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Oxidative Metabolism of Protocatechuic Acid by Certain Soil Pseudomonads: A New Ring-Fission Mechanism

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Dagley, Evans & Ribbons (1960) have recently drawn attention to the diversity of the intermediary pathways by which micro-organisms metabolize aromatic compounds. These divergences may occur at three different stages:

(1) Before ring fission; benzenoid structures are modified in different ways, depending on the micro-organism and cultural conditions, before the cleavage step. Substituent groups may be added or eliminated, e.g. by hydroxylation or decarboxylation or both (Ribbons & Evans, 1960).

(2) After ring fission; e.g. protocatechuic acid (I) gives rise to *cis-cis*- β -carboxymuconate (*cis-cis*-3-carboxyhexa-2,4-dienedioic acid) (II) by the action of protocatechuate oxidase (protocatechuate oxygenase, EC 1.99.2.3) present in a variety of micro-organisms; its further conversion into β -oxoadipate (III), however, may occur via at least three alternative routes. Examples of these pathways may be found among the following organisms: *Neurospora crassa* [β -carboxymuconolactone intermediate (Gross, Gafford & Tatum, 1956)]; *Pseudo-*

monas fluorescens A3.12 [unidentified intermediate (Macdonald, Stanier & Ingraham, 1954; Cain, Ribbons & Evans, 1961)]; *Vibrio* O1 [muconolactone intermediate (Cain, 1961)]; and *Nocardia erythropolis* [unidentified lactone intermediate (Cain & Cartwright, 1960)].

(3) During ring fission. Cleavage of catechol to *cis-cis*-muconic acid (*cis-cis*-hexa-2,4-dienedioic acid), by rupture of the C-C bond between the hydroxyl groups, is effected by pyrocatechase (catechol oxygenase, EC 1.99.2.2) (Hayaishi & Hashimoto, 1950; Evans, Smith, Linstead & Elvidge, 1951).

Until recently this last reaction was the only known mechanism by which catechol could be enzymically transformed into aliphatic compounds. Dagley & Stopher (1959), however, discovered that some *Pseudomonas* strains contained an enzyme which oxidatively opened the catechol nucleus between C-2 and C-3, giving α -hydroxymuconic semialdehyde (2-hydroxyhexa-2,4-dienedioic 6-aldehyde). It transpired that these organisms were not unique, and a soil pseudomonad with the new ring-fission mechanism for the cleavage of protocate-

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chuic acid was isolated (Trippett, Dagley & Stopher, 1960; Dagley *et al.* 1960). The work described here compares the new cleavage enzyme protococatechuate 4,5-oxygenase (cf. Mason, 1957) with protococatechuate oxidase (Stanier & Ingraham, 1954). The partial characterization and subsequent metabolism of the new ring-fission product, γ -carboxy- α -hydroxymuconic semialdehyde (4-carboxy-2-hydroxyhexa-2,4-dienedioic 6-aldehyde) (2), are also described. Fig. 1 illustrates the two pathways.

EXPERIMENTAL

Organisms. The *Pseudomonas* strain N.C.I.B. 8893 (Dagley & Patel, 1957) was used initially as a source of the new ring-cleavage system; it was designated D1. Compositions of culture media are as described by them, except where indicated. *Pseudomonas* sp. P1 was isolated by Ribbons & Evans (1960) and used as a source of the protococatechuate-oxidase system; it was grown as previously described. The third micro-organism, designated D2, was isolated by perfusion of garden soil with a mineral-salts medium containing phthalate as sole source of organic carbon. The perfusion medium, neutralized to pH 7 with NaOH, contained the following constituents (g./l.): phthalic acid, 1; KH_2PO_4 , 2; $(\text{NH}_4)_2\text{SO}_4$, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; NaCl, 0.25; CaCl_2 , 0.25; FeCl_3 , 0.02; MnSO_4 , 0.002;

ZnSO_4 , 0.002; CuSO_4 , 0.002. *Pseudomonas* D2 was a Gram-negative motile rod, and showed fluorescence; it was maintained on nutrient-agar slopes by subculturing every 6 weeks; liquid cultures were grown on 0.1% phthalate-mineral-salts medium.

Liquid cultures of all organisms were grown with forced aeration in their appropriate media at room temperature. Samples (100 ml.) of medium contained in 1 l. baffled conical flasks were inoculated from slopes; the flasks were placed in a rotary shaker, and after 2–3 days' growth the whole culture was used to inoculate 10 l. of sterile medium; after 36–48 hr. these were either harvested or used to inoculate 40 l. of non-sterile medium contained in 55 l. polythene bottles. The polythene bottles (55 l.) were sterilized for 2–3 days with toluene or chloroform and the medium introduced just before inoculation. The 50 l. cultures were grown for a further 2 days.

Cultures (10 and 50 l.) were harvested at 13 000g in a bench model Sharples supercentrifuge with a cellophan lining to the rotor. The cells were washed with 10 vol. of 0.2M-phosphate buffer, pH 7.0, and either used immediately or stored at -15° until required.

Cell-free extracts. Cell-free extracts were prepared with the Hughes (1951) bacterial press without abrasive at -15° . Each 1 g. (wet wt.) of crushed cells was suspended in 2 ml. of buffer or water as indicated in a Potter-Elvehjem homogenizer at $0-3^\circ$. Cell suspensions (1 g. wet wt. of cells suspended in 2 ml.) were also disintegrated with the M.S.E.-Mullard Ultrasonic Disintegrator at $0-4^\circ$. The

Fig. 1. Pathways of aerobic dissimilation of protococatechuate by micro-organisms. The ring-fission mechanism determines the subsequent pathway, as follows: (a) Action of protococatechuate oxidase on protococatechuate (I) gives *cis-cis*- β -carboxymuconate (II); conversion into β -oxoadipate (III) occurs through a variety of intermediates; a C_3 - C_4 split of (III) affords acetyl-CoA and succinate (IV). (b) Action of protococatechuate 4,5-oxygenase on protococatechuate (I) gives γ -carboxy- α -hydroxymuconic semialdehyde (2) which shows keto-enol tautomerism; an internal oxido-reduction of (2) affords 4-carboxy-2,3-dihydro-2-hydroxymuconate (3); a C_4 - C_3 split of (3) could produce malate and pyruvate (5) through a coupled oxido-reduction. Action of ammonia on (2) gives 2,4-lutidinic acid (6).

disrupted cell suspensions were centrifuged at 1000g for 10 min. to remove unbroken cells and debris, and the supernatant was again centrifuged at 20 000g for 30 min. at 0° to remove smaller particulate matter.

Materials. Protocatechuic acid was synthesized by the method of Pearl (1949); it was also obtained from L. Light and Co. Ltd., and recrystallized twice from water before use. 2,3-Dihydroxybenzoic acid was prepared by the method of Perkin & Robinson (1914), 2,3,4-trihydroxybenzoic acid as described by Kostanecki (1885), and 2,4,5-trihydroxybenzoic acid according to Gattermann & Eggers (1899). Homoprotocatechuic acid (3,4-dihydroxyphenylacetic acid) was prepared by the method of Pictet & Gams (1909). 2,4-Lutidinic acid (pyridine-2,4-dicarboxylic acid) was obtained from L. Light and Co. Ltd.

Analytical reagents were used where available. All melting points are uncorrected. Microanalyses were by Weiler and Strauss, Oxford.

Estimations. Spectrophotometric determinations were carried out in the Unicam SP. 500 with silica cuvettes of 1 cm. light path. Protein was estimated by the method of Warburg & Christian (1942). Protocatechuic acid and *cis-cis*- β -carboxymuconic acid were estimated spectrophotometrically (Stanier & Ingraham, 1954). Protocatechuic acid was also estimated by the Folin-Ciocalteu reagent and the Evans (1947) reagent.

Manometric experiments were conducted in the conventional Warburg constant-volume apparatus at 30°. Oxygen uptake and carbon dioxide evolution were estimated by the direct method (Dixon, 1952). β -Oxadipate was estimated by catalytic decarboxylation with 4-aminoantipyrine (Sistrom & Stanier, 1953).

Acid production during enzymic reactions was measured titrimetrically. The automatic titrator (Radiometer Ltd., Copenhagen) consisted of an electrometric titration assembly, an automatic titrator and a recording unit. The reaction cuvette was a double-walled glass vessel with an internal capacity of 10 ml. Temperature control was effected by circulating water from a constant-temperature bath with a Circotherm (Braun, Melsungen, West Germany) unit controlled by a contact thermometer. For measurements of acid production during enzymic reactions the burette (0.5 ml.) contained 0.1N-NaOH. The reaction mixtures contained 1 ml. of enzyme solution in water and 4 ml. of water, and the pH was adjusted to 7 with NaOH (the pH of the enzyme solution was usually 6.7–6.8). After 3–4 min. had been allowed for equilibration to 30° and a further 2 min. to estimate acid production (usually negligible) in the absence of added substrate, reactions were initiated by addition of protocatechuate (1 ml., neutralized to pH 7 with NaOH) at the concentrations indicated in the text. The stirring did not permit sufficient aeration for the oxidizing enzymes to show maximum activity, and consequently reaction rates were limited by aeration and were erratic. The stirrer was adjusted so that it beat air into the reaction mixture to obtain linear progress curves, although the aeration was still limiting the rate of reaction.

Chromatography. 2,4-Lutidinic acid was chromatographed in: (a) butan-1-ol-acetic acid-water (4:5:1, by vol.); (b) ethanol-aq. ammonia (sp.gr. 0.88)-water (18:1:3, by vol.). The ascending technique with Whatman no. 4 paper was used.

2,4-Dinitrophenylhydrazones of keto acids were chro-

matographed in: (c) butan-1-ol-ethanol-water (4:1:5, by vol.) (Cavallini & Frontali, 1954); (d) butan-1-ol-ethanol-aq. ammonia (sp.gr. 0.88)-water (16:4:1:19, by vol.); (e) pentan-1-ol-propan-1-ol-aq. ammonia (sp.gr. 0.88) (13:1:7, by vol.). Both ascending and descending techniques were used with Whatman no. 4 paper.

All chromatograms were run at room temperature.

Detection reagents. Ultraviolet-absorbing substances were detected with a mercury lamp (253.7 m μ). Acids were detected by spraying with bromocresol green (Lugg & Overall, 1948), and dipping in the aniline-xylose reagent (Smith, 1958). 2,4-Lutidinic acid was detected by spraying with 3% (w/v) FeSO₄, giving red spots (characteristic of 2-carboxypyridine compounds).

The initial ring-fission product of the 4,5-cleavage γ -carboxy- α -hydroxymuconic semialdehyde) was detected on acid-solvent chromatograms by spraying with N-NaOH.

2,4-Dinitrophenylhydrazones of the keto acids were generally visible, but spraying the air-dried chromatograms with 10% (w/v) Na₂CO₃-N-NaOH (1:1, v/v) increased the sensitivity. Fresh authentic samples of 2,4-dinitrophenylhydrazones of keto acids were always used as markers, since older samples streaked and invariably separated into three or more spots.

Buffers. Incubations were carried out in Na₂HPO₄-KH₂PO₄, tris-HCl, veronal-HCl or sodium acetate-acetic acid buffers. The proportions of each constituent were varied according to pH required, and the molarity of the resulting buffer is indicated in the text.

NaHCO₃ (20 mM), equilibrated with CO₂ + O₂ (50:50) mixture at 30°, was also used.

Preparation of the bis-2,4-dinitrophenylhydrazone of the initial ring-fission product. Two Erlenmeyer flasks (1 l.) each contained: 0.2M-phosphate buffer, pH 7.0 (50 ml.); dialysed (18 hr. against tap water at 4°) Hughes-press extract of *Pseudomonas* D1 (20 ml., approx. 30 mg. of protein/ml.); water (60 ml.); FeSO₄ (5 ml. to give a final concn. of 1 mM); cysteine hydrochloride (5 ml. to give a final concn. of 1 mM). They were equilibrated for 10 min. at 30°. Protocatechuic acid (10 ml., containing 500 μ moles, neutralized to pH 7 with NaOH) was added to each flask to initiate the reaction. The flasks were incubated at 30° for 14 min. on a reciprocating shaker to ensure adequate aeration. (The rate of reaction was previously determined by a pilot experiment in the Warburg apparatus.) After completion of the reaction, the flask contents were pooled, acidified with 6N-HCl (6 ml.), cooled to 0° until the protein flocculated and centrifuged; the precipitate was discarded. The supernatant was evaporated under reduced pressure to about 50 ml.; care was taken that the temperature did not exceed 30°, since the initial ring-fission product is very unstable.

2,4-Dinitrophenylhydrazine solution (500 mg. in 50 ml. of 1% (v/v) HCl in ethanol) was added to the aqueous concentrate, and after a few minutes a hydrazone started to precipitate. This was allowed to stand at room temperature for 6 hr. and then overnight at 0°, and was recrystallized twice from aq. 30% (v/v) acetic acid.

Isoelectric precipitation of protocatechuate 4,5-oxygenase. Hughes-press extracts were subjected to ultrasonic waves for 20–30 min. at 0° or treated with deoxyribonuclease (1 mg./100 ml. of extract) and MgSO₄ (10 mM) for 10 min. at 30° to reduce the high viscosity. The resulting extract was centrifuged at 22 000g for 30 min. at 0°. The super-

nantant was diluted with ice-cold water to give a protein concentration of 10 mg./ml., and the pH lowered to 5.25 by dropwise addition of 0.1N-acetic acid with constant stirring at 0°. After leaving for 10–15 min. at –5 to 0° a precipitate flocculated, and was discarded after centrifuging. The supernatant was brought to pH 4.5 with 0.2N-acetic acid and the centrifuged flocculent precipitate resuspended in ice-cold water (volume of original Hughes-press extract) in a Potter-Elvehjem homogenizer.

Effect of enzyme concentration on protocatechuate oxidation. Each Warburg flask contained: cell-free extract (*Pseudomonas* D2, ultrasonically disintegrated, approx. 18 mg. of protein/ml.), 0.1–0.6 ml. by 0.1 ml. increments; 0.2M-phosphate buffer, pH 7.0 (1 ml.); protocatechuate, neutralized to pH 7.0 with NaOH, in the side arm (5 μ moles in 0.2 ml.); 20% (w/v) KOH in the centre well (0.2 ml.); and water to a final reaction volume of 3.0 ml. Incubation was at 30° in air.

Substrate specificity of protocatechuate 4,5-oxygenase. Each Warburg flask contained: cell-free extract (ultrasonically disintegrated, 0.5 ml.); 0.2M-phosphate buffer, pH 7.0 (1 ml.); substrate in the side arm (5 μ moles in 0.2 ml.); 20% (w/v) KOH in the centre well (0.2 ml.); and water to a final reaction volume of 3.0 ml. For inhibition studies with structural analogues of protocatechuate, potential inhibitors were added to the main compartment (5 μ moles in 0.2 ml.) 15 min. before the addition of protocatechuate from the side arm (5 μ moles in 0.2 ml.).

RESULTS

The pathways of metabolism of *p*-cresol by *Pseudomonas* D1 and of phthalate by *Pseudomonas* P1 converge at the protocatechuic acid stage (Dagley & Patel, 1957; Ribbons & Evans, 1960).

Pathway of metabolism of phthalate by Pseudomonas D2

The pathway of phthalate metabolism by *Pseudomonas* D2 was shown to proceed through 4,5-dihydroxyphthalate and protocatechuate, as

was previously demonstrated by Ribbons & Evans (1960) for *Pseudomonas* P1.

Protocatechuic acid was produced in liquid cultures and was identified by chromatography and u.v.-absorption spectroscopy. When Fe²⁺ ion was deficient in the culture media, larger amounts of protocatechuic acid accumulated. Washed suspensions of phthalate-grown cells rapidly oxidized phthalate, 4,5-dihydroxyphthalate and protocatechuate without lag. The anaerobic decarboxylation of 4,5-dihydroxyphthalate to protocatechuate was demonstrated with soluble extracts of *Pseudomonas* D2, prepared by ultrasonic disintegration.

Oxidation of protocatechuic acid

Gaseous exchange. Protocatechuate was oxidized by crude cell-free extracts of all three strains with the consumption of 1 mole of oxygen/mole of protocatechuate. No carbon dioxide was evolved when extracts of *Pseudomonas* D1 or *Pseudomonas* D2 were used. With extracts of *Pseudomonas* P1, 1 mole of carbon dioxide/mole of protocatechuate was evolved and β -oxoadipate was produced in stoichiometric amounts (Table 1).

Acid production. The results quoted below represent the net increase of carboxyl groups after allowing for the one present initially in the protocatechuate molecule. Crude cell-free extracts of *Pseudomonas* D1 and of *Pseudomonas* D2 metabolizing protocatechuate produced 2 equiv. of acid/mole of protocatechuate. Crude cell-free extracts of *Pseudomonas* P1 metabolizing protocatechuate produced between 1.15 and 1.5 equiv. of acid/mole of protocatechuate. Since *Pseudomonas* P1 oxidizes protocatechuate (I) through *cis-cis*- β -carboxymuconate (II) to β -oxoadipate (III), the variable value obtained with several different extracts is attributed to the relative activities of

Table 1. *Gaseous exchange during protocatechuate metabolism by crude cell-free extracts*

Each Warburg flask contained: protocatechuate (neutralized to pH 7.0 with sodium hydroxide) as indicated; cell-free extract (1.0 ml. of Hughes-press extracts, containing approx. 27 mg. of protein); 0.2M-phosphate buffer, pH 7.0 (1 ml.); and water to a final reaction volume of 3.0 ml. Potassium hydroxide [0.2 ml. of 20% (w/v)] or hydrochloric acid (0.2 ml. of 2N) was used in the centre well or second side arm for oxygen and carbon dioxide measurements respectively. Incubation was at 30° in air for 60 min. Values recorded are corrected for endogenous levels and retention is estimated in control vessels containing all constituents except protocatechuate. β -Oxoadipate was estimated by catalytic decarboxylation with 4-aminoantipyrine. One sample (2 ml.) from each flask, after incubation for 60 min., was pipetted into a new Warburg flask containing N-acetic acid (1 ml.) in the main compartment and 0.1M-4-aminoantipyrine in the side arm. The β -oxoadipate was estimated immediately after equilibration at 30°.

Expt. no.	Cell-free extract (organism)	Protocatechuate (μ moles)	O ₂ uptake (μ moles)	CO ₂ evolved (μ moles)	β -Oxoadipate formed (μ moles)
1	D1	5	5.1	0.53	0.2
2		10	10.05	0.44	0.23
3	D2	5	5	0.32	0.17
4		10	10.1	0.22	0.2
5	P1	5	5.05	5.26	4.76
6		10	10.2	10.2	9.65

protocatechuate oxidase and the 'decarboxylase complex'. The subsequent decarboxylation of *cis-cis*- β -carboxymuconate to β -oxoadipate is then responsible for the value being less than 2, although theoretically ring cleavage between C-3 and C-4 results in the production of two carboxyl groups/molecule.

Dialysed extracts of *Pseudomonas* D1 and of *Pseudomonas* D2 oxidized protocatechuic acid with the consumption of 1 mole of oxygen and the production of 1 equiv. of acid/mole of substrate supplied. The reaction solution in the cuvette assumed an intense yellow colour which could be discharged in acid solution and reproduced again at neutral or alkaline pH [reminiscent of the keto-enol tautomerism of 'fumarylacetoacetate' (Ravdin & Crandall, 1951)].

When dialysed extracts of *Pseudomonas* P1 were incubated with protocatechuate, *cis-cis*- β -carboxymuconate accumulated (Ribbons & Evans, 1960), and 2 equiv. of acid was produced/mole of substrate (Table 2).

Acid production was also measured manometrically. A discrepancy in the results from these two techniques was observed. The production of the second equivalent of H^+ ion/mole of protocatechuate by *Pseudomonas* D1 and by *Pseudomonas* D2 could not be detected manometrically. The apparent disparity would be explained if the pK of the second carboxyl group produced is higher than that of carbonic acid.

Changes in absorption spectra. With crude extracts of *Pseudomonas* D1 or of *Pseudomonas* D2 (1 ml.) and 5 μ moles of protocatechuate in the

reaction vessels (reaction vol. 3 ml.), the oxygen uptake was complete in 8–12 min., and the reaction mixture became yellow. During the next 30–60 min. the yellow colour disappeared without any further gaseous exchange, and a compound accumulated which absorbed maximally at 284 $m\mu$ (ϵ_{max} , 5×10^3). Dialysed extracts, supplemented with Fe^{2+} ions and reducing compounds, metabolized protocatechuate to the yellow compound with the concomitant oxygen consumption. The yellow compound absorbed maximally at 410 $m\mu$ (ϵ_{max} , 29×10^3) in neutral or alkaline solution and at 293 $m\mu$ (ϵ_{max} , 2.3×10^3) in acid solution.

Partial characterization of the initial ring-fission product of protocatechuate by extracts of Pseudomonas D1 and of Pseudomonas D2 as γ -carboxy- α -hydroxymuconic semialdehyde

At no time could we detect a compound absorbing at 276 $m\mu$ (see Dagley & Patel, 1957) during the oxidation of protocatechuate (I) by extracts of *Pseudomonas* D1 or of *Pseudomonas* D2. The compound absorbing at 276 $m\mu$ was shown to be 2,4-lutidinic acid (6) by Trippett *et al.* (1960), and was obtained by oxidation of protocatechuate catalysed by undialysed ammonium sulphate-precipitated extracts of *Pseudomonas* D1.

The yellow compound [γ -carboxy- α -hydroxymuconic semialdehyde (2)] which accumulates during oxidation of protocatechuate by dialysed extracts contains keto groups and could be extracted into ether or ethyl acetate from an acidic aqueous solution. Re-extraction into the aqueous phase with either sodium hydroxide or potassium

Table 2. *Stoichiometry of acid production by crude undialysed and dialysed extracts*

The reaction cuvette contained enzyme solution (1 ml.) of extracts prepared in water, containing approx. 25 mg. of protein, water (4 ml.), and protocatechuate (neutralized to pH 7.0) as indicated. The cell-free extracts [from Hughes press (Expts. 1 and 2) or ultrasonically disintegrated (Expts. 3–6)] and water were placed in the cuvette first and titrated to pH 7.0 (original pH was between 6.75 and 6.9). The reaction was initiated by addition of protocatechuate from a walking-stick pipette. Incubation was at 30° (for detailed procedure see Experimental section) for 45 min. β -Oxadipate was estimated as detailed in Table 1; *cis-cis*- β -carboxymuconate was estimated spectrophotometrically after suitable dilution. —, Not determined.

Expt. no.	Extract (organism)	Protocatechuate (μ moles)	H^+ ion formed (μ equiv.)	H^+ ion formed/protocatechuate oxidized (μ equiv./ μ mole)	<i>cis-cis</i> - β -Carboxymuconate formed (μ moles)	β -Oxadipate formed (μ moles)
<i>Pseudomonas</i> D1						
1	Crude extract	10	19.6	2	—	0.1
2	Dialysed extract	10	9.44	1	—	—
<i>Pseudomonas</i> D2						
3	Crude extract	5	9.9	2	—	0.15
4	Dialysed extract	5	4.95	1	—	—
<i>Pseudomonas</i> P1						
5	Crude extract	10	13.3	1.3*	0.2	9.2
6	Dialysed extract	10	19.6	2	9.3	0.3

* See text for discussion of this value.

hydroxide restored the yellow colour. Ammonia (0.1–6N) also brought the ring-fission compound into the aqueous phase, but within 2–8 min. the absorption peak at 410 m μ disappeared, and a new maximum at 276 m μ appeared. The new spectrum was almost identical with that of 2,4-lutidinic acid. Chromatography revealed spots identical with those of 2,4-lutidinic acid, both in R_F values and colour reactions. Eluted spots possessed u.v. absorption spectra identical with that of authentic 2,4-lutidinic acid (Table 3).

The initial ring-fission product, γ -carboxy- α -hydroxymuconic semialdehyde, was unstable at room temperature in neutral or alkaline solution.

Table 3. *Chromatographic identification of 2,4-lutidinic acid as the product of reaction between γ -carboxy- α -hydroxymuconic semialdehyde and ammonia*

See text for details of solvent systems.

Solutions applied	R_F	
	Solvent (a)	Solvent (b)*
(1) Ethyl acetate soln. of γ -carboxy- α -hydroxymuconic semialdehyde	0.59†	0.75†
(2) Aq. NH ₃ extract of (1)	0.64†	0.7†
(3) KOH extract of (1)	0.52†	0.69†
(4) Authentic 2,4-lutidinic acid	0.67†	0.73†

* Ammoniacal solvent.

† Detected by bromocresol green indicator, NaOH soln. and by FeSO₄ soln. after exposure to NH₃ fumes.

‡ Detected by u.v., bromocresol green indicator, aniline-xylose reagent and FeSO₄ soln.; also possessed u.v. absorption spectra identical with that of 2,4-lutidinic acid.

It was, however, possible to prepare an acidic bis-2,4-dinitrophenylhydrazone (see Experimental section); the twice-recrystallized derivative had m.p. 170–172° (decomp.); chromatography revealed one major component (Table 4) (Found: C 43.5; H, 3.5; N, 20.6. C₁₉H₁₄N₈O₁₂ requires C, 41.8; H, 2.6; N, 20.6%).

Formation of pyruvate and oxaloacetate from protocatechuate by whole cells

Washed suspensions of *Pseudomonas* D1 or of *Pseudomonas* D2 oxidized protocatechuate rapidly, with the uptake of 5–6 atoms of oxygen/mole of substrate. At the termination of oxygen uptake no keto acids could be detected in the supernatant after removal of cells. In the presence of appropriate concentrations of arsenite, protocatechuate acid oxidation was slower with substantial accumulation of keto acids. Pyruvate was present as the major component, with smaller amounts of oxaloacetate and γ -carboxy- α -hydroxymuconic semialdehyde. Partial inhibition of protocatechuate oxidation in whole cells by pyridine-3-sulphonic acid, a known DPN inhibitor (Euler, 1942), caused substantial accumulation of oxaloacetate, and of pyruvate to a less extent. The keto acids were identified by chromatography of their 2,4-dinitrophenylhydrazones (Table 4).

Properties of protocatechuate 4,5-oxygenase

Stability. Concentrated cell-free extracts were stable for several days at 0° with respect to oxidase activity, but dilution of extracts with water or phosphate buffer diminished the stability. When some extracts (ultrasonically disintegrated) were

Table 4. *Identification of the products of protocatechuate oxidation by whole cells when inhibited with arsenite and pyridine-3-sulphonate*

Each Warburg flask contained: cell suspension of *Pseudomonas* D1 or of *Pseudomonas* D2 in 0.1M-phosphate buffer, pH 7.0 (2.0 ml., approx. 13 mg. dry wt. of cells); protocatechuate in side arm (10 μ moles in 0.2 ml.); and water to a final reaction volume of 3.0 ml. Potassium hydroxide [0.2 ml. of 20% (w/v)] was placed in the centre well. Sodium arsenite (final concn. 5 mM) or pyridine-3-sulphonic acid neutralized to pH 7.0 (final concn. 20 mM) was added as indicated. Incubation was at 30° in air. Incubations were initiated by addition of the protocatechuate from the side arm and terminated after 2 hr. by centrifuging the flask contents. Excess (1 ml.) of 2,4-dinitrophenylhydrazine soln. (1 mg./ml. of 2N-sulphuric acid) was added to the supernatant (2 ml.) and left to stand for 2 hr. at room temperature. Solvent extraction separated the acidic 2,4-dinitrophenylhydrazones, and ethyl acetate solutions were applied to the chromatograms. See text for details of the solvent systems.

Solution applied	R_F		
	Solvent (c)	Solvent (d)	Solvent (e)
γ -Carboxy- β -hydroxymuconic semialdehyde bis-2,4-dinitrophenylhydrazone	0.42	0.3*	0.15*
Pyruvate 2,4-dinitrophenylhydrazone	0.48; 0.61	0.55; 0.6	0.31
Oxaloacetate 2,4-dinitrophenylhydrazone	0.34	0.34	0.07
Arsenite-inhibited oxidation	0.33†; 0.43; 0.59	0.36*†; 0.5	0.05†; 0.15*; 0.3
Pyridine-3-sulphonate-inhibited oxidation	0.35; 0.49; 0.59	0.35; 0.5; 0.6	0.05; 0.15; 0.33

* Striking spots.

† Faint spots.

diluted 40–50 times, activity was lost at a rate of 30–40%/hr. at 30°; this did not appear to be due to surface effects or oxidation of thiol groups, since shaking or bubbling oxygen through these diluted extracts did not affect the rate of loss of activity.

Substrate specificity. Protocatechuate was the only substrate of those tested which was oxidized by protocatechuate 4,5-oxygenase in crude cell-free extracts. Compounds that were inactive as substrates, but non-inhibitory, were: *m*-hydroxybenzoate, *p*-hydroxybenzoate, 2,3-dihydroxybenzoate, 2,4-dihydroxybenzoate and vanillate. Compounds inactive as substrates, but inhibiting protocatechuate oxidation were: catechol, protocatechuic aldehyde, 2,3,4-trihydroxybenzoate, 2,4,5-trihydroxybenzoate, gallate, homoprotocatechuate and 4,5-dihydroxyphthalate. *p*-Hydroxybenzoate and 4,5-dihydroxyphthalate were tested with extracts from *Pseudomonas* D2 and *Pseudomonas* D1 respectively. The protocol for these experiments is contained in the Experimental section.

Co-substrates. Molecular oxygen appeared to be essential for activity; methylene blue and ferricyanide could not be substituted. The enzyme had a very high affinity for oxygen; in an O₂+N₂ (1:99) atmosphere, 20% activity was recorded. Hydrogen peroxide (3 or 15 mM) did not stimulate activity, nor did catalase (1 mg. in 3.0 ml.) inhibit it.

Effect of enzyme concentration. The rate of protocatechuate oxidation was proportional to enzyme concentration, up to a limit due to inadequate aeration. When the rate of reaction (scale units: 2 μ l. of oxygen/min.) was plotted against enzyme concentration (scale units: 0.1 ml. of enzyme soln.), a straight line with a slope of 45° was obtained.

Effect of pH. The pH optimum for activity was 7.0–7.5, irrespective of the buffer used (Fig. 2). Included in Fig. 2 is the effect of pH on protocatechuate-oxidase activity.

Effect of cation additions. Protocatechuate-4,5-oxygenase activity, in some crude extracts (ultrasonically disintegrated or from the Hughes press), could be stimulated by the addition of Fe²⁺ ions. This effect was more pronounced with extracts prepared by ultrasonic disintegration and aged extracts; Fe³⁺ ions had no effect, and Mn²⁺, Co²⁺, Ni²⁺, and Mg²⁺ ions were slightly inhibitory. Complete inhibition was observed on the addition of Hg²⁺ and Cu²⁺ ions. The concentration of all these additions (as sulphates, except mercuric chloride) was 1 mM.

Effect of reducing compounds. Oxidase activity in aged extracts was markedly stimulated by cysteine and ascorbate, but not appreciably by reduced glutathione.

Effect of inhibitors. Table 5 summarizes the results obtained from inhibition and reactivation studies on

protocatechuate 4,5-oxygenase. Inhibition was not observed with thiol-group inhibitors other than the mercurials; iodoacetate, alloxan, oxidized glutathione or cystine at a concentration of 1 mM did not inhibit. The reactivation of *p*-hydroxymercuribenzoate inhibition by cysteine, or glutathione plus Fe²⁺ ions, or ascorbate plus Fe²⁺ ions, showed a similarity to homogentisate oxidase (Crandall, 1955).

Effect of dialysis. Dialysis of crude extracts against tap water, distilled water or phosphate buffer for 24 hr. at 5° with agitation resolved protocatechuate 4,5-oxygenase with respect to Fe²⁺ ions. Full activity could be restored by supplements of Fe²⁺ ions and cysteine at a concentration of 1 mM.

Reactivation of isoelectric precipitates. The optimum concentrations of Fe²⁺ ions and cysteine required to reactivate isoelectric precipitates of protocatechuate 4,5-oxygenase are shown in Figs. 3 and 4.

Fig. 2. Effect of pH on protocatechuate 4,5-oxygenase activity. Each Warburg flask contained: cell-free extract (*Pseudomonas* D2 for protocatechuate 4,5-oxygenase, and *Pseudomonas* P1 for protocatechuate oxidase, ultrasonically disintegrated, in water, approx. 15 mg. of protein/ml.; 0.4 ml.); 0.2M-buffer as indicated (1 ml.); protocatechuic acid adjusted to the desired pH with NaOH (5 μ moles in 0.2 ml.); 20% (w/v) potassium hydroxide in the centre well (0.2 ml.); and water to a final reaction volume of 3.0 ml. Incubation was at 30° in air. Buffer solutions used were: acetate (○); phosphate (△); tris-HCl (▲); veronal-HCl (□). Full lines indicate protocatechuate 4,5-oxygenase, broken lines protocatechuate oxidase.

Table 5. *Effect of various inhibitors on protocatechuate 4,5-oxygenase*

Each Warburg flask contained: cell-free extract (*Pseudomonas* D2, ultrasonically disintegrated, approx. 20 mg. of protein/ml.; 0.3 ml.); 0.2M-phosphate, pH 7.0 (1 ml.); protocatechuic acid, neutralized to pH 7.0, in side arm (5 μ moles in 0.2 ml.); 20% (w/v) potassium hydroxide in centre well (0.2 ml.); and water to a final reaction volume of 3.0 ml. Inhibitors were added 15 min. before reactions were initiated. Where other additions were made they were added 15 min. after the inhibitor simultaneously with initiation of the reaction. Incubation was at 30° in air. When potassium cyanide was used as inhibitor, it was also placed in the centre well. *p*-Hydroxymercuribenzoate was used as supplied by Sigma Chemical Co. The rate of oxygen uptake in absence of inhibitors was 6.5 μ l./min. Percentage inhibitions were calculated over the first 15 min. after the reactions were initiated.

Additions	Concn. (mM)	Inhibition (%)
Potassium cyanide	1	0
$\alpha\alpha'$ -Bipyridyl	1	0
Potassium fluoride	1	8
Ethylenediaminetetra-acetic acid	1	10
8-Hydroxyquinoline	1	50
8-Hydroxyquinoline + ferrous sulphate (2 mM)	1	0
HgCl ₂	1	100
Phenyl mercuric nitrate	1	100
<i>p</i> -Hydroxymercuribenzoate	1	100
<i>p</i> -Hydroxymercuribenzoate	0.08	50
<i>p</i> -Hydroxymercuribenzoate + cysteine (mM)	0.08	0
<i>p</i> -Hydroxymercuribenzoate + cysteine (mM) + ferrous sulphate (mM)	0.08	0
<i>p</i> -Hydroxymercuribenzoate + glutathione (mM)	0.08	30
<i>p</i> -Hydroxymercuribenzoate + glutathione (mM) + ferrous sulphate (mM)	0.08	0
<i>p</i> -Hydroxymercuribenzoate + ascorbate (mM)	0.08	40
<i>p</i> -Hydroxymercuribenzoate + ascorbate (mM) + ferrous sulphate (mM)	0.08	0

Fig. 3. Effect of Fe²⁺ ion concentration on protocatechuate 4,5-oxygenase activity in isoelectric precipitates. Each Warburg flask contained: enzyme solution (isoelectric ppt. between pH 5.25 and 4.5, approx. 15 mg. of protein/ml., 0.2 ml.); 0.2M-tris-HCl buffer (pH 7.1, 1 ml.); 20% (w/v) potassium hydroxide in the centre well (0.2 ml.); protocatechuate neutralized to pH 7.0 in the side arm (5 μ moles in 0.2 ml.); ferrous sulphate and cysteine hydrochloride as indicated; and water to a final reaction volume of 3.0 ml. Incubation was at 30° in air. O, In the presence of cysteine (1 mM); ●, in the absence of cysteine.

Fig. 4. Effect of cysteine concentration on protocatechuate 4,5-oxygenase activity in isoelectric precipitates. Warburg flask contents and conditions are as in Fig. 3 except that a different enzyme preparation was used. ●, In the presence of Fe²⁺ ions (1 mM); O, in the absence of Fe²⁺ ions.

DISCUSSION

In a study of the detoxication of the cresols, Dagley & Stopher (1959) obtained extracts of a Gram-negative bacterium which oxidized catechol by a new pathway: ring cleavage occurred between C-2 and C-3 of the nucleus. The ring-fission product was characterized as α -hydroxymuconic semialdehyde (Dagley *et al.* 1960).

Attention had also been focused on an apparently new acidic intermediate of protocatechuate (I) metabolism (Dagley & Patel, 1957). This compound had m.p. 235°, λ_{max} 276 m μ , and two carboxyl groups/molecule; it contained no phenol or enol groups, and when formed from protocatechuate 1 mole of oxygen/mole of substrate was consumed but no carbon dioxide was evolved. The compound was later identified as 2,4-lutidinic acid (6) but its relationship to protocatechuate metabolism was obscure (S. Dagley, personal communication). Re-investigation of this particular bacterial oxidation of protocatechuate (I) has revealed that *cis-cis*- β -carboxymuconate is not formed; the ring-fission product showed a resemblance to α -hydroxymuconic semialdehyde, which suggested that a new mode of ring-fission had occurred. Although the initial oxidation product, γ -carboxy- α -hydroxymuconic semialdehyde (2), proved too unstable to crystallize, an acidic bis-2,4-dinitrophenylhydrazone derivative was prepared and analysed. The position of ring cleavage was decisively shown to be at the 4,5-bond of protocatechuate by the non-enzymic reaction of γ -carboxy- α -hydroxymuconic semialdehyde and ammonia to form 2,4-lutidinate (6). (A ring cleavage at the 2,3-position would yield a product that could form 3,5-lutidinate.) The formation of a pyridine derivative from a benzene-ring structure is not unknown, for 3-hydroxyanthranilate is converted into α -picolinate enzymically and into nicotinate and quinolinate non-enzymically (Mehler, 1958). α -Picolinate is also formed non-enzymically by the action of ammonia on α -hydroxymuconic semialdehyde, although at a much lower rate than that observed for 2,4-lutidinate formation (Trippett *et al.* 1960). The rapidity of the latter reaction may account for the negative results obtained with Tollens and Rothera reagents on γ -carboxy- α -hydroxymuconic semialdehyde, although the *o*-dianisidine (aldehyde test) was positive.

The further metabolism of γ -carboxy- α -hydroxymuconic semialdehyde is not so well established. Pyruvate is one end product of protocatechuate oxidation by cell extracts, and it appears without further gaseous exchange after the ring-fission step (Dagley *et al.* 1960). The extracts used in this work (ultrasonically disintegrated or from the Hughes press) always accumulated the compound with λ_{max} 284 m μ , which is presumably an earlier intermediate than pyruvate in the reaction chain. It is formed from protocatechuate with the consumption of 1 mole of oxygen and the appearance of two carboxyl groups/mole of substrate. Since keto groups could not be detected, and in view of its u.v. absorption, we suggest that it is 4-carboxy-2,3-dihydro-2-hydroxymuconic acid (3). It could be formed from γ -carboxy- α -hydroxymuconic semi-

aldehyde (2) by an internal oxidation-reduction. Production of pyruvate from this presumed intermediate, without further gaseous exchange, would then be achieved by an aldolase-type reaction giving lactate and oxaloacetate (4), followed by oxidation of lactate to pyruvate with concomitant reduction of oxaloacetate to malate (5). Inhibition experiments with washed cell suspensions support this hypothesis, since oxaloacetate and pyruvate accumulate during protocatechuate oxidation in the presence of pyridine-3-sulphonate or arsenite.

The nomenclature of the new ring-cleavage enzymes for *o*-dihydroxyphenol oxidation requires consideration; it is suggested that the position of rupture be indicated, i.e. protocatechuate 4,5-oxygenase and catechol 2,3-oxygenase respectively. The previously characterized enzymes that cleave the nucleus between the carbon atoms bearing hydroxyl groups have become well known as protocatechuate oxidase and pyrocatechase. The term oxygenase is not entirely justified, since this implies transfer of molecular oxygen to the substrate [cf. pyrocatechase (Hayaishi, Katagiri & Rothberg, 1957); 3-hydroxyanthranilate oxidase (Hayaishi, Rothberg & Mehler, 1956); tryptophan pyrrolase (Hayaishi *et al.* 1956); homogentisate oxidase (Crandall, Yasunobu, Kruger & Mason, 1958)]. This point has not been investigated for the new ring-cleavage enzymes discussed here; rigid proof awaits a tracer study with ^{18}O . However, comparison of the properties of protocatechuate 4,5-oxygenase with other well established oxygenases which attack phenols does support this terminology, e.g. Fe^{2+} ions and thiol groups are required for full activity, molecular oxygen is essential, and other electron acceptors will not substitute.

Some differences between protocatechuate 4,5-oxygenase and protocatechate oxidase have also been revealed; the pH optima for the two enzymes are quite different. Protocatechuate oxidases obtained from *Pseudomonas fluorescens* A3.12 (Stanier & Ingraham, 1954) or *Pseudomonas* P1 do not possess pH optima, but activity increases with pH until protocatechuate is auto-oxidized (pH 9). Protocatechuate oxidase from *Nocardia erythropolis* (Cain & Cartwright, 1960), however, does possess a definite pH optimum, as does protocatechuate 4,5-oxygenase. The binding of Fe^{2+} ions by the two enzymes oxidizing protocatechuate is markedly different. Protocatechuate 4,5-oxygenase is easily resolved with respect to Fe^{2+} ions by dialysis, aging, enzyme precipitation or the use of inhibitors; resolution of Fe^{2+} ions in protocatechuate oxidase is difficult to achieve, the only reported instance being by enzyme precipitation in the presence of nitroso-R Salt (Sugiyama, Yano, Tanaka, Komagata & Arima, 1958).

Protocatechuate 4,5-oxygenase appears to be very substrate-specific; several compounds, all containing *o*-dihydroxyphenol groupings, inhibited protocatechuate oxidation, suggesting that this structure is essential for obtaining a site on the enzyme. 2,3-Dihydroxybenzoate is not inhibitory, possibly because the nearness of the adjacent carboxyl group sterically hinders the molecule from attaching itself to an active site.

So far, no bacterial strain has been encountered containing both types of ring-cleavage enzymes. It is, however, apparent that the occurrence of bacteria possessing the new ring-fission enzymes is not uncommon since Kojima, Itada & Hayaishi (1961) have reported on a soil *Pseudomonas* capable of oxidizing catechol in a manner identical with the one originally described (Dagley & Stopher, 1959; Dagley *et al.* 1960; Dagley & Morgan, 1962), although the Japanese workers have named the enzyme *m*-pyrocatechase.

SUMMARY

1. Pathways of metabolism of phthalate by *Pseudomonas* P1 and by *Pseudomonas* D2, and of *p*-cresol by *Pseudomonas* D1, converge at the protocatechuate stage.

2. Extracts of *Pseudomonas* P1 oxidized protocatechuate via *cis-cis*- β -carboxymuconate to β -oxoadipate, with the consumption of 1 mole of oxygen and the evolution of 1 mole of carbon dioxide/mole of substrate.

3. Protocatechuate is oxidized by cell-free extracts of *Pseudomonas* D1 and of *Pseudomonas* D2 with the consumption of 1 mole of oxygen/mole of substrate, but no carbon dioxide is evolved. The product, γ -carboxy- α -hydroxymuconic semialdehyde, results from an oxidative cleavage at the 4,5-bond; the new ring-fission enzyme has been called protocatechuate 4,5-oxygenase.

4. The structure assigned to the new ring-fission product, γ -carboxy- α -hydroxymuconic semialdehyde, is based on the following evidence: (a) production of one new carboxyl group/molecule during ring fission; (b) analysis of a crystalline acidic bis-2,4-dinitrophenylhydrazone derivative; (c) nature of the absorption spectrum curve in acid and alkali, suggesting tautomeric change of keto and enol forms; (d) production of 2,4-lutidinic acid by the action of NH_4^+ ions.

5. A scheme for the further metabolism of γ -carboxy- α -hydroxymuconic semialdehyde to pyruvate and malate via 4-carboxy-2,3-dihydro-2-hydroxymuconate is proposed.

6. The pH optimum of protocatechuate 4,5-oxygenase is 7.0–7.5; Fe^{2+} ions and a reducing compound are essential for full activity.

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Synergistic Effects in the Activation of a Streptococcal Deoxyribonuclease by Bivalent Metal Ions

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This paper is a full account of our finding (Stone & Burton, 1961) that suitable pairs of bivalent metal ions show large synergistic effects on both the initial rate and the final extent of hydrolysis of deoxyribonucleic acid by a streptococcal deoxyribonuclease. The enzyme preparation was the same as that used by Potter & Laskowski (1959), whose results indicate that the enzyme is a deoxyribonuclease oligonucleotidohydrolase (EC 3.1.4.5). Streptodornase has often been used as a trivial name for the streptococcal deoxyribonucleases, of which Wannamaker (1958) has distinguished three types, produced in different proportions by different strains.

MATERIALS AND METHODS

Streptococcal deoxyribonuclease. This was Streptodornase (Lederle Laboratories, Pearl River, N.Y., U.S.A.), prepared from the culture medium of a group C strain (H46A) of *Streptococcus haemolyticus*. The preparation differed from Varidase, which contains both streptokinase and streptodornase. The material contained 4% of protein as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin (Armour Laboratories, Eastbourne) as standard. Phosphate salts form the bulk of the enzyme preparation, contributing 40 μ M-phosphate to the incubation mixtures. Solutions were prepared in water and stored at 4° for a maximum period of 2 days.

Bovine pancreatic deoxyribonuclease. Crystalline enzyme was purchased from Sigma Chemical Co. (St Louis 18, Mo., U.S.A.).

Snake-venom diesterase and 5'-nucleotidase. A preparation that contained both these enzymes was kindly supplied by Dr P. Esnouf. It had been obtained from Russell's viper venom and was inactive on deoxyribonucleoside 3'-phosphates.

Deoxyribonucleic acid. Unless otherwise indicated, DNA (P content, 7.9%) had been prepared from herring testes essentially according to Emanuel & Chaikoff (1953). Solutions (2 mg./ml.) were prepared in 0.01 M-NaCl. Heat-denatured DNA refers to such a solution heated in a boiling-water bath for 30 min. and then cooled rapidly to room temperature. The extinction coefficient of this solution at 260 m μ was 136% of that before heating. Heated DNA was stored at 4° for up to 1 month. During this period the extinction was not altered and the DNA did not become susceptible to streptococcal deoxyribonuclease in the presence of Mg²⁺ as the sole activating metal ion. DNA from *Alcaligenes faecalis* and *Pseudomonas aeruginosa* was prepared as described by Burton (1960). Calf-thymus DNA was prepared according to Chargaff (1955).

Metal salts. These were analytical-reagent grade: Mg, Mn, Ca, Sr, and Ba were added as the chlorides (standardized by titration against AgNO₃); Fe²⁺ and Zn were added as the sulphates.

Buffers. A M solution of tris was adjusted to pH 7.2 at room temperature with N-HCl and diluted to the required molarity of tris. Similarly, m-glycine was adjusted to pH 8.6 with N-NaOH and diluted to the required molarity of glycine.

Glass-distilled water was used for the preparation of all solutions used.

Measurement of deoxyribonuclease activity. For all experiments except those in Table 1, the activity was measured by the release of acid-soluble products. Streptodornase (10 μ g. of the preparation), tris-HCl buffer, pH 7.2 (10 μ moles of tris), DNA (0.6 mg.) and metal salts as indicated were incubated at 37° in a volume of 1.3 ml. After a suitable time the reaction was stopped by the addition of 0.15 ml. of 2.5 N-perchloric acid. The mixture was stored at 4° for 30 min., centrifuged and the precipitate discarded. The extinction coefficient of the supernatant was measured at 260 m μ .

Estimation of metals as described below indicated that about 5 μ M-Ca and less than 0.2 μ M-Mg were contributed to the incubation mixture by the DNA [in Stone & Burton