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Multiphasic Kinetics for Transformation of Methyl Parathion by *Flavobacterium* Species

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Transformation rates of an insecticide, methyl parathion, in pure cultures of *Flavobacterium* sp. followed multiphasic kinetics involving at least two systems (I and II). System I was a high-affinity, low-capacity system, and system II was a low-affinity, high-capacity system. Data from rate experiments suggested that metabolites formed via system II inhibited system I such that only one system operated at a time. System I operated at approximately 20 μg liter^{-1} and less; system II operated at approximately 4 mg liter^{-1} and less. These results show that xenobiotic chemicals, like naturally occurring substrates, can be transformed via multiple uptake and transformation systems even by a pure culture. Furthermore, computer simulation models of pollutant transformation rates based on kinetic constants determined in this study show that large errors can occur in predicted rates when the multiphasicity of kinetics is neglected.

As part of an effort to determine maximum safe levels of toxic chemicals in natural aquatic systems, the U.S. Environmental Protection Agency is developing and testing mathematical models for predicting the transport and fate of pollutants. For chemicals transformed or degraded via microbial processes, models typically employ rate coefficients based on Michaelis-Menten kinetics for substrate-enzyme reactions (8, 10). More specifically, current models incorporate the assumption that a single set of kinetic constants, including a half-saturation (K_m) value and a maximum velocity (V) value, governs reaction rates for a particular pollutant. For naturally occurring substrates, utilization rates may be determined by multiple systems; hence, multiphasic kinetics maximize uptake rates over a wide range of substrate concentrations (2, 6, 7, 12). Because natural microbial populations have only been exposed to xenobiotic chemicals for a few decades, we might not expect a prevalence of multiple systems specific for xenobiotic chemicals in natural systems. If xenobiotic chemicals are commonly transformed via systems used for naturally occurring substrates, however, transformations of xenobiotics via multiphasic systems may be no less common than for naturally occurring substrates.

Multiphasicity may result either from the presence of different species within mixed populations having systems with different affinities and capacities for the same substrate, or from a single species possessing systems with different affinities and capacities for the substrate. We decided to investigate the latter possibility because multiphasicity in a single species presupposes that an even greater degree of multiphasicity probably exists among mixed populations having numerous species capable of transforming the substrate. We chose *Flavobacterium* sp. and methyl parathion (MP), an insecticide, for these studies because earlier work (9) indicated that this organism and compound may exhibit multiphasic kinetics.

Should multiphasic kinetics exist for transformations of xenobiotic pollutants, predictive models may have to incorporate a range of kinetic constants with corresponding pollution concentration ranges. General trends in the kinetics of multiple uptake and transformation systems may be discovered, however, that would allow estimation of transformation rates over wide ranges of pollutant concentrations with a simple model.

MATERIALS AND METHODS

Culture conditions. *Flavobacterium* sp., isolated from a local river, was identified by Southeast Laboratories, Inc., Atlanta, Ga., and was maintained on nutrient agar (BBL Microbiology Systems) slants. For kinetic experiments, sterile nutrient broth (BBL) that was diluted 1:10 with distilled water was inoculated and then incubated at 20°C in a shaker incubator (180 rpm). After 48 h of incubation, the cultures were centrifuged, and the bacteria were suspended in fresh sterile nutrient broth that was diluted 1:100 with distilled water. This procedure yielded approximately 10^{12} CFU of *Flavobacterium* sp. per liter.

Inhibition of MP transformation by chloramphenicol was tested by incubating cultures that had about 10^{12} CFU liter^{-1} in nutrient broth that was diluted 1:10 with distilled water and contained 100 mg of chloramphenicol per liter. Cultures were incubated for 24 h at 20°C in the chloramphenicol solution before spiking with MP to test for inhibition.

Quantitative procedures. MP concentrations were determined with a Tracor 222 (Southern Analytical Inc., Oakwood, Ga.) gas-liquid chromatograph equipped with a ^{63}Ni detector and a 6-m column (internal diameter, 4 mm) packed with 3% OV-1 on 80/100 mesh Supelcoport. For gas-liquid chromatographic analyses, cultures were extracted with 2,2,4-trimethyl pentane at an extraction efficiency of 96.9% (standard deviation ± 3.3; n = 10).

With nutrient agar (BBL), concentrations of *Flavobacterium* sp. were estimated by total plate counts at 25°C with a 48-h incubation time.

Kinetic experiments. Experiments designed to determine whether loss of MP followed multiphasic kinetics were done by two methods. First, replicate batch cultures were spiked with MP (99.9%; Monsanto Chemical Co., Inc.) and sampled over a short period of time (usually about 20 min), during which only a small fraction of the initial MP concentration was taken up by the microorganisms (Fig.1). Second, single batch cultures were similarly spiked, and loss of MP was

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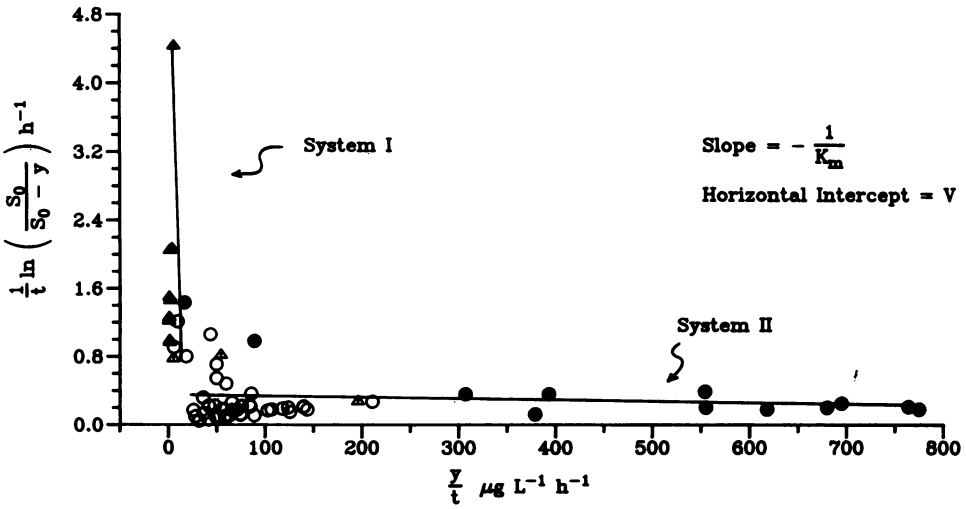

FIG. 1. Integrated Michaelis-Menten plot showing the two systems for MP in *Flavobacterium* sp. Each datum represents 1 of 62 batch cultures sampled for loss of MP for up to 1 h. Replicate cultures spiked with MP concentrations that elicited uptake rates near the intersecting slopes had uptake rates that followed either one slope or the other, showing that only one system operated at a time. Different symbols represent different experiments. Bacterial concentrations ranged from 1.0×10^{12} to 2.5×10^{12} CFU liter^{-1} among experiments; however, no measurable growth occurred during experiments. Data were normalized to 1.8×10^{12} CFU liter^{-1} for direct comparison of slopes and intercepts.

followed for up to 8 h, at which time MP had decreased to undetectable concentrations (Fig. 2 and 3). Before spiking, none of the cultures had been exposed to MP in the laboratory. It is not known whether the organism had been exposed to MP in the field before it was isolated. (Isolation occurred several years before these experiments.) All cultures contained approximately 10^{12} CFU liter^{-1} with sufficiently low concentrations of nutrients (nutrient broth diluted 1:100) such that no detectable growth occurred during the experiments.

Graphical representations of rate data were based on equation 1, in which S_0 is the initial MP (substrate) concentration, and $S_0 - y$ is the concentration at time t (1, 5).

$$\frac{1}{t}\ln\frac{S_0}{S_0 - y} = \frac{V}{K_m} - \frac{1}{K_m} \times \frac{y}{t} \quad (1)$$

This integrated form of the Michaelis-Menten equation allows data from both of our experimental methods to be plotted together for direct comparison of initial transformation kinetics of replicate cultures with kinetic data from the

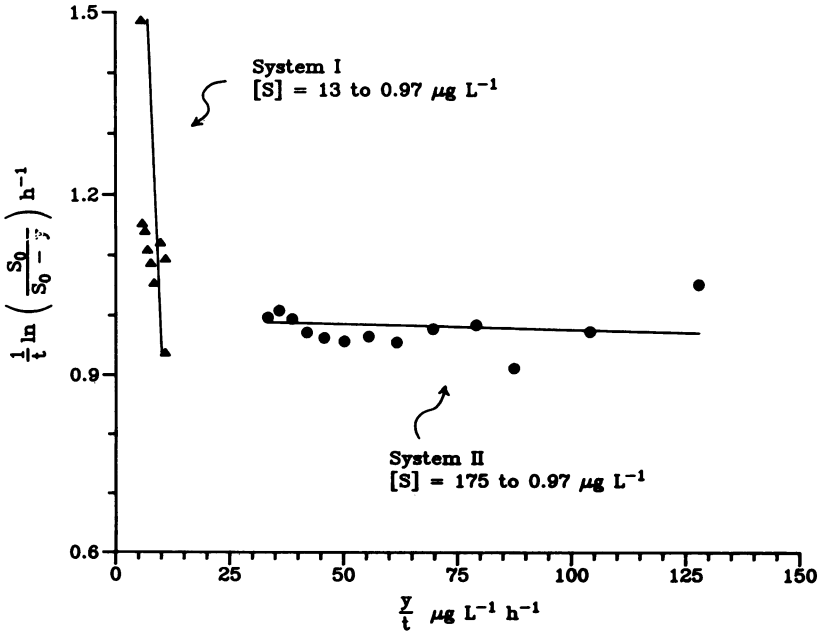

FIG. 2. Progressive transformation of MP by duplicate batch cultures containing $1.5 \times 10^{12} \pm 0.1 \times 10^{12}$ CFU of *Flavobacterium* sp. liter^{-1} spiked with different initial MP concentrations. Once system II was in operation at a high initial MP concentration, MP was transformed to undetectable limits without switching to system I.

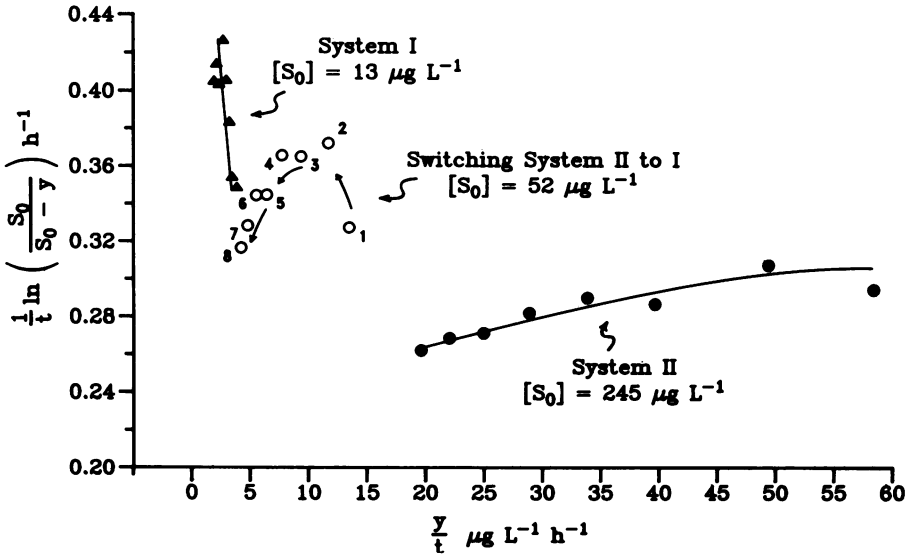

FIG. 3. Triplicate batch cultures of *Flavobacterium* sp. (ca. 10^{12} CFU liter^{-1}) were spiked with various initial MP concentrations, S_0, such that systems I and II transfomed low and high concentrations, respectively. The intermediate concentration was transformed initially by system II, but rapidly switched to system I. The numbers associated with the points indicate from where in the sequence of sampling each point came.

progressive transformation of single batch cultures. When $1/t\ \ln[S_0/(S_0 - y)]$ is plotted against y/t, a straight line is obtained in which the slope equals $-1/K_m$ and the horizontal intercept is V. Slopes and intercepts used to determine kinetic constants for computer simulations (Fig. 4 and 5) were estimated by using least-squares regression. Equation 1, as with all such expressions based on data from in vitro studies of pure enzyme-substrate reactions, is useful for studying rates that conform to Michaelis-Menten kinetics. The application of such equations to whole cell and particularly to mixed culture systems, however, should not be done indiscriminately without consideration of numerous factors that may affect the kinetics of in vivo studies.

The integrated Michaelis-Menten plots were useful for differentiating systems having widely different kinetic constants, although precise determinations of the constants were not possible. Plotting kinetic data from separate experiments over wide ranges of MP concentrations required normalizing the data to a particular bacterial concentration. Estimates of bacterial concentrations are very imprecise indicators of biological reactant concentrations. This imprecision is evident in Fig. 1, where the normalized hollow-circle data fell below the slope of the line indicated by other data for system II.

For single-culture progression experiments, a different

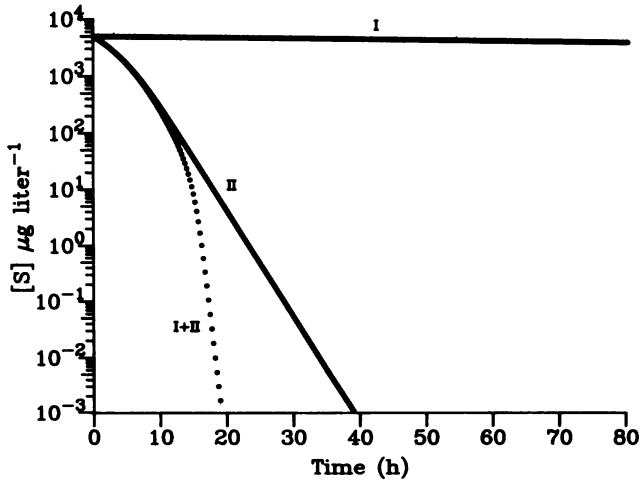

FIG. 4. Computer-calculated data for loss of high initial concentrations of MP in a *Flavobacterium* sp. culture (10^{12} CFU liter^{-1}). The plots include rates of loss based on K_m and V values for systems I and II separately and for rates based on both systems operating simultaneously (I + II).

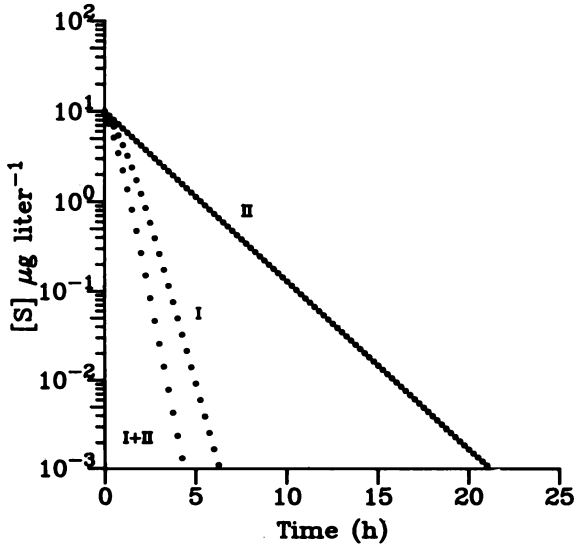

FIG. 5. Computer-calculated data for loss of low initial concentrations of MP in a *Flavobacterium* sp. culture (10^{12} CFU liter^{-1}). The plots include rates of loss based on K_m and V values for systems I and II separately and for rates based on both systems operating simultaneously (I + II).

problem resulted from our experimental condition of low nutrients that had been designed to preclude growth. We assumed that cultures rapidly depleted these nutrients, causing velocities of MP transformation to sometimes decrease with time faster than what first-order kinetics would dictate, such that slopes of $1/t \ln[S_0/(S_0 - y)]$ versus y/t were positive (i.e., K_m was negative) as is evident in Fig. 3. Nevertheless, these procedures consistently distinguished uptake and transformation systems having widely different kinetic constants.

Because our data fit linearized Michaelis-Menten plots, we did not use any of the commonly available programs for nonlinear curve fitting (11). Such programs either require initial estimations of kinetic constants by the user or internally linearize the plots to obtain initial estimates of the constants. The disadvantage of such programs is that they will diverge if initial estimates of the constants are not close enough to the best fit for the data and may converge in areas that do not yield the best estimates of constants. More recent attempts to use the simplex model for estimating such constants hopefully will greatly improve our ability to make conclusions concerning the significance of differences among data sets representing nonlinear processes (4).

RESULTS AND DISCUSSION

Based on rate data from replicate batch cultures, it was evident that loss of MP over a wide range of initial MP concentrations (7.0 to 3,700 μg liter^{-1}) followed kinetics involving at least two uptake or transformation systems (Fig. 1). These uptake or transformation systems are referred to as system I, which was a high-affinity, low-capacity system, and system II, which was a low-affinity, high-capacity system. Analysis of kinetic data with higher initial MP concentrations of up to 10 mg liter^{-1} indicated that a third system existed. Kinetic experiments with whole cells, however, involve considerable errors in addition to those already discussed above, and we were probably able to only resolve a difference in kinetic constants of a factor of approximately 5 or more at the very high MP concentrations. Therefore, the existence of a third system operating between 3.7 and 10 mg of MP per liter was a point of conjecture.

When MP was added to cultures that had been treated for 24 h with chloramphenicol, an inhibitor of protein synthesis, MP transformation initally proceeded at about 20% or less of the rates of untreated cultures, and then transformation rates quickly approached zero within less than 1 h. This inhibition showed that loss of MP was biologically mediated and may have resulted from the inability of the organisms to synthesize new protein and maintain a steady-state concentration of MP-transforming enzymes.

The multiphasicity of *Flavobacterium* sp. response to MP would not have been evident had loss of MP been studied by simply spiking a batch culture with a high MP concentration and following the loss of the compound to lower concentrations. This observation is shown by Fig. 2, which illustrates that once system II was in operation, *Flavobacterium* sp. did not use system I as MP concentrations decreased. Furthermore, kinetic data obtained with replicate cultures spiked with different MP concentrations did not show curvature, which would have indicated the additive effects of multiple systems operating simultaneously. Rather, the data showed that cultures spiked with MP concentrations that elicited uptake or transformation rates near the two intersecting slopes were widely scattered between the slopes (Fig. 1).

Scattering of data near the intersecting slopes for the two systems apparently resulted from cultures switching systems during the course of rate experiments (Fig. 3). Apparently, *Flavobacterium* sp. can switch from system II to system I unless concentrations of metabolites from system II are sufficiently high to inhibit system I (Fig. 2).

The exclusive operation in batch pure cultures of one system or another at a given time probably does not reflect what would happen in natural systems. Microenvironments in natural systems most likely are exposed to a wide range of substrate and metabolite concentrations; therefore, the loss of any substrate would be mediated by all systems operating simultaneously. Such simultaneous operation has been demonstrated by other researchers using naturally occurring substrates in a mixed-organism system (6).

Computer plots of the rates of MP transformation for systems I and II over a wide range of MP concentrations (Fig. 4 and 5) illustrate the importance of multiphasic kinetics with regard to predictive modeling of microbial transformation rates. Using kinetic constants determined at low MP concentrations (system I) to predict MP transformation rates at MP concentrations much higher than K_m for system I (Fig. 4) resulted in gross errors. At MP concentrations below the K_m for both systems (Fig. 5), however, predictions with either set of kinetic constants were within an acceptable range for environmental models. Constants for system I best approximated the net effect of both systems operating simultaneously. The similarity of transformation rates below the K_m among systems is determined by similarities in the ratios of K_m to V. The ratios of K_m to V for systems I and II were within a factor of 3; therefore, transformation rates below the K_m were within a factor of 3 (Fig. 5).

Our results with *Flavobacterium* sp. and MP and the results of other researchers using naturally occurring substrates (2, 6) indicate that the general trend for multiphasic systems is that lower K_m systems have lower values of V and the lowest K_m system has the optimum ratio of K_m to V, therefore producing the most rapid transformation of substrate below the K_m. Based on this generalization, we can at least predict the maximum transformation rate for microbial populations by determining values of K_m and V at very low substrate concentrations and using the ratio of values of K_m to V up to a K_m value equal to environmental concentrations of the substrate. Using this approach for xenobiotic chemicals assumes that natural systems possess a wide range of affinities and capacities for transforming xenobiotics.

With regard to the latter assumption, it seems unlikely that systems that transform xenobiotic chemicals are specific for the xenobiotics. MP and other xenobiotic chemicals are not common substrates in natural systems; therefore, no general selective pressure exists whereby organisms would typically possess systems specific for such chemicals. Bacteria capable of transforming these chemicals can be isolated from most any natural system, however (10a). It seems more likely that xenobiotic chemicals are generally, if not exclusively, transformed by systems normally used for naturally occurring substrates.

This conclusion implies that natural systems, which normally are not saturated by environmental concentrations of naturally occurring substrates, should not be saturated by xenobiotic chemicals that generally occur in much lower concentrations. Therefore, microbially mediated transformation rates of xenobiotic chemicals should be pseudo-first order to chemical concentrations over their entire range of concentrations in natural aquatic systems. This conclusion is disputed by data published by other researchers, who show that transformation rates should approach saturation in

field-collected samples spiked with very low concentrations of xenobiotic chemicals (3, 8). As demonstrated in our experiments with MP, however, only by spiking replicate samples with a very wide range of initial substrate concentrations can the multiphasic potential of organisms be elucidated.

Regardless of various exceptions that may exist for accurately predicting transformation rates by using the ratio of K_m to V, the approach should still be useful for estimating the maximum microbial transformation rate possible for a natural system. Although basing V on total plate counts or direct counts may be improved by basing it on concentrations of protein or other estimates of bacterial biomass, such data are not commonly available to mathematical modelers. Upper limits of total plate counts and direct counts for various aquatic systems are generally known and would probably provide a reasonable approach to estimating the maximum possible microbial transformation velocity of a system, assuming that all counts represent bacteria capable of transforming the chemical. More accurate estimates could be made in cases where the proportions of transformer bacteria to total bacterial concentrations are known. This approach at least provides a screening procedure for evaluating whether microbial processes could be significant in natural systems when compared with rates of physical and chemical processes involved in the transport and fate of the chemical.

In summary, we have demonstrated that a multiphasic system can exist for xenobiotic chemicals even in a pure bacterial culture. Furthermore, we expect that xenobiotic chemicals are generally transformed via systems normally used for naturally occurring substrates and that their transformations commonly obey multiphasic kinetics as do natural substrates. We also have shown that in some cases the multiphasicity of a culture may only be expressed in experiments with replicate cultures spiked with a wide range of initial substrate concentrations. This constraint was evident because metabolites from system II inhibited the expression of system I. It was also evident that batch cultures spiked with a certain range of initial MP concentrations switched systems during the course of experiments. Such data could lead to very confusing results if only that range of substrate concentrations were studied. Finally, the multiphasicity of *Flavobacterium* sp. to MP transformation shows that we cannot assume that environmental systems and subsamples thereof have a single K_m and V for transformations of xenobiotics. Therefore, studies that treat such samples as if they possess a single K_m and V are of dubious value unless the investigators demonstrate the absence of multiphasicity in their studies.

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