The Decomposition of 4-(2,4-Dichlorophenoxy)butyric Acid by *Flavobacterium* sp.

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SUMMARY

A *Flavobacterium* sp. isolated from soil and grown in media containing 4-(2,4-dichlorophenoxy)butyric acid (4-(2,4-DB)) metabolized 2,4-dichlorophenol and 4-chlorocatechol without a preliminary period of induction. The initial oxidation of 4-(2,4-DB) was rapid, but the rate declined to a value equivalent to that observed for butyric and crotonic acid oxidation. The bacterium produced 2,4-dichlorophenol, 4-chlorocatechol and butyric and crotonic acids when grown in the presence of 4-(2,4-DB). It is proposed that the initial step in 4-(2,4-DB) oxidation involves a cleavage of the ether linkage rather than β -oxidation of the aliphatic moiety.

INTRODUCTION

Several of the phenoxyalkyl carboxylic acids which are used as herbicides are decomposed in soil by microbial action. By means of a plant bioassay technique, Burger, MacRae & Alexander (1962) established a relationship between molecular structure and the persistence in treated soil of phytotoxic phenoxyalkyl carboxylic acids, but such studies do not indicate whether the inhibitory effects result solely from residual pesticide or whether new phytotoxic substances appear as a result of microbial degradation of the added compound. To establish the pathway of decomposition, investigations have been made with cultures of some of the bacteria responsible for the detoxication in natural environments. For example, Steenson & Walker (1957) proposed that 2,4-dichlorophenol and 4-chlorocatechol were intermediates in the metabolism of 2,4-dichlorophenoxyacetic acid (2,4-D) by strains of *Achromobacter* and *Flavobacterium peregrinum*, and Evans & Smith (1954) reported the production of 2-hydroxy-4-chlorophenoxyacetate and 4-chlorocatechol in cultures of a soil bacterium which metabolized 4-chlorophenoxyacetic acid. Webley, Duff & Farmer (1957) demonstrated the conversion of 4-(4-chlorophenoxy)-butyric acid to β -hydroxy-4-(4-chlorophenoxy)butyric acid by *Nocardia opaca*; these results, together with the more recent observations of Taylor & Wain (1962), suggest that the aliphatic moiety of phenoxyalkyl carboxylic acids is metabolized by β -oxidation. The present paper is concerned with work on the decomposition of 4-(2,4-dichlorophenoxy)butyric acid (4-(2,4-DB)) by a *Flavobacterium* isolated from soil. The data suggest that the molecule is initially degraded by cleavage of the ether linkage rather than by β -oxidation of the fatty acid.

METHODS

The bacterium, which was isolated and described by Burger *et al.* (1962), was grown in a medium containing: 4-(2,4-DB), 0.5 g.; $(\text{NH}_4)_2\text{HPO}_4$, 0.5 g.; yeast extract (Difco), 0.5 g.; KCl, 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 g.; and distilled water, 1.0 l. For the isolation of intermediates, the same medium was used, but 4-(2,4-DB) was provided as sole source of carbon and energy. For manometric investigations, 5-day cultures grown with forced aeration were harvested by centrifugation, washed twice in 0.02M phosphate buffer (pH 7.0) and suspended in the same buffer. Oxygen uptake was measured at 30° with air as gas phase (Umbreit, Burris & Stauffer, 1957). Each respirometer flask contained 1.0–2.5 μmole substrate, 50 μmole phosphate buffer (pH 7.0) and the equivalent of about 15 mg. dry wt. bacteria in a total volume of 3.0 ml. To the centre well was added 0.2 ml. of 20 % (w/v) KOH. The indicated rates of oxygen uptake have been corrected for endogenous gas exchange. Following completion of the manometric measurements, the contents of each flask were centrifuged to remove the bacteria, and the supernatant fluid was analysed for chloride and the decrease in ultraviolet (u.v.) absorbency at the wavelength at which the compound showed maximum light absorption.

Chloride was determined by the colorimetric method of Bergmann & Sanik (1957). Ultraviolet absorption analyses were made with a Beckman spectrophotometer, Model DU. Phenolic substances were detected by the chromatographic method of Lederer (1949), the spots being located either by photographing the chromatograms under u.v. radiation in the range 275–300 m μ , or by spraying the chromatograms with diazotized sulphanilic acid followed by sodium carbonate (Lederer, 1949). The technique of Reid & Lederer (1951) was used for showing the presence of fatty acids.

Samples to be characterized by gas chromatography were dissolved in diethyl ether and introduced into a Barber–Coleman gas chromatograph (Model 10) fitted with an argon ionization detection cell containing 56 μc . ^{226}Ra . A U-shaped column of heavy walled borosilicate glass tubing, 5 mm. i.d. and 6 ft. long, was packed with the partitioning medium, which employed Silicone 710 on Chromasorb W, 80–100 mesh, in a ratio of 1:5 (w/w). The operating parameters were: column temperature, 162°; cell temperature, 235°; flash heater, 206°; and argon flow rate, 46 ml./min.

RESULTS

Sequential induction

In preliminary trials it was observed that 4-(2,4-DB)-grown *Flavobacterium* oxidized the herbicide most readily in the vicinity of pH 7.0, and this pH value was therefore used in subsequent studies. The oxidation was rapid at pH 8.0 but was nil at pH 6.0. The rate of O_2 uptake was linear with time around pH 7.

Previous work (Burger *et al.* 1962) indicated that the enzymes concerned with 4-(2,4-DB) degradation by this organism were inducible. Using the technique of sequential induction (Stanier, 1947), several compounds were examined as possible intermediates in the metabolism of 4-(2,4-DB) by *Flavobacterium* cells grown in the liquid medium containing 4-(2,4-DB) as carbon source. The substrates tested

included 2,4-dichlorophenol, 4-chlorocatechol, 2,4-D, hydroxyhydroquinone and chlorohydroquinone. Oxygen uptake, carbon dioxide evolution, chloride release and disappearance of the u.v. absorption specific for the substrate were used as criteria for the oxidation of the test compound.

Non-proliferating bacteria which had been grown in a 4-(2,4-DB) medium consumed O_2 at a rapid linear rate with 4-(2,4-DB) and 2,4-dichlorophenol as substrate, and no induction period was required (Fig. 1). This suggests that 2,4-dichlorophenol may be an intermediate in the degradation of the herbicide. The u.v. absorption characteristic of these two compounds was lost, and 102 and 113 % of the bound chlorine in 4-(2,4-DB) and 2,4-dichlorophenol, respectively, was recovered as free

Fig. 1

Fig. 2

Fig. 1. Oxidation by 4-(2,4-DB)-grown *Flavobacterium* of 4-(2,4-DB) (●—●), 2,4-dichlorophenol (▲—▲) and 2,4-D (○—○). The three substrates were supplied in quantities of 2.0, 1.05 and 2.0 μ mole, respectively.

Fig. 2. Oxidation by 4-(2,4-DB)-grown *Flavobacterium* of 4-(2,4-DB) (●—●), 4-chlorocatechol (▲—▲), hydroxyhydroquinone (○—○) and chlorohydroquinone (△—△). The substrates were supplied in quantities of 2.18, 1.14, 1.70, and 2.43 μ mole, respectively.

chloride. The latter substrate was completely metabolized, the theoretical value of 6 μ mole O_2 being consumed per μ mole of 2,4-dichlorophenol. The oxidation of 4-(2,4-DB) was incomplete, by contrast, and only about 6 μ mole of O_2 were utilized per μ mole of this substrate; the theoretical value is 10.5.

If the initial steps of degradation of 4-(2,4-DB) involve β -oxidation of the aliphatic moiety, it would be expected that the bacterium should have been induced to metabolize 2,4-D. The results in Fig. 1 show no significant O_2 consumption in the presence of 2,4-D. Moreover, there was neither loss of u.v. absorption nor liberation of chloride when the 4-(2,4-DB)-induced organisms were exposed to 2,4-D. The lack of activity on exogenously supplied 2,4-D may have resulted from a permeability barrier.

In the presence of 4-chlorocatechol, the rate of O_2 consumption was rapid, and no period of induction was detected (Fig. 2). For each μ mole 4-chlorocatechol

supplied, 6 μ mole of O_2 disappeared; this is the theoretical value for complete oxidation. The gas consumed before the marked decrease in the rate of 4-(2,4-DB) oxidation was equal to about 6 μ mole O_2/μ mole substrate. At the end of the experimental period, 96 and 105 % of the bound chlorine of 4-(2,4-DB) and 4-chlorocatechol was liberated, and the u.v. absorption of the aromatic nuclei of the two molecules had disappeared. There was no change of optical density in the u.v. region when the bacteria were incubated with chlorohydroquinone or hydroxyhydroquinone, and no chloride was liberated by the organism from the former compound. The O_2 consumption in the presence of the quinones was only slightly above the endogenous gaseous exchange, which was 63 μ l. O_2 in 2 hr., and may have resulted from a stimulation of endogenous respiration.

Fig. 3. Oxidation by 4-(2,4-DB)-grown *Flavobacterium* of 4-(2,4-DB) (●—●), butyrate and crotonate (▲—▲), β -hydroxybutyrate (○—○) and acetate (△—△). The substrates were supplied in quantities of 2.00, 1.95, 1.95, 2.44 and 6.75 μ mole, respectively.

Oxidation of aliphatic acids

Cells of 4-(2,4-DB)-grown *Flavobacterium* were tested for ability to oxidize aliphatic acids. None of the compounds examined was metabolized readily (Fig. 3). There was no difference in activity between butyrate and crotonate. It is interesting that following the disappearance of sufficient O_2 to account for the complete metabolism of the aromatic portion of the herbicide molecule, the rate of O_2 uptake upon 4-(2,4-DB) declined to a value equivalent to that obtained for butyrate, crotonate and β -hydroxybutyrate. The initial rapid oxidation may thus have reflected the degradation of the aromatic ring, while the subsequent slow rate may have represented the decomposition of the aliphatic moiety.

Isolation and identification of intermediates

To demonstrate the presence of metabolic intermediates, the *Flavobacterium* was inoculated into a medium containing 4-(2,4-DB) as the sole carbon source. Samples of the cultures were taken at intervals and tested for phenolic substances by using diazotized sulphanilic acid and sodium carbonate. When a strong yellow

colour was obtained with these reagents, the cultures were adjusted to pH 2.0 with hydrochloric acid and steam distilled. The distillate was brought up to pH 8.0 with sodium hydroxide and evaporated to dryness in vacuum, the residue taken up in diethyl ether and applied to paper chromatograms, which were developed in *n*-butanol saturated with 5N-NH₄OH. Parallel chromatograms were run using authentic 2,4-dichlorophenol and 4-chlorocatechol. When the development of the chromatograms was complete, the spots were located either by photographing under u.v. radiation or by spraying with diazotized sulphanilic acid followed by sodium carbonate. Two spots were revealed, one of *R_f* value 0.74, equivalent to that of 2,4-dichlorophenol, and a second of *R_f* 0.82. The *R_f* value of an authentic sample of 4-chlorocatechol was 0.81.

Fig. 4

Fig. 5

Fig. 4. Gas chromatograms of authentic 2,4-dichlorophenol (A) and extracts of culture filtrate (B).

Fig. 5. Gas chromatograms of the phenolic fraction (C) and the acid fraction (D) obtained from *Flavobacterium* culture filtrates.

Additional steam distillates of the culture were prepared in the same way, and the dried material was taken up in 0.1 N-hydrochloric acid. The u.v. absorption spectrum of the sample was compared with that of authentic 2,4-dichlorophenol; the spectra were identical with symmetrical peaks at about 285 m μ .

Gas chromatograms were prepared from an ethereal solution of the dried steam distillate obtained in an identical manner. Samples (10 μ l.) of the standard 2,4-dichlorophenol in diethyl ether were injected into the gas chromatographic column. The authentic compound yielded a normal peak with a retention time of 3.5 min., or a retention volume of 161 ml., and no tailing. In contrast, the material from the culture revealed a peak at 3.5 min., a shoulder at 4.25 min. and a considerable degree of tailing (Fig. 4).

The dried material was taken up in 5 % sodium hydroxide, the solution saturated with carbon dioxide and then extracted with ether to separate the phenolic com-

ponent. The remaining solution was adjusted to pH 2.0 with hydrochloric acid and again extracted with ether to obtain a second fraction. The water in the ether fractions was removed by drying over anhydrous sodium sulphate; 10 μ l. samples were used for gas chromatography. The chromatograms obtained for these two fractions are shown in Fig. 5. The phenolic fraction showed a peak identical with that of the authentic sample of 2,4-dichlorophenol. The second fraction showed a peak at 4.25 min. with a small shoulder at 3.5 min., suggesting incomplete removal of the phenolic constituent. Although the identity of the peak at 4.25 min. had not been established by gas chromatographic analysis, the crystalline material did exhibit an odour similar to crotonic acid. A sample of the original 4-(2,4-DB) substrate showed no peaks in a 30 min. development, establishing that the herbicide sample was free of 2,4-dichlorophenol.

Metabolism of the butyrate moiety

Washed suspensions of *Flavobacterium* which had been grown in the 4-(2,4-DB) medium were incubated in 50 ml. of solution containing 100 μ mole unlabelled 4-(2,4-DB), 0.09 μ g. 4-(2,4-DB) carrying a ^{14}C label on the carboxyl carbon (50.6 mc./mg.) and 1.0 mmole phosphate buffer (pH 7.0). Carbon dioxide-free air was passed through this suspension and samples were withdrawn periodically to determine the quantity of ^{14}C remaining in the reaction vessel. At the time at which only half of the ^{14}C remained in the bacterial suspension, sufficient concentrated sodium hydroxide was added to give a final concentration of 5% (w/v) NaOH. This solution was saturated with carbon dioxide, extracted with ether, the aqueous residue adjusted to pH 2.0 and then re-extracted with ether to give a fraction containing organic acids. The acids were converted to ammonium salts, and these were applied to paper chromatograms together with authentic 4-(2,4-DB), butyrate, crotonate, acetate, propionate and β -hydroxybutyrate and the chromatograms developed with *n*-butanol that had been equilibrated previously with an equal volume of aqueous 1.5 N- NH_4OH . Only two spots appeared on chromatograms of the acid fraction after spraying with 0.04% (w/v) brom cresol purple (Reid & Lederer, 1951). These two spots had R_f values of 0.87 and 0.66, the known 4-(2,4-DB), butyrate and crotonate giving R_f values of 0.87, 0.65 and 0.66, respectively. By passing chromatogram strips through a chromatograph strip counter (Atomic Accessories, Bellerose, N.Y., Model RSC-5B), it was found that both spots carried the ^{14}C -label.

The paper was sprayed with a dilute solution of potassium permanganate, and the spot with R_f value 0.66 was resolved into two portions. The first of these, which decolorized the permanganate, had an R_f value of 0.66 and corresponded with the standard for crotonate. The second portion, which did not decolorize the permanganate, had an R_f value of 0.65, identical with that of butyrate.

DISCUSSION

The manometric data indicate that 4-(2,4-DB)-induced *Flavobacterium* metabolized 2,4-dichlorophenol and 4-chlorocatechol without a preliminary induction phase, but not 2,4-D, chlorohydroquinone or hydroxyhydroquinone. The oxidation of 2,4-dichlorophenol and 4-chlorocatechol was complete, whereas the initially

rapid rate of O_2 disappearance in the presence of 4-(2,4-DB) decreased when the quantity of O_2 consumed was about equal to that required for the oxidation of only the aromatic portion of the molecule. The new rate was the same as that obtained whether the substrate supplied to the bacterium was butyrate or crotonate. Consistent with these findings was the evidence for the formation of 2,4-dichlorophenol, 4-chlorocatechol, butyrate and crotonate by suspensions of the organism incubated with the herbicide.

On the basis of these observations the most plausible hypothesis for the mechanism of 4-(2,4-DB) decomposition by the organism used is that the initial attack on the molecule involves a cleavage of the ether linkage, with the formation of 2,4-dichlorophenol and butyric acid. The 2,4-dichlorophenol is then dehalogenated at the *o*-position, and the resultant 4-chlorocatechol is readily and completely degraded, possibly with the formation of β -chloro-cis, cis-muconic acid. The butyric acid is probably metabolized at a slow rate by β -oxidation.

Fig. 6

The present findings differ from those of Webley *et al.* (1957) and Taylor & Wain (1962), whose isolates of *Nocardia*, *Pseudomonas* and *Micrococcus* metabolized the aliphatic moiety of phenoxyalkyl carboxylic acids while the fatty acid was still attached to the aromatic ring. It would thus appear that such molecules can be metabolized by at least two pathways, one being initiated by a β -oxidation sequence, as in the strains of *Nocardia* examined, the second by a cleavage of the ether linkage. The existence of two pathways may have an ecological significance, since β -oxidation of herbicides of this type would lead to the formation of new phytotoxic compounds. The alternate pathway would result in a detoxication of the applied herbicide by microbial action.

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