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Adaptive Patterns in the Bacterial Oxidation of 2:4-Dichloro- and 4-Chloro-2-methyl-phenoxyacetic Acid

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SUMMARY: Organisms of a strain of *Flavobacterium peregrinum* and an *Achromobacter* strain, capable of decomposing 2:4-dichlorophenoxyacetic acid (2:4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) respectively, have been obtained by growing cultures on peptone agar plates containing either 2:4-D or MCPA. Glucose did not prevent adaptation. Organisms of the *F. peregrinum* strain were adapted to oxidize 2:4-D when grown on peptone agar containing either MCPA or 2-chloro-4-methylphenoxyacetic acid; they did not decompose MCPA. The effects of some related compounds on adaptation to 2:4-D by these bacteria were examined. Growth of the *Achromobacter* strain on peptone agar containing either 2:4-dichlorophenol or 5-chloro-2-cresol gave organisms which were adapted to oxidize both 2:4-D and MCPA as well as the inducing compound.

Evidence was obtained by Steenson & Walker (1956, 1957) that the oxidation of 2:4-dichlorophenoxyacetic acid (2:4-D) and 4-chloro-2-methylphenoxyacetic acid (MCPA) by two different soil bacteria was effected by adaptive enzyme systems and that 2:4-dichlorophenol and 5-chloro-2-cresol were intermediates in the dissimilation of 2:4-D and MCPA respectively. Organisms of *Flavobacterium peregrinum* n.sp. (Stapp & Spicher, 1954) grown on 2:4-D could oxidize 2:4-D, 4-bromo-2-chloro- and 2:4-dibromophenoxyacetic acid and the corresponding phenols from which these acids are derived, but none of the isomeric dichlorophenoxyacetic acids. The power of an *Achromobacter* sp. to oxidize MCPA became decreased by repeated subculture in media containing 2:4-D in place of MCPA, but was restored when the organism was again grown in MCPA-containing media. On the other hand, MCPA-grown organisms of this strain could oxidize both MCPA, 2:4-D and the corresponding phenols, 5-chloro-2-cresol and 2:4-dichlorophenol.

Walker & Newman (1956) described the isolation of strains of a soil micro-organism, tentatively identified as a *Mycoplana* sp., which decomposed 2:4-D and the 2:4-D-grown organisms also oxidized 2:4-dichlorophenol. Oxidation of 2:4-dichlorophenol by a 2:4-D-decomposing *Corynebacterium* sp. has been reported by Rogoff & Reid (1956) and Bell (1957) obtained a similar finding with an *Achromobacter* sp. Earlier work by Audus (1951) with soil-enrichment cultures in a percolator had shown that there was some adaptation between MCPA and 2:4-D. Hitherto, most workers have used culture media in which 2:4-D or MCPA was the sole carbon source, with perhaps supplementary amounts of yeast or soil extracts as sources of growth factors. Indeed, Audus (1951) found that all adaptation to oxidize 2:4-D was lost if he grew his strain of *Bacterium globiforme* on media containing sugars, but pre-

sumably not 2:4-D. We have found that adapted organisms may be obtained by growth of our two bacterial strains on peptone agar or peptone glucose agar in the presence of moderate amounts of 2:4-D or MCPA. The effect on adaptive enzyme formation of a number of related aromatic compounds has been studied therefore by incorporating them at suitable concentrations in peptone media for the growth of the bacteria.

METHODS

Micro-organisms. A strain of *Flavobacterium peregrinum*, n.sp. (Stapp & Spicher) and an *Achromobacter* strain, both of which were isolated from Rothamsted soil and described by us (Steenson & Walker, 1956) previously, were used.

Media and cultural conditions. Agar plates were poured from a sterilized solution of peptone (0·2–1·0 g.), 2:4-D or MCPA (5–50 mg.) and agar (2·0 g.) in distilled water (100 ml.) adjusted to pH 7·3 by adding aqueous sodium carbonate solution. As necessary, other compounds were used in place of 2:4-D. All cultures were incubated at 25° for 2–4 days.

Manometeric methods. Organisms were washed in 0·02 M-phosphate buffer solution (pH = 6·98) and resuspended in the same buffer solution. The total nitrogen content of the suspension was determined by the usual micro-Kjeldahl procedure. Oxygen uptake by 1 ml. cell suspension (containing usually 0·15–0·4 mg. total N) was measured in Warburg manometers at 30°. The main cup of the Warburg vessel contained the suspension of organisms and enough 0·02 M-phosphate buffer solution to give a total volume of 3 ml.; the substrates were added from the side bulb, after equilibration. The centre cup contained 0·2 ml. 20 % (w/v) aqueous potassium hydroxide and the gas phase was air.

Reference compounds. 2:3-, 2:5- and 3:5-Dichloro-phenoxyacetic acids and 2:4-difluorophenoxyacetic acid were given to us by Professor R. L. Wain, F.R.S. 2-Methyl-, 4-methyl- and 2:4-dimethyl-phenoxyacetic acids were prepared by reacting the sodium salts of the corresponding phenols with sodium monochloroacetate. 2-Chloro-4-methylphenoxyacetic acid was synthesized for us by Mr J. Heidemeyer. Other compounds were obtained commercially.

RESULTS

The effect of peptone and glucose on adaptation

Cultures of the *Achromobacter* strain were grown on peptone (0·5 %) glucose (0·5 %) agar plates for 3 days with and without 2:4-D (0·05 %). The rates of oxygen uptake by the harvested organisms were measured in the presence of 2:4-D or 2:4-dichlorophenol as substrate. The results, given in Table 1, showed that the organisms were adapted to oxidize the substrates only after growth in the presence of 2:4-D; neither peptone nor glucose prevented adaptation from taking place.

In another experiment, organisms of *Flavobacterium peregrinum* adapted to

oxidize 2:4-D were obtained by growth in a peptone (0·5%) agar medium containing 2:4-D (0·05%). When grown on peptone agar alone, the organisms obtained were not adapted to oxidize 2:4-D (Table 2).

Table 1. *Respiration of organisms of* Flavobacterium peregrinum *grown on different media, in the presence of various substrates*

Oxygen uptakes were determined in Warburg manometers at 30° as described in the text. q_{O_2} represents μl. of O_2/mg. cell N/hr. The endogenous respiratory rate was not subtracted in calculating the q_{O_2} values in this table.

Medium	Substrate	q_{O_2}
Peptone	None	40
	2:4-D	50
Peptone + 2:4-D	None	40
	2:4-D	600
Peptone + MCPA	None	125
	2:4-D	1050
	MCPA	275
	5-Chloro-2-cresol	400
Peptone + 2-chloro-4-methylphenoxyacetic acid	None	100
	2:4-D	260
	MCPA	125
	2-Chloro-4-methylphenoxyacetic acid	195

Table 2. *Respiration of* Achromobacter *organisms, grown on different media, in the presence of various substrates*

Oxygen uptakes were determined in Warburg manometers at 30° as described in the text. q_{O_2} represents μl. of O_2/mg. cell N/hr. The endogenous respiratory rate was not subtracted in calculating the q_{O_2} values in this table.

Medium	Substrate	q_{O_2}
Peptone + glucose	None	214
	2:4-D	285
	2:4-Dichlorophenol	190
Peptone + glucose + 2:4-D	None	220
	2:4-D	860
	2:4-Dichlorophenol	810
Peptone + 2:4-dichlorophenol	None	207
	2:4-D	1070
	MCPA	570
	2:4-Dichlorophenol	750
	5-Chloro-2-cresol	336
Peptone + 5-chloro-2-cresol	None	86
	2:4-D	666
	MCPA	566
	2:4-Dichlorophenol	140
	5-Chloro-2-cresol	233

The effect of MCPA on Flavobacterium peregrinum

Cultures of *Flavobacterium peregrinum* were grown on 0·5% peptone agar containing MCPA (0·05%) for 3 days. These organisms were not adapted to oxidize MCPA but gave a considerable oxygen uptake in the presence of

2:4-D or 5-chloro-2-cresol. Thus, although MCPA itself is not metabolized by this organism, it can induce in *F. peregrinum* the enzymes necessary for the oxidation of 2:4-D.

The effect of related compounds on Flavobacterium peregrinum

The activity of organisms grown on peptone agar with a series of substituted phenoxyacetic acids was examined.

2-Chloro-4-methylphenoxyacetic acid behaved like the isomeric MCPA in that it induced the adaptation of the organisms to oxidize 2:4-D (Table 2). The organisms also oxidized the inducing compounds to some extent.

None of the following substances caused any adaptation to 2:4-D oxidation: phenoxyacetic acid, 2-methyl-, 4-methyl- and 2:4-dimethyl-phenoxyacetic acid; 2-chloro-, 4-chloro-, 2:3-, 2:5- and 3:5-dichloro-phenoxyacetic acid; 2:4-difluoro-phenoxyacetic acid.

The effect of 2:4-dichlorophenol on the Achromobacter *strain*

Cultures of the *Achromobacter* strain were grown on peptone (0·2%) agar medium containing 2:4-dichlorophenol (0·004%) for 3–4 days. The harvested organisms were adapted to oxidize 2:4-D, 2:4-dichlorophenol, 4-chloro-catechol, and, at a slower rate MCPA and 5-chloro-2-cresol. 2:4-Dichlorophenol was oxidized only at low concentrations, viz. 1 μmole/3 ml., and twice this concentration was inhibitory.

The effect of 5-chloro-2-cresol on the Achromobacter *strain*

Similar experiments to the above were carried out in which cultures were grown on peptone (0·2%) agar containing 5-chloro-2-cresol (0·004%). 2:4-Dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid were oxidized most rapidly, 5-chloro-2-cresol was oxidized more slowly and 2:4-dichlorophenol scarcely at all.

The effect of 2:4-dichlorophenol on Flavobacterium peregrinum

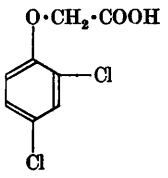
It was found that 2:4-dichlorophenol was rather inhibitory to the growth of this strain even at a concentration of 50 parts per million, and it was difficult to obtain enough organisms in 3–4 days for manometry. However, in one experiment, organisms grown on peptone (0·25%) agar containing 2:4-dichlorophenol (32 parts per million), showed a moderate rate of oxygen uptake with 2:4-dichlorophenol (1 μmole/3 ml.), a slower one with 5-chloro-2-cresol and very feeble oxidation of 2:4-D.

DISCUSSION

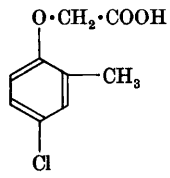
The fact that organisms adapted to oxidize 2:4-D or MCPA could be obtained from cultures grown on peptone media, provided that small concentrations of 2:4-D or MCPA were also present suggested that the effect of other compounds might be tested similarly. It seemed that growth in the presence of a

suitable inducing compound was all that was necessary to cause the formation of the adaptive enzyme systems involved and the inducing compound need not be the only substance metabolized by the bacteria during their growth. It is interesting that both 4-chloro-2-methylphenoxyacetic acid (MCPA) and the isomeric 2-chloro-4-methylphenoxyacetic acid could also induce in *Flavobacterium peregrinum* the adaptive enzymes for oxidizing 2:4-D. This organism has not been shown to decompose MCPA in soil cultures whereas it readily attacks 2:4-D.

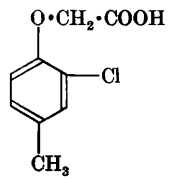
The effect of 2:4-dichlorophenol or 5-chloro-2-cresol in adapting organisms of the *Achromobacter* strain to oxidize 2:4-D and MCPA is not contrary to the theory of simultaneous adaptation outlined by Stanier (1947). It was pointed out by Stanier that in a complex dissimilation an enzyme may act at more than one stage in the process. Hence when two intermediates, say D and E, are separated by one enzymic step, the possibility exists that the enzyme catalysing that particular step (D → E) may also function later on in the oxidation of E, and that growth on E will also adapt the cells completely for the attack on D. If two intermediates, say B and E, are separated by several intervening steps (B → C → D → E), growth on E is not likely to produce cells completely adapted for the oxidation of B. In the present case, the above phenols may be assumed to be the first oxidation products of 2:4-D and MCPA respectively and can apparently induce adaptation of bacteria to oxidize their immediate precursors.



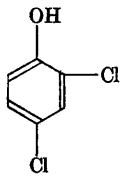
2:4-D



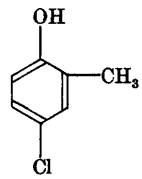
MCPA



2-Chloro-4-methyl-phenoxyacetic acid



2:4-Dichlorophenol



5-Chloro-2-cresol

In all these cases, the inducing compounds are 2:4-disubstituted phenols or phenoxyacetic acids, and it is clear that position isomerism is the dominant factor determining the specificity of the adaptation.

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REFERENCES

AUDUS, L. J. (1951). The biological detoxication of hormone herbicides in soil. *Plant & Soil*, **3**, 170.

BELL, G. R. (1957). Some morphological and biochemical characteristics of a soil bacterium which decomposes 2:4-dichlorophenoxyacetic acid. *Canad. J. Microbiol.* **3**, 821.

ROGOFF, M. H. & REID, J. J. (1956). Bacterial decomposition of 2:4-dichlorophenoxyacetic acid. *J. Bact.* **71**, 303.

STANIER, R. Y. (1947). Simultaneous adaptation: a new technique for the study of metabolic pathways. *J. Bact.* **54**, 339.

STAPP, C. & SPICHER, G. (1954). Untersuchungen über die Wirkung von 2:4-D im Boden. IV. Mitteilung: *Flavobacterium peregrinum* n.sp. und seine Fähigkeit zum Abbau des Hormones. *Z. Bakt.* (II. Abt.), **108**, 113.

STEENSON, T. I. & WALKER, N. (1956). Observations on the decomposition of chlorophenoxyacetic acids by soil bacteria. *Plant & Soil*, **8**, 17.

STEENSON, T. I. & WALKER, N. (1957). The pathway of breakdown of 2:4-dichloro- and 4-chloro-2-methyl-phenoxyacetic acid by bacteria. *J. gen. Microbiol.* **16**, 146.

WALKER, R. L. & NEWMAN, A. S. (1956). Microbial decomposition of 2:4-dichlorophenoxyacetic acid. *Appl. Microbiol.* **4**, 201.

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