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Relationship between Properties of a Series of Anilines and Their Transformation by Bacteria

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The effect of compound structure on the microbial transformation of a series of substituted anilines was investigated. For the pure-culture and environmental water samples studied, the rate of transformation of the compounds decreased in the following order: aniline > 3-bromoaniline > 3-chloroaniline > 3-methylaniline > 3-methoxyaniline > 3-nitroaniline > 3-cyanoaniline. Second-order rate constants (k_b) for each compound was calculated by using bacterial and compound concentrations measured as a function of time. The rate constants correlated with steric parameters. Water samples also were used in kinetic studies with three of the compounds (aniline, 3-chloroaniline, and 3-nitroaniline) to test the relationships with mixed bacterial populations. A simple linear regression of van der Waals radius of the substituent group with log k_b gave correlation coefficients (r^2) of 0.924 for the river isolate and 0.99 for the mixed populations. Analyses of pure-culture and mixed-population samples by thin-layer chromatography indicate that the primary products are catechols. This finding suggests that the transformation pathway involves oxidative deamination of the anilines.

Quantitative relationships between microbial reactivity and the physical or chemical properties of a group of congeners have been reported for selected pesticides, phthalates, phenols, and esters of chlorinated carboxylic acids (1, 22–24, 32). In earlier studies (22) of microbial oxidation of eight phenols, 96% of the variance in the data could be accounted for in the correlation of microbial transformation rate constants and van der Waals radii. Such correlations provide a powerful research tool for assessing the environmental fate of chemicals and for ranking them for more in-depth evaluation. The structure-activity correlation studies reported in this paper include those for aniline and a series of ring-substituted anilines. Anilines were selected because we wanted to study a group of microbially oxidizable single-ring compounds to determine whether they have transformation rate constants that correlate with the van der Waals radii of the substituent groups. Phenols are transformed by monooxygenases incorporating one atom of molecular oxygen, whereas anilines are transformed by diooxygenases incorporating two atoms of molecular oxygen (7, 10).

Anilines are ubiquitous in the environment. They are used in the manufacture of pesticides, dyes, and pharmaceuticals (2, 12, 16, 17). They are also common metabolites of the microbial transformation of nitroaromatic compounds, such as explosives and dinitroaniline herbicides (8, 13, 20, 30). Although they are ubiquitous, very little quantitative data on the microbial transformation of these compounds have been reported in the literature.

The objective of our studies is to establish a mathematical equation relating biological reactivity to some molecular property or properties of a series of compounds. In this paper, we report microbial rate constants for the oxidation of anilines and examine the relationship between steric properties as assessed by van der Waals radius (γ_{vdw}) and microbial reactivity.

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MATERIALS AND METHODS

We monitored the rate of disappearance of aniline and of substituted anilines in reaction vessels containing axenic bacterial cultures or concentrated mixed microbial cultures derived from pond and river waters. Residual levels of the anilines were measured by high-pressure liquid chromatography, and bacterial populations were enumerated by standard pour plate techniques and reported as CFU.

Organism. The bacterium used in these studies was isolated from samples collected from the Oconee River in North Georgia. Cultures were grown in 1:10 nutrient broth that had been amended with 1 mg of aniline per liter and inoculated with 5 ml of Oconee River water per liter and incubated for 48 h. Inocula from cultures were streaked on nutrient agar. Isolated colonies were then transferred to fresh nutrient agar. Nutrient agar transfers were repeated until a pure culture was obtained. Oxi/Ferm tubes (Roche Diagnostics, Div. Hoffmann-La Roche, Inc., Nutley, N.J.) were used in the identification of the isolate. Stock cultures of the organism were maintained as slant cultures on tryptone-glucose extract agar. No tests were made to determine whether the organism could utilize aniline as a carbon source.

Before each experiment, the isolate was cultured in a 6-liter Erlenmeyer flask containing 4 liters of sterile nutrient broth (autoclaved at 121°C for 15 min) and 1 mg of aniline per liter and incubated at 22°C on a reciprocal shaker. After 48 h, the bacteria were harvested, washed three times with sterile dilution water, and suspended in 1 liter of the basal salts medium (pH 6.7) described by Payne and Feisal (25). The suspension contained 1 mg of aniline/liter to allow utilization of endogenous materials and to reduce the lag period. After 24 h, the bacteria were harvested, washed three times with sterile dilution water, and suspended in 20 ml of the basal salts medium. Approximately 2 ml of this bacterial suspension was transferred to a series of 500-ml flasks each containing 200 ml of basal salts medium. The resulting bacterial concentrations were approximately 10^{12} CFU/liter.

High concentrations were used to reduce transformation times for the less reactive compounds.

Field samples. Water samples were collected from two ponds and one river within 10 km of Athens, Ga., and transported to the laboratory in polyethylene bottles. Bacterial populations in water samples were concentrated 10-fold by filtering 10 liters of water through membrane filters (pore diameter, 0.22 μm; Nuclepore Corp., Pleasanton, Calif.) that had been prewashed with 500 ml of distilled water. After filtration, the filters were placed in 1 liter of the original water, and NH_4Cl (0.5 g), $(NH_4)_2SO_4$ (0.5 g), Na_2HPO_4 (0.05 g), KH_2PO_4 (0.05 g), $MgSO_4$ (0.001 g), and $FeCl_3$ (0.001 g) were added from sterile aqueous stock solutions. These bacterial suspensions were incubated overnight at 22°C in a temperature-controlled shaker before each experiment was begun. This procedure enhances the bacterial population 10- to 100-fold (21).

Liquid chromatography. Aniline substrates and metabolic products were measured by using a liquid chromatograph (no. 970A; Tracor Instruments, Austin, Tex.) equipped with an octadecylsilane-2 column (Whatman, Inc., Clifton, N.J.) and with a UV detector. Samples from reaction vessels were centrifuged (4,000 × *g*) for 20 min before analysis. The mobile phase for measuring the anilines during a kinetic study was acetonitrile-water (40:60, vol/vol) and that for identifying the microbial products was acetonitrile-water-acetic acid (35:64.8:0.2, vol/vol/vol). The flow rate was 2 cm^3/min, and the pressure was 1,800 lb/in^2. The wavelength was 227 nm for detecting the anilines, 272 nm for detecting catechol, 4-methylcatechol, and 4-chlorocatechol, and 250 nm for detecting 4-nitrocatechol. The wavelengths for analyses of metabolites of 3-cyanoaniline, 3-bromoaniline, and 3-methoxyaniline were those used for analyses of the parent compounds.

Thin-layer chromatography. Reaction mixtures were adjusted to pH 2 with 6 N hydrochloric acid and extracted twice with ethyl acetate. Products in the extract were separated by thin-layer chromatography on plates coated with silica gel and fluorescent indicator. The plates were developed in solvent mixtures containing petroleum ether, benzene, and acetic acid at a ratio of 1:2:1 (vol/vol/vol) and chloroform, ethanol, and 0.1 N sodium hydroxide at a ratio of 100:5:1 (vol/vol/vol). Compounds were detected by using a UV chromatographic viewer and by spraying the plates with ninhydrin followed by heating at 100°C for 5 min.

Experimental design Individual experiments with the isolate or enriched field samples were initiated by the addition of a 10- to 20-μl acetone solution of each compound to the basal salts medium or concentrated field sample. All compound concentrations were 1 mg or less per liter of water. Autoclaved and filter-sterilized water samples served as controls for measurement of abiotic processes. Control experiments without added anilines served to detect changes in bacterial population that were not due to the presence of the anilines. Bacterial levels were enumerated at prescribed times by pour plate counts. Biological activity was terminated in the 5-ml samples for chromatographic analysis by the addition of 50 μl of a 37% Formalin solution.

Concentrations of residual anilines were calculated from data obtained by high-pressure liquid chromatography analysis of centrifuged samples. At the end of each experiment, organic extracts of the flask contents were analyzed by thin-layer chromatography and liquid chromatography to identify products.

Transformation kinetics. Pseudo-first-order rate constants (k_1) were obtained from a least-squares regression of the plot of log substrate concentration versus ti ıe (Fig. 1). If there was a lag period, the data were not used in the regression. Only the portion of the curve showing active transformation was used. Second-order rate constants (k_b) were obtained by dividing k_1 by the average bacterial concentration in organisms per liter, where an organism is the unit used in this paper and has been used previously (21 to 24) for bacterial concentration in the rate constant k_b. For the enumeration method used, however, CFU or cell is more fitting. The bacterial populations did not change significantly during the experiments, and the changes were random and were not indicative of bacterial growth. The averaged bacterial con-

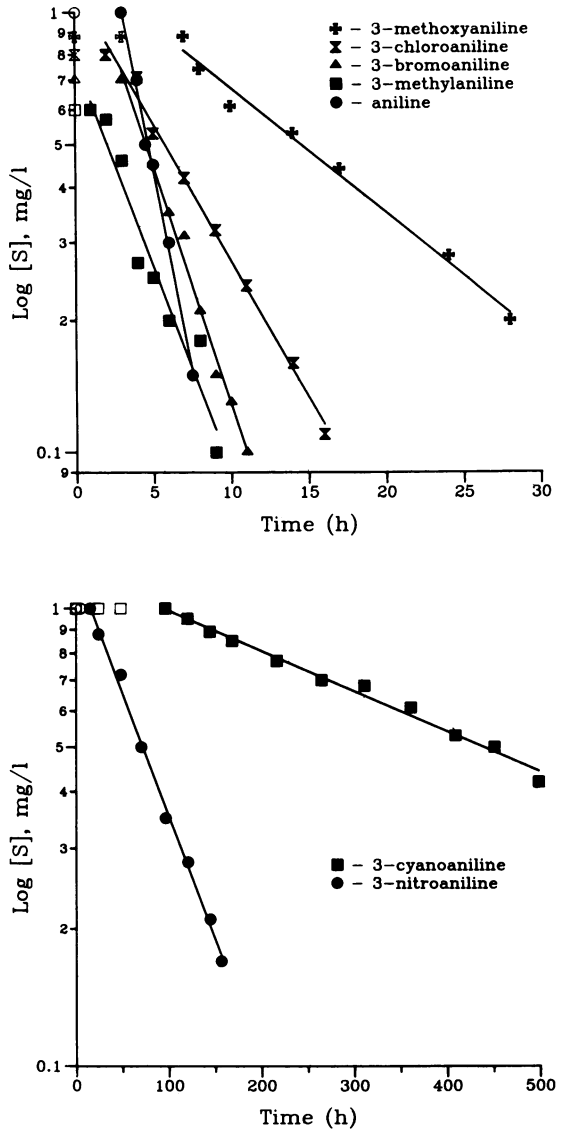

FIG. 1. Log concentration versus time for the disappearance of the seven compounds investigated. Open datum points represent lag periods, and closed datum points represent active transformation periods. The bacterial population for each compound in these experiments (in CFU $liter^{-1}$) (A) 3-methylaniline, 5.6×10^{12}; aniline, 1.4×10^{12}; 3-methoxyaniline, 7.5×10^{12}; 3-bromoaniline, 3.2×10^{12}; 3-chloroaniline, 2.6×10^{12}; (B) 3-cyanoaniline, 8×10^{12}; 3-nitroaniline, 6.7×10^{12}.

TABLE 1. Rate constants (k_b) and selected physical properties of test compounds

Compound	k_b[a] (liter organism^{-1} h^{-1})	$t_{1/2}$[b] (h)	γ_{vdw} (10^{-8} m)	pK_a
Aniline	$(2.1 \pm 1.3) \times 10^{-13}$	0.3	0.12	4.6
m-Methylaniline	$(1.7 \pm 1.3) \times 10^{-14}$	4.0	0.17	4.8
m-Chloroaniline	$(2.3 \pm 1.5) \times 10^{-14}$	3.0	0.18	3.5
m-Bromoaniline	$(3.7 \pm 2.2) \times 10^{-14}$	2.0	0.19	4.0
m-Methoxyaniline	$(5.0 \pm 1.9) \times 10^{-15}$	14.0	0.26	4.3
m-Nitroaniline	$(1.2 \pm 0.8) \times 10^{-15}$	58.0	0.26	2.5
m-Cyanoaniline	$(2.1 \pm 0.9) \times 10^{-16}$	330.0	0.32	2.8

[a] Mean ± standard deviation of 10 determinations.
[b] $t_{1/2}$ calculated assuming 10^{13} organisms liter^{-1}.

centration values, therefore, were used for the rate expression.

Materials. Aniline, 3-nitroaniline, 3-cyanoaniline, 3-chloroaniline, 3-methoxyaniline, 3-methylaniline, and 3-bromoaniline were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.; 4-methylcatechol, 4-nitrocatechol, and catechol were purchased from Pfaltz and Bauer, Inc., Waterbury, Conn.; 4-chlorocatechol was purchased from CTC Organics, Atlanta, Ga. Analytical thin-layer chromatographic plates precoated with Silica Gel F were from Analtec, Inc., Newark, Del. Organic solvents were pesticide grade, distilled in glass (Burdick and Jackson, Inc., Muskegon, Mich.).

RESULTS

The isolate from the Oconee River was a yellow-pigmented, mucoid, gram-negative, oxidase-positive rod. With the Oxi/Ferm tube system, negative results were observed for anaerobic dextrose, arginine dihydrolase, nitrogen gas production, xylase, indole, and citrate. Only the aerobic dextrose and urea tests were positive.

The results demonstrate that the isolate was capable of transforming the selected anilines (Table 1). The calculated half-lives of the compounds range from 0.3 h for aniline to 330 h for 3-cyanoaniline. The concentration of the test anilines in sterile controls did not change during the course of the experiment.

In Table 2, mean values for the disappearance rate constants of three selected compounds (aniline, 3-chloroaniline, and 3-nitroaniline) in field samples are shown. The values ranged from 4.5×10^{-13} liter organism^{-1} h^{-1} for 3-nitroaniline to 1.1×10^{-11} liter organism^{-1} h^{-1} for aniline. For organisms in these samples and for the Oconee isolate, the rate constants of similar compounds were in the same relative order.

Thin-layer chromatography and liquid chromatography (Table 3) showed that catechol, 4-methylcatechol, 4-chlorocatechol, 4-nitrocatechol, 4-methoxycatechol, 4-bromocatechol, and 4-cyanocatechol were the intermediates of the metabolism of aniline, 3-methylaniline, 3-chloroaniline, 3-nitroaniline, 3-methoxyaniline, 3-bromoaniline, and 3-cyanoaniline, respectively. Only trace levels were found in earlier samples, and none was found in the later samples. None was detected in the controls. Samples of the first four compounds were cochromatographed with authentic standards of the catechols in two different solvent systems. The ratio of R_f values for the products and parent compounds was 0.36 ± 0.06 in the chloroform–ethanol–0.1 N sodium hydroxide (100:5:1, vol/vol/vol) solvent system. In the petroleum ether-benzene-acetic acid (1:2:1, vol/vol/vol) solvent system, anilines with pK_as greater than 4.0 (aniline, 3-methylaniline, and 3-methoxyaniline) had lower R_f values than anilines with pK_as of 4.0 and below. R_f values of the four catechol standards ranged from 0.25 to 0.29, whereas those of the corresponding anilines ranged from 0.09 to 0.41. The wide range in aniline R_fs resulted in two ratio groups. Ratios of aniline and 3-methylaniline were used for comparison of 3-methoxyaniline products, and ratios of 3-chloroaniline and 3-nitroaniline were used for the other two compounds. The absence of other products from the samples, as well as the fact that aniline-grown isolates could oxidize the respective catechols (catechol, 4-chlorocatechol, 4-methylcatechol, and 3-nitrocatechol) without a lag, suggests that catechols are intermediates in the metabolism of anilines. Other re-

TABLE 3. R_f values of anilines and catechols in two solvent systems (thin-layer chromatography) and liquid chromatography retention times

Compound	R_f		Liquid chromatography[c] R_T (min)
	Solvent system 1[a]	Solvent system 2[b]	
Catechol	0.26	0.23	2.1
Aniline	0.09	0.58	6.7
4-Methylcatechol	0.29	0.24	2.3
3-Methylaniline	0.10	0.59	7.8
4-Chlorocatechol	0.27	0.20	3.7
3-Chloroaniline	0.40	0.63	9.5
4-Nitrocatechol	0.25	0.16	2.9
3-Nitroaniline	0.41	0.55	5.6
X[d]	0.38	0.22	2.5
3-Methoxyaniline	0.13	0.60	7.3
X[d]	0.17	0.25	4.1
3-Bromoaniline	0.29	0.62	11.8
X[d]	0.21	0.19	2.1
3-Cyanoaniline	0.36	0.57	4.0

[a] Ether-benzene-acetic acid at 1:2:1.
[b] Chloroform–ethanol–0.1 N sodium hydroxide at 100:5:1.
[c] See Materials and Methods for liquid chromatography conditions.
[d] X is a metabolite for which we had no authentic sample.

TABLE 2. Bacterial transformation rate constants and half-lives for three anilines and three sites

Compound	k_b (liter organism^{-1} h^{-1})[a] at:			Mean k_b for all sites	$t_{1/2}$[b] (yr)
	Oconee River	Hickory Hills Pond	Memorial Park Pond		
Aniline	$(1.1 \pm 0.8) \times 10^{-11}$	$(1.9 \pm 1.8) \times 10^{-11}$	$(1.4 \pm 0.4) \times 10^{-11}$	$(1.1 \pm 0.8) \times 10^{-11}$	0.07
3-Chloroaniline	$(4.1 \pm 3.2) \times 10^{-12}$	$(1.3 \pm 0.9) \times 10^{-12}$	$(1.1 \pm 1.0) \times 10^{-12}$	$(2.2 \pm 1.7) \times 10^{-12}$	0.4
3-Nitroaniline	$(4.1 \pm 1.5) \times 10^{-13}$	$(4.5 \pm 2.7) \times 10^{-13}$	$(4.6 \pm 2.3) \times 10^{-13}$	$(4.6 \pm 0.1) \times 10^{-13}$	1.7

[a] Mean value of eight determinations per site ± standard error of the estimate.
[b] $t_{1/2}$ calculated from mean k_b assuming a bacterial population of 10^8 liter^{-1}.

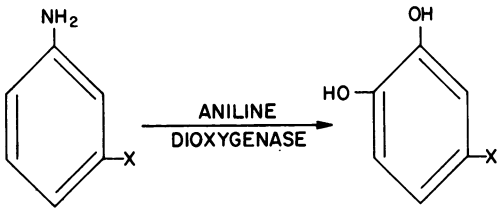

FIG. 2. Intermediate catechol formation in the bacterially mediated oxidation of aniline.

searchers (11, 15, 28, 31, 34) have reported catechol and 4-chlorocatechol as metabolites of bacterial transformation of aniline and 3-chloroaniline. No reports on metabolites of bacterial degradation of the other five anilines were found.

DISCUSSION

The common transformation pathway for the anilines in this investigation, as indicated by the product studies, enables us to use our kinetic data to develop a structure activity relation. The trace levels of the catechols produced from the anilines shown in Fig. 2 have been reported (15, 28, 34) previously as intermediates in the pathway of aniline degradation. Although the anilines have *meta* substituents and the products have *para* substituents, no shift of the substituent (X) occurs as has been reported for monooxygenases (7). Reber et al. (28), in their studies on the metabolism of chloroanilines, reported 3-chlorocatechol as an intermediate product of 2-chloroaniline and 4-chlorocatechol as an intermediate metabolite of 3-chloroaniline and 4-chloroaniline. You and Bartha (33) reported a similar finding for 3,4-dichloroaniline, with 4,5-dichlorocatechol being the transformation product.

In these studies, the presence of a Br or Cl atom decreased the rate of aniline disappearances 5- and 10-fold, respectively, whereas Reber et al. (28) and McClure (19) reported that a Cl atom decreased aniline disappearance rates only 2- and 4-fold. Shukat et al. (29) also found that a *Rhodococcus* sp. was active in degrading chloroanilines. From their results, we calculated that the disappearance rate constant for 3-chloroaniline was 2.9×10^{-14} liter organism^{-1} h^{-1}. The rate constant value for the Oconee River isolate cultures, 2.3

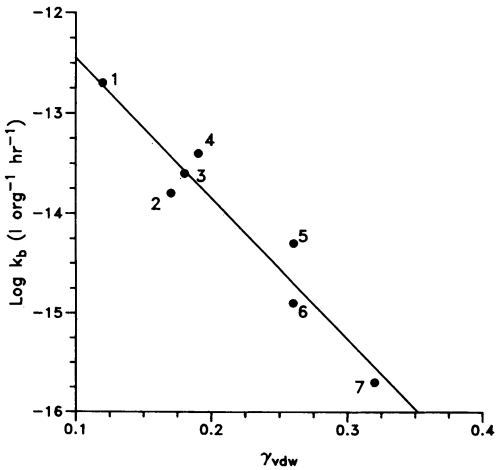

FIG. 3. Correlation of microbial transformation rate constants with van der Waals radii. Compounds: 1, aniline; 2, 3-methylaniline; 3, 3-chloroaniline; 4, 3-bromoaniline; 5, 3-methoxyaniline; 6, 3-nitroaniline; 7, 3-cyanoaniline.

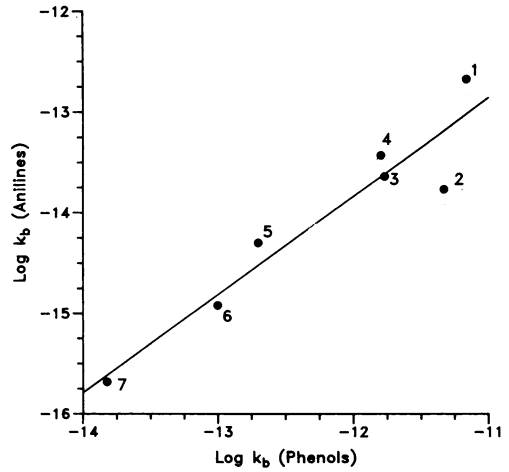

FIG. 4. Relationship between microbial transformation rate constants for aniline and rate constants for phenols. Compounds are anilines and phenols with the following ring substitutions: 1, —H; 2, —CH_3; 3, —Cl; 4, —Br; 5, —OCH_3; 6, —NO_2; 7, —CN.

$\times 10^{-14}$ liter organism^{-1} h^{-1}, is similar. Zeyer et al. (34) reported that a *Moraxella* sp. utilized aniline and 3-choroaniline as sole sources of carbon and nitrogen.

In our studies the methyl, methoxy, nitro, and cyano groups had a greater effect on the disappearance rate constants than did the halogen substituents. These groups reduced the rate constants 12- to 1,000-fold. Only a few reports (4, 18, 34) on the bacterial degradation of anilines with methyl, methoxy, or nitro substitutions were found in the literature. Chambers et al. (4) reported that a mixture of several bacterial species adapated to phenol oxidized 3-nitroaniline to a very limited extent but little if any activity was observed with 4-nitroaniline. In contrast to our results, Malaney (18) reported that 3-and 4-methylanilines are oxidized as readily as aniline by aniline-acclimated sludge; the NO_2 group, however, markedly increased the resistance of the molecule to biological degradation, and degradation was four times slower than for aniline.

On the basis of earlier studies (22, 24) with a series of phenols, it was assumed that the steric properties of the anilines would be related to their transformation rates. A simple linear regression (Fig. 3) of van der Waals radius (γ_{vdw}), a measure of the steric bulk of the substituent, with log k_b, the bacterial transformation rate constant, gave a correlation coefficient of 0.924 and resulted in equation 1.

$$\log k_b = (-11.0 \pm 0.4) - (14.1 \pm 1.8)\gamma_{vdw} \quad (1)$$

where γ_{vdw} is the van der Waals radius (in 10^{-8} meters). The values are given plus or minus the standard errors of the estimates from the least-squares analyses. The correlation coefficient suggests that our assumption was correct. Although the enzyme systems for transformation of phenols (monooxygenase) and of anilines (dioxygenase) are different (7, 10), it appears that in our studies, the steric effects control the relative bacterial oxidation rates of the aniline and phenol. The slopes of the two groups of compounds are the same (Fig. 4). The mathematical description of this relationship is shown in equation 2.

$$\log k_b \text{ (an)} = (0.98 \pm 0.14)\log k_b\text{(ph)} - (2.14 \pm 1.73) \quad (2)$$

where k_b is the transformation rate constant (in liter organism^{-1} hour^{-1}), (an) is aniline, and (ph) is phenol.

The correlation coefficient is 0.905. For any single-ring compound, regardless of the degradation process, the specificity of the active site may control the rate of the reaction. Bulky substituents not fitting well and binding poorly could decrease the rate of bacterial reactivity.

Substitution of an amino group for a hydroxyl group decreased the biotransformation rate constants approximately 100-fold, as shown by the constant (−2.14) in equation 2. Pitter et al. (26, 27), studying the relation between structure and biodegradability of several phenols and anilines, reported that an amino group, as well as a nitro group, can have a negative effect on the ability of the compound to be degraded. Because the steric and hydrophobic values of the hydroxyl and amino groups are close, the 100-fold difference between phenol and aniline rate constants is probably due to the different organisms used in the studies and the different enzymes active in the transformations.

Knackmuss (14), Dorn and Knackmuss (6), and Gibson (7) have suggested that some oxygenases function by electrophilic attack and, as a result, have only weak activity for substrates carrying electron-withdrawing substituents. Knackmuss (14) also has attributed the biological persistence of halogenated aromatic substances to sterical substituent effects. Our studies and those of Zeyer et al. (34) with a *Moraxella* sp. indicate that the rates of transformation of the substituted anilines correlated better with the size of the substituent than with its electron-withdrawing effect. Zeyer et al. (34) reported that small substituents like hydrogen or fluorine (γ_{vdw}, 0.12 and 0.135, respectively) allowed a fast turnover of the anilines, whereas bulky substituents like a methyl group or iodine (γ_{vdw}, 0.2 and 0.215, respectively) prevented rapid metabolism. Our data (Table 2) show the same trend in the rate constants. Zeyer et al. (34) used mainly *para*-substituted anilines, whereas the anilines used in this study were all *meta*-substituted. Zeyer et al. (34) reported a maximum γ_{vdw} value of 0.2 for the methyl group. We used a minimum value of 0.17 in the phenol study (22, 24) and in this study on the basis of the assumption by Charton (5) that for correlating E_s (the Taft steric parameter) values with γ_{vdw}, a minimum value was better. Charton (5) reasoned that a minimum value would be appropriate because a group would be oriented in such a way as to minimize the degree of steric interaction. Hansch and Leo (9) have stated that because one has little or no idea of what to expect in steric effects in biological correlation analysis, one must generally try both parameters to discover which yields the best fit. The minimum was the best fit for their data and ours. Zeyer et al. (34) have stated that the oxygenase activities correlated with the size of the substituents, but they did not develop an equation describing this relationship. Their data (34) for *para*-substituted anilines (except iodine) and the γ_{vdw} values given by Charton (5) (—OCH_3 value from reference 22) gave a good correlation ($r^2 = 0.86$) and resulted in equation 3:

$$\log k_1 = (-0.06 \pm 0.05) - (1.93 \pm 0.41)\gamma_{vdw} \quad (3)$$

where $\log k_1$ is the log of substrate (milligrams) removed $hour^{-1}$.

Aniline, 3-chloroaniline, and 3-nitroaniline were selected for the environmental water studies because the rate constant values for these compounds had a broad range in the pure-culture studies. The transformation rate constants were approximately 2 orders of magnitude higher than in the pure-culture studies (Table 2); the response, however, of the bacteria in both systems to changes in the molecular structure was very similar, as indicated by the slopes of the regression (equation 1) and in the equation for the field samples

$$\log k_b = (-9.83 \pm 0.17) - (9.78 \pm 0.89)\gamma_{vdw} \quad (4)$$

where k_b is the second-order rate coefficient for bacterial transformation of the anilines. This phenomenon has been observed previously in this and other laboratories (3, 23, 29). The presence of a carbon source in the water sample could contribute to the differences observed. Schukat et al. (29) reported that utilization of glucose or of aniline stimulated the degradation of 3-chloroaniline by aniline-grown cells of *Rhodococcus* sp. in 0.1 M phosphate buffer (pH 6.9).

As the data base increases for bacterial oxidative reactions and as these data are included in the correlations, it will become possible to use more than one molecular parameter. This would better define the effect of the different parameters on bacterial oxidation processes, thus expanding and enhancing the usefulness of this predictive tool.

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