTN utilization by a defined microbial culture, as well as its utilization by symbiotic microbial activity.

Symbiosis. After more than 2 years of growth in continuous culture with PTN as the sole substrate, the acclimated PTN-utilizing culture

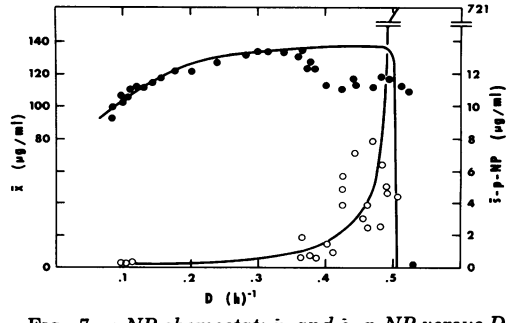

FIG. 7. *p-NP chemostat: $\bar{x}_1$ and $\bar{s}_1$-p-NP versus D. Steady-state dry-cell mass in St-1 (micrograms per milliliter) (●) and steady-state concentration of p-NP in St-1 (micrograms per milliliter) (○) versus dilution rate (per hour). Solid lines are theoretical, generated from the equations: $\bar{x} = [(DY_g)(S_r - \bar{s})]/(mY_g + D) = [(DY_g)/(mY_g + D)][S_r - DK_s/(\mu_{max} - D)]$ and $\bar{s} = (DK_s)/(\mu_{max} - D)$, where $K_s = 0.37$, $\mu_{max} = 0.50$, $Y_g = 0.21$, $m = 0.21$, $S_r = 721.15$.*

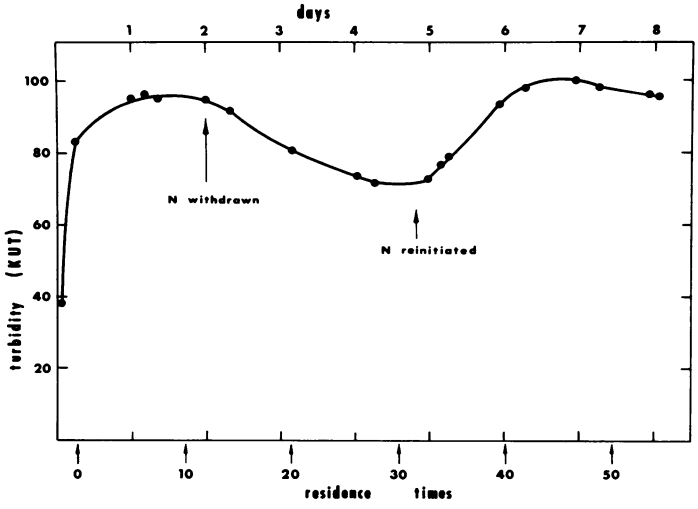

FIG. 8. *Effects of nitrogen limitation on the utilization of p-NP in the chemostat. Steady-state turbidity (KUT) versus days and residence times. Times are marked at which $(NH_4)_2SO_4$ was removed from the minimum salts influent supply (N withdrawn) and at which $(NH_4)_2SO_4$ was returned to the supply (N reinitiated).*

remained a multispecies association. Since competition, as the only interaction between species, would necessarily lead to mutual exclusion of all but one species (30), we assumed that a symbiotic interaction existed. This assumption proved correct when we showed that the PTN was hydrolyzed by a strain of *P. stutzeri* and the resultant *p*-NP was utilized by a strain of *P. aeruginosa*. Neither of these species could fulfill the role of the other, but they could grow symbiotically on PTN in batch and continuous culture. Therefore, *P. stutzeri* probably grew on metabolic products from *P. aeruginosa*. Griffiths and Walker (21) also isolated a pseudomonad that could grow on *p*-NP but could not hydrolyze PTN; Munnecke and Hsieh (46) reported a similar phenomenon. Only once before has the synergistic microbial degradation of an organophosphorus insecticide (i.e., diazinon) been shown, but its utilization for growth was not demonstrated (24).

The exceptionally high dissimilation rate achieved for PTN (8 g/liter per day) is apparently the highest ever reported for a pesticide. This rate is nearly 10,000 times faster than the reported hydrolysis rates in water or seawater under comparable conditions (pH 7.4 to 7.8, 23°C) (C. G. Daughton, Ph.D. thesis, University of California, Davis, 1976.) Notably, this biodegradative process does not result in the end-product formation of a stoichiometric amount of toxic *p*-NP (51), and the low, steady-state *p*-NP concentrations permitted bacterial growth on *p*-NP for the first time at pH <7.0.

Nonoxidative hydrolysis of parathion. The most widely reported mechanism for PTN hydrolysis is oxidative, involving an oxygenated-sulfur complex of PTN (58). Nakatsugawa et al. (48, 49) reported that DETP was released from PTN in eucaryotes primarily by microsomal oxygenases but not by non-oxidative hydrolytic enzymes. The hydrolysis of PTN by "thionase" enzymes was reported by Matsumura and Hogendijk (42) to occur in PTN-resistant houseflies, but this postulated non-oxidative hydrolysis has since been questioned (48, 71). One of the only reports of desulfuration of a thioate to an oxon by a microorganism concerned the intra-cellular desulfuration of Dyfonate to dyfoxon by soil fungi (16). The role of non-oxidative metabolism of organophosphates is largely unknown, except for the reductive reactions that yield such products as aminoparathion. Oxidative desulfuration of a thionate in procaryotes has never been shown, although the absence of the oxon, during metabolism of a thionate, has been noted in nearly all investigations of bacterial (2, 5, 41, 43, 44) and fungal (41, 67) transformations of phosphorothionates.

We conclude that *P. stutzeri* cometabolically hydrolyzes PTN non-oxidatively probably with an exocellular enzyme. Munnecke and Hsieh (47) reported that the hydrolysis of PTN by undefined acclimated bacteria was by an enzyme(s) predominantly associated with the cells. Paris et al. (54) concluded that malathion was hydrolyzed exo (ecto)-cellularly; Clark and Wright (8) concluded that the slightly water-

soluble carbamate propham was hydrolyzed exocellularly by *Arthrobacter* and *Achromobacter* spp. Exocellular hydrolysis of organophosphorothionates by bacteria would explain the absence of oxon formation, due to the unavailability of the requisite oxygenases outside the cell membrane.

Since phosphotriesters may occur naturally as phospholipids (17, 18) and since phospholipases can act at the supersubstrate interface of non-water-soluble substances, we propose that the non-oxidative exocellular hydrolysis of PTN could be mediated by phospholipases. This enzyme class could have particular importance in the hydrolysis of organophosphates in the environment, especially soil, since it has recently been shown that phospholipase production could be a major characteristic of soil actinomycetes (35). Phosphatases, on the other hand, act upon phosphates rather than phosphorothionates as preferred substrates (45), and rarely hydrolyze phosphotriesters (25).

Fate of the dialkyl thiophosphoryl moiety. Even though DETP was found to reach steady-state concentrations in St-1 equimolar to the influent PTN concentration, we hypothesized that perhaps the DETP would support diauxic growth in St-2, where the concentrations of other more preferred substrates would be low. DETP, though, was found to remain unaltered in St-2, regardless of the presence of inorganic phosphate. Wolfenden and Spence (72) reported the isolation of an "*Aerobacter*" *aerogenes* that could utilize DMP, but only as a sole phosphorus source. This is the only report of utilization of an ionic dialkyl phosphate by a defined microbial culture; never has the utilization of an ionic dialkyl thiophosphate been demonstrated, and the only reported instances of dialkyl phosphate utilization have occurred when the compound was the sole source of phosphate (4, 23, 24, 72).

Our results indicate that DETP and DMTP are exceedingly stable, not only to chemical hydrolysis, but also to attack by the highly acclimated PTN-utilizing bacteria. The inability of the PTN-utilizing bacteria to hydrolyze DETP and DMTP was probably due to the lack of or inaccessibility of a suitable phosphodiesterase and the toxicity of the substrate. We have calculated that in California alone (1974 to 1975) a potential 3×10^6 kg of ionic dimethyl and diethyl phosphates and thiophosphates could have been generated in the environment by release from only the organophosphates applied to crops. In addition, these compounds are used in very large quantities in several industries (1, 27).

Ionic dialkyl phosphates and especially thiophosphates are exceedingly unreactive and highly unusual molecules to biological systems (13, 19, 33, 50, 64). They are not without toxicity to eucaryotes. Some ionic dialkyl phosphates are quite toxic to fish, and, more importantly, they can be potent synergists for the toxic action of their parent insecticides (3). Plants have been shown capable of assimilating ionic dialkyl phosphates and biosynthetically reconvert them to cholinesterase inhibitors (52). *O,O*-diisopropyl phosphorothioic acid has been shown to be a persistent fungal hydrolysis product of Kitazin P (67); Masuda and Kanazawa (40) isolated significant quantities of *O,O*-diisopropyl *S*-methyl phosphorothiolate from rice straw and grains and from soils treated with Kitazin P. Methylation of ionic phosphates and phosphonates in biological systems has also been reported (37, 60). The photolysis of PTN and paraoxon was also recently shown to yield stable trialkyl esters (22). Certain trialkyl phosphoesters have proved to be potent potentiators of the toxicity of some organophosphates in warm-blooded animals (55).

In view of these results, concern about the environmental fates of ionic dialkyl phosphates and thiophosphates seems justified. In addition, these results further support the idea that pesticide "nonpersistence" is too often equated simply with "disappearance" of the parent compound (32).

The use of microorganisms in continuous culture could provide another means of determining the possible environmental fates of synthetic xenobiotics; the chemostat could be used as a "model ecosystem." Compounds that would ordinarily prove toxic to microorganisms in batch culture could serve as growth substrates, since low, steady-state substrate concentrations are achievable.

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