BACTERIAL DECOMPOSITION OF 2,4-DICHLOROPHENOXYACETIC ACID[1]

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Numerous researches have established that 2,4-dichlorophenoxyacetic acid (2,4-D) exhibits a gradual disappearance from the soil (Nutman *et al.*, 1945; Audus, 1949; Thorup, 1951; and Rogoff, 1952) and that a biological factor is the agent of inactivation (Audus, 1949; Norman, 1950; Jensen and Petersen, 1952).

Newman and Thomas (1949) reported crude cultures of bacteria which inactivated 2,4-D and some undefined pure cultures exhibiting a slight detoxifying effect. Enrichment techniques employed by Audus (1949, 1951) yielded an organism of the type *Bacterium globiforme* which in pure culture decomposed 2,4-D. Jensen and Petersen (1952) isolated two organisms, one identified as *Flavobacterium aquatile*, the other similar to that of Audus, which would carry out the decomposition. Neither investigator found it possible to effect the decomposition with the pure cultures when carried out in a synthetic medium.

These studies were made in an attempt to identify the organisms active in the decomposition of 2,4-D in typical Pennsylvania field soils, study the metabolism of these organisms in synthetic media containing 2,4-D, and determine the nature of the end products of their activity.

MATERIALS AND METHODS

Isolations of microorganisms capable of metabolizing 2,4-D were made from modified Lees and Quastel (1946) percolation units which had been used for studies of the rate of inactivation of 2,4-D in Pennsylvania soils. These had received several treatments of 500 ppm of 2,4-D and were capable of rapidly inactivating that amount. Such units might thus be considered as enrichments. Subcultures were made by streaking 0.1 ml of the leachate of such a unit over the surface of an agar plate containing a medium of the following composition: 2,4-D, 0.1 per cent; $(NH_4)_2HPO_4$, 0.05 per cent; KCl, 0.02 per cent; $MgSO_4$, 0.02 per cent; glutamic acid, 0.01 per cent; $FeSO_4 \cdot 7H_2O$, 0.005 per cent; and agar, 1.5 per cent. The medium was prepared with tap water and adjusted to pH 7.2 before sterilization by autoclaving at 121 C for 15 min. The broth medium used in subsequent experiments contained the same ingredients, with the omission of the agar. Broth for chloride recovery experiments was made by substituting NH_4NO_3 for $(NH_4)_2HPO_4$, K_2HPO_4 for KCl, and ion-free water for tap water. Ion-free water was obtained by passing distilled water through a Crystalab ion exchange Deminizer, which delivered water containing less than 1 ppm of free ions.

Analytical methods. Quantitative analysis of 2,4-D was made spectrophotometrically using a Beckman model DU spectrophotometer at 282 mμ. Samples taken for analysis were treated with an equal volume of 1.2 per cent trichloroacetic acid, made to volume with distilled water, centrifuged, and decanted. The supernatant was used for the spectrophotometric determination. In routine determinations density readings were taken at 270, 280, 282, 284, 286, and 290 mμ. Density at 282 mμ was used for the quantitative estimation. The other readings served as a check on the identity of the compound being determined, the 282 and 290 mμ readings bearing the constant relationship D 282/D 290 = 1.16 $\pm$ 0.01, for 2,4-D.

Chloride ion was determined gravimetrically by precipitation as silver chloride according to the method described by Fales and Kenny (1939).

Cultures for resting cell suspensions. Cultures were maintained in stock on 100-ml portions of the 2,4-D broth in 500-ml Erlenmeyer flasks. These were shaken on a reciprocating type shaker at 25 C for 72 hr and stored at 5 C. To

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obtain cell crops, each of two 4-L Erlenmeyer flasks containing 750 ml of the 2,4-D broth, with an additive of 0.002 per cent soil extract, was inoculated with 50 ml of a stock broth culture and shaken at 25 C for 48 hr. The cells were removed by centrifugation in the cold, and washed once with M/50 phosphate buffer, then suspended in 10 ml of buffer. The pH of the buffer was 7.52. The Warburg apparatus was used in the conventional manner to measure O_2 uptake and CO_2 evolution.

EXPERIMENTAL RESULTS

Identity of microorganism. The bacterium isolated from the 2,4-D agar streak plates appeared in practically a pure culture and was identified as a member of the genus *Corynebacterium*. It was a gram positive irregularly staining rod appearing singly and in clusters, with a tendency for the rods to become coccoid in older cultures. On nutrient agar it produced yellow, smooth, round, entire colonies, while in nutrient broth it produced moderate turbidity and sediment. No hydrogen sulfide or indole was produced and nitrate was reduced to nitrite. The methyl red and Voges-Proskauer tests were negative. In litmus milk it produced no change, or very slow reduction. Gelatin was not liquefied. Neither acid nor gas was produced from the following: glucose, sucrose, maltose, lactose, salicin, mannitol, and glycerol. The organism gave a positive catalase test. None of the species in the genus *Corynebacterium* described in the current edition of *Bergey's Manual* (1948) correspond to this organism; therefore, a species name has not been assigned. The organism differs from the *B. globiforme* of Audus (1951) in its colonial characteristics on agar and in its growth in broth. *B. globiforme* liquefies gelatin; this organism did not do so, even on prolonged incubation. Agar colonies of the *Corynebacterium* produce a yellow pigment, while no pigmentation on agar is mentioned in *Bergey's Manual* for *B. globiforme*.

Decomposition of 2,4-D. Pure cultures of the organism obtained from the streak plates were used to inoculate 20-ml portions of 2,4-D agar in 8-oz prescription bottles, in which the agar was allowed to gel with the bottles on their sides. After 48 hr of incubation at 25 C, the growth from the surface of each bottle culture was washed off with 5 ml of sterile distilled water. This material was then used to inoculate 100-ml aliquots of the 2,4-D broth in 500-ml Erlenmeyer flasks. The flasks were shaken at 25 C, and tested periodically for the disappearance of 2,4-D. Decomposition of 2,4-D in such a flask culture and decomposition on subsequent transfer are seen in table 1.

TABLE 1

Decomposition of 2,4-D by a Corynebacterium sp. in a synthetic medium

Days	Transfer Number				
	0	1	2	3	Control
	mg/ml 2,4-D				
1	—	1.055	1.085	1.085	1.075
2	0.990	1.075	0.980	0.930	—
3	—	1.020	0.800	0.845	1.075
4	—	0.760	0.479	0.660	—
5	1.090	0.470	0.085	0.358	1.060
6	—	0.245	—	0.115	—
7	1.070	0.195	—	—	—
11	—	—	—	—	1.076
22	0.110	—	—	—	—

The use of small amounts (0.02 per cent) of additives, such as tryptose or yeast extract, to the medium did not serve to increase significantly the rate of decomposition, nor did the addition of materials such as sterile agar, glass wool, or asbestos fiber, which would provide surface area. The fermentation was conducted at various starting pH's and the optimum established in the range pH 7 to 9, the most complete decomposition taking place at pH 8.0. Similarly when oxygen for the fermentation was provided in varying amounts by provision of more or less exposed surface of the medium, or mechanically by shaking or sparging, it was found that the rate of decomposition was proportional to the amount of oxygen available.

When various nitrogen sources were substituted for $(NH_4)_2SO_4$ in the medium, it was found that certain of the amino acids provided for the most rapid and complete decomposition. Tryptose increased the rate of fermentation, but to a lesser degree. Results of such an experiment may be seen in table 2.

On increasing the concentration of 2,4-D by increments of 500 ppm in serial transfer, decomposition of the compound took place up to a level of 3,000 ppm. At a level of 3,500 ppm of 2,4-D, there was no evidence of either growth or

decomposition of the compound following an incubation period of 45 days.

Recovery of 2,4-D chlorine as chloride ion. In attempting to determine the end products of 2,4-D decomposition, it had been noted that no compounds detectable spectrophotometrically had appeared. To determine the degree of destruction of the ring structure, the release of chlorine from the compound was determined. The following procedure was used: One hundred ml aliquots of the chloride-free medium were inoculated with 1 ml of a stock broth culture. Control flasks received 1 ml of sterile distilled water. Samples were withdrawn from the flasks immediately, and determinations of 2,4-D made. This determination served as the basis for the calculation of organic chlorine before fermentation. The flasks were incubated on the shaker. On removal they were made to volume with ion-free water, centrifuged at 3,000 g and the supernatants filtered through Whatman No. 2 filter paper, with suction. Aliquots of the filtrate were taken for analysis. The results of two such studies are summarized in table 3. The data are typical of those obtained from long- and short-term experiments to determine the release of organic chlorine at various stages of 2,4-D decomposition.

TABLE 2

The effect of nitrogen source on the fermentation of 2,4-D by Corynebacterium sp. in broth culture

Days	Nitrogen Source				
	$(NH_4)_2SO_4$	$NaNO_3$	Tryptose	Asparagine	Alanine
	*mg/ml 2,4-D**				
1	1.416	1.403	1.490	1.389	1.376
2	1.060	1.048	1.115	1.043	1.023
3	0.935	0.773	0.695	0.685	0.530
4	0.453	0.216	0.250	0.207	0.153
5	0.135	0.142	0.238	0.120	0.110

* Averages of duplicate flasks.

TABLE 3

Recovery of 2,4-D chlorine as chloride ion after fermentation in a medium free of chloride ion

	Hours of Fermentation	
	96	48
	mg	*mg*
Organic Cl before fermentation	14.20	13.70
Organic Cl after fermentation	1.08	9.65
Organic Cl freed (theoretical)	13.12	4.05
Chloride recovered	12.50	3.90
Per cent recovery	95.50	96.50

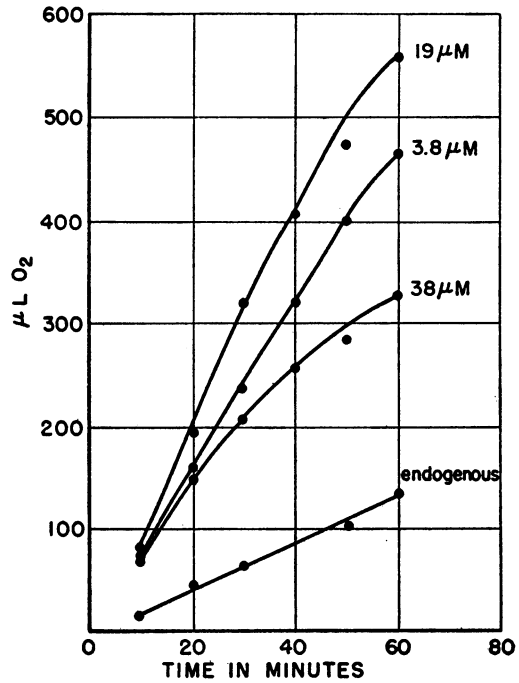

Figure 1. Oxidation of 2,4-D by cells of *Corynebacterium* sp. at pH 7.5.

Manometric studies. A series of experiments were conducted using 3.8 μM, 19 μM, and 38 μM of 2,4-D as substrate. As seen in figure 1, the cells oxidized 2,4-D at all levels of concentration, but at the higher levels there was a toxic effect evidenced. In all subsequent experiments a level of 3.8 μM, or lower, of 2,4-D was used. Experiments in which CO_2 evolution, as well as O_2 uptake, was measured showed a respiratory quotient, at completion, of 1.12. Calculations showed more than 95 per cent of the 2,4-D added to be oxidized in these experiments.

Other experiments in which 2,4-D, 2,4-dichlorophenol, α-phenoxyacetamide, 2-phenoxyethanol, and phenoxyacetic acid were used as substrates showed oxidation of 2,4-D and 2,4-

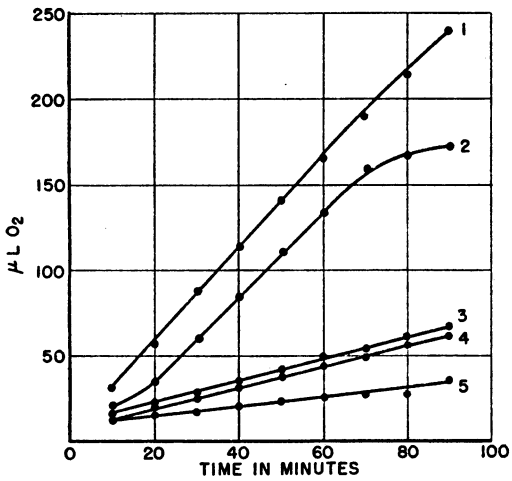

Figure 2. Oxidation of 2,4-D and related compounds by cells of *Corynebacterium* sp. Line 1, 2,4-D; line 2, 2,4-dichlorophenol; line 3, 2-phenoxyethanol; line 4, phenoxyacetic acid; line 5, endogenous.

dichlorophenol, and some stimulation of respiration with 2-phenoxyethanol and phenoxyacetic acid (figure 2).

DISCUSSION

The organism found to possess the ability to decompose 2,4-D has been placed in the genus *Corynebacterium* without species assignment. Members of this genus are quite numerous in soils and often have been implicated in the attack of various aromatic compounds. The organism isolated in this study bears a certain resemblance to the *B. globiforme* of Audus (1951), and that of Jensen and Petersen (1952). Both organisms attacked 2-methyl-2-chlorophenoxyacetic acid (MCPA) as well. The attack of other aromatic compounds by organisms of this type has been reported. Jensen and Gundersen (1954) isolated a culture of *Corynebacterium simplex* which attacked a series of dinitro compounds including 4,6-di-nitro-orthoeusol, picric acid, and 2,4-dinitrophenol. Choman *et al.* (1954) isolated several strains of the genus *Corynebacterium* which actively decomposed nicotine in pure culture. Apparently the organism is highly adaptive in its oxidative abilities.

The isolate is able to decompose relatively large amounts of 2,4-D (1,000 ppm) in from 3 to 5 days. Apparently the molecule is attacked as a whole, the ring being ruptured, and complete destruction following. This is evidenced in the manometric experiments where CO_2 equivalent to the 2,4-D carbon was recovered, and the R.Q. of 1.12 would account for oxidation of all but the carboxyl carbon of the compound. Organic chlorine was released from the molecule as chloride, equivalent to that amount of 2,4-D decomposed by the culture, as determined spectrophotometrically. The residual 2,4-D was apparently unchanged. This, too, seems to substantiate complete destruction. The ability of this organism to oxidize 2,4-dichlorophenol and the determination by previous investigators (Audus, 1951; Jensen and Petersen, 1952) that organisms of this type attack MCPA as well tend to accentuate the role of the para substitution group as a prerequisite for attack, this being the constant factor in these cases. It is of interest that the *para*-nitro group is necessary for the attack of certain dinitrocresols and dinitrophenols (Jensen and Gundersen, 1954) by *Corynebacterium simplex*.

SUMMARY

An organism belonging to the genus *Corynebacterium* has been isolated. This organism decomposes 2,4-dichlorophenoxyacetic acid (2,4-D) in relatively large amounts in a synthetic medium. Indications are that complete destruction of the molecule follows ring rupture.

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