METABOLISM OF PHENOXYALKYL CARBOXYLIC ACIDS BY A *FLAVOBACTERIUM* SPECIES[1]

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ABSTRACT

MACRAE, I. C. (Cornell University, Ithaca, N.Y.), AND M. ALEXANDER. Metabolism of phenoxyalkyl carboxylic acids by a *Flavobacterium species*. J. Bacteriol. **86**:1231–1235. 1963.—A *Flavobacterium* sp. isolated from soil and grown in media containing 4-(2,4-dichlorophenoxy) butyric acid metabolized ω-linked 2,4-dichlorophenoxyalkyl carboxylic acids in the series from 3-(2,4-dichlorophenoxy)propionic acid through 11-(2,4-dichlorophenoxy)undecanoic acid rapidly and without a preliminary induction phase. There was no detectable oxidation of 2,4-dichlorophenoxyacetic acid. Phenols and the fatty acids corresponding to the aliphatic side chains were liberated during the decomposition of the dichlorophenoxy alkanoates from propionate to octanoate. The data indicate that the initial step in the degradation of ω-linked 2,4-dichlorophenoxyalkyl carboxylic acids by the bacterium involves a cleavage of the ether linkage, a new mechanism for the microbial metabolism of these compounds.

Previous studies by MacRae, Alexander, and Rovira (1963) have indicated that a *Flavobacterium* sp. was able to degrade the herbicide 4-(2,4-dichlorophenoxy)butyric acid [4-(2,4-DB)] by cleavage of the ether linkage to yield 2,4-dichlorophenol and butyric acid. The resulting phenol was metabolized rapidly, while the butyric acid was oxidized slowly. Such a mechanism would lead to the immediate detoxication of the compound, a substance having potential herbicidal activity. This mechanism is in contrast with the oxidation pathway proposed by Webley, Duff, and Farmer (1957) and Taylor and Wain (1962), whose isolates of *Nocardia* initiated the degradation of phenoxyalkyl carboxylic acids by beta oxidation of the aliphatic side chain. The latter mechanism would lead to the production of phytotoxic intermediates from the original substrate.

The present investigation was designed to determine whether an ether-cleaving mechanism is involved in the decomposition of a series of ω-linked 2,4-dichlorophenoxyalkyl carboxylic acids by the flavobacterium.

MATERIALS AND METHODS

The microorganism used in these studies was the *Flavobacterium* sp. originally isolated from soil and described previously (Burger, MacRae, and Alexander, 1962). The 4-(2,4-DB)-containing medium employed for the growth of the organism was that of MacRae, Alexander, and Rovira (1963).

For manometric investigations, 5-day-old cultures grown with forced aeration were harvested by centrifugation and washed twice in 0.02 M phosphate buffer (pH 7.5); the cells were then suspended in the same buffer. Oxygen uptake was measured at 30 C with air as the gas phase (Umbreit, Burris, and Stauffer, 1957). Each respirometer flask contained 1.0 μmole of substrate, 50 μmoles of phosphate buffer (pH 7.5), and approximately 20 mg (dry weight) of bacterial cells in a total volume of 3.0 ml. To the center well was added 0.2 ml of 20% KOH.

After completion of the manometric measurements with the phenoxyalkyl carboxylic acids, the contents of each flask were centrifuged to remove the microbial cells, and the supernatant was analyzed for the decrease in ultraviolet-light absorption at the wavelength at which the compound exhibited maximal light absorption. Measurements of the ultraviolet absorption were performed with a Beckman DU spectrophotometer. All oxygen-uptake rates have been corrected for endogenous gas consumption.

To isolate and identify intermediates formed during the metabolism of phenoxyalkyl carboxylic

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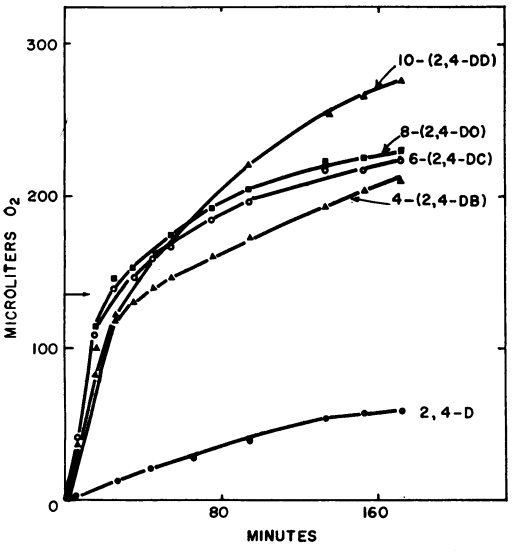

FIG. 1. *Oxidation of ω-linked 2,4-dichlorophenoxyalkyl carboxylic acids with even numbers of carbons.*

acids by *Flavobacterium* sp., the following procedure was adopted. Cells were harvested from the 4-(2,4-DB) medium, washed twice in 0.02 M phosphate buffer (pH 7.5), and then approximately 80 mg (dry weight) of the cells were placed in flasks containing 0.002 M solutions of each of the phenoxy acids in 0.02 M phosphate buffer (pH 7.5). The flasks were incubated on a rotary shaker at 30 C, and samples were removed periodically for analysis of phenols by the method of Fawcett et al. (1958). Samples were also taken for paper and gas chromatographic identification of fatty acids. The fatty acids were obtained by adding NaOH to give a 5% solution of NaOH, saturating the solution with carbon dioxide, extracting with ether, adjusting the residue to pH 2.0, and then re-extracting with ether to yield a fraction containing organic acids.

The organic acids were converted to the ammonium salts prior to their characterization by paper chromatography. The solvent system used was 5% ethyl alcohol in *n*-butanol, the butanol having been previously equilibrated with an equal volume of aqueous 1.5 N NH_4OH (Block, Durrum, and Zweig, 1958). Ascending chromatograms were prepared, and the acids were detected by spraying with 0.04% bromocresol purple (Reid and Lederer, 1951).

Samples to be characterized by gas chromatography were dissolved in diethyl ether and introduced into a Barber-Colman gas chromatograph (model 10) fitted with an argon ionization-detection cell containing 56 μc of Ra^{226}. A U-shaped column of heavy-walled borosilicate glass tubing (5 mm inner diameter and 6 ft long) was used. Prior to chromatographic characterization, the fatty acids were converted to their methyl esters by the microtechnique of Roper and Ma (1957). The esters were dissolved in diethyl ether and introduced into the chromatograph. The column was packed with a partitioning medium which employed 20% diethylene glycol succinate on siliconized Chromasorb W (80 to 100 mesh). The operating parameters were: column temperature, 79 C; cell temperature, 25 C; flask heater, 185 C; and argon flow rate, 55 ml/min.

Samples of 5-(2,4-dichlorophenoxy)valeric acid [5-(2,4-DV)], 6-(2,4-dichlorophenoxy)caproic acid [6-(2,4-DC)], 7-(2,4-dichlorophenoxy)-heptanoic acid [7-(2,4-DH)], 8-(2,4-dichlorophenoxy)octanoic acid [8-(2,4-DO)], 9-(2,4-dichlorophenoxy)nonanoic acid [9-(2,4-DN)], 10-(2,4-dichlorophenoxy)decanoic acid [10-(2,4-DD)], and 11-(2,4-dichlorophenoxy)undecanoic acid [11-(2,4-DU)] were supplied by May and Baker Ltd., Dagenham, Essex, England. Samples of 3-(2,4-dichlorophenoxy)propionic acid [3-(2,4-DP)] were obtained from the U.S. Department of Agriculture, and 4-(2,4-DB) was provided by Chipman Chemical Co., Inc., Portland, Ore., and Amchem Products, Inc., Ambler, Pa. The chemicals were shown to be free from contaminating chlorinated phenoxy compounds by gas chromatography and free from fatty acids by paper chromatography.

RESULTS

The patterns of oxidation of ω-linked phenoxyalkyl carboxylic acids having an even number of carbon atoms in the side chain are shown in Fig. 1. The results for the series having an odd number of carbon atoms are presented in Fig. 2. It is apparent that all the substrates with the exception of 2,4-dichlorophenoxyacetic acid (2,4-D) were readily metabolized. The initial phase of the oxidation of both groups of compounds proceeded at a rapid, linear rate. The linear phase of gas consumption terminated when the amount of oxygen consumed was approximately equal to the theoretical value for oxidation of the aromatic portion of the molecules, a value

for oxygen uptake indicated by the arrows in Fig. 1 and Fig. 2. The subsequent rates of oxidation were far slower than the first phase. The data indicate, moreover, that cells induced to metabolize 4-(2,4-DB) oxidized phenoxyalkyl carboxylic acids having alkanoate moieties larger than butyrate with no lag period.

Spectrophotometric analysis of the contents of the reaction vessels at the completion of the manometric measurements revealed that the ultraviolet-light absorption characteristic of all but one of the molecules was lost (Table 1). There was no discernible activity with the smallest molecule of the group, 2,4-D. These results demonstrate that the aromatic ring of each of the compounds except 2,4-D was cleaved. On the basis of ring rupture with the nine larger molecules, the decline in oxidation rate when the amount of oxygen consumed was approximately equal to that needed for oxidation of the aromatic nucleus, and the observation that 4-(2,4-DB)-induced cells metabolized all higher phenoxyalkyl carboxylic acids with no lag, it seems likely that the linear portions of the oxidation curves represent the dissimilation of the aromatic moieties of the molecules and that the subsequent slow rates of gas consumption reflect a slow decomposition of the aliphatic side chains.

A relationship between the rate of oxidation and the size of the fatty acid substrate was noted when 4-(2,4-DB)-grown *Flavobacterium* cells were supplied with 1 μmole of each of the fatty acids in the series from acetic to undecanoic acid (Fig. 3 and Fig. 4). Not only the amount of oxygen consumed but also the rate of gas disappearance was directly correlated with fatty acid chain length. A similar relationship was observed in the secondary, slow phase of dissimilation of the phenoxy compounds.

Phenolic substances were detected at various sampling intervals when the bacterium was incubated with all phenoxyalkyl carboxylic acids tested in the series 3-(2,4-DP) through 11-(2,4-DU). No attempt was made to identify the individual phenols, however. The phenol concentration ranged from trace amounts to approximately 100 μg/ml, depending upon the substrate and the length of incubation; the quantity formed was consistently greater from phenoxy compounds with an odd number of carbons in the aliphatic chain than from those substrates with an even number of carbons. These results showing greater phenol yield from the odd-number compounds resemble those of Taylor and Wain (1962) with *N. coeliaca* and Gutenmann et al. (Soil Sci. Soc. Am. Proc., *in press*) with a mixed microbial population of soil, and they may indicate that the bacterium can also effect a slow beta oxidation of the aliphatic chain while it is still linked to the aromatic moiety, the beta

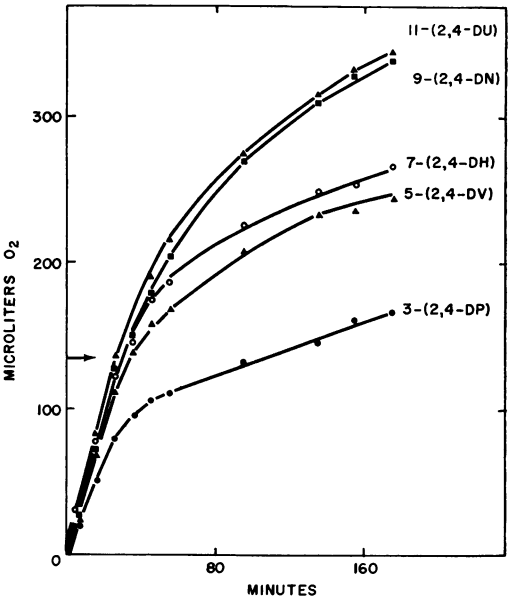

FIG. 2. *Oxidation of ω-linked 2,4-dichlorophenoxyalkyl carboxylic acids with odd numbers of carbons.*

TABLE 1. *Changes in ultraviolet absorption during degradation of 2,4-dichlorophenoxyalkyl carboxylic acids by Flavobacterium*

Substrate	Wavelength	Optical density*	
		0 min	205 min
	mμ		
2,4-D	283	0.246	0.230
3-(2,4-DP)	283	0.206	0.010
4-(2,4-DB)	283	0.223	0.020
5-(2,4-DV)	283	0.223	0.015
6-(2,4-DC)	283	0.240	−0.005
7-(2,4-DH)	285	0.200	0.020
8-(2,4-DO)	283	0.210	0.030
9-(2,4-DN)	285	0.215	0.015
10-(2,4-DD)	285	0.196	0.000
11-(2,4-DU)	283	0.183	0.045

* Corrected for ultraviolet-light absorption in the absence of substrate.

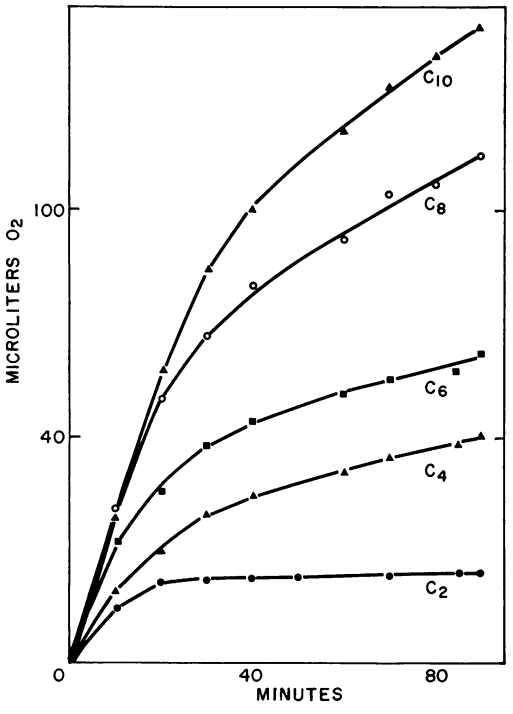

FIG. 3. *Oxidation of free fatty acids with even numbers of carbons. C_2, acetic; C_4, butyric; C_6, caproic; C_8, octanoic; and C_{10}, decanoic acid.*

oxidation of the odd series proceeding to yield 2,4-dichlorophenoxyformate which might spontaneously decompose to the corresponding phenol.

Paper chromatography of the reaction mixture after incubation of the bacterium with 6-(2,4-DC), 7-(2,4-DH), and 8-(2,4-DO) revealed that a single component was released in appreciable amounts from each of the three substrates. The R_F values of these three products were 0.55 for the metabolite released from 6-(2,4-DC), 0.61 for the product liberated from 7-(2,4-DH), and 0.63 for that formed from 8-(2,4-DO). The R_F values of authentic caproate, heptanoate, and octanoate with the solvent system and conditions employed were 0.55, 0.61, and 0.63, respectively.

Gas chromatographic analyses of the methylated ether extracts after 24 hr of incubation of bacterial cells with the substrates revealed the presence of free propionic, butyric, valeric, caproic, heptanoic, and octanoic acids in cell suspensions metabolizing 3-(2,4-DP), 4-(2,4-DB), 5-(2,4-DV), 6-(2,4-DC), 7-(2,4-DH), and 8-(2,4-DO), respectively. Retention volumes for methyl esters of authentic fatty acids and for the peaks obtained with ether extracts of the incubation mixtures are given in Table 2. A plot of the logarithm of the retention volumes against the number of carbons in the fatty acids

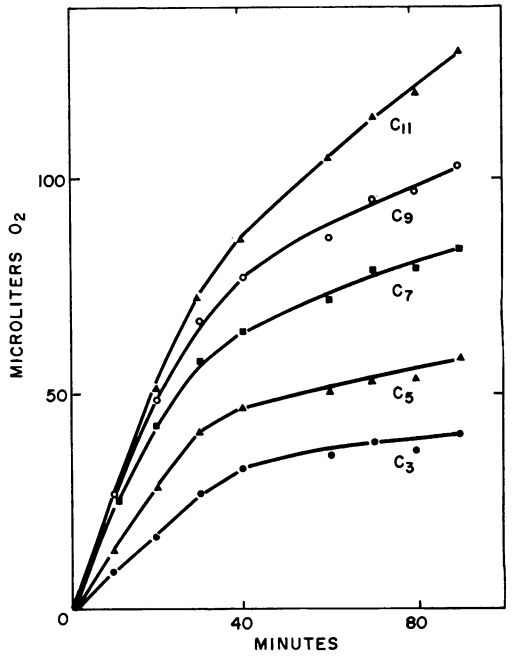

FIG. 4. *Oxidation of free fatty acids with odd numbers of carbons. C_3, propionic; C_5, valeric; C_7, heptanoic; C_9, nonanoic; and C_{11}, undecanoic acid.*

TABLE 2. *Gas chromatographic analysis of methylated fatty acids and acid fractions of Flavobacterium cultures after incubation with phenoxyalkyl carboxylic acids*

Authentic chemical or test substrate	Retention volume
	ml
Authentic chemicals	
Propionate, methyl	57.7
Butyrate, methyl	94
Valerate, methyl	173
Caproate, methyl	297
Heptanoate, methyl	510
Octanoate, methyl	900
Test substrates	
3-(2,4-DP)	57.7*
4-(2,4-DB)	99.5, 137
5-(2,4-DV)	173, 137
6-(2,4-DC)	302, 137
7-(2,4-DH)	302, 520, 137
8-(2,4-DO)	302, 920, 137

* Retention volumes of products formed from the indicated test substrates.

gives the anticipated straight line for the authentic acids; a similar plot of the logarithm of retention volumes of one of the metabolites produced in the decomposition of each of the test substrates against the number of carbons in the alkanoate moiety of the substrate yields a straight line identical with the first.

An unidentified component with a retention volume of 137 ml was observed in extracts from cultures metabolizing phenoxy acids from 4-(2,4-DB) through 8-(2,4-DO). The finding of peaks with retention volumes of 920 and 302 ml in cultures incubated with 8-(2,4-DO) demonstrates the formation of both octanoic and caproic acids, the latter presumably by a beta oxidation mechanism from the former. The finding of a substance with retention characteristics identical with that of caproate in cultures incubated with 7-(2,4-DH) is rather surprising, however.

Discussion

The data reported in the present investigation suggest that the initial attack upon ω-linked 2,4-dichlorophenoxyalkyl carboxylic acids by a *Flavobacterium* sp. involves cleavage of the ether linkage. This cleavage results in the production of a phenol and the fatty acid corresponding to the aliphatic moiety of the original molecule. Although no attempt was made to characterize the phenol, the formation of 2,4-dichlorophenol from 4-(2,4-DB) by the flavobacterium was previously demonstrated (MacRae et al., 1963).

Little oxygen was consumed in the presence of 2,4-D, and even this slight activity may have resulted from a stimulation of endogenous respiration since no decrease in ultraviolet-light absorption was detected. The next higher member of the series, 3-(2,4-DP), was metabolized, but the rate of degradation was slower than that observed with any of the larger molecules. It is likely, therefore, that short side chains may interfere with the ether-cleaving enzyme system.

Enzymatic cleavage of ether linkages, though often considered to pose difficulties because of the energy barrier, has been demonstrated to occur in animal tissues. Axelrod (1956), for example, observed that rabbit liver homogenates were able to effect the hydrolytic cleavage of aromatic ethers. The observations reported previously (MacRae et al., 1963) and in the present communication indicate either a reductive cleavage or a hydrolytic cleavage followed by a reduction to give rise to the phenol and the fatty acid.

These findings provide evidence for a second mechanism for the microbial metabolism of phenoxyalkyl carboxylic acids. The first pathway involves beta oxidation of the aliphatic moiety (Webley et al., 1957; Taylor and Wain, 1962); the second pathway is initiated by cleavage of the ether linkage.

Acknowledgments

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