Structure-Biodegradability Correlations Among Xenobiotic Industrial Amines

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ABSTRACT

Using acclimated sewage sludge enrichment cultures, 60 amines with potential uses in industrial processes were evaluated for their biodegradability. Biodegradation was measured as cell protein yield with 0.05% amine serving as the sole source of carbon, nitrogen and energy in a mineral solution. Inhibitory properties of the amines, measured by their ability to decrease protein yield on a glucose medium, were unrelated to biodegradability. Under the specified test conditions, all amines that contained tertiary carbon atoms were recalcitrant. Ring N-substituted heterocyclics were readily degraded, but ring C-substituted piperidines, with the exception of pipecolinic acid, were not. When amines lacked other functional groups, tertiary amino groups hindered degradation. Amines with alkyldiamine moieties shorter than propyl did not serve as growth substrates. Predictive generalizations of this type about biodegradability serve as an aid in selecting environmentally safe compounds for industrial processes.

INTRODUCTION

Xenobiotic industrial amines are produced in the US on the scale of millions of metric tons per year (1). Some of their major uses include polymer (nylon) manufacture and the scrubbing of acidic compounds (CO_2, H_2S) from natural gas. They are used as corrrosion inhibitors and antioxidants in metal working lubricants, engine coolants and gasoline, as tanning agents for animal hides and finishing agents for textiles. They also serve as solvents, chelators, emulsifiers, plasticizers and catalysts in a variety of processes and products. Considering the volume and diversity of their uses, the routine and accidental release of industrial amines into the environment appears unavoidable. Nevertheless, the assessment of their environmental impact is still in a very preliminary stage (2).

Concern over the potential impact of large-scale accidental spills involving gas-scrubbing amines that are either in current use or have a potential for future use gave the initial impetus for this study. Other available xenobiotic amines were included to explore more fully some of the emerging structure-biodegradability correlations. This study intends to arrive at some predictive conclusions concerning the biodegradation of xenobiotic amines. Biodegradation being the most crucial parameter of environmental safety next to technical and cost factors, this feature should be taken into consideration when selecting amines for large-scale industrial uses.

EXPERIMENTAL PROCEDURES

Materials

For brevity, the tested amines are referred to by code number (Figs. 1-5). They were obtained from the following sources: Abbott Labs (North Chicago, IL): A7, A13, A14, A21, A24, A28, A29, A39, A40, A41, A45, A46, A51; Aldrich Chem. Co. (Milwaukee, WI): A1, A3, A4, A5, A8, A9, A10, A11, A31, A32, A33, A37, A38, A43, A50; Chem. Procurement Labs (College Point, NY): A6, A17;

[1] Present address: Merck, Sharp & Dohme Research Laboratories, P.O. Box 2000 R80W250, Rahway, NJ 07065.

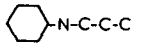
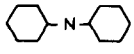
Amine(A)	Prot.Yld. (µg/ml)	Amine(A)	Prot.Yld. (µg/ml)
1 	47	7 	68
2 	62	8 	29
3 	48	9 	170
4 	27	10 	29
5 	69	11 	73
6 	62	12 	(2)

FIG. 1. Biodegradability of monoamines (500 µg/mL) by acclimated sewage sludge enrichment cultures as measured by protein yield. Numbers enclosed in brackets signify negative test results.

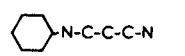
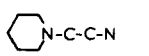
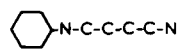
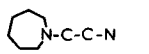
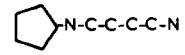
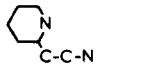
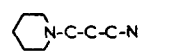
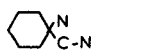
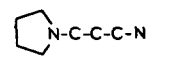
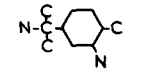
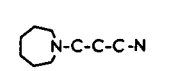
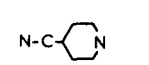
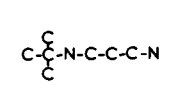
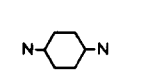
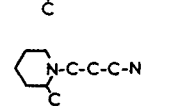
Amine(A)	Prot.Yld. (µg/ml)	Amine(A)	Prot.Yld. (µg/ml)
13 	90	22 	(1)
14 	64	23 	(0)
15 	54	24 	(2)
16 	65	25 	(14)
17 	77	26 	(0)
18 	52	27 	(0)
19 	(2)	28 	(0)
20 	(10)	29	(0)
21 	(0)	30	(0)

FIG. 2. Biodegradability of diamines (500 µg/mL) by acclimated sewage sludge enrichment cultures as measured by protein yield. Numbers enclosed in brackets signify negative test results.

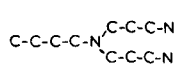
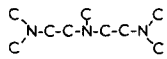
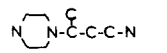
Amine(A)	Prot.Yld. (µg/ml)	Amine(A)	Prot.Yld. µg/ml)
31 N-C-C-C-N-C-C-C-C-N	42	34 	(0)
32 N-C-C-C-N-C-C-C-C-N-C-C-C-N	31	35 	(0)
33 N-C-C-N-C-C-N	(3)	36 	(0)

FIG. 3. Biodegradability of polyamines (500 µg/mL) by acclimated sewage sludge enrichment cultures as measured by protein yield. Numbers enclosed in brackets signify negative test results.

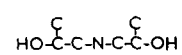
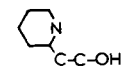
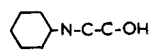
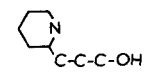
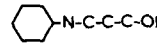
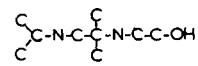
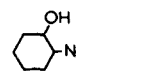
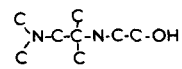
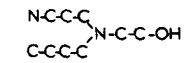
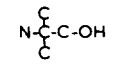
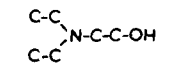
Amine(A)	Prot.Yld. (µg/ml)	Amine(A)	Prot.Yld. (µg/ml)
37 N-C-C-OH	60	44 	(1)
38 HO-C-C-N-C-C-OH	81	45 	(5)
39 	56	46 	(2)
40 	56	47 	(1)
41 	26	48 	(0)
42 	22	49 	(10)
43 	70		

FIG. 4. Biodegradability of amine alcohols (500 µg/mL) by acclimated sewage sludge enrichment cultures as measured by protein yield. Numbers enclosed in brackets signify negative test results.

Eastman Org. Chemicals (Rochester, NY): A2, A12; ICN (Cleveland, OH): A23. Compounds A15, A16, A18, A19, A20, A22, A25, A26, A27, A30, A34, A35, A36, A42, A44, A47, A48, A49, A52, A53, A54, A55, A56, A57, A58, A59 and A60 were provided by EXXON R & E Company. The purity of the test compounds, determined by the suppliers, using a variety of gas chromatographic (GC) and thin layer chromatographic (TLC) techniques was 97% or higher with the exception of A41. This compound had a purity of 90% and its small quantity made further purification impractical. In our biodegradation tests, marginal protein yields that could have resulted from the use of impurities were interpreted as negative results. All amines were neutralized with HCl before testing. All other chemicals and solvents used were of reagent grade or better.

Acclimation and Test Procedure

All enrichments and tests were conducted in a basal salts solution containing 0.4% Na_2HPO_4, 0.15% KH_2PO_4, 0.02% $MgSO_4.7\ H_2O$ (A) and ferric ammonium citrate, 0.01% $CaCl_2$ (B). Solutions A and B were autoclaved separately and 10 mL of B was added aseptically to 1 L of A. Three 50 mL Erlenmeyer flasks, each containing 20 mL

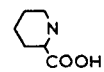
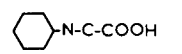
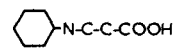
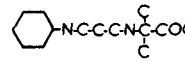
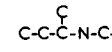
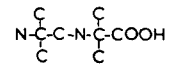
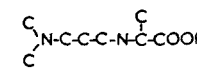
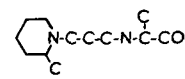
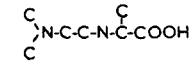
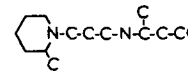
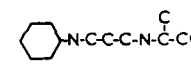
Amine(A)	Prot.Yld. (µg/ml)	Amine(A)	Prot.Yld. (µg/ml)
50 	74	56 	(0)
51 	73	57 	(2)
52 	20	58 	(2)
53 	56	59 	(0)
54 	20	60 	(0)
55 	(2)		

FIG. 5. Biodegradability of amino acids (500 µg/mL) by acclimated sewage sludge enrichment cultures as measured by protein yield. Numbers enclosed in brackets signify negative test results.

basal salts solution were used for each test compound. Two flasks received, in addition, 0.05% amine as the sole organic carbon and nitrogen source. The third flask contained 0.05% amine, 1% glucose and 0.1% NH_4Cl to determine any inhibitory effects of the amine on growth. Protein yields in this treatment were compared with a control with 1% glucose and 0.1% NH_4Cl, but no amine. Each flask was inoculated with 1 mL of fresh sewage sludge obtained from the Somerset-Raritan Valley Sewage Treatment Facility (Bridgewater, NJ). These enrichment cultures were incubated aerobically, with agitation, at 28 C. At weekly intervals, 0.1 mL of each culture was transferred to fresh medium (20 mL) of the same composition. One week after the third transfer, cultures were assayed for cell protein yield by the Lowry method (3). To eliminate interference by polyamines in the medium or on cell surfaces, triplicate 1 mL samples were washed twice with 0.9% NaCl before protein determination. Control cultures without amine or glucose contained 1-7 µg protein per mL. An increase in cellular protein to at least 20 µg/mL was considered positive for biodegradability. The significance of protein yields below 20 µg/mL was ambiguous; these values were considered negative. The protein values presented in the figures represent the average of 3 determinations. For clarity of the figures, no individual standard deviations are noted. The coefficients of variation for all sets of protein determinations were within 10%.

RESULTS AND DISCUSSION

Selection and Limitations of the Biodegradability Test

The test was chosen to fulfill the following criteria: (a) to be quantitative yet rapid and thus suitable for screening numerous compounds; (b) to be relevant to aquatic environments in which amines are likely to have a greater impact than in soils; (c) to produce results that closely predict the actual environmental fate of the compounds.

The requirement for speed and simplicity dictated a common measurement approach. The widely used biological oxygen demand (BOD) measurement approach was evaluated in a preliminary experiment using 11 test amines. It was rejected because it gave negative results for 4 amines that were definitely used by enrichment cultures. The biocidal properties of some amines toward nonadapted microbial populations may have been responsible for some of the negative BOD results. The test procedure we finally selected resembles, in a simplified form, the test system of Pitter (4), who used acclimated sewage sludge in combination with dissolved organic carbon measurements. Our acclimiation process was less elaborate and our protein yield measurements could detect only use of amines as sole carbon and energy source. Initial attempts to measure utilization of amines as a nitrogen source only in presence of another carbon source (glucose) were frustrated by enrichment for N_2-fixing microorganisms, mainly *Azomonas*. Consequently, a lack of biodegradation in our test system does not necessarily imply an absolute lack of biodegradation in the environment. Biodegradation of compounds with negative tests may involve initial cometabolic steps (5) and is expected to be very slow. Because this screen requires over 10% degradation in 7 days to be considered positive, less extensive degradation, e.g., limited to deamination, may not be detectable.

To compare protein yields, all test amines were supplied on equal weight rather than on a molar basis. The C/N ratios of the test amines ranged from 2:1 (A37) to 12:1 (A8). More typically, C/N ratios fell between 3:1 and 5:1. Assuming an average of 35% amine to protein conversion efficiency and a 12% N-content for protein, all enrichment cultures were expected to be C- rather than N-limited, and a maximum protein yield of ca. 175 μg/mL could be expected. This maximum was approached by only 1 compound (A9). The typically lower protein yields were caused by lower conversion efficiencies, incomplete or very slow utilization or a combination of these factors.

Structure-Biodegradability Correlations

Deamination is known to be performed by 4 enzyme classes: oxidases, monooxygenases, dehydrogenases and transaminases (6). The latter 2 systems also operate under anaerobic conditions. The initial product of amine degradation by procaryotes is an aldehyde or a ketone. Deaminating enzymes generally exhibit broad substrate ranges. For example, an amine dehydrogenase isolated from *Mycobacterium convolutum* oxidized *n*-propylamine, *iso*-propylamine and 1,3-propanediamine (7,8). The methylamine dehydrogenase of *Pseudomonas* AM1 oxidizes primary aliphatic mono-, di- and polyamines as well as histamine and ethanolamine (9). Secondary and tertiary amines and aminoalcohols are oxidized by the trimethylamine monooxygenase of *Pseudomonas aminovorans* (10). Putrescine oxidase isolated from *Micrococcus rubens* oxidizes di-, tri-, and tetraamines (11). The xenobiotic amines that supported microbial growth in our experiments were probably deaminated by one or more of the above mechanisms, but our screening process did not distinguish between these mechanisms.

Monoamines (Fig. 1). Alicyclic amines A1, A2 and A3 were readily utilized. A 2-methyl analog (A4) of cyclohexylamine, because of branching, was a less suitable substrate. Cyclohexylamine and some related alicyclic primary amines are deaminated to the corresponding alicyclic ketone by the cyclohexylamine oxidase of a *Pseudomonas* strain grown on cyclamates (12,13). All straight-chain N-alkyl analogs (A5, A6, A7) of cyclohexylamine were utilized with substantial protein yields, but the introduction of a second N-cyclohexyl-substitution (A8) reduced utilization. The heterocyclic amines pyrrolidine (A9) piperidine (A10) and homopiperidine (A11) were utilized, but the 2-methyl analog (A12) of piperidine (A12) was recalcitrant, as were all tested amines that included this structure (see Figs. 2, 4 and 5). The only 2-substituted piperidine subject to biodegradation was pipecolinic acid (Fig. 5, A50).

Based on catabolic pathways for pipecolinic acid, the most likely mechanism for catabolism of piperidine is ring cleavage via hydrolysis of the 3,4,5,6-tetrahydropyridine intermediate. This provides a possible explanation for the recalcitrance of 2-substituted piperidines. Substitution at a carbon adjacent to the nitrogen atom would impede formation of the double bond involving the substituted carbon and may cause steric hindrance to double bond formation on the opposite side of the heteroatom.

Diamines (Fig. 2). Diamine analogs of biodegradable monoamines were readily utilized as long as the 2 nitrogen atoms were separated by 3 or more carbons. Thus, propane- (A13, A16, A17, A18) and butane-diamine (A14, A15) derivatives were readily utilized unless some other feature of the molecule interfered with biodegradation. The *t*-butyl group of A19 and the 2-methyl-piperidine moiety of A20 constituted such interference. Ethane-diamine derivatives (A21, A22, A23) were consistently recalcitrant in our test system. However, the recalcitrance of the ethanediamine moiety is not absolute. Pitter (4) reported its biodegradation in an activated sludge test system. The more prolonged acclimation of Pitter's sludge system may account for the positive results obtained by this author. In our laboratory, A21 served as an N- but not as a C-source for a *Nocardia rhodochrous* strain (George and Bartha, unpublished results).

Diamines A24-A30 were recalcitrant. A24 may be regarded as a 2-methylpiperidine analog, A25 and A26 are highly branched structures. 1,4-Diaminocyclohexylamine (A28) was also recalcitrant as was A30 that had a terminal tertiary amine group. The causes for recalcitrance are less clear in case of A27 and A29.

Polyamines (Fig. 3). In this group, only the naturally occurring polyamines, spermidine (A31) and spermine (A32), served as growth substrates. Factors contributing to the recalcitrance of the other structures included ethanediamine moiety (A33), tertiary amines with 2 or more carbon chains longer than ethyl (A34, A35) and multiple heteroatoms (A36).

Tertiary amines (Figs. 2, 3, 4 and 5). In our tests, tertiary polyamines (Fig. 3, A34, A35) and diamines (Fig. 2, A30) were recalcitrant unless the tertiary amine was a heteroatom. In the latter case, the compounds were biodegradable (Fig. 2, A16, A17, A18). Other tertiary amines that were subject to microbial attack were structures that contained a more highly oxidized functional group: the amino-alcohols A42 and A43 and the amino acids A53 and A54 in Figs. 4 and 5, respectively.

Although tertiary amines are less biodegradable than primary or secondary amines (14), the presence of a tertiary amino group in a structure is not sufficient to render an amine nonbiodegradable. Trimethylamine and triethylamine are easily metabolized (15, 4), as are derivatives with one long *n*-alkyl chain (14). However, tertiary amines with 3 long-chain substituents resist degradation.

Amino alcohols (Fig. 4). The majority of the tested compounds were derivatives of ethanolamine (A37). In general, ethanolamines and N-substituted ethanolamines are easily metabolized, as determined in several biodegradation test systems (16,4). Diethanolamine (A38) was used extensively in our test as it was in the above biodegradation studies.

The N-cyclohexyl-substituted analog (A39) of ethanolamine was used extensively; N-cyclohexylpropanolamine (A40) to a lesser degree. 2-Aminocylohexanol (A41) and a diaminoalcohol containing a tertiary amino group (A42) were moderately good substrates, while N,N-diethylethanolamine (A43) supported strong growth. For tertiary amines, the presence of a hydroxyl group enhanced biodegradability. A structure similar to A42, without a hydroxyl group (Fig. 3, A34), was recalcitrant.

When amino alcohols were not utilized, this could usually be explained by the presence of a moiety demonstrated to block microbial attack. A45 and A46 are both 2-substituted piperidines, a category of compounds that were all recalcitrant. Three amino alcohols, A47, A48 and A49, contained a tertiary carbon and such structures are known to resist degradation (17). A44 lacks a tertiary carbon but is branched in 2 places and proved to be recalcitrant.

Amino acids (Fig. 5). Pipecolinic acid (A50), a carboxyl-substituted derivative of piperidine, was readily degraded although other less highly oxidized 2-substituted piperidine structures were consistently associated with recalcitrance. The biodegradability of N-cyclohexylamino acids depended on side-chain length. N-cyclohexyl-β-alanine (A51) with propionic acid side chain was a good growth substrate, whereas N-cyclohexylglycine (A56) with a 2-carbon side chain was recalcitrant. The lack of a β-carbon in the shorter side chain precludes metabolism via β-oxidation. In addition, initial attack at the nitrogen of A56 may have been inhibited by the proximity of 2 bulky substituents, carboxyl and cyclohexyl groups. The exchange of a smaller *sec*-butyl moiety for the cyclohexyl group (A52) resulted in a usable molecule. However, the low protein yield suggested that degradation was slow or limited.

Among 7 diamino acids tested, 2 (A53 and A54) were degradable. As demonstrated in the diamine category, a short N-to-N distance (A54) was associated with lower protein values. When diamino acids gave negative results, their structures included features associated with features that caused recalcitrance in other categories. Highly branched (A57 and A58) and 2-methylpiperidine-substituted (A59 and A60) compounds did not support growth. The explanation for recalcitrance of A55 is less obvious. Although β-oxidation is blocked by lack of a β-carbon, the cyclohexyl-substituted nitrogen should be accessible as an alternate site of attack. Possibly degradation may have been inhibited by steric factors.

For the majority of the amino acids, the report (17) previously cited in connection with aminoalcohols is relevant also for the interpretation of these structures. Monomethyl-substituted acids are readily degraded, but dimethyl-substituted acids are usually resistant, especially when both methyl groups are attached to the same carbon. The closer a substituent is located to the α-carbon, whether it is a methyl- or an amino group, the less susceptible is the acid to microbial attack. Aminoacetic acid (glycine), 3-aminopropionic acid (β-alanine) and 4-aminobutyric acid (GABA) are readily degraded. More resistant to degradation are 2- and 3-aminobutyric acids, but they are utilized to some extent. These findings were predictable because substitution of the α-carbon blocks β-oxidation, a major route for dissimilation of aliphatic acids.

Inhibitory Properties of Amines

Polyamines and synthetic alkylamines exhibit a wide spectrum of antimicrobial activity (18-22). Our test included flasks with 0.05% amine in presence of glucose and NH_4Cl. The purpose of these controls was to show whether biocidal effects rather than structural recalcitrance prevented the utilization of some of the tested amines.

At 0.05%, only 1 compound (Fig. 2, A26) inhibited glucose control severely (90%). This compound also had structural features that interfered with biodegradation. Only 2 compounds inhibited glucose metabolism between 50-60% (Fig. 2, A21; Fig. 3, A32). Of these, the former was recalcitrant and the latter (spermine) was biodegradable. One half of the amines tested caused 25-50% inhibition and ¾ at least a 10% inhibition of glucose metabolism. However, no consistent correlation was found between these moderate inhibitory effects and the ability of the amines to serve as growth substrates for enrichments. The inhibition data are available elsewhere (23) and because of their apparent irrelevance to structure-biodegradability correlations, are not presented here in detail.

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