Effect of Chemical Structure on the Biodegradability of Aliphatic Acids and Alcohols

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Sewage microorganisms readily degraded unsubstituted aliphatic acids, but the rate of decomposition was much slower with substituted acids as substrates. The type, number, and position of the substituents governed the rate of the oxidation. A single halogen, particularly if on the \( \alpha \)-carbon, decreased the rate of biodegradation, but the dihalogenated compounds tested were especially resistant. Dimethyl-substituted aliphatic acids and alcohols were also poorly utilized. Bacteria unable to grow on certain brominated fatty acids were capable of oxidizing and dehalogenating \( \omega \)-but not \( \alpha \)-bromooliphatic acids.

The widespread use of synthetic chemicals has been the direct cause of an increased pollution of many environments. Some of these compounds are readily degraded by microorganisms, but others are resistant to biological decomposition and tend to accumulate, resulting in a potential hazard to public health and environmental quality. Attempts to minimize pollution of water or soil, where much of the degradation of synthetic chemicals results from microbial activities, would be facilitated if it were possible to predict the influence of the structure of particular chemicals on their susceptibility to microbial attack. The introduction of the linear alkylbenzene sulfonate detergents to replace the branched alkylbenzene sulfonates illustrates how such investigations lead to means of minimizing pollution of waterways. Ample additional precedents exist to show the relation between the structure of synthetic chemicals and their susceptibility to rapid microbial decomposition (1, 11).

The present study was designed to determine the effect of type and position of substituents on the biodegradability of substituted aliphatic acids and alcohols. The compounds were selected with a view to determining whether substituents near the carboxyl group of aliphatic acids or the hydroxyl group of alcohols might render the compounds refractory to microbial attack.

MATERIALS AND METHODS

Microbial degradation of the test compounds was determined by measuring the depletion of dissolved oxygen (DO) at regular intervals by using a method identical to the biochemical oxygen demand (BOD) test (3, 11), except for differences in temperature and incubation period. Standard BOD dilution water (3) contained in 300-ml bottles was used except when a halide-free medium was needed; in this instance, the calcium chloride was replaced by 5.0 ml of a saturated solution of calcium sulfate per liter, and the other chlorides in the dilution water were replaced with sulfate salts of the various cations in molarities equal to that of the original chloride salts. The test compound was used as a carbon and energy source at a concentration of 2.0 mg of carbon per liter, unless stated otherwise. Solutions containing acidic substrates were adjusted to pH 6.5 to 7.5 with KOH.

To assess whether the lack of biodegradation resulted from the toxicity of a chemical rather than its resistance to microbial attack, each compound was also tested in combination with glucose (both at concentrations of 2.0 mg of carbon per liter) in the usual medium. Comparisons of the rate of glucose oxidation in the presence and absence of the test substances revealed that none was toxic at the concentrations employed. BOD bottles with no added carbon were also included in all experiments to measure the oxygen depletion caused by the domestic sewage used as inoculum, and all values have been corrected for the oxygen depletion by the sewage seed. Bottles were incubated at 25 C in the dark. Before use, the raw sewage was stored at 25 C for 24 to 48 hr; it was then filtered through cotton, and 5.0 ml was added per liter of dilution water.

DO was measured with a polarographic electrode (Biochemical Oxygen Analyzer, model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio). The electrode was mounted in a rubber stopper allowing it to fit the neck of the BOD bottle. The instrument was calibrated with water whose DO was determined by an iodometric technique (3). Nitrate was measured as described by Montgomery and Dymock (10) and nitrite by means of naphthylamine and sulphanilic acid (3). Samples for nitrate determination were concentrated 5- to 10-fold before analysis. Bromide was determined by the method of Bergmann and Sanik (4).

1 Present address: Anil Starch Products Ltd., Ahmedabad, India.
The extent of oxygen depletion caused by nitrification was calculated from the data of Wezernak and Gannon (12). Preliminary investigations showed no significant nitrification in 10 days, and nitrite and nitrate determinations were, therefore, performed only at 30 days. All oxygen depletion values recorded have been corrected for the oxygen consumed in nitrification.

Except as otherwise indicated, the optical isomerism of the compounds which exist as two isomers was not listed by the manufacturer, and the substrates are thus assumed to contain both forms.

To prepare resting cells, cultures were grown in 250 or 500 ml of medium contained in 1.0- or 2.0-liter flasks maintained on a rotary shaker at 25 C for 48 hr. The cells were collected by centrifugation, washed once with 0.10 M phosphate buffer (pH 7.1), and then resuspended in the same buffer. Oxygen uptake was measured at 30 C by standard manometric techniques. The Warburg flasks contained 1.0 ml of cell suspension, 100 μmoles of phosphate buffer (pH 7.1), and 3.0 μmoles of substrate. The center well contained 0.2 ml of 20% KOH. The reported values are corrected for endogenous respiration. When halide was determined, the reaction was stopped by adding 1.0 ml of 2 N HNO3 to the Warburg flask, the contents of the flask were pipetted out, and the flasks were rinsed with two additional 1.0-ml quantities of acid. The cells were then removed by centrifugation prior to analysis.

RESULTS

The microbial community of raw sewage readily utilized unsubstituted fatty acids ranging from two to eight carbons in chain length. With the exception of α-substituted acids with more than three carbons, the introduction of a halogen substituent rendered all of the test compounds more refractory to attack. The four disubstituted compounds tested were more resistant to degradation than the corresponding monosubstituted acids (Table 1). The position of the substituent in the monosubstituted acids, moreover, greatly influenced their resistance to decomposition.

With the sole exception of the chloropropionic acids, the α-substituted acids were more resistant than the corresponding compounds having halogens on other carbon atoms; the degree of recalcitrance of these α-substituted acids seemed to decrease as the chain length increased for compounds having more than three carbon atoms. This is also indicated in Table 2, in which instance the dembrimation of the α-substituted acids was also determined. The release of bromide was essentially complete for compounds with seven and eight carbons. The solutions containing α-bromo-substituted butyric, pentanoic, hexanoic, heptanoic, and octanoic acids had 6.6, 5.2, 4.4, 3.8, and 3.3 mg of organic bromine per liter so that the bromide liberated in 11 days was equivalent to 12, 13, 18, 79, and 85% of the theoretical yield, respectively. On the other hand, the debromination of the α-substituted acids was generally more rapid than the α-substituted compounds, and most of the bromine in substrates of the former group was released in 3 days.

The monomethyl-substituted acids investigated were all readily degraded. The dimethyl-substituted compounds were particularly resistant, however (Table 3). The dicarboxylic acids with a quaternary carbon, namely 2,2- and 3,3-dimethylglutaric acids, were degraded more rapidly than the monocarboxylic acids having a

TABLE 1. Oxygen consumption resulting from the microbial degradation of halogenated aliphatic acids

| Organic acid | Source | Amt of O2 consumed (mg/liter) on day |
|--------------|--------|-----------------------------------|
|              |        | 2      | 5      | 10     | 30     |
| Acetic       | B      | 3.0    | 4.1    | 4.1    |
| Monochloroacetic | F | 0.0    | 1.5    | 3.1    |
| Dichloroacetic | E | 0.0    | 1.0    | 2.5    |
| Trichloroacetic | E | 0.0    | 0.0    |        |
| Propionic    | F      | 3.6    | 4.3    | 4.5    | 4.6    |
| 2-Chloropropionic | E | 0.4    | 3.5    | 3.3    | 4.8    |
| 3-Chloropropionic | E | 0.3    | 2.4    | 3.5    | 4.3    |
| 2,3-Dichloropropionic | K | 0.0    | 0.3    | 1.4    |
| 3-Bromopropionic | E | 1.1    | 2.7    | 4.2    | 4.9    |
| 2,3-Dibromopropionic | K | 0.0    | 0.2    | 0.3    |         | 0.9    |
| Butyric      | F      | 3.1    | 3.2    | 3.9    |
| 2-Chlorobutyric | K | 0.5    | 0.6    | 0.0    | 1.7    |
| 3-Chlorobutyric | K | 0.7    | 2.8    | 2.2    | 2.9    |
| 4-Chlorobutyric | K | 2.8    | 3.3    | 2.5    | 3.4    |
| 2-Bromobutyric | E | 0.0    | 0.4    | 0.4    | 0.3    |
| 3-Bromobutyric | K | 2.0    | 3.3    | 2.5    | 4.2    |
| Pentanoic    | E      | 3.5    | 4.7    | 5.7    | 5.1    |
| 2-Chloropentanoic | D | 0.0    | 0.2    | 0.6    | 1.1    |
| 5-Chloropentanoic | K | 1.0    | 4.0    | 4.4    |
| 2-Bromopentanoic | K | 0.5    | 1.0    | 1.2    | 3.9    |
| 5-Bromopentanoic | K | 3.2    | 3.7    | 4.7    | 4.2    |
| Hexanoic     | E      | 3.0    | 3.4    | 3.8    | 4.6    |
| 2-Bromohexanoic | K | 0.5    | 0.5    | 1.9    | 3.6    |
| 6-Bromohexanoic | E | 3.2    | 3.7    | 4.7    | 4.2    |
| 2,6-Dibromohexanoic | K | 0.1    | 0.6    | 0.4    |
| Heptanoic    | K      | 3.2    | 4.4    | 3.9    | 4.2    |
| 2-Bromoheptanoic | K | 0.6    | 0.8    | 3.9    | 4.0    |
| 7-Bromoheptanoic | K | 0.6    | 3.7    | 4.8    | 4.6    |
| Octanoic     | N      | 3.2    | 3.9    | 4.7    | 4.6    |
| 2-Bromoocanoic | P | 1.1    | 2.2    | 5.0    | 4.7    |
| 8-Bromoocanoic | K | 3.3    | 4.3    | 5.6    | 6.1    |

* Source of chemicals: J. T. Baker (B), Fisher Scientific Co. (F), Eastman Organic Chemicals (E), K & K Laboratories (K), Dow Chemical Co. (D), Pfaltz & Bauer (P), and Nutritional Biochemicals Corp. (N).

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quaternary carbon. The only dimethyl-substituted acid with the substituents on different carbon atoms which was tested (2,3-dimethylbutyric acid) proved to be less recalcitrant than the acids with a quaternary carbon, but it was more resistant than the monosubstituted acids.

Unsubstituted alcohols were readily degraded by the heterogeneous sewage community, and monomethyl substitution did not make them much more refractory. However, the tertiary alcohols were quite resistant (Table 4). The only alcohol with two methyl substituents on different carbons (2,3-dimethyl-1-pentanol) which was tested was also quite slowly attacked. The tertiary alcohols seemed to be less suitable substrates than acids with a quaternary carbon.

If an amino or phenyl group was present on the α-carbon of butyric acid, the molecule was not prone to rapid microbial attack. By contrast, the identical group on the omega carbon did not prevent quick microbial destruction (Table 5). These results are similar to those already reported for the halogenated aliphatic acids. Aminoacetic (glycine) and the aminopropionic acids were readily degraded.

Attempts to isolate pure cultures were made by selective culture techniques with BOD dilution water supplemented with 0.02% NH₄Cl and 0.1% of the test substrate. No isolates able to grow on 2,2-dimethylpropionic, 2-chlorobutyric, and 2-bromohexanoic were obtained, even if yeast extract, selected B vitamins, or trace elements were added to the enrichment solution. On the other hand, bacteria able to debrominate halogenated aliphatic acids were readily obtained when the enrichment and isolation media contained unsubstituted pentanoic, hexanoic, or heptanoic acid as carbon sources (strains C₅, C₆, and C₇, respectively). The substrates were sterilized by filtration. The first two of the isolates were identified as strains of Arthrobacter, but the last one was not characterized to the genus level.

The three isolates were not able to grow on the α- or ω-brominated derivatives (0.02%) of the unsubstituted acid used for their enrichment and isolation. The lack of growth on the brominated compounds was not due to their toxicity, as indicated by the ability of the bacteria to multiply in media containing both the unsubstituted and the substituted (0.02%) acids. The cultures grew well in media containing the unsubstituted compounds at a concentration of 0.05%.

Strains C₅, C₆, and C₇ were grown on pentanoic, hexanoic, and heptanoic acid, respectively, and resting cell suspensions (2.7 to 3.5 mg of cells) were incubated with 3.0 μmoles of halogenated acids for 60 min at 30°C. In this period, isolates C₅, C₆, and C₇ showed little or no O₃ uptake and released no bromide from the α-bromo-pentanoic, hexanoic, and heptanoic acids, respectively, and C₅ also was inactive on 5-bromopentanoic acid.
TABLE 4. Oxygen consumption resulting from the microbial degradation of aliphatic alcohols

| Compound                  | Source | Amt of O$_2$ consumed (mg/liter) on day |
|---------------------------|--------|---------------------------------------|
|                           |        | 2     | 5     | 10    | 30    |
| 1,1-Dimethyl-1-ethanol    | F      | 0.3   | 0.0   | 0.2   | 0.0   |
| (tert-butanol)            |        |       |       |       |       |
| 2-Methyl-1-propanol       | MC     | 3.4   | 4.9   | 6.0   | 4.4   |
| 1,1-Dimethyl-1-propanol   | F      | 0.0   | 0.0   | 0.5   | 0.0   |
| (tert-pentanol)           |        |       |       |       |       |
| 1-Butanol                 | M      | 3.4   | 4.4   | 4.9   | 4.3   |
| 3-Methyl-1-butanol        | K      | 3.8   | 4.9   | 5.9   | 4.1   |
| 3,3-Dimethyl-1-butanol$^b$| A      | 0.0   | 0.0   | 0.3   | 0.3   |
| 1-Pentanol                | F      | 2.7   | 4.1   | 4.9   | 4.1   |
| 2-Methyl-1-pentanol$^b$   | A      | 1.3   | 3.9   | 4.4   | 3.8   |
| 3-Methyl-1-pentanol$^b$   | A      | 0.0   | 4.1   | 5.2   |       |
| 4-Methyl-1-pentanol$^b$   | A      | 0.5   | 3.6   | 4.4   | 3.6   |
| 1,1-Dimethyl-1-pentanol$^b$| 2-      | 0.0   | 0.0   | 0.0   | 0.0   |
| methyl-1-hexanal$^b$      |        |       |       |       |       |
| 2,2-Dimethyl-1-pentanol$^b$| E      | 0.0   | 0.0   | 0.6   | 0.3   |
| 2,3-Dimethyl-1-pentanol$^b$| A      | 0.0   | 0.0   | 0.3   | 1.1   |
| 1,1-Dimethyl-1-hexanol$^b$| 2-      | 0.0   | 0.0   | 0.3   | 0.6   |

$^a$ F, Fisher Scientific Co.; MC, Mallinckrodt Chemical Works; M, Matheson, Coleman and Bell; K, K & K Laboratories; A, Aldrich Chemical Co.; E, Eastman Organic Chemicals.

$^b$ Carbon in the methyl substituents was not considered in calculating the amount of substrate added (2 mg of carbon per liter).

By contrast, strain C6 released 125 µg of bromide when supplied with 3.0 µmoles of 6-bromo-hexanoic acid, and strain C7 released 175 µg of bromide when provided with 7-bromo-heptanoic acid; small amounts of O$_2$ were consumed in the process. Resting cells of each organism rapidly oxidized the growth substrate. Thus, although two of the three ω-substituted fatty acids could be co-metabolized, the α-substituted compounds were resistant to metabolism by bacteria induced to use the free aliphatic acids.

Strain C5 was grown on pentanoic acid, and resting cell suspensions were prepared. When a suspension containing 3.5 mg of these cells was incubated with 3.0 µmoles of 2-bromo hexanoic or 2-bromo heptanoic, little or no O$_2$ was utilized and little or no bromide was liberated. On the other hand, 3.5 and 4.6 µmoles of O$_2$ disappeared in 60 min, and 185 and 150 µg of bromide appeared in 4 hr when the substrates were 3.0 µmoles of 6-bromo hexanoic and 7-bromo heptanoic acids, respectively.

### DISCUSSION

The results show that the type, position, and number of substituents on an aliphatic chain determine whether a compound will be refractory to microbial attack. The data also suggest that a substituent near the end of a chain bearing the carboxyl or hydroxyl group, at which end microbial metabolism presumably is initiated, makes the molecule less susceptible to attack. Exceptions to this generalization are the methyl-substituted compounds, but the methyl group may be considered to be common in catabolic and anabolic sequences, in contrast to the halogens; possibly, mechanisms to cleave or metabolize such substituents are widespread. It is interesting to note that α-methyl-substituted acids are as readily oxidized by animals as the unsubstituted compounds, but other substituents retard oxidation (6).

Other investigators have also noted the resistance of α-substituted compounds. The slow degradation of α- as compared with ω-substituted phenoxyalkyl carboxylic acids is a case in point (2). The resistance of compounds containing a quaternary carbon as well as tertiary alcohols has also been reported (7-9), but probably the first recognition of the lack of susceptibility of molecules of these sorts was based on studies of the slowly biodegradable synthetic detergents (5, 11). It is also of interest that Williams et al. (13) found...
that the alcohol dehydrogenase of a *Pseudomonas* species was inactive on tertiary alcohols but did act on monomethyl-substituted alcohols.

The resistance of \( \alpha \)-substituted compounds probably is attributable to a hindrance to \( \beta \)-oxidation imposed by the substituent adjacent to the carboxyl group, a reaction sequence which requires the presence of two protons on the \( \alpha \)-carbon. Evidence to support this view has been obtained by providing bacteria with dimethyl-substituted fatty acids in which the methyl groups were somewhat removed from the carboxyl end; the cultures metabolized these compounds partially and apparently by \( \beta \)-oxidation to yield an \( \alpha,\alpha \)-dimethyl fatty acid (Hammond and Alexander, unpublished data).

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