Quantitative Structure–Activity Relationships in Bacterial and Abiotic Alkaline Hydrolyses of Para-Substituted Acetanilides

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Quantitative structure–activity relationships were established for bacterial and abiotic alkaline hydrolysis of acetanilide, 4-bromoacetanilide, 4-cyanoacetanilide, 4-fluoroacetanilide, 4-methoxyacetanilide, 4-methylacetanilide, and 4-nitroacetanilide. The influence of ring substituents on hydrolysis rates was investigated using alkaline solutions and pure bacterial cultures isolated from soil. For an *Arthrobacter* species, second-order rate constant, k_b, for bacterial hydrolysis ranged from $(1.09 \pm 0.01) \times 10^{-14}$ to $(2.62 \pm 0.03) \times 10^{-14}$ L organism^{-1} s^{-1} for 4-cyanoacetanilide and acetanilide, respectively, showing little sensitivity to substituent effects. Acetanilides were hydrolyzed to the corresponding anilines. Regression of log k_b with van der Waals radii of individual substituents yielded a correlation coefficient (r^2) of 0.904. Substituent size and geometry, rather than any electronic property, infuenced biologically mediated hydrolysis rates. Alkaline hydrolysis rates were controlled by electronic effects ($r^2 = 0.847$). Biotic and abiotic hydrolysis rates did not correlate ($r^2 = 0.37$), indicating these processes are controlled by different parameters.

INTRODUCTION

The widespread application of acetanilide herbicides such as alachlor, metolachlor, propanil, and propachlor to cropland for weed control has resulted in concern regarding the environmental fate of these chemicals. McClure (1974) has shown microbial hydrolysis to be the primary degradation pathway of acetanilide herbicides in natural ecosystems. Understanding the physical and chemical parameters controlling biological hydrolysis of acetanilides will facilitate models that predict reactivity and transformation rates in nature. Quantitative structure–activity relationships (QSAR) may also aid in the design and synthesis of more effective herbicides possessing optimal selectivity and persistence.

As early as 1961, researchers related microbial transformation rates in the natural environment to chemical structure (Alexander and Aleem, 1961). Linear free energy relationships (LFER) have been generated using rate data for reactions of organophosphate and organophosphorothioate esters (Wolfe, 1980), phthalate esters (Wolfe et al., 1980a,b), carbamate pesticides (Wolfe et al., 1978), and carboxylic acid esters (Paris et al., 1984). Reactions and processes studied were abiotic and microbial hydrolysis, sediment–water partitioning, and biosorption.

Paris et al. (1982, 1983) measured the effects of the molecular structure of a series of para-substituted phenols on bacterial transformation rates using a pure culture of *Pseudomonas putida* U and mixed cultures from pond and river water samples. In both cases, a good correlation was obtained by plotting the log of the second-order bacterial oxidation rate constant vs the van der Waals radius, a steric parameter, of individual substituents. Similar results were obtained in an investigation of meta-substituted anilines (Paris and Wolfe, 1987). A pure culture isolated from river water, as well as a mixed culture from a pond water sample, transformed the anilines to their corresponding oxidative deamination products. Simple linear regression of the van der Waals radii with log k_b gave correlation coefficients (r^2) of 0.924 for the pure culture and 0.99 for the mixed populations. Rate constants for microbial transformations often correlate with abiotic hydrolysis rate constants, suggesting that many biodegradation reactions are enzyme-mediated hydrolyses controlled by similar molecular parameters (Wolfe et al., 1980c).

The present study was conducted to investigate the influence of ring substituents on the rates of bacterial hydrolysis of para-substituted acetanilides by pure cultures isolated from soil. In addition, correlation analysis was employed to determine the physicochemical parameters controlling biolysis of para-substituted acetanilides. Comparisons between biotic and abiotic alkaline hydrolysis rates were made to determine if a relationship exists between the processes.

MATERIALS AND METHODS

Chemicals. All para-substituted acetanilides were purchased commercially and used as received. Purities were reported as >97%. Melting point determinations confirmed compound purity. As well, gas chromatographic (GC) analysis confirmed that there were no extraneous or interfering peaks above background. Acetanilide, 4-bromoacetanilide, 4-fluoroacetanilide, 4-methoxyacetanilide, and 4-methylacetanilide were obtained from Lancaster Synthesis, Inc., Windham, NH. 4-Nitroacetanilide was from Eastman Kodak Co., Rochester, NY, and 4-cyanoacetanilide was purchased from Pfaltz and Bauer, Inc., Waterbury, CT. 4-Chloroacetanilide and 4-methylacetanilide (Lancaster Synthesis) were employed throughout the investigation as external standards for gas chromatographic analysis. Corresponding para-subsituted anilines, purchased as commercially synthesized compounds and used as received, included aniline (Baker Analytical, Inc., Phillipsburg, NJ), 4-methylaniline, 4-bromoaniline, and 4-nitroaniline (Eastman Kodak), and 4-fluoroaniline, 4-methoxyaniline, and 4-cyanoaniline (Aldrich Chemical Co., Milwaukee, WI). Purities were reported to be >98%. The anilines were used as calibration standards for study of hydrolysis reactions to confirm product identity and determine concentrations in the kinetic studies.

Methyl isobutyl ketone (redistilled in glass) from Eastman Kodak was used for solvent extractions of all para-substituted acetanilides and corresponding aniline hydrolysis products. All other chemicals were of the highest purity commercially available and were used as received.

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Hydrolysis Reactions. *Abiotic Alkaline Hydrolysis.* Kinetic experiments on the alkaline hydrolysis of para-substituted acetanilides were initially performed to measure rate constants for 4-bromo-, 4-methoxy-, 4-methyl-, 4-nitro-, 4-cyano-, and 4-fluoroacetanilide and the unsubstituted acetanilide. Sodium hydroxide solutions were used for the specific base-catalyzed hydrolysis of all seven para-substituted acetanilides. To avoid unwanted catalytic effects, hydrolysis reactions were conducted using unbuffered solutions. Standardization of the alkaline solutions was performed at the start of reactions and after generation of kinetic data (2–3 half-lives of hydrolysis), by potassium hydrogen phthalate (KHP) titration using phenolphthalein as indicator.

Kinetic Experiments. All para-substituted acetanilide solutions were prepared at 2.0×10^{-4} M using filtered (0.45 μm) deionized water. Hydrolysis reactions were initiated by pipetting 100 mL of each of the para-substituted acetanilide and sodium hydroxide solutions into glass-stoppered, 250 -mL iodine flasks wrapped in aluminum foil to minimize photolytic effects. Initial solution concentrations after mixing were 0.1 mM acetanilide and 0.1, 0.032, or 0.01 M hydroxide ion. Reaction vessels were immediately placed into a thermostated water bath and maintained at 25.0 ± 0.2 °C. Duplicate reaction flasks for each para-substituted acetanilide were run to determine precision of hydrolysis rate constants. 4-Cyanoacetanilide was selected as a model compound to determine pH dependence of alkaline hydrolysis at hydroxide molarities of 0.1, 0.032, and 0.01.

At each sampling period, a measured portion of the alkaline solution was pipetted into a glass-stoppered test tube, and then an equal volume of MIBK was added to extract the acetanilides and aniline products into the organic phase. Extraction was completed by mixing the sample for 2.0 min at high speed on a vortex mixer. Extraction efficiencies into MIBK were >94% for all seven para-substituted acetanilides over the concentration range 10^{-5}–10^{-4} M. Calculations to determine concentrations of compounds were performed assuming 100% extraction efficiency for both reactants and products in all cases. In a clean test tube, equal volumes of the extract and external standard were combined, and an aliquot of the mixture was analyzed by gas chromatography as described below.

Biotic Hydrolysis. Kinetic experiments on the biolysis of para-substituted acetanilides were performed using pure bacterial cultures isolated from soil and sediment samples collected in Golden, CO.

Isolation and Maintenance of Pure Cultures. Culture tubes containing 20.0 mL of inorganic salts medium amended with 10 mM acetanilide were inoculated with 0.5–1.0 g (wet weight) of soil or sediment. The inorganic salts medium was prepared by dissolving 1.0 g of NH_4Cl, 1.0 g of K_2HPO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 0.01 g of $CaCl_2$ in 1.0 L of deionized water (Stanier et al., 1976). Solution pH was 7.2. Pure cultures of acetanilide-utilizing bacteria were isolated by transferring portions of the enrichment cultures to inorganic salts agar plates containing 10 mM acetanilide. Cultures were successively transferred to ensure purity of the isolates as distinguished by color and morphology of colonies (Krieg, 1981). Four different pure cultures of bacteria demonstrated the ability to utilize acetanilide as a sole source of carbon and energy. Their ability to utilize the remaining para-substituted acetanilides as a sole source of carbon and energy was also demonstrated qualitatively as evidenced by turbid bacterial growth in culture tubes after 5 days of incubation. The bacterial cultures were tentatively identified to the genus level by employing various techniques including colony and cellular morphology, microscopic characterization, and Gram staining (Stanier et al., 1966; Doudoroff and Palleroni, 1974; Rugosa et al., 1974; Palleroni, 1984). The use of two strains of *Arthrobacter* sp. (strains CSMNS and DSMP), a *Bacillus* sp. (strain B.EPA donated from the U.S. EPA, Athens, GA), and a *Pseudomonas* sp. (strain CCS) allowed for a comparison of biolysis kinetics using different genera of bacteria.

Cells used in the kinetic studies were initially grown in 600 mL of medium prepared by combining equal volumes of nutrient broth (Difco, Inc., Detroit, MI), 0.10 M phosphate buffer adjusted to pH 7, and 0.1 mM acetanilide solution. The cultures were incubated for 14 days at 30 °C. Cells were then harvested by centrifugation at 5 °C, washed twice with 0.10 M phosphate buffer to remove excess nutrients, and then resuspended in 20 mL of phosphate buffer and refrigerated until used in kinetic studies less than 24 h later.

Biological Kinetic Experiments. Resting cell experiments were designed to minimize bacterial growth and maintain constant cell concentrations throughout the kinetic studies by limiting available nutrients for cell metabolism. Cultures of the bacterial isolates described above were used in the resting cell experiments.

Preliminary kinetic experiments were performed and bacterial cell concentrations were adjusted to yield half-lives on the order of 3–5 h for the biolysis of acetanilide. Initial cell concentrations for all biolysis experiments were determined by direct microscopic counting using a Nikon type 104 phase contrast microscope and a Neubauer-Helber counting chamber. Bacterial concentrations were reported in organisms per liter. For kinetic studies with the *Bacillus* and *Pseudomonas* cultures, direct microscopic counts were also performed periodically during the experiments to detect changes in cell concentration. Since cell concentrations in kinetic studies using the two *Arthrobacter* strains were too low for direct counting, changes in cell concentration were monitored by plate counts at 0, 4, and 8 h (Krieg, 1981). All counting was performed on reaction mixtures containing the unsubstituted acetanilide. It was assumed that, after reaction vessels containing the other para-substituted acetanilide solutions were spiked, equivalent cell concentrations were present in these, as well. Precision and accuracy for the volumetric cell transfer were significantly greater than the actual cell concentration determinations. Random checks on selected solutions of substituted acetanilides were in general agreement with reported cell concentrations.

Biolysis experiments were performed by combining 50 mL each of 2.0×10^{-4} M para-substituted acetanilide and 0.2 M phosphate buffer solutions in glass-stoppered 250-mL iodine flasks wrapped in aluminum foil to minimize photolytic effects. Reactions were initiated by pipetting 1.0 mL of the concentrated bacterial cell suspension into the reaction vessels which were then immediately placed into a thermostated water bath maintained at 30.0 ± 0.2 °C. Initial concentrations were 1.0×10^{-4} M acetanilide and 5.69×10^{9}, 1.83×10^{9}, 1.01×10^{13}, and 5.90×10^{12} organisms/L for the two strains of *Arthrobacter* sp. (strains CSMNS and DSMP), the *Bacillus* sp. (strain B.EPA), and the *Pseudomonas* sp. (strain CCS), respectively. Neutral pH was maintained in all reaction vessels by the phosphate buffer solution. Duplicate reaction flasks for each para-substituted acetanilide were run to determine the precision of biolysis rate constants. Sample aliquots were taken at 1.0-h intervals, unless otherwise noted. All solution volume transfers were performed using glass pipets heat-sterilized for 20 min at 150 °C.

A sample (4.0 mL) of the reaction mixture was pipetted into a glass-stoppered 20-mL test tube, and then 2.0 mL of MIBK was added to extract the acetanilides and aniline hydrolysis products into the organic phase. Extraction was completed by mixing the sample for 2.0 min at high speed on a vortex mixer. If an emulsion formed upon extraction with MIBK, as it did with the reaction mixtures containing *Bacillus* and *Pseudomonas*, sample test tubes were centrifuged to separate layers. In a clean test tube, equal volume of the extract and the external standard were combined, and an aliquot (1.0 μL) of the mixture was analyzed by gas chromatography as described below.

Analytical Methods. A Hewlett-Packard (HP) 5890A gas chromatograph, equipped with a nitrogen–phosphorus detector (NPD), was employed for the analysis of the series of para-substituted acetanilides and their corresponding aniline hydrolysis products. A capillary column, 25 m × 0.53 mm, coated with cross-linked 5% phenylmethyl siloxane (HP), was used to separate products and reactants. For reactant and product identification and confirmation, retention times relative to para-substituted acetanilide and aniline standards were compared using the same GC system. Zero-grade helium was used as the carrier gas and makeup gas. Flow rates were 5.0 and 25 mL/min, respectively. Hydrogen and air, both of zero-grade, were supplied to the NPD, at flow rates of 3.0 and 100 mL/min, respectively. For the analysis of acetanilide, 4-bromoacetanilide, 4-fluoroacetanilide, 4-methoxyacetanilide, 4-methylacetanilide, 4-cyanoacetanilide, and their corresponding para-substituted aniline hydrolysis products, the initial oven temperature was set at 100 °C. After a holding time

Table I. Summary of Results for the Alkaline Hydrolysis of Para-Substituted Acetanilides

		disappearance of reactant		appearance of product	
compound	σ	k hydrolysis, s^{-1} M^{-1}	$-\log k$ hydrolysis	k hydrolysis, s^{-1} M^{-1}	$-\log k$ hydrolysis
acetanilide	0.000	7.70 ± 0.02 10E–06	5.1135	4.19 ± 0.17 10E–06	5.3778
4-bromoacetanilide	0.232	7.40 ± 0.16 10E–06	5.1308	1.05 ± 0.04 10E–05	4.9788
4-cyanoacetanilide	1.000[a]	3.77 ± 0.19 10E–05	4.4237	4.87 ± 0.02 10E–06	5.3125
4-fluoroacetanilide	0.062	8.34 ± 0.12 10E–06	5.0788	1.13 ± 0.21 10E–05	4.9469
4-methoxyacetanilide	–0.268	8.90 ± 0.10 10E–06	5.0506	5.89 ± 0.41 10E–06	5.2299
4-methylacetanilide	–0.170	7.25 ± 0.04 10E–06	5.1397	6.42 ± 0.10 10E–06	5.1925
4-nitroacetanilide	1.270[a]	1.15 ± 0.04 10E–03	2.9393	1.32 ± 0.10 10E–03	2.8794

[a] Sigma values for 4-CN and 4-NO_2 are from Yukawa and Tsuno (1959). All others are from Jaffe (1953).

Table II. Bacterial Hydrolysis Results for Para-Substituted Acetanilides

		k biolysis (10E14), s^{-1} org^{-1} L			
compound	van der Waals radius, nm	*Arthrobacter* (CSMNS)	*Arthrobacter* (DSMP)	k biolysis (10E17), s^{-1} org^{-1} L *Pseudomonas* (CCS)	k biolysis (10E18), s^{-1} org^{-1} L *Bacillus* (B.EPA)
acetanilide	0.120	2.14 ± 0.02	2.62 ± 0.03	2.60 ± 0.03	8.32 ± 0.20
4-bromoacetanilide	0.185	2.09 ± 0.10	1.63 ± 0.02	2.11 ± 0.02	7.59 ± 0.02
4-cyanoacetanilide	0.320	1.16 ± 0.31	1.09 ± 0.01	1.24 ± 0.10	5.28 ± 0.04
4-fluoroacetanilide	0.147	1.89 ± 0.05	2.04 ± 0.01	3.24 ± 0.17	10.60 ± 0.10
4-methoxyacetanilide	0.260	1.47 ± 0.02	1.44 ± 0.00	1.11 ± 0.18	5.20 ± 0.06
4-methylacetanilide	0.200	1.93 ± 0.11	1.82 ± 0.01	1.56 ± 0.00	7.32 ± 0.04
4-nitroacetanilide	0.259	1.77 ± 0.02	1.57 ± 0.01	1.37 ± 0.04	6.23 ± 0.20

[a] van der Waals radii values are from Bondi (1964) and Charton (1969).

of 1.0 min, the temperature was increased at a rate of 25 °C/min to a final temperature of 250 °C. 4-Chloroacetanilide was used as an external standard. For the analysis of 4-nitroacetanilide and 4-nitroaniline, the initial oven temperature was set at 150 °C. After a holding time of 1.0 min, the temperature was increased at a rate of 25 °C/min to a final temperature of 250 °C. 4-Methylacetanilide was used as the external standard. Signal output from the NPD was connected to an HP 3392A integrator. Integrated peak areas, relative to the external standard, were used to calculate compound concentrations.

Data Analysis. *Abiotic Alkaline Hydrolysis.* Kinetic data were generated to estimate the pseudo-first-order rate constants, k_{obs}, by plotting ln C vs time, where C equals the concentration of the corresponding para-substituted acetanilide. The slope of the line yielded k_{obs}. Second-order rate constants, k_{OH}, were calculated by dividing k_{obs} by the mean hydroxide ion concentration. Quantitative structure–activity relationships were generated by correlating rate data with substituent constants for electronic effects, i.e., Hammet sigma values (Jaffe, 1953). For electron-withdrawing substituents located in the para position, such as 4-CN- and 4-NO_2, sigma values termed σ_p^- (Yukawa and Tsuno, 1959) were employed in the correlation analysis. All values are presented in Table I.

Biotic Hydrolysis. Correlation analysis was also performed to relate fundamental physicochemical parameters of molecular structure with biolysis rate constants. Kinetic data were generated to determine rates of enzyme-catalyzed hydrolysis of the series of para-substituted acetanilides. A modification of the Monod equation describing the metabolism of a single organic carbon source by a pure bacterial culture is illustrated in the empirical rate law (Paris et al., 1975; Banerjee et al., 1984)

$$-d[S]/dt = k_b[B][S] \quad (1)$$

where $-d[S]/dt$ is the change in organic substrate concentration [S] as a function of time, [B] is the bacterial cell concentration or density (organisms/L), and k_b is the second-order rate constant for bacterial metabolism of the substrate. Kinetic data were used to estimate pseudo-first-order biolysis rate constants, k_{obs}, from a least-squares regression of the log of substrate concentration, [S], vs time. Second-order rate constants, k_b, were calculated by dividing k_{obs} by the average bacterial cell concentration, [B], as in eq 2. The units on k_b were s^{-1} org^{-1} L.

$$k_b = k_{obs}/[B] \quad (2)$$

Correlating the negative of log k_b with various substituent constants for steric effects, i.e., van der Waals radii (Bondi, 1964; Charton, 1969), established the quantitative structure–activity relationships for this series of para-substituted acetanilides. Substituent constant values are presented in Table II.

RESULTS

Alkaline Hydrolysis. Table I summarizes results for the alkaline hydrolysis of seven para-substituted acetanilides, showing both the k_{OH} for disappearance of reactant and appearance of product. Mean ± half-range values are reported. A plot of hydrolysis rate vs hydroxide ion concentration for 4-cyanoacetanilide over the range 0.01–0.1 M is linear (r^2 = 0.996), suggesting the alkaline hydrolysis of para-substituted acetanilides is first-order in hydroxide ion over this pH range. Under alkaline conditions, hydrolysis of the amide linkage was the primary rection for all para-substituted acetanilides except 4-cyanoacetanilide, as evidenced by the formation of stoichiometric amounts of the corresponding substituted anilines. Second-order hydrolysis rate constants for the compounds 4-bromo-, 4-fluoro-, 4-methoxy-, and 4-methylacetanilide and the unsubstituted acetanilide ranged from (7.25 ± 0.04) × 10^{-6} to (8.90 ± 0.10) × 10^{-6} s^{-1} M^{-1}, showing little substituent effect. 4-Cyanoacetanilide hydrolyzed more rapidly, yielding a k_{OH} equal to (3.77 ± 0.19) × 10^{-5} s^{-1} M^{-1}. However, the rate of appearance of 4-cyanoaniline was markedly slower (approximately 1 order of magnitude) than the disappearance of the reactant. This suggested a parallel reaction was consuming the reactant. A large substituent effect was observed for the hydrolysis of 4-nitroacetanilide. The second-order rate constant for 4-nitroacetanilide of (1.15 ± 0.04) × 10^{-3} s^{-1} M^{-1} was almost 100 times larger than those of the other five substituted acetanilides studied, suggesting that effects other than electronic or steric influence hydrolysis rate of this substituted acetanilide.

Bacterial Hydrolysis. Figure 1 illustrates the bacterial hydrolysis of 4-methylacetanilide by the *Arthrobacter* (DSMP) and *Bacillus* (B.EPA) species. Enzyme-catalyzed hydrolysis forming the corresponding para-substituted aniline products was the primary reaction observed. An apparent lag phase preceding biotransformation of the substrate was observed with the genera *Bacillus* and *Pseudomonas*. Only active biolysis data were used to generate rate constants for these experiments. After kinetic data were collected, pseudo-first-order biolysis rate

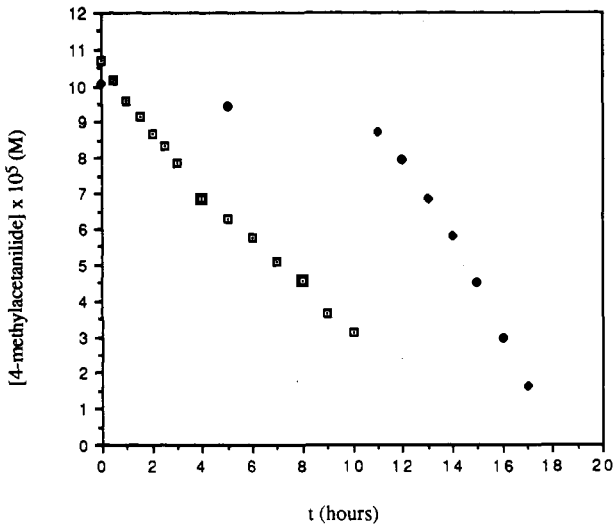

Figure 1. Plot of the disappearance of 4-methylacetanilide in the presence of *Arthobacter* sp. strain DSMP (⊡) and *Bacillus* sp. strain B.EPA (◆).

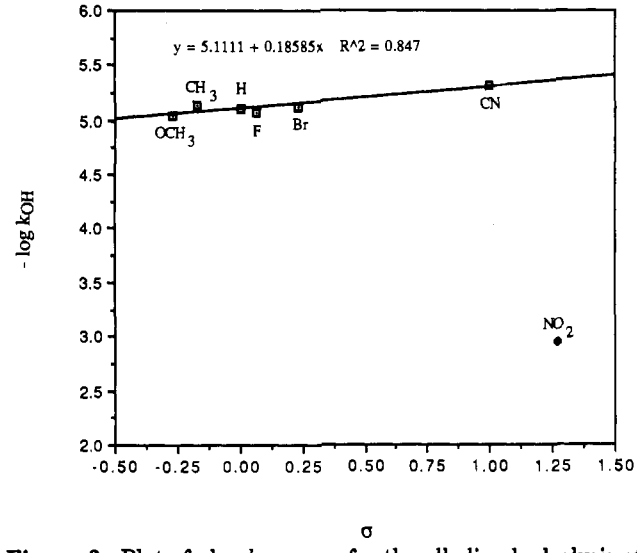

Figure 2. Plot of $-\log k_{OH}$ vs σ_p for the alkaline hydrolysis of para-substituted acetanilides using the rate of appearance of the aniline, rather than the rate of disappearance of the parent compound for 4-cyanoacetanilide. The solid diamond represents 4-nitroacetanilide, which was not included in the regression.

constants, k_{obs}, were estimated from a least-squares regression of the log of substrate concentration, [S], vs time. Second-order rate constants, k_b, were calculated from eq 2. No significant change in cell concentrations was observed throughout the course of resting cell biolysis experiments, as determined by comparing initial and final counts. Thus, average cell concentrations were used to calculate second-order rate constants for bacterial hydrolysis.

Results of the kinetics for bacterial biolysis of seven para-substituted acetanilides using the four different pure bacterial cultures are presented in Table II. Again, the mean ± half-range values of duplicate runs are reported. For the *Arthrobacter* species (strain CSMNS), calculated second-order biolysis rate constants for the seven substrates ranged from 1.16×10^{-14} to $2.14 \times 10^{-1}14$ s^{-1} org^{-1} L. Similar results were obtained for *Arthrobacter*, strain DSMP. The k_b ranged from 1.09×10^{-14} to 2.62×10^{-14} s^{-1} org^{-1} L for the seven para-substituted acetanilides. Substantially smaller second-order biolysis rate constants were noted for both the *Pseudomonas* (CCS) and *Bacillus* (B.EPA) species. Results using *Pseudomonas* CCS yielded k_b ranging from 1.11×10^{-17} to 3.24×10^{-17} s^{-1} org^{-1} L for the seven compounds. For *Bacillus* strain B.EPA, calculated second-order biolysis rate constants for the seven substrates ranged from 5.20×10^{-18} to 10.60×10^{-18} s^{-1} org^{-1} L. In all biolysis experiments, substituents in the para position had little effect on the biotransformation rate of the acetanilide molecule.

DISCUSSION

Alkaline Hydrolysis. Correlation analysis was used to relate the rates of alkaline hydrolysis of the para-substituted acetanilides with physicochemical properties of the substituents. Figure 2 illustrates the correlation between $-\log k_{OH}$ and the Hammett electronic substituent constant, σ_p, for all seven para-substituted acetanilides. A linear free energy relationship (LFER) was established for five of the seven compounds: 4-bromo-, 4-fluoro-, 4-methoxy-, and 4-methylacetanilide and the unsubstituted acetanilide. From the slope of the line, a value of 0.082 for ρ, the reaction constant, was calculated for the alkaline hydrolysis of five para-substituted acetanilides. The small value of ρ reflects low sensitivity to substituent effects, implying a small charge redistribution in the transition state. These results are consistent with those of Bender and Thomas (1961), who showed that the electronic substituent parameter, σ, rather than any steric parameter, controlled the alkaline hydrolysis of para-substituted acetanilides. In their results, a reaction constant, ρ, of 0.1 indicated only a small influence of substituents on hydrolysis rates.

Results for five of the seven substituted acetanilides, including 4-bromo-, 4-fluoro-, 4-methoxy-, and 4-methylacetanilide and the unsubstituted acetanilide demonstrated that the abiotic alkaline hydrolysis of para-substituted acetanilides followed second-order kinetics and was insensitive to substituent effects. Hydrolysis rates were slow, even at high pH. The primary hydrolysis products were the corresponding para-substituted anilines. These products showed hydrolytic stability over the 28-day experiment at hydroxide ion concentrations of 0.01 M, as evidenced by mass balance of parent and product compounds.

For 4-cyano- and 4-nitroacetanilide, $-\log k_{OH}$ and Hammett σ values were not linearly related to those for the other five compounds; thus, these electronic effects do not explain the observed larger rate constants. Resonance of these conjugative substituents cannot account fully for the large k_{OH} noted in this investigation. Different mechanisms or competing reactions for these two compounds may explain such large hydrolysis rate constants. Linear regression using the $-\log k_{OH}$ for the appearance of the hydrolysis product, 4-cyanoaniline, vs Hammett σ results in a reaction constant, ρ, of 0.186 and a correlation coefficient (r^2) of 0.847 for all para-substituted acetanilides, excluding 4-nitroacetanilide. This provides further evidence that the alkaline hydrolysis of para-substituted acetanilides is controlled by electronic effects. For 4-cyanoacetanilide, hydrolysis of the nitrile substituent could account for the 10-fold greater observed rate constant for disappearnce of reactant compared to the rate constant for appearance of the aniline product. Hydrolysis products for the nitrile hydrolysis were not analyzed for chromatographically. The reaction mechanism proposed for the hydrolysis of the nitrile substituent on 4-cyanoacetanilide is adapted from March (1985).

The mechanism for 4-nitroacetanilide hydrolysis under alkaline conditions proposed by Pollack and Bender (1970) involves the formation of an intermediate dianion of the

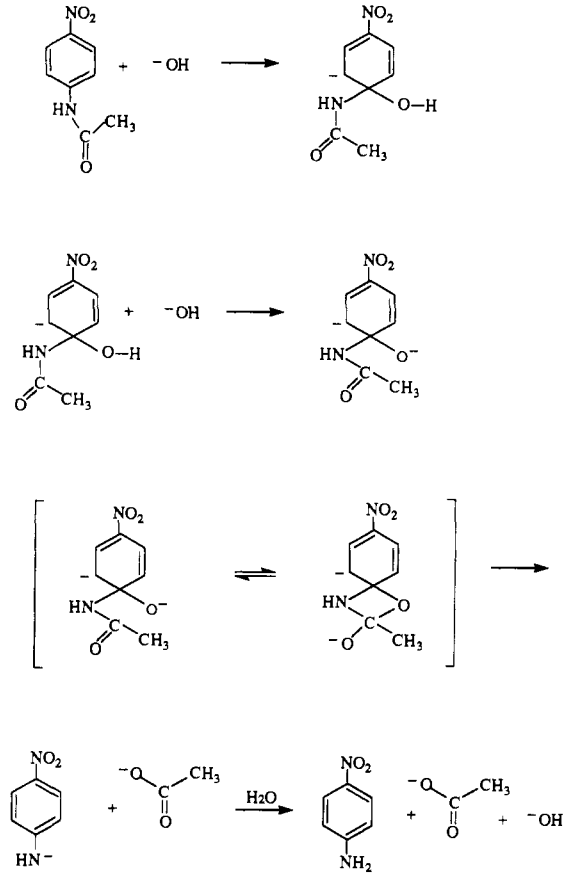

Figure 3. Proposed reaction mechanism for the alkaline hydrolysis of 4-nitroacetanilide [adapted from March (1985)].

amide carbon. This relatively stable intermediate could explain the nearly 100-fold increase in hydrolysis rate for this compound, relative to other para-substituted acetanilides. However, Figure 3 illustrates an alternative to the mechanism proposed by Pollack and Bender for the alkaline hydrolysis of 4-nitroacetanilide, which could also explain the observed rate enhancement. The ring carbon, upon attack by hydroxide ion, could form a dianionic ring complex intermediate stabilized by several resonance structures. Similar reaction mechanisms of analogous 4-nitro aromatics involving the formation of cyclic Meisenheimer complexes as intermediates have been proposed (March, 1985).

Bacterial Hydrolysis. Results demonstrated that common soil bacteria not only have the ability to utilize acetanilide compounds as a sole source of carbon and energy but also are able to transform these compounds initially by mechanisms of enzyme-catalyzed hydrolysis. Relative activity for biolysis of para-substituted acetanilides by bacteria used in this investigation was as follows: *Arthrobacter* ≫ *Pseudomonas* > *Bacillus*. Rate constants differed by less than a factor of 3 for each genus, indicating a small substituent effect on the bacterial biolysis of these seven para-substituted acetanilides. Rate constants calculated from kinetic data for the disappearance of para-substituted acetanilides were used in quantitative structure–activity analyses. Table III summarizes correlations of $-\log k_b$ with physicochemical parameters for kinetic data generated using the *Arthrobacter* strain DSMP. Attempts to correlate second-order biolysis rate constants with physicochemical substituent properties of the para-substituted acetanilide molecules such as pK_a (Harris and Mayes, 1982), $\log K_{OW}$ (Chou and Jurs, 1979), molecular weight, Hansch's π (Hansch and Fujita, 1964; Hansch et al., 1973), Taft's E_s (Taft, 1952), and Hammett's σ (Jaffe, 1953) resulted in linear regressions with correlation coefficients (r^2) of less than 0.50. It is unlikely that diffusion of the acetanilide molecules through the phospholipid cell membrane is controlling the biolysis kinetics, as evidenced by the poor correlations with $\log K_{OW}$ and molecular weight. As well, rates of appearance of product anilines were equivalent with rates of disappearance of the parent compounds. Experiments with free enzymes would show more conclusively whether such mass transfers would be rate-limiting. Nevertheless, a good correlation ($r^2 = 0.904$) was demonstrated with the steric substituent contants, van der Waals radii. The relationship between $-\log k_b$ and van der Waals radii (γ_{vdW}) using the *Arthrobacter* strain DSMP is illustrated in Figure 4.

Structure–activity correlation analysis using γ_{vdW} was performed on biolysis kinetic data from all four bacterial cultures. For the bacterial hydrolysis of this series of parasubstituted acetanilides, second-order biolysis rate constants correlated well with van der Waals radii for all strains tested. The correlations were statistically significant at the 99% confidence level. A summary of the regression equations is as follows:

for *Arthrobacter* strain DSMP

$$-\log k_b = 1.6211\gamma_{vdW} + 13.4273$$
$$r^2 = 0.9040, F = 113.0, P = 0.0001$$

for *Arthrobacter* strain CSMNS

$$-\log k_b = 1.2503\gamma_{vdW} + 13.4944$$
$$r^2 = 0.6171, F = 19.34, P = 0.0009$$

for *Pseudomonas* strain CCS

$$-\log k_b = 2.2149\gamma_{vdW} + 16.2839$$
$$r^2 = 0.7579, F = 37.57, P = 0.0001$$

for *Bacillus* strain B.EPA

$$-\log k_b = 1.3994\gamma_{vdW} + 16.8558$$
$$r^2 = 0.7836, F = 43.45, P = 0.0001$$

The small range of slopes for the correlations of second-order biolysis rate constants with van der Waals radii suggests that the same enzyme-catalyzed hydrolysis mechanism is operative in all genera of bacteria investigated. Thus, a similar hydrolase enzyme or enzyme system may be responsible for the bacterial hydrolysis of para-substituted acetanilides in all cases. Lag phases observed with the genera *Pseudomonas* and *Bacillus* may be attributed to an inducible hydrolase enzyme or enzyme system.

Bacterial hydrolysis has been demonstrated as an important process in the degradation of these chemicals under simulated environmental conditions. This research is consistent with the work of Paris and Wolfe (1987) and Paris et al. (1982), indicating correlations of microbial degradation rates and van der Waals radii. Second-order rate constants for the microbial oxidation of substituted phenols and anilines correlated with van der Waals radii. Correlation analysis using steric parameters including size and geometrical configuration of atoms or groups of atoms could have utility in modeling the environmental fate of para-substituted acetanilides and herbicides of similar molecular structure.

Related research by Steen and Collette (1989) has shown a correlation ($r^2 = 0.962$) of the microbial transformation rate constants of seven amide herbicides and fungicides

Table III. Parameters for and Results of Correlation Analysis

compound	−log k biolysis *Arthrobacter* (DSMP)	correlation parameter: van der Waals radius,[a] nm	pK_a[b]	π	log K_{OW}	E_s	σ	−log k hydrolysis
acetanilide	13.5817	0.120	4.70	0.00	1.16	0.05	0.000	5.1135
4-bromoacetanilide	13.7878	0.185	3.83	0.86	2.02	0.00	0.232	5.1308
4-cyanoacetanilide	13.9626	0.320	1.69	−0.57	0.59	−0.51	1.000	4.4237
4-fluoroacetanilide	13.6904	0.147	4.32	0.14	1.30	0.49	0.062	5.0788
4-methoxyacetanilide	13.8416	0.260	4.69	−0.02	0.79	0.99	−0.268	5.0506
4-methylacetanilide	13.7399	0.200	4.89	0.56	1.82	0.00	−0.170	5.1397
4-nitroacetanilide	13.8041	0.259	1.00	−0.28	0.90	−0.75	1.270	2.9393
correlation coefficient (r^2)		0.9049	0.3534	0.1504	0.1795	0.0592	0.2531	0.1180

[a] van der Waals radii values are from Bondi (1964) and Charton (1969). [b] pK_a values are estimated from Lyman et al. (1982). [c] π values are from Hansch and Fujita (1964) and Hansch and Leo (1979). [d] log K_{OW} values are taken from Chou and Jurs (1979). [e] Es values are from Taft (1952). [f] σ values are from Jaffe (1953) and Yukawa and Tsuno (1959).

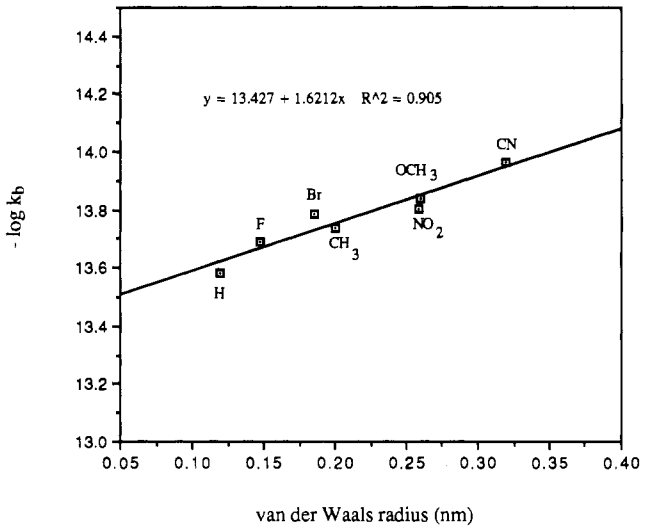

Figure 4. Structure–activity correlation for the bacterial hydrolysis of para-substituted acetanilides.

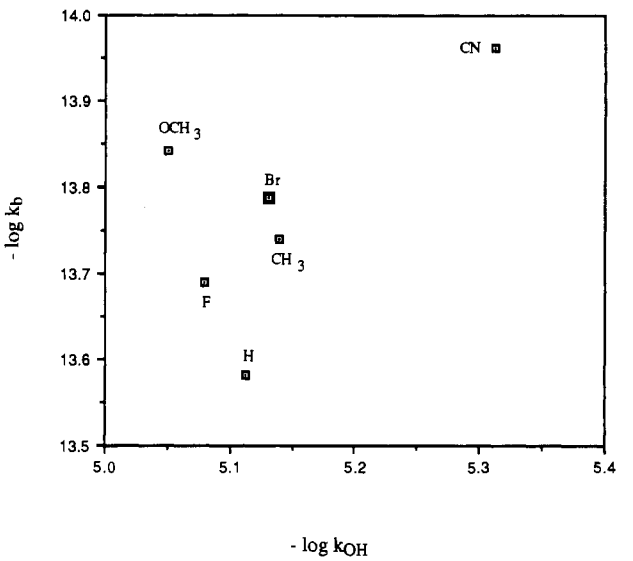

Figure 5. Plot of −log k_{OH} vs −log k_b. Bacterial hydrolysis data were obtained from studies with *Arthrobacter* sp. strain DSMP.

in natural pond water with the infrared carbonyl-stretching frequency of each compound. Apparently, the carbonyl bond strength controls the rate of hydrolysis of the amide linkage. The infrared carbonyl-stretching frequency may therefore be used to predict microbial transformation rate constants of structurally related organic compounds in natural aquatic systems.

Abiotic vs Biotic Hydrolysis. The kinetics of alkaline hydrolysis were compared with bacterial hydrolysis using results from the *Arthrobacter* strain DSMP. Linear regression of the negative logarithm of second-order hydrolysis rate constants (−log k_{OH}, using the rate of appearance of 4-cyanoaniline) vs the negative logarithm of second-order biolysis rate constants (−log k_b) for six of the seven para-substituted acetanilides (excluding 4-nitroacetanilide) gave a correlation coefficient, r^2, of 0.370 (Figure 5). The lack of a correlation suggests that the rates of these two processes are controlled by different physicochemical parameters. Both alkaline hydrolysis and bacterial hydrolysis are largely insensitive to substituent effects for these para-substituted acetanilides. These results contrast with those of Wolfe et al. (1980c), who showed good correlations of second-order alkaline hydrolysis and biolysis rate constants using 1-butoxyl ethyl esters of 2,4-D, malathion, methyl benzoate, methyl anisate, methoxychlor, and chlorpropham and several phthalate esters.

Conclusions. A quantitative structure–activity relationship (QSAR) was established for the bacterial hydrolysis of para-substituted acetanilides correlating second-order biolysis rate constants with the steric parameter, van der Waals radius. The biolysis process showed little sensitivity to substituent effects for these compounds. Abiotic hydrolysis of para-substituted acetanilides at environmental pH's is expected to be very slow, suggesting that degradation by microorganisms, via enzyme-mediated bacterial hydrolysis, is the dominant mechanism of elimination under environmental conditions. On the basis of considerations of substituent size and geometry, microbial degradation rates could be predicted for analogous compounds having the acetanilide structure.

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