Cometabolism of m-Chlorobenzoate by an Arthrobacter

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Twenty isolates representing nine bacterial genera were obtained from enrichment cultures and were shown to cometabolize one or more of 22 substituted benzoates. One of the isolates, an Arthrobacter sp., cometabolized m-chlorobenzoate to a product identified as 4-chlorocatechol by thin-layer chromatography and ultraviolet and infrared spectroscopy. The data indicate that cometabolism by the arthrobacter results from the formation of products by its benzoate-oxidizing enzyme system that are not acted upon by the catechol-metabolizing enzymes of the bacterium.

Synthetic aromatic compounds are constantly being added to the biosphere in the form of pesticides and industrial wastes. Some of these chemicals are apparently acted upon microbiologically, but the products of the reaction are seemingly refractory to further degradation, and they accumulate in nature. The available data indicate that cooxidation or cometabolism accounts for the incomplete metabolism and degradation of a number of ecologically important pesticides (1). Foster (5) proposed the term cooxidation to describe the oxidation of a compound by a microorganism unable to utilize the energy derived from the oxidation to sustain its growth. Jensen (10) suggested the more general term cometabolism because bacteria occasionally dehalogenate pesticides although they are unable to utilize these chlorine-substituted compounds as carbon sources.

Evidence exists that cometabolism is common to many microorganisms. Leadbetter and Foster (12) noted cometabolism of alkanes by Pseudomonas methanica growing at the expense of methane, and Davis and Raymond (4) observed the identical phenomenon in the transformation of alkyl-aryl hydrocarbons by Nocardia sp. Halogen-substituted organic compounds have likewise been reported to be subject to cometabolism by a variety of bacteria unable to multiply at the expense of these substrates (8, 14, 16).

Although a widespread occurrence of cometabolism in nature has been postulated, data to support this view are lacking. The present investigation was designed to identify some of the microbial genera capable of cometabolizing a class of ecologically important molecules, specifically aromatic molecules of the type found in many pesticides and industrial pollutants, and to determine the basis for the inability of one organism to utilize for growth a compound that it was capable of metabolizing.

MATERIALS AND METHODS

Isolates were obtained from enrichment cultures (200 ml/500-ml Erlenmeyer flask) inoculated with lake or ditch water and incubated at 30 C on a rotary shaker. The enrichment medium contained 0.5 g of a chloro- or aminobenzoate, 0.4 g of KH2PO4, 1.6 g of K2HPO4, 0.5 g of NH4NO3, 0.2 g of MgSO4·7H2O, 0.025 g of FeCl3·6H2O, and 1 mg of sodium salt of penicillin G per liter of distilled water. When half of the organic compound had been metabolized, as determined spectrophotometrically, samples were centrifuged at 5,000 X g for 10 min. The cells were washed twice in 0.02 M phosphate buffer (pH 7.2) and suspended in the same buffer. For the isolation of the bacteria, each suspension was streaked on a medium containing 1.0 g of sodium benzoate, plus the inorganic salts listed above, and 15 g of Noble agar per liter of distilled water. Cultures were grown on a rotary shaker at 30 C in media containing the same inorganic ingredients and 0.1 g of sodium benzoate. The isolates were identified according to the scheme of Skerman (15).

The respiration of resting cells was determined at 30 C in air by standard manometric techniques (19). All results were corrected for endogenous activity. Unless otherwise noted, resting cell suspensions consisted of a 48-hr culture of benzoate-grown cells suspended in 0.02 M phosphate buffer (pH 7.2) adjusted to an optical density at 545 nm of 2.0. Each Warburg flask contained KOH in the center well, 100 µg of substrate in 1.0 ml of the phosphate buffer, and 2.0 ml of cell suspension.

The amount of substrate which was metabolized was determined spectrophotometrically (2), and 4-chlorocatechol was measured by the method of Arnow (3). For the identification of 4-chlorocatechol, thin-layer chromatography was employed by using
Eastman Chromagram Sheets 6060 and chloroform and chloroform-methanol (10:1, v/v) solvent systems. The spots were developed under ultraviolet (UV) light. UV spectra of the sample and of authentic 4-chlorocatechol in water were obtained in a Beckman spectrophotometer, model DB. Lead derivatives of the sample and of authentic 4-chlorocatechol were prepared by the procedure of Helling and Bollag (7). The infrared spectra of these derivatives were determined as KBr macropellets in a Beckman spectrophotometer, model IR-10.

To prepare cell extracts, suspensions containing 0.1 g of cell paste in 5.0 ml of 0.02 m phosphate buffer (pH 7.2) were treated for 5 min at 0 C with a Biosonic disintegrator (Bronwill Scientific, Rochester, N.Y.). The extracts were clarified by centrifugation at 20,000 × g for 60 min, and their activity was determined manometrically. The Warburg flasks contained 2.0 ml of the extract, KOH in the center well, and 100 µg of catechol or 4-chlorocatechol. Uninoculated controls were included in all studies.

The three monochloro- and 2,4- and 3,4-dichlorobenzoates were obtained from Eastman Organic Chemicals (Rochester, N.Y.). 2,5-Di-, 2,3,4-tri-, 2,3,5-tri-, 2,4,5-tri-, 2,3,4,5-tetra-, 2,3,5,6-tetra-, pentachloro-, and 2,5-dichloro-3-amino benzoates were supplied by Amchem Products (Ambler, Pa.). Aldrich Chemical Co. (Milwaukee, Wis.) was the source of 2,6-dichloro-, m- and p-amino-, 3,5-diamino-, 2-chloro-5-amino-, 4-chloro-2-amino- and 5-chloro-2-aminobenzoates and of 4-chlorocatechol. K & K Laboratories (Plainview, N.Y.) supplied 2,3,6-tri-chlorobenzoate and 2-chloro-4-aminobenzoate. Catechol was obtained from Fisher Scientific Co. (Pittsburgh, Pa.). Catechol, m-chlorobenzoate, and 4-chlorocatechol were purified by vacuum sublimation. The purity of all substrates was assessed by thin-layer chromatography.

RESULTS

The 20 organisms isolated from enrichment cultures were members of nine genera. Six were identified as strains of Pseudomonas, four as Bacillus, three as Arthrobacter, two as Flavobacterium, and one each as strains of Achromobacter, Brevibacterium, Microbacterium, Micrococcus, and Xanthomonas. All 20 grew on and hence metabolized unsubstituted benzoate, but none of the 20 bacteria multiplied in media with any of the 22 substituted benzoates as sole carbon sources. However, although the latter compounds were not suitable as carbon sources to support proliferation, all were metabolized by one or more of the isolates (Table 1). The oxidation was incomplete in every instance, and the tabulated values of moles of O₂ per mole of substrate metabolized represent calculations from the time the rate of gas exchange fell to the endogenous rate. Each of the 20 isolates was able to metabolize several of the test compounds.

**Table 1. Metabolism of substituted benzoates by 20 bacterial isolates**

| Substituted benzoate | No. of inactive cultures | Moles of O₂ consumed per mole of substrate metabolized* |
|----------------------|--------------------------|------------------------------------------------------|
|                      |                          | 0.5 ± 0.1 | 1.0 ± 0.1 | 1.5 ± 0.1 | 2.0 ± 0.1 | 2.5 ± 0.1 | 3.0 ± 0.1 |
| o-Chloro             |                          | 15        | 3         | 0         | 0         | 2         | 0         | 0         |
| m-Chloro             |                          | 14        | 6         | 0         | 2         | 3         | 0         | 0         |
| p-Chloro             |                          | 12        | 7         | 2         | 1         | 1         | 0         | 0         |
| 2,4-Dichloro         |                          | 17        | 10        | 1         | 1         | 1         | 0         | 0         |
| 2,5-Dichloro         |                          | 15        | 6         | 0         | 2         | 0         | 0         | 0         |
| 2,6-Dichloro         |                          | 15        | 8         | 0         | 0         | 1         | 0         | 0         |
| 3,4-Dichloro         |                          | 16        | 9         | 0         | 2         | 0         | 0         | 0         |
| 2,3,4-Trichloro      |                          | 16        | 9         | 0         | 2         | 0         | 0         | 0         |
| 2,3,5-Trichloro      |                          | 15        | 9         | 0         | 1         | 0         | 0         | 0         |
| 2,3,6-Trichloro      |                          | 11        | 9         | 0         | 2         | 0         | 0         | 0         |
| 2,4,5-Trichloro      |                          | 10        | 9         | 0         | 2         | 0         | 0         | 0         |
| 2,3,4,5-Tetrachloro  |                          | 9         | 9         | 0         | 2         | 0         | 0         | 0         |
| Pentachloro          |                          | 9         | 9         | 0         | 2         | 0         | 0         | 0         |
| m-Amino              |                          | 18        | 9         | 0         | 1         | 0         | 0         | 0         |
| p-Amino              |                          | 18        | 9         | 0         | 1         | 0         | 0         | 0         |
| 3,5-Diamino          |                          | 17        | 10        | 1         | 0         | 2         | 0         | 0         |
| 2-Chloro-4-amino     |                          | 18        | 9         | 0         | 0         | 1         | 0         | 0         |
| 2-Chloro-5-amino     |                          | 18        | 9         | 0         | 0         | 1         | 0         | 0         |
| 4-Chloro-2-amino     |                          | 16        | 9         | 0         | 0         | 1         | 0         | 0         |
| 5-Chloro-2-amino     |                          | 16        | 9         | 0         | 0         | 1         | 0         | 0         |
| 2,5-Dichloro-3-amino |                          | 17        | 10        | 1         | 0         | 2         | 0         | 0         |

* Figures in the columns represent the number of cultures metabolizing the compound with the consumption of the quantity of O₂ indicated.
Samples were taken at the end of the incubation period from a reaction mixture containing m-chlorobenzoate and cells of Arthrobacter strain 1 and were examined by thin-layer chromatography. The results suggested that 4-chlorocatechol had been generated from m-chlorobenzoate. $R_f$ values for the product from the chlorobenzoate and for authentic 4-chlorocatechol were 0.23 in chloroform and 0.70 in chloroform-methanol. No other products were detected on thin-layer chromatograms. This organism was selected for further study.

Resting cell suspensions of this arthrobacter consumed 1.1 μmoles of $O_2$ and 0.9 μmole of $CO_2$ was formed per μmole of m-chlorobenzoate metabolized in 1 hr, at which time the oxidation had terminated. The value for $O_2$ consumed and the evolution of $CO_2$ likewise suggested the formation of a catechol. Because halide was not released during the oxidation of m-chlorobenzoate by the isolate, the presumed catechol appears to have contained a chlorine substituent.

A suspension of the isolate previously grown on benzoate was incubated at 30 C in the presence of 0.1 mg of m-chlorobenzoate per ml and the inorganic salts for seven days. The UV spectrum of the supernatant obtained from this incubation mixture was similar to that of authentic 4-chlorocatechol. A lead derivative of the metabolite was prepared, and the supernatant was found to have lost the characteristic UV absorbancy. The infrared spectrum of the lead derivative was similar to that of authentic lead-4-chlorocatecholate, except for peaks at 1,630 cm$^{-1}$ and 1,020 cm$^{-1}$ (Fig. 1). These peaks resulted from the contaminating lead carbonate (7).

The changes in m-chlorobenzoate and 4-chlorocatechol concentration were measured quantitatively to determine the stoichiometry of the reaction. The results of four experiments show that in 1 hr 1 μmole of oxygen was taken up and 1 μmole of $CO_2$ and of 4-chlorocatechol was formed for each μmole of m-chlorobenzoic acid metabolized. Recoveries ranged from 90 to 100% in the four tests.

The inability of the bacterium to metabolize m-chlorobenzoate beyond the catechol level may have resulted from the microbial conversion of the substrate to a toxic end-product. The possibility of the inhibitory action of catechol on benzoate oxidation was tested in incubation mixtures containing 0.64 mM m-chlorobenzoate in the presence and absence of 0.69 mM 4-chlorocatechol. The data showed that the rates and extent of $O_2$ consumption and $CO_2$ evolution by the bacterium were the same, regardless of whether the catechol was present; hence, 4-chlorocatechol showed no toxicity under the test conditions.

The failure of the organism to grow upon m-chlorobenzoate and the presumed cometabolism of the same compound could result from levels of the chemical toxic to multiplication but yet not sufficiently high to abolish substrate oxidation; alternatively, the accumulation of 4-chlorocatechol may have been so large that further bacterial replication was prevented. This possibility was examined by inoculating the arthrobacter into the liquid salts medium containing 0.1% unsubstituted benzoate and 0.01% of m-chlorobenzoate, 4-chlorocatechol, or both. Growth was rapid in media containing benzoate alone and was neither retarded nor enhanced by the chlorinated compounds, either singly or in combination.

Oxygen uptake by benzoate-grown cells commenced immediately upon the addition of m-chlorobenzoate to the cell suspensions. This indicated that the same enzyme system was involved both in the oxidation of benzoate and the conversion of m-chlorobenzoate to 4-chlorocatechol. This view was supported by the finding that cells grown in nutrient broth exhibited a lag period before initiation of the oxidation of benzoate or m-chlorobenzoate, a lag similar in duration for both

![Infrared spectra of authentic lead-4-chlorocatecholate (broken line) and of the lead derivative of a product of metabolism of m-chlorobenzoate by Arthrobacter sp. (solid line).](image-url)
cometabolism of \( m \)-chlorobenzoate by a strain of *Arthrobacter* employed in the present study was characterized as 4-chlorocatechol, and the stoichiometry of its formation suggested that it was the only major product of \( m \)-chlorobenzoate cometabolism.

Cometabolism could result from an accumulation of some toxic product, as suggested by Tranter and Cain (18), or an inability of the organism to carry the metabolism to a stage where the carbon could be assimilated, as suggested by Hughes (8). Gibson et al. (6) obtained evidence that halogenated catechols inhibited the enzyme system of *P. putida* which catalyzed the incorporation of \( \text{O}_2 \) into the aromatic nucleus. The present data, however, show that 4-chlorocatechol was not toxic to the isolate at the concentrations employed; hence, product toxicity does not appear to account for the inability of the arthrobacter to grow upon compounds which it can oxidize.

Since benzoate-grown cells oxidized \( m \)-chlorobenzoate without a lag, because the duration of the lag on \( m \)-chloro- and unsubstituted benzoate was the same with noninduced cells, and inasmuch as preincubation with \( m \)-chlorobenzoate induced the cells to metabolize both benzoate and \( m \)-chlorobenzoate, it seems likely that the two compounds are metabolized by the same enzyme system. Hughes (12) also showed that 5-fluoronicotinic acid induced the formation of enzymes which oxidized both 5-fluoronicotinic acid and nicotinic acid.

The inability of the catechol-degrading enzyme to act on 4-chlorocatechol coupled with the activity of the benzoate enzyme on \( m \)-chlorobenzoate appears to account for cometabolism by *Arthrobacter* sp. The failure of the bacterium to cleave the chlorine substituent from the benzene ring and the substrate specificity of the ring-cleaving enzyme prevented the metabolism of \( m \)-chlorobenzoate from proceeding to a point at which the carbon could be assimilated.

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