Degradation of the Selective Herbicide 2,2-Dichloropropionate (Dalapon) by a Soil Bacterium

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(Received 10 May 1978)

A bacterium capable of utilizing the selective herbicide 2,2-dichloropropionate (Dalapon) as sole source of carbon and energy was isolated from soil and tentatively identified as a fast-growing species of *Rhizobium*. 2-Chloropropionate also supported good growth of the organism but 3-chloropropionate, monochloroacetate and dichloroacetate were not utilized. Bacteria grown in the presence of either 2,2-dichloropropionate or 2-chloropropionate oxidized these compounds and a variety of non-chlorinated substrates but not monochloroacetate.

Cell-free extracts of 2,2-dichloropropionate-grown bacteria converted 2,2-dichloropropionate into pyruvate with the concomitant release of two chloride ions for each molecule of pyruvate formed, indicating the presence of dehalogenase activity. Dichloroacetate and 2-chloropropionate were also inducers and substrates for the dehalogenase. Monochloroacetate was a substrate for the dehalogenase but did not serve as an inducer whereas 3-chloropropionate was a non-substrate inducer.

INTRODUCTION

The selective herbicide 2,2-dichloropropionate (Dalapon) is widely used against monocotyledonous plants. It is readily removed from the soil by a variety of micro-organisms including species of *Pseudomonas*, *Agrobacterium*, *Nocardia*, *Alcaligenes*, *Arthrobacter* and *Bacillus* (Foy, 1975). However, the conversion of 2,2-dichloropropionate to intermediates of the central metabolic pathways has not been examined in detail. Bacteria able to utilize 2,2-dichloropropionate are known to release chloride ions from this herbicide (Jensen, 1957, 1960; Hirsch & Alexander, 1960; Senior *et al.*, 1976) and cell-free extracts of an *Arthrobacter* species able to grow on 2,2-dichloropropionate have been reported to convert it into pyruvate (Kearney *et al.*, 1964). The present investigation examines some properties of a newly isolated bacterium able to utilize 2,2-dichloropropionate as sole source of carbon and energy. A brief report of part of this work has appeared previously (Berry *et al.*, 1976).

METHODS

Media. The mineral salts medium was that of Brammar & Clarke (1964). Sterile solutions of carbon sources were added separately to give a final concentration of 0.5% (w/v) unless otherwise stated. When a chlorinated aliphatic acid was added as a second carbon source its final concentration was 0.2% (w/v) and the final concentration of the non-chlorinated carbon source was reduced to 0.4% (w/v). Free acids were neutralized to pH 7.0 with NaOH prior to sterilization by filtration. Media were solidified by the addition of either Oxoid Ionagar no. 2 or Difco Bacto-agar at a final concentration of 1.5% (w/v).

Growth conditions. Bacteria were grown in Universal screw-capped bottles containing either 10 ml nutrient broth or mineral salts medium plus an appropriate carbon source. When larger volumes were required, 100 ml quantities of medium in 250 ml conical flasks were inoculated with 0.2 ml of culture from the corresponding Universal bottle and incubated on an orbital shaker at 250 rev. min⁻¹. Bacteria were harvested by centrifugation (6000g, 10 min). Bacterial dry weight was determined from a standard curve relating the absorbance at 680 nm to dry weight. All cultures were grown at 30 °C unless otherwise stated.

Isolation of 2,2-dichloropropionate-utilizing micro-organism. Garden soil (10 g) was added to 50 ml mineral salts medium containing 2,2-dichloropropionate at a final concentration of 0.1 % (w/v) and incubated at 25 °C. After repeated subculture, an organism capable of utilizing 2,2-dichloropropionate as the sole source of carbon was isolated and purified.

Identification of isolate. Tests were carried out using the methods of Cowan (1974) unless otherwise stated.

Respirometry. Stationary phase bacteria (100 ml) were harvested, washed once with 20 ml 100 mM-phosphate buffer, pH 7.2, and resuspended to give 5 to 10 mg dry wt bacteria ml⁻¹. Oxygen uptake was followed at 30 °C using a Gilson respirometer. The manometer flasks contained: 0.5 ml cell suspension; 100 µmol phosphate buffer, pH 7.2; 0.2 ml 10 % (w/v) KOH in the centre well; distilled water to a final volume of 2.8 ml. Substrates (30 µmol in 0.2 ml) were added from the side-arm after equilibration. Rates of O₂ uptake are expressed as µl O₂ consumed h⁻¹ (mg dry wt)⁻¹.

Preparation of cell-free extracts. Bacteria were harvested, washed once in 20 ml 100 mM-phosphate buffer, pH 7.2, and resuspended in 10 ml of the same buffer. Ice-cold cell suspensions were disrupted at 0 °C by sonication in an MSE disintegrator (100 W) for 45 s, followed by centrifugation (12000g, 10 min, 4 °C). The resulting supernatant solutions, containing 2 to 5 mg crude bacterial protein ml⁻¹, were retained. Soluble protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Enzyme assays. The assay for glucose-6-phosphate dehydrogenase [D-glucose-6-phosphate:NADP⁺ 1-oxidoreductase; EC 1.1.1.49] was based on that of Kuby & Noltman (1966). The assay mixture contained (in 3 ml): 150 µmol Tris/HCl buffer, pH 8.0; 15 µmol MgCl₂; 0.75 µmol NADP; cell-free extract (containing 530 to 850 µg soluble protein). The reaction was started by the addition of 3 µmol glucose 6-phosphate to the test cuvette. The reduction of NADP was followed at 340 nm and 30 °C using a Pye Unicam SP1800 recording spectrophotometer. In order to assay 6-phosphogluconate dehydrogenase [6-phospho-D-glucuronate:NADP⁺ 2-oxidoreductase (decarboxylating); EC 1.1.1.44], 3 µmol 6-phosphogluconate replaced glucose 6-phosphate in the previous assay. For both enzymes, specific activities were calculated as nmol NADP reduced min⁻¹ (mg protein)⁻¹.

Pyruvate estimation. Pyruvate was estimated colorimetrically according to Friedemann & Haugen (1943), assuming the molar absorption coefficient of alkaline pyruvate 2,4-dinitrophenylhydrazone to be 18000 at 445 nm (Kornberg & Morris, 1965). Pyruvate formation from 2,2-dichloropropionate by cell-free extracts was followed continuously at 315 nm and 30 °C using a Pye Unicam SP1800 recording spectrophotometer. The assay mixture contained (in 3 ml): 100 µmol 2,2-dichloropropionate; 1.74 mg phenylhydrazine hydrochloride; 0.48 ml RIM buffer. The RIM buffer contained: 200 µmol EDTA, pH 6.8; 100 µmol MgCl₂; 500 µmol imidazole buffer, pH 6.8; distilled water to a final volume of 4 ml. The reaction was started by the addition of cell-free extract (containing 50 to 800 µg soluble protein) to the test cuvette. Specific activities were calculated as nmol pyruvate formed min⁻¹ (mg protein)⁻¹, assuming the molar absorption coefficient of pyruvate phenylhydrazone to be 13100 at 315 nm (Cooper *et al.*, 1965).

Chloride ion estimation. The ability of cell-free extracts of bacteria to release chloride ions from various compounds was followed in an incubation mixture containing 360 µmol chlorinated aliphatic acid, 360 µmol phosphate buffer, pH 7.2, and distilled water to a final volume of 6 ml. Samples (1.0 ml) were removed at appropriate intervals and assayed for chloride ions, using a Corning EEL (model 920) chloride meter.

Chemicals. 2,2-Dichloropropionate was obtained as the sodium salt from Fluka, CH-9470 Buchs, Switzerland. 2-Chloropropionic and 3-chloropropionic acids were obtained from Ralph N. Emanuel, Wembley. Monochloroacetic and dichloroacetic acids were obtained from BDH. All other chemicals were of the highest purity commercially available.

RESULTS

Characterization of a 2,2-dichloropropionate-utilizing micro-organism

The only other chlorinated aliphatic acid to support good growth of the 2,2-dichloropropionate-utilizing organism was 2-chloropropionate. The isolate utilized a number of non-chlorinated compounds as sole source of carbon including pyruvate, lactate, acetate, mannitol, glycerol, glucose, ribose, sucrose and serine. There was little or no growth on

3-chloropropionate, monochloroacetate, dichloroacetate, citrate, malate, succinate, propionate, glyoxylate, gluconate or acetamide. The doubling time in mineral salts medium with 2,2-dichloropropionate as sole source of carbon was 12.4 h at 30 °C. The addition of yeast extract at a final concentration of 0.05% (w/v) reduced the doubling time with 2,2-dichloropropionate to 5 h at 30 °C. The doubling times in mineral salts medium with pyruvate, lactate and glucose as sole sources of carbon were 3.8, 3.5 and 4.0 h, respectively, at 30 °C. The isolate did not grow at 5 or 37 °C.

The organism was a Gram-negative rod, 0.42 to 0.83×1.7 to $2.5 \mu\text{m}$ in size when grown in mineral salts medium with 2,2-dichloropropionate or glucose as sole source of carbon. Cells from young cultures were occasionally motile. Colonies, which were 1 to 4 mm in diameter after 4 d at 30 °C, had an entire edge, were convex, circular, opaque, shining and white to cream in colour. Older colonies developed a much darker centre. On yeast extract mannitol agar (Fred *et al.*, 1932) copious amounts of free-flowing gum were produced. Congo red (Hahn, 1966) was not absorbed when added to this medium but the addition of NaCl (Kleczkowska *et al.*, 1968) prevented growth of the isolate. The organism was catalase, oxidase and urease positive but lecithinase, deoxyribonuclease and β -galactosidase were not produced. An oxidative reaction was given in the medium of Hugh & Leifson (1953). In the ammonium salt sugar medium of Smith *et al.* (1952) acid was produced from arabinose, cellobiose, fructose, glucose, glycerol, inositol, maltose, mannitol, sorbitol, sucrose, trehalose and xylose, but not from adonitol, lactose, raffinose, rhamnose, salicin or ethanol. The methyl red and Voges-Proskauer tests were negative. There was no hydrolysis of arginine, aesculin, lysine, ornithine, Tween 20 or 80. Nitrate was reduced to nitrite but neither indole nor H_2S were produced. Gelatin was not liquefied. Gluconate was not converted to 2-ketogluconate and phenylalanine was not converted to phenylpyruvate. Nile blue was not reduced in the medium of Skinner (1977). The organism did not grow on MacConkey agar, in selenite broth or in the presence of either cetrимide or KCN. These results suggest that the 2,2-dichloropropionate-utilizing isolate is a fast-growing species of *Rhizobium* (Buchanan & Gibbons, 1974). In support of this view, the isolate was found to possess both glucose-6-phosphate dehydrogenase [specific activity $140 \text{ nmol NADP reduced min}^{-1} (\text{mg protein})^{-1}$] and 6-phosphogluconate dehydrogenase [specific activity $31 \text{ nmol NADP reduced min}^{-1} (\text{mg protein})^{-1}$]. This latter enzyme is present in fast-growing but not slow-growing rhizobia, whilst both groups of bacteria possess glucose-6-phosphate dehydrogenase (Martinez-de Drets & Arias, 1972; Mulongoy & Elkan, 1977).

Oxidation of substrates by bacteria

Washed suspensions of 2,2-dichloropropionate-grown bacteria readily oxidized 2,2-dichloropropionate, pyruvate, lactate and acetate (Table 1). 2-Chloropropionate and alanine were oxidized more rapidly than 2,2-dichloropropionate, after an initial lag period. Propionate and 3-chloropropionate were oxidized less readily than 2,2-dichloropropionate but monochloroacetate, dichloroacetate and propionamide were not oxidized. Bacteria grown on 2-chloropropionate were also able to oxidize 2,2-dichloropropionate. In contrast, washed suspensions of the organism grown on glucose, pyruvate or lactate did not oxidize 2,2-dichloropropionate at a significant rate, although other substrates were readily oxidized by these bacteria (Table 1). Growth on these media in the presence of 2,2-dichloropropionate as a second carbon source enabled the bacteria to oxidize this compound but only at 55 to 79% of the rate observed for bacteria grown on 2,2-dichloropropionate alone.

Pyruvate formation from 2,2-dichloropropionate by cell-free extracts

Cell-free extracts prepared from bacteria grown on 2,2-dichloropropionate as sole source of carbon readily converted this compound to pyruvate (Table 2). There was no pyruvate formation when propionate or 2-chloropropionate replaced 2,2-dichloropropionate in the

Table 1. *Oxidation of substrates by bacteria grown on various carbon sources*

Oxygen uptake was followed at 30 °C using a Gilson respirometer as described in Methods.
Oxygen uptake rates were corrected for endogenous respiration.

Substrate	Rates of oxidation [μ l O ₂ consumed h ⁻¹ (mg dry wt) ⁻¹] by bacteria grown on:			
	2,2-Dichloro- propionate	2-Chloro- propionate	Pyruvate	Pyruvate + 2,2-Dichloro- propionate
2,2-Dichloropropionate	18.1	9.3	1.4	12.4
2-Chloropropionate	24.7	28.3	NT	NT
3-Chloropropionate	3.5	3.1	NT	NT
Acetate	9.5	29.1	34.8	15.8
Propionate	4.5	16.6	NT	NT
Pyruvate	10.3	9.9	12.6	34.5
Lactate	10.9	3.8	22.2	16.2
Glucose	0.9	1.5	3.5	1.7
Alanine	25.5	65.2	34.4	41.4

Substrate	Rates of oxidation [μ l O ₂ consumed h ⁻¹ (mg dry wt) ⁻¹] by bacteria grown on:			
	Lactate	Lactate + 2,2-Dichloro- propionate	Glucose	Glucose + 2,2-Dichloro- propionate
2,2-Dichloropropionate	—	14.3	0.4	9.9
2-Chloropropionate	NT	NT	NT	NT
3-Chloropropionate	NT	NT	NT	NT
Acetate	30.6	10.9	13.3	11.3
Propionate	NT	NT	NT	NT
Pyruvate	11.0	18.7	—	12.0
Lactate	24.9	22.4	3.3	11.3
Glucose	2.1	1.6	22.5	6.3
Alanine	43.6	25.6	22.8	19.7

—, No net O₂ uptake; NT, not tested.

Table 2. *Pyruvate formation from 2,2-dichloropropionate by cell-free extracts of bacteria grown on various carbon sources*

Carbon source(s) for growth	Pyruvate formed [nmol min ⁻¹ (mg protein) ⁻¹]
2,2-Dichloropropionate	64.4
2-Chloropropionate	10.3
Lactate	ND
Lactate + 2,2-dichloropropionate	35.9
Pyruvate	ND
Pyruvate + 2,2-dichloropropionate	16.0
Mannitol	ND
Mannitol + 2,2-dichloropropionate	17.9
Glycerol	ND
Glycerol + 2,2-dichloropropionate	23.7
Glucose	ND
Glucose + 2,2-dichloropropionate	22.2
Lactate + 2-chloropropionate	11.1
Lactate + 3-chloropropionate	10.4
Lactate + dichloroacetate	21.4
Lactate + monochloroacetate	ND

ND, Not detectable.

Table 3. Chloride ion release from chlorinated aliphatic acids by cell-free extracts of 2,2-dichloropropionate-grown bacteria

Substrate	Chloride ion released [nmol min ⁻¹ (mg protein) ⁻¹]
2,2-Dichloropropionate	129
2-Chloropropionate	419
3-Chloropropionate	ND
Monochloroacetate	335
Dichloroacetate	76

ND, Not detectable.

incubation mixture. Extracts of bacteria grown on 2-chloropropionate were also able to convert 2,2-dichloropropionate to pyruvate but only at one-sixth of the rate observed using extracts of 2,2-dichloropropionate-grown cells (Table 2). Extracts of bacteria grown on lactate, pyruvate, mannitol, glycerol and glucose did not form pyruvate from 2,2-dichloropropionate. However, growth on these non-chlorinated compounds in the presence of 2,2-dichloropropionate resulted in induction of the pyruvate-forming system (Table 2).

Some chlorinated aliphatic acids, which did not support growth, nevertheless induced the pyruvate-forming system when added to bacteria growing on lactate as the sole carbon source. Extracts prepared from cells grown on lactate in the presence of dichloroacetate converted 2,2-dichloropropionate to pyruvate at twice the rate observed using extracts of cells grown on lactate in the presence of either of the monochloropropionates (Table 2). However, monochloroacetate did not induce the pyruvate-forming system.

Chloride ion release by cell-free extracts

The liberation of chloride ions from 2,2-dichloropropionate would be expected to accompany the formation of pyruvate from 2,2-dichloropropionate. Cell-free extracts of bacteria grown on 2,2-dichloropropionate as sole source of carbon readily liberated chloride ions from this compound when added to an incubation mixture containing only 60 mM-phosphate buffer, pH 7.2, and 2,2-dichloropropionate (Table 3). The specific activity of the dehalogenase was 129 nmol chloride ion released min⁻¹ (mg protein)⁻¹. When samples of the incubation mixture were assayed for both chloride ion and pyruvate (Friedemann & Haugen, 1943), one molecule of the latter compound was present for each two chloride ions.

Chloride ions were also released from other chlorinated aliphatic acids (Table 3). The rate with 2-chloropropionate was about three times that with 2,2-dichloropropionate but there was no chloride ion release from 3-chloropropionate. Although monochloroacetate was not oxidized by whole bacteria, chloride ions were released from this compound more rapidly than from 2,2-dichloropropionate. Chloride ions were also liberated from dichloroacetate but at about half the rate observed for 2,2-dichloropropionate. The Michaelis constants for 2,2-dichloropropionate, 2-chloropropionate, monochloroacetate and dichloroacetate were 1.95, 3.8, 16.4 and 1.69 mM, respectively.

DISCUSSION

The results of a number of identification tests, together with the comparatively rapid growth rate of the 2,2-dichloropropionate-utilizing bacterium, indicate that this organism is a fast-growing species of *Rhizobium*. The presence of 6-phosphogluconate dehydrogenase in the isolate adds weight to this conclusion as this enzyme is present in fast-growing but not slow-growing rhizobia (Martinez-de Drets & Arias, 1972; Mulongoy & Elkan, 1977).

Species of *Rhizobium* have not previously been reported to degrade 2,2-dichloropropionate but these bacteria are known to utilize a wide range of carbon sources for growth (Buchanan & Gibbons, 1974). At least some species are closely related to pseudomonads and agrobacteria (Elkan, 1971; White, 1972), which have been reported to grow on 2,2-dichloropropionate (Jensen, 1957, 1960; Magee & Colmer, 1959; Senior *et al.*, 1976).

The formation of pyruvate from 2,2-dichloropropionate by extracts of bacteria grown on this latter compound as sole source of carbon was readily demonstrable in agreement with the observation of Kearney *et al.* (1964). As two chloride ions were released from 2,2-dichloropropionate for each molecule of pyruvate formed, the conversion of 2,2-dichloropropionate to pyruvate may be taken as a measure of dehalogenase activity in this bacterium. 2-Chloropropionate, 3-chloropropionate and dichloroacetate served as inducers of dehalogenase activity but were not as effective as 2,2-dichloropropionate under the same conditions. 2-Chloropropionate is also an inducer of the dehalogenase from *Pseudomonas putida* p3 (Slater *et al.*, 1976), but the inducer specificity of the dehalogenase from an *Arthrobacter* species was not examined by Kearney *et al.* (1964).

Studies on the substrate specificities of dehalogenases from 2,2-dichloropropionate-utilizing micro-organisms are confined to the brief reports by Kearney *et al.* (1964) and Slater *et al.* (1976). In the present study it was found that chloride ions were liberated from 2-chloropropionate more readily than from 2,2-dichloropropionate, which could account for the more rapid oxidation of the former compound by whole cells. As two chloride ions are released from 2,2-dichloropropionate compared with one from 2-chloropropionate, this latter compound is effectively dehalogenated 6.5 times more readily than 2,2-dichloropropionate. In contrast, the partially purified dehalogenase from an *Arthrobacter* species liberated chloride ions from 2-chloropropionate less readily than from 2,2-dichloropropionate (Kearney *et al.*, 1964). The dehalogenase from *P. putida* p3 also showed activity towards 2-chloropropionate but the relative rates of chloride ion release from this compound and 2,2-dichloropropionate were not reported (Slater *et al.*, 1976).

Dichloroacetate and monochloroacetate were also substrates for the dehalogenase but did not support growth of the isolate. In the case of dichloroacetate this may be due to the inability of the organism to utilize glyoxylate which is formed from dichloroacetate (Goldman, 1972). The inability of monochloroacetate to induce the dehalogenase is one explanation for the observed lack of growth on this chlorinated compound. Dichloroacetate was a substrate for the dehalogenase from an *Arthrobacter* species (Kearney *et al.*, 1964) but no activity was reported with monochloroacetate. The corresponding enzyme from *P. putida* p3 possessed activity towards dichloroacetate and monochloroacetate (Slater *et al.*, 1976). Although 3-chloropropionate induced the dehalogenase it was not a substrate for the enzyme. The dehalogenase from *Arthrobacter* also showed no activity with 3-chloropropionate (Kearney *et al.*, 1964) whereas the enzyme from *P. putida* showed slight activity towards this substrate (Slater *et al.*, 1976).

The present study has shown that a species of *Rhizobium* capable of growth on 2,2-dichloropropionate can readily convert this substance to pyruvate by means of an inducible dehalogenase. This enzyme shows activity with a variety of chlorinated aliphatic acids and can be induced not only by 2,2-dichloropropionate but also by other chlorinated compounds. Additional information on the properties of the enzymes from cells grown under various conditions will lead to a greater understanding of the degradation of 2,2-dichloropropionate and related compounds.

We gratefully acknowledge the valuable contributions of Mr B. Holmes of the Central Public Health Laboratory, London, and Dr K. Feltham of the University of Leicester in performing some tests used in the identification of the isolate. We thank Mrs E. Parkin for help in the isolation of the 2,2-dichloropropionate-utilizing micro-organism.

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