The Metabolism of β -Phenylpropionic Acid by an *Achromobacter*

By S. DAGLEY, P. J. CHAPMAN AND D. T. GIBSON

Department of Biochemistry, University of Leeds, and Division of Biochemistry,
Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Ill., U.S.A.

(Received 17 May 1965)

1. When a species of *Achromobacter* grew with β -phenylpropionate as carbon source, 2-hydroxy- β -phenylpropionate and 2,3-dihydroxy- β -phenylpropionate appeared in the growth medium. The concentrations of these compounds were maximal during exponential growth. 2. The cells contained an oxygenase that required Fe^{2+} ions and cleaved the benzene nucleus between the adjacent carbon atoms that bear the side chain and one hydroxyl group of 2,3-dihydroxy- β -phenylpropionate. 3. The ring-fission product, formed with the consumption of 1mol. of oxygen/mol. of substrate, was isolated and a chemical structure assigned. Sephadex-treated cell extracts converted 1mol. of this compound into 1mol. of 4-hydroxy-2-oxovalerate without oxygen consumption; succinic acid was also formed. 4. When Mn^{2+} ions or Mg^{2+} ions were added, dialysed extracts converted 4-hydroxy-2-oxovalerate into pyruvate and acetaldehyde, but the reaction did not proceed to completion.

β -Phenylpropionic (hydrocinnamic) acid and *trans*-cinnamic acid are both compounds that support the growth of certain soil micro-organisms. β -Phenylpropionic acid is found amongst the products of putrefaction of proteins in soil (Hickinbottom, 1954) and *trans*-cinnamic acid occurs either in the free state or esterified in various oils and resins of higher plants (Miller, 1957). Webley, Duff & Farmer (1955) showed that *Nocardia opaca* oxidized β -phenylpropionic acid to cinnamic acid and benzoic acid, probably by β -oxidation of the side chain. In contrast, Coulson & Evans (1959) found that 2-hydroxy- β -phenylpropionate (*o*-hydroxy- β -phenylpropionate) and 2,3-dihydroxy- β -phenylpropionate accumulated in cultures of a *Pseudomonas* during growth with cinnamate as source of carbon. These observations of Coulson & Evans (1959), which have been confirmed by Blakley & Simpson (1964), suggested that when β -phenylpropionate is metabolized by pseudomonads hydroxylation of the benzene nucleus must precede any attack that might occur upon the side-chain substituent. Hitherto, further progress has been hindered by failures to prepare cell-free extracts capable of attacking any of the reaction intermediates that have been proposed (Blakley & Simpson, 1964). However, in the present work we have shown that extracts of an *Achromobacter* grown with β -phenylpropionate contain enzymes that catalyse the oxidative fission of the benzene nucleus of 2,3-dihydroxy- β -phenylpropionic acid and then convert the reaction product into succinate and 4-hydroxy-2-oxovalerate, a compound which is also formed when catechol is metabolized by

species of *Pseudomonas* (Nishizuka, Ichiyama, Nakamura & Hayaishi, 1962; Dagley & Gibson, 1964, 1965). A preliminary report of some of this work has been given (Dagley, Chapman & Gibson, 1963).

MATERIALS AND METHODS

Maintenance and growth of organisms. The organism was isolated from soil in Leeds by elective culture in a medium that supplied β -phenylpropionic acid as sole source of carbon for growth. Dr J. M. Wood kindly examined the organism and identified it as a species of *Achromobacter*: it forms short, stout Gram-negative rods and, when viewed in the electron microscope or when stained by the method of Rhodes (1958), no flagella were revealed. Stock cultures were maintained on nutrient-agar slopes sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) and subcultured monthly.

For experimental use cells were grown with forced aeration at 30° in medium adjusted with NaOH to pH 7.2 and containing (per l.): KH_2PO_4 , 2g.; $(\text{NH}_4)_2\text{SO}_4$, 1g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4g.; β -phenylpropionic acid, 0.5g. After growth for 18hr. cells were harvested in a Sharples centrifuge and washed once by resuspending in phosphate buffer (KH_2PO_4 , 2g./l.; pH 7.2) followed by centrifuging at 3° for 10 min. at 4300g. All phosphate buffers contained KH_2PO_4 brought to the required pH value by addition of NaOH. The methods used to prepare cell extracts and to measure the gas exchanges which they catalysed were those described by Dagley & Gibson (1965). All reactions in Warburg respirometers took place in 3 ml. of solution.

Partial purification of cell extracts. Crude cell extracts (25–30 mg. of protein/ml.) were treated as follows to give three preparations that are referred to as 'heat-treated', 'Sephadex-treated' and 'dialysed'. When crude extracts

were held at 55° for 5 min., and the precipitate so formed was removed by centrifuging, the heat-treated extract still oxidized 2,3-dihydroxy- β -phenylpropionate when Fe²⁺ ions were added, but no longer degraded the product of ring-fission. Crude extracts were also treated with 0.2 vol. of 2% (w/v) protamine sulphate and the precipitate was centrifuged and discarded; the solution was then brought to 70% saturation by addition of a saturated neutralized solution of (NH₄)₂SO₄ and the resulting precipitate (60 mg. of protein) was collected on the centrifuge. When a Sephadex-treated extract was required, this precipitate was dissolved in 2 ml. of phosphate buffer, pH 7.5 (KH₂PO₄, 2 g./l.), the solution was applied to a column (20 cm. $\times$ 2 cm.) of Sephadex G-25 (100–270 mesh) and the extract (15–20 mg. of protein/ml.) was eluted with the same phosphate buffer. Alternatively, a dialysed extract could be prepared from the pellet obtained after treatment with (NH₄)₂SO₄ by dissolving in phosphate buffer and dialysing the solution (15–20 mg. of protein/ml.) with stirring against three changes of phosphate buffer.

Chromatography. Compounds formed during growth of a culture of 251. were extracted into solution for chromatographic examination by centrifuging the cells, acidifying the liquid by addition of 25 ml. of 2N-H₂SO₄ to each 10 l. and reducing the volume to 500 ml. by evaporation at 25–30° in a Laboratory Circulating Evaporator (J. Towers Ltd., London). Carboxylic acids were extracted with ether, then taken into 5% (w/v) NaHCO₃ and finally, after acidification, into ether again. The ether was removed by evaporation, the residue dissolved in ethyl acetate and the solution applied to chromatograms.

For phenolic acids, one-dimensional ascending systems on Whatman no. 4 papers were used with the following solvents: A, butan-1-ol-ethanol-(NH₄)₂CO₃ buffer (40:11:19, by vol.) (Dagley, Fewster & Happold, 1952); B, benzene-acetic acid-water (25:5:1, by vol.); C, 5% (w/v) sodium formate-formic acid-water (200:1, v/v) (Smith, 1960). For experiments in which succinic acid was identified: E, butan-1-ol-acetic acid-water (12:3:5, by vol.); F, phenol-formic acid-water (500:13:167, w/v/v) (Kornberg, 1958); G, ether-benzene-formic acid-water (7:3:1:4:1, by vol.) (Weimberg, 1959). Thin-layer chromatography was carried out on Kieselgel G (E. Merck A.-G., Darmstadt, Germany) as described by Dagley & Gibson (1965), with solvent H for the isolation and identification of the 2,4-dinitrophenylhydrazones of 4-methyl-2-oxobutylolactone [solvent H: light petroleum (b.p. 60–90°)-ethyl formate-propionic acid (70:30:15:4, by vol.) (Ronkainen, 1963)] and solvent K (acetic acid-chloroform; 1:10, v/v) for the isolation of succinic acid. The instrument used for gas-liquid chromatography of methyl esters of carboxylic acids was a Hy-Fi model 600C (Wilkins Instrument and Research Inc., Walnut Creek, Calif., U.S.A.), which has been described by Dagley & Trudgill (1965). The stationary phases were 5% silicone gum rubber at a column temp. of 98° and 5% butane-2,3-diol succinate at 130°, both on Chromosorb W sieved to 60–80 mesh (Johns-Manville, Celite Division, New York, N.Y., U.S.A.). Nitrogen was used as the carrier gas at flow rate of 25 ml./min. Carboxylic acids were esterified with diazomethane generated from *N*-nitrosomethylurea in the apparatus of Roper & Ma (1957).

Detection reagents. Phenolic acids were located on paper chromatograms by spraying with the following solutions: (a) Folin-Ciocalteu reagent (British Drug Houses Ltd.,

Poole, Dorset) followed by exposure to ammonia vapour (Mitchell & Davies, 1954); (b) the reagent of Evans (1947) adapted for use in chromatography by Coulson & Evans (1959); (c) diazotized *p*-nitroaniline (Smith, 1960); (d) diazotized sulphonic acid (Smith, 1960); (e) α -nitroso- β -naphthol (Smith, 1960). Lactones were detected by treating chromatograms as described by Cain (1961), and keto acids by spraying with 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2N-HCl; a solution of aniline (1%, v/v) in 95% (v/v) methanol (Nordmann & Nordmann, 1960) was used as a general reagent for carboxylic acids.

Chemical analyses. Determinations of phenolic compounds (Folin & Ciocalteu, 1927) and of *o*-dihydroxyphenols (Evans, 1947) were made with reference to calibration curves prepared from standard solutions of 3-hydroxycinnamic acid and catechol respectively. 4-Hydroxy-2-oxovalerate was determined by converting the compound into its lactone and measuring extinctions at 230 m μ (Dagley & Gibson, 1965). Pyruvate was determined in the presence of 4-hydroxy-2-oxovalerate by a modification of the method of Friedemann & Haugen (1943) used by Dagley & Gibson (1965).

Infrared-absorption spectra. Samples (3 mg.) of 2,4-dinitrophenylhydrazones were mullied in Nujol and their spectra were recorded by a Unicam SP.200 spectrometer.

Materials. Compounds that were not commercially available were prepared as follows. 2-Hydroxy- β -phenylpropionic acid and 3-hydroxy- β -phenylpropionic acid (*m*-hydroxy- β -phenylpropionic acid) were obtained by reducing 2-hydroxycinnamic acid and 3-hydroxycinnamic acid respectively with 3% (w/w) sodium amalgam (Tiemann & Ludwig, 1882). A sample of 2,3-dihydroxy- β -phenylpropionic acid was kindly given by Professor W. C. Evans. Later, the compound was obtained from 2,3-dimethoxy- β -phenylpropionic acid, which was synthesized either from 2,3-dimethoxybenzaldehyde (from Koch-Light Laboratories Ltd., Colnbrook, Bucks.) according to Haworth (1927) or, more conveniently, by reduction of 2,3-dimethoxycinnamic acid (from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.) by means of 3% (w/w) sodium amalgam. The method used to convert 2,3-dimethoxy- β -phenylpropionic acid into 2,3-dihydroxy- β -phenylpropionic acid was due to Professor W. C. Evans and has been described in detail by Blackley & Simpson (1964).

Aqueous solutions of 2,3-dihydroxycinnamic acid were obtained by a series of reactions in which 2-hydroxy-3-methoxybenzaldehyde (from Koch-Light Laboratories Ltd.) was first demethylated by means of HBr in acetic acid (Pauly, Schubel & Lockemann, 1911), to give 2,3-dihydroxybenzaldehyde, of which 2 g. was dissolved in 5 ml. of pyridine and acetylated by slow addition of 3 ml. of acetic anhydride at 0°. The mixture was then allowed to stand at 20° for 2 hr., poured into 15 ml. of ice-water and the solid which separated overnight was recrystallized from water: yield, 1.5 g. of 2,3-diacetoxybenzaldehyde, m.p. 97–98°. This compound (1.5 g.) was refluxed for 7 hr. with a mixture of 4 g. of anhydrous sodium acetate and 8 ml. of acetic acid; 20 ml. of 5N-H₂SO₄ was then added and refluxing continued for 1 hr. The solution was extracted three times with equal volumes of ether and the combined ethereal solutions were extracted with 25 ml. of 5% (w/v) NaHCO₃. This solution was acidified and extracted with ether; the ethereal solution was dried over anhydrous Na₂SO₄ and the ether removed by distillation to give a

pink oil. This oil could not be crystallized but single spots were given on chromatograms with solvents A, B, C and D and its reactions with detection reagents were similar to those of 2,3-dihydroxy- β -phenylpropionic acid (Table 1) and 2,3-dihydroxybenzoic acid. From the method of preparation used, the compound was assumed to be 2,3-dihydroxycinnamic acid. Aqueous solutions were prepared by eluting bands on chromatograms, and their concentrations were determined by the method of Evans (1947) for *o*-dihydroxyphenols. After the completion of this work a sample of authentic 2,3-dihydroxycinnamic acid (Blakley & Simpson, 1964) was kindly supplied by Dr E. R. Blakley and shown to have chromatographic properties identical with those of the material we prepared.

Solutions of 4-hydroxy-2-oxovalerate were prepared from synthetic 4-methyl-2-oxobutyrolactone (Rossi & Schinz, 1948) as described by Dagley & Gibson (1965).

RESULTS

Washed suspensions of *Achromobacter* grown with β -phenylpropionate as source of carbon oxidized β -phenylpropionate, 2- and 3-hydroxy- β -phenylpropionate, 2,3-dihydroxy- β -phenylpropionate, cinnamate and 3-hydroxycinnamate at approximately equal rates; 2-hydroxycinnamate was not oxidized. Since, from these observations alone, it is not possible to decide between the various pathways that may be suggested for oxidation of β -phenylpropionate by whole cells, filtrates of a culture growing with β -phenylpropionate were examined for reaction intermediates. First, the concentrations of phenolic compounds and of *o*-dihydroxyphenols were determined as they appeared in samples withdrawn at intervals during growth of a culture in 3l. of medium (Fig. 1). Similar determinations were then made for a culture of 25l., and, when the concentration of phenolic compounds was maximal, cells were

removed by centrifuging and acidic compounds present in the medium were concentrated into a small volume of ethyl acetate, as described in the Materials and Methods section, and then chromatographed (Table 1). The only phenolic acids shown to be present were 2-hydroxy- β -phenylpropionic acid and 2,3-dihydroxy- β -phenylpropionic acid: no derivative of cinnamic acid was detected.

Fission of the benzene nucleus of 2,3-dihydroxy- β -phenylpropionate. Crude cell extracts oxidized 2,3-dihydroxy- β -phenylpropionate rapidly, no added cofactor being required; but the following

Fig. 1. Formation of phenolic acids in a culture of *Achromobacter* growing with β -phenylpropionate. —, Turbidity ($E_{400m\mu}$) of the culture; ○, phenolic compounds; ●, *o*-dihydroxyphenolic compounds.

Table 1. *Chromatographic identification of 2-hydroxy- β -phenylpropionic acid and 2,3-dihydroxy- β -phenylpropionic acid as compounds present in culture media*

See text for details of solvent systems and detection reagents used as sprays.

Solution applied	Solvent...	R_F				Reaction with spray		Colour with spray		
		A	B	C	D	(a)	(b)	(c)	(d)	(e)
2-Hydroxycinnamic acid		0.44	0.68	0.58	0.25	+	—	Mauve	Orange	Grey
2-Hydroxy- β -phenylpropionic acid		0.63	0.73	0.86	0.61	+	—	Mauve	Orange	Grey
3-Hydroxycinnamic acid		0.44	0.63	0.58	0.27	+	—	Red	Orange	Grey
3-Hydroxy- β -phenylpropionic acid		0.51	0.65	0.87	0.44	+	—	Red	Orange	Grey
Compound I from culture		0.62	0.72	0.87	0.60	+	—	Mauve	Orange	Grey
2,3-Dihydroxycinnamic acid		0.23	0.31	0.42	0.06	+	+	Purple	Mauve	Orange
2,3-Dihydroxy- β -phenylpropionic acid		0.48	0.43	0.83	0.32	+	+	Purple	Mauve	Orange
Compound II from culture		0.44	0.43	0.83	0.30	+	+	Purple	Mauve	Orange

compounds were not attacked: β -phenylpropionate, 2- and 3-hydroxy- β -phenylpropionate, 2,3-dihydroxybenzoate and protocatechuic acid. Catechol was oxidized at about half the rate of 2,3-dihydroxy- β -phenylpropionate, but when extracts were heat-treated they no longer oxidized catechol whereas ability to oxidize 2,3-dihydroxy- β -phenylpropionate was retained. When 5 μ moles of 2,3-dihydroxy- β -phenylpropionate were incubated in a reaction mixture that contained phosphate buffer, pH 7.5 (potassium dihydrogen phosphate, 2g./l.), mM-ferrous sulphate and heat-treated extract (20mg. of protein), 5 μ moles of oxygen were taken up in 10 min.; there was no reaction unless Fe^{2+} ions were added. At pH 7.2 a solution of the reaction product was yellow and absorbed strongly to give a single peak at 400 $m\mu$, which was removed to 315 $m\mu$ on acidification (Fig. 3). Accordingly, the formation of this compound from 2,3-dihydroxy- β -phenylpropionate by the action of a heat-treated extract and its degradation by a Sephadex-treated extract could be followed by measurement of the changes in extinction at 400 $m\mu$ (Fig. 2). Heat-treated extracts also oxidized 2,3-dihydroxycinnamic acid to give a solution that was red in alkali and colourless in acid.

A sample of the product of ring-fission of 2,3-dihydroxy- β -phenylpropionate was isolated in an experiment in which ten 250ml. flasks were incubated on a rotatory shaker (150 rev./min.) at 30°. Each flask contained 60ml. of a mixture of 0.6m-mole of 2,3-dihydroxy- β -phenylpropionate, 18ml. of heat-treated extract, 60 μ moles of ferrous sul-

Fig. 2. Formation and degradation by cell extracts of the product of ring-fission of 2,3-dihydroxy- β -phenylpropionate. Cuvettes contained, in 3 ml., 0.1m-mole of phosphate buffer (pH 7.5), 3 μ moles of FeSO_4 and 0.1ml. of mM-2,3-dihydroxy- β -phenylpropionate. O, The reaction at 20° was started by addition of heat-treated extract (1mg. of protein) and, at the point shown by the arrow, Sephadex-treated extract (1mg. of protein) was added. ●, The reaction was started by addition of 0.1ml. of an untreated extract (2mg. of protein).

phate and 4.5m-moles of phosphate buffer, pH 7.2. After 1hr., 20ml. of 20% (w/v) metaphosphoric acid was added to each flask, precipitates were removed by centrifuging and the clear supernatant solutions were pooled and extracted continuously with ether for 24hr. The ethereal solution was shaken with 5% (w/v) sodium bicarbonate until no further yellow compound was extracted; the aqueous solution was then acidified with 2N-sulphuric acid and extracted continuously with ether for 24hr. After drying overnight with anhydrous sodium sulphate, the ether was removed by distillation to leave 700mg. of a microcrystalline powder. This compound (see Scheme 1) had m.p. 115–117° (decomp.), light-absorption max. 315 $m\mu$ (ϵ 9870) at pH 1 and 400 $m\mu$ (ϵ 31 300) at pH 13 (Fig. 3) (Found: C, 50.3; H, 4.7; $\text{C}_9\text{H}_{10}\text{O}_6$ requires C, 50.6; H, 4.7%). A titration curve showed inflexions at pH 4.0–4.3 (small) and at pH 6.0–8.0 (pronounced), indicating a dibasic acid of mol. wt. 218; calc. for $\text{C}_9\text{H}_{10}\text{O}_6$, 213. The following chemical properties were consistent with the formula proposed in Scheme 1. Addition of bromine-water to an aqueous solution, followed by potassium iodide and starch, gave a blue colour due to liberated iodine as expected for a compound possessing enolizable carbonyl groups (Feigl, 1960). An insoluble acidic 2,4-dinitrophenylhydrazone was also given, but there was no reaction with the following three reagents for aldehydes (Feigl, 1960): ammoniacal silver oxide; a methanolic solution of 1,2-dianilinoethane; a saturated solution of *o*-dianisidine in acetic acid. Carbonyl-group properties were lost when the compound was catalytically reduced by hydrogen as follows. The side arms of three Warburg flasks contained respectively 5 μ moles of succinic acid, maleic acid and fumaric acid, each dissolved in 0.2ml. of ethanol; each main compartment contained 1mg. of platinum oxide and 2.8ml. of ethanol. Flasks were flushed with hydrogen for 15 min. and shaken at 30° until uptake of hydrogen ceased; the acids were then tipped from the side arms and each reaction was followed to completion. The amounts of hydrogen taken up by 1 μ mole of succinic acid, maleic acid and the ring-fission compound were respectively 0, 1.0 and 2.9 μ moles; reduction of two ethylenic bonds and one carbonyl group of $\text{C}_9\text{H}_{10}\text{O}_6$ requires 3.0 μ moles of hydrogen.

Conversion of 2,3-dihydroxy- β -phenylpropionate into succinate and 4-hydroxy-2-oxovalerate. The experiment of Fig. 2 showed that the ring-fission product was metabolized by enzymes that were destroyed by heating but not by treatment with protamine and ammonium sulphate and passage down Sephadex G-25. No carbon dioxide was evolved and 10 μ moles of oxygen were consumed when 10 μ moles of 2,3-dihydroxy- β -phenylpro-

Fig. 3. Spectra of the product of enzymic ring-fission of 2,3-dihydroxy- β -phenylpropionate. The solution was 0.02 mM, in 0.3 N-HCl (○) and in 0.3 N-NaOH (●).

pionate were incubated in a Warburg flask for 50 min. with 120 μ moles of phosphate buffer, pH 7.5, 3 μ moles of ferrous sulphate and Sephadex-treated extract (15 mg. of protein). After addition of 0.5 ml. of 5 N-hydrochloric acid the solution was held at 100° for 3 min. to convert 4-hydroxy-2-oxovalerate into its lactone; precipitated protein was then removed by centrifuging. The spectrum of the solution, which showed $\lambda_{\max}$ 228 m μ when acid and 262 m μ when alkaline, was identical with that of authentic 4-methyl-2-oxobutyrolactone; measurement of the extinction at 230 m μ (Dagley & Gibson, 1965) showed that 9.5 μ moles of 4-hydroxy-2-oxovalerate had been formed from 10.0 μ moles of 2,3-dihydroxy- β -phenylpropionate. A solution obtained by incubating 5 μ moles of isolated ring-fission product for 15 min. with the same Sephadex-treated extract was similarly acidified, heated and freed from protein. The spectrum was again that of 4-methyl-2-oxobutyrolactone, of which 5 μ moles were formed as calculated from the measured extinction at 230 m μ . No oxygen was consumed, nor carbon dioxide evolved, as the ring-fission product disappeared; the reaction rate was not affected by replacement of the oxygen in the Warburg flask by nitrogen.

To confirm the identity of 4-hydroxy-2-oxovalerate ten Warburg flasks were assembled, each containing the reaction mixture in the experiment described above in which 10 μ moles of 2,3-dihydroxy- β -phenylpropionate were oxidized by a Sephadex-treated extract. After incubating the mixed

reactants for 50 min., protein was precipitated, the acidified solutions were held at 100° for 3 min., the solutions were pooled and the precipitate was removed by centrifuging. To the clear solution was added 25 ml. of 0.1% (w/v) 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid and the yellow precipitate formed after incubating for 24 hr. at 30° was collected on the centrifuge, dissolved in ethyl acetate and applied as a band to the base of a layer (0.02 in. thick) of Kieselgel G. When chromatographed with solvent H two bands were obtained with R_F values the same as those of the 2,4-dinitrophenylhydrazone of synthetic 4-methyl-2-oxobutyrolactone. The bands were removed from the plate and the compound was eluted with ethyl acetate, the solution was dried over anhydrous sodium sulphate and the ethyl acetate was evaporated to leave 6 mg. of yellow crystals, m.p. 171–173°. All absorption peaks of the infrared-absorption spectrum of this compound were also shown in the spectrum of the 2,4-dinitrophenylhydrazone of synthetic 4-methyl-2-oxobutyrolactone (m.p. 172–174°).

In a similar experiment 100 μ moles of 2,3-dihydroxy- β -phenylpropionic acid were again distributed between ten Warburg flasks and oxidized by Sephadex-treated extract. Protein was then precipitated by addition of 0.6 ml. of 20% (w/v) metaphosphoric acid to each flask and, after centrifuging, supernatant solutions were combined and extracted continuously with ether for 24 hr. The ethereal solution was dried over anhydrous sodium sulphate, the ether removed by evaporation and the residue was taken up in ethanol. Thin-layer chromatography of a small portion of this solution gave two spots in solvent K: one, reacting only as a carboxylic acid with the aniline and xylose spray, had the same R_F (0.36) as succinic acid; the other, which behaved as a lactone possessing a reactive carbonyl group, had the R_F (0.52) of 4-hydroxy-2-oxobutyrolactone. Paper chromatography in solvents E, F and G gave single spots having the respective R_F for succinic acid: in these solvents, 4-methyl-2-oxobutyrolactone runs with the solvent front. The remainder of the ethanolic solution of reaction products was applied as a band to the base of a layer of Kieselgel G, chromatographed in solvent K and the material containing the band that ran as succinic acid was removed and extracted continuously with ether for 24 hr. The ether was then evaporated and the residue treated with diazomethane. The product of this treatment was examined by gas-liquid chromatography and its retention times in two stationary phases were compared with those of eight dimethyl esters of dibasic carboxylic acids (Table 2). The measured values for the compound isolated were those expected for the dimethyl ester of succinic acid.

Table 2. Identification of the dimethyl ester of succinic acid by gas-liquid chromatography

Carboxylic acid converted into dimethyl ester and applied to column	Retention time (min.)	
	Stationary phase 5% silicone rubber (SE-30) at 98°	Stationary phase butane-2,3-diol succinate at 130°
Malonic acid	1.20	1.82
Methylmalonic acid	1.40	1.50
Dimethylmalonic acid	1.60	1.10
Succinic acid	2.40	2.76
Methylsuccinic acid	2.74	2.68
2,3-Dimethylsuccinic acid	3.15	2.23
Glutaric acid	4.60	4.58
2-Methylglutaric acid	5.60	4.38
Enzymic reaction product	2.42	2.80

Conversion of 4-hydroxy-2-oxovalerate into pyruvate and acetaldehyde. Species of *Pseudomonas* grown with phenol contain an enzyme which has a pH optimum about 8.8, is stimulated by Mg^{2+} ions and converts one of the enantiomers of synthetic 4-hydroxy-2-oxovalerate into pyruvate and acetaldehyde (Dagley & Gibson, 1965). Preliminary experiments showed that, if Mg^{2+} ions were included in the reaction mixture, some pyruvate was formed in addition to 4-hydroxy-2-oxovalerate when 2,3-dihydroxy- β -phenylpropionate was oxidized by extracts of *Achromobacter* grown with β -phenylpropionate. When the extracts used were dialysed, yields of pyruvate were greater than when they were Sephadex-treated. Stimulation of activity was increased by about one-third when Mn^{2+} ions replaced Mg^{2+} ions, but Zn^{2+} , Co^{2+} and Fe^{2+} ions were without effect when added at the same concentration (1.7 mM). A dialysed extract converted synthetic 4-hydroxy-2-oxovalerate into pyruvate, which was determined by a modification (Dagley & Gibson, 1965) of the method of Friedemann & Haugen (1943). The yield of pyruvate (1.88 μ moles) obtained after incubating for 1 hr. was less than that expected from the metabolism of one enantiomer of the substrate (2.5 μ moles) and the rate of the reaction decreased with time: thus the amount of pyruvate present after 20 min. was nearly 70% of that after 1 hr. (Table 3). Since acetaldehyde is a volatile compound, a no. 1 Conway (1947) unit was used to study its formation from synthetic 4-hydroxy-2-oxovalerate. The reaction mixture described in Table 3 was placed in the outer chamber of the unit and the inner chamber contained 3 ml. of 0.1 M-sodium pyrosulphite. Immediately after the addition of substrate the unit was sealed with a ground-glass plate coated with silicone grease and incubated at 30°. Acetaldehyde collected in the centre chamber and was

Table 3. Formation of pyruvate from synthetic 4-hydroxy-2-oxovalerate

Reaction mixtures at 30° contained, in 3 ml., 120 μ moles of tris (pH 8.5), 5 μ moles of $MnSO_4$, 5 μ moles of 4-hydroxy-2-oxovalerate and dialysed extract (30 mg. of protein).							
Time (min.)	0	10	20	30	40	50	60
Pyruvate (μ moles)	0	0.86	1.28	1.52	1.66	1.80	1.88

determined by the method of Barker & Summerson (1941): from 5 μ moles of 4-hydroxy-2-oxovalerate, 1.8 μ moles of acetaldehyde were formed in 2 hr.

DISCUSSION

When grown with β -phenylpropionate, *Achromobacter* contained an enzyme that catalysed fission of the benzene nucleus of 2,3-dihydroxy- β -phenylpropionate but did not attack catechol, protocatechuate or 2,3-dihydroxybenzoate. 2,3-Dihydroxycinnamic acid was oxidized by heat-treated extracts, and this might be taken to indicate that the compound is a reaction intermediate in the metabolism of β -phenylpropionate. However, this conclusion is at variance with the observation that although 2-hydroxy- β -phenylpropionate and 2,3-dihydroxy- β -phenylpropionate were detected in culture media (Table 1) and also behaved kinetically as reaction intermediates (Fig. 1), we could detect no derivatives of cinnamic acid. Similar observations were made by Coulson & Evans (1959) and by Blakley & Simpson (1964) for culture media from the pseudomonads they studied. Unlike these pseudomonads, *Achromobacter* did not grow with cinnamic acid despite the fact that this substrate was oxidized by cells grown with β -phenylpropionate and their extracts also oxidized 2,3-dihydroxycinnamate. Accordingly we suggest that 2,3-dihydroxy- β -phenylpropionate oxygenase does not discriminate between this substrate and 2,3-dihydroxycinnamate, and that the enzyme is not induced or de-repressed by cinnamic acid in *Achromobacter*. The results of Blakley & Simpson (1964) indicated that when growth does occur at the expense of cinnamic acid, as with their *Pseudomonas*, the growth substrate must first be reduced to β -phenylpropionic acid before degradation can be initiated. The failure of our extracts to metabolize either β -phenylpropionate or 2-hydroxy- β -phenylpropionate is evidently due to the fact that the initial reactions would be catalysed by hydroxylases which require a continuous supply of NADH to convert these substrates into 2,3-dihydroxy- β -phenylpropionate: this requirement has been established for other bacterial hydroxylases (Ichiara, Adachi, Hosokawa & Takeda, 1962; Taniuchi & Hayaishi, 1963; Taniuchi *et al.* 1964).

Scheme 1.

The ring-fission enzyme might be designated as '2,3-dihydroxy- β -phenylpropionate 1,2-oxygenase', since fission takes place between C-1 and C-2. This nomenclature is not entirely satisfactory, however, because the name 'catechol 1,2-oxygenase' (EC 1.99.2.2) indicates that fission occurs between the carbon atoms bearing hydroxyl groups, whereas in the reaction we have studied it does not; accordingly we suggest the name '3-carboxyethylcatechol 2,3-oxygenase'. This oxygenase resembles protocatechuate 4,5-oxygenase (Dagley, Evans & Ribbons, 1960) and differs from protocatechuate 3,4-oxygenase (EC 1.99.2.3) (Stanier & Ingraham, 1954) insofar as the Fe^{2+} ions required for activity are readily removed by dialysis or heat-treatment. The structure proposed for the product of ring-fission (Scheme 1) agrees with the chemical analysis and properties of the material isolated and was confirmed by its enzymic conversion into succinate and 4-hydroxy-2-oxovalerate; further, 1 mol. of the last-named compound was formed from 1 mol. of either 2,3-dihydroxy- β -phenylpropionate or the ring-fission product.

Cell extracts contained an enzyme system that converted 4-hydroxy-2-oxovalerate into pyruvate (Table 3). It resembled an enzyme that catalyses this reaction in *Pseudomonas* grown with phenol (Dagley & Gibson, 1965) in showing a requirement for Mg^{2+} (or Mn^{2+}) ions; however, with extracts of *Achromobacter* the reaction did not proceed to completion and dialysis may have removed another cofactor not yet identified. The reaction cannot be studied conveniently in untreated extracts since the pyruvate formed is rapidly metabolized.

Dagley, Chapman, Gibson & Wood (1964) have drawn attention to the general nature of the reactions of Scheme 1. Thus both catechol and 2,3-dihydroxy- β -phenylpropionate are converted into 4-hydroxy-2-oxovalerate; but whereas C-3 of catechol bears a hydrogen atom and gives rise to formate, the corresponding carbon atom of 2,3-dihydroxy- β -phenylpropionate bears a carboxyethyl group and appears in the carboxyl group of succinate. Reactions of this type have recently been shown to occur during the degradation by *Nocardia restrictus* of androst-4-ene-3,17-dione (Sih, Wang, Gibson & Whitlock, 1965). When a metabolic intermediate in the degradation, namely 3,4-dihydroxy-9,10-

secoandrosta-1,3,5(10)-triene-9,17-dione, was incubated with cell extracts that required Fe^{3+} ions for activity, the aromatic A ring was oxidatively cleaved between C-4 and C-5. One product of the reaction was 4-hydroxy-2-oxohexanoate, corresponding to the conversion of 2,3-dihydroxy- β -phenyl propionate into 4-hydroxy-2-oxovalerate; the other product was a carboxylic acid analogous to succinate in the system we have studied.

We thank the Department of Scientific and Industrial Research for Research Studentships to P. J. C. and D. T. G., and the National Science Foundation of the U.S.A. for a Senior Foreign Scientist Fellowship to S. D. held at the University of Illinois in 1963-1964.

REFERENCES

- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **133**, 535.
 Blakley, E. R. & Simpson, F. J. (1964). *Canad. J. Microbiol.* **10**, 175.
 Cain, R. B. (1961). *Biochem. J.* **79**, 298.
 Conway, E. J. (1947). *Microdiffusion Analysis and Volumetric Error*, 2nd ed., p. 8. London: Crosby Lockwood and Son Ltd.
 Coulson, C. B. & Evans, W. C. (1959). *Chem. & Ind.* p. 543.
 Dagley, S., Chapman, P. J. & Gibson, D. T. (1963). *Biochim. biophys. Acta*, **78**, 781.
 Dagley, S., Chapman, P. J., Gibson, D. T. & Wood, J. M. (1964). *Nature, Lond.*, **202**, 775.
 Dagley, S., Evans, W. C. & Ribbons, D. W. (1960). *Nature, Lond.*, **188**, 560.
 Dagley, S., Fewster, M. E. & Happold, F. C. (1952). *J. Bact.* **63**, 327.
 Dagley, S. & Gibson, D. T. (1964). *J. biol. Chem.* **239**, 1284.
 Dagley, S. & Gibson, D. T. (1965). *Biochem. J.* **95**, 466.
 Dagley, S. & Trudgill, P. W. (1965). *Biochem. J.* **95**, 48.
 Evans, W. C. (1947). *Biochem. J.* **41**, 373.
 Feigl, F. (1960). *Spot Tests in Organic Analysis*, 6th ed., pp. 229 and 236. Amsterdam: Elsevier Publishing Co.
 Folin, O. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
 Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
 Haworth, R. D. (1927). *J. chem. Soc.* p. 2281.
 Hickinbottom, W. J. (1954). In *Chemistry of Carbon Compounds*, vol. 3, part A, p. 595. Ed. by Rodd, E. H. Amsterdam: Elsevier Publishing Co.
 Ichihara, A., Adachi, K., Hosokawa, K. & Takeda, Y. (1962). *J. biol. Chem.* **237**, 2296.

- Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
- Miller, E. V. (1957). *The Chemistry of Plants*, p. 89. New York: Reinhold Publishing Corp.
- Mitchell, F. L. & Davies, R. E. (1954). *Biochem. J.* **56**, 690.
- Nishizuka, Y., Ichiyama, A., Nakamura, S. & Hayaishi, O. (1962). *J. biol. Chem.* **237**, rc268.
- Nordmann, J. & Nordmann, R. (1960). In *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1, p. 279. Ed. by Smith, I. London: William Heinemann Ltd.
- Pauly, H., Schubel, K. & Lockemann, K. (1911). *Liebigs Ann.* **383**, 288.
- Rhodes, M. E. (1958). *J. gen. Microbiol.* **18**, 639.
- Ronkainen, P. (1963). *J. Chromat.* **11**, 228.
- Roper, R. & Ma, T. S. (1957). *Microchem. J.* **1**, 245.
- Rossi, A. & Schinz, H. (1948). *Helv. chim. acta*, **31**, 473.
- Sih, C. J., Wang, K. C., Gibson, D. T. & Whitlock, H. W. (1965). *J. Amer. chem. Soc.* **87**, 1386.
- Smith, I. (1960). In *Chromatographic and Electrophoretic Techniques*, 2nd ed., vol. 1, p. 291. Ed. by Smith, I. London: William Heinemann Ltd.
- Stanier, R. Y. & Ingraham, J. L. (1954). *J. biol. Chem.* **210**, 799.
- Taniuchi, H., Hatanaka, M., Kuno, S., Hayaishi, O., Nakajima, M. & Kurihara, N. (1964). *J. biol. Chem.* **239**, 2204.
- Taniuchi, H. & Hayaishi, O. (1963). *J. biol. Chem.* **238**, 283.
- Tiemann, F. & Ludwig, R. (1882). *Ber. dtsh. chem. Ges.* **15**, 2043.
- Webley, D. M., Duff, R. B. & Farmer, V. C. (1955). *J. gen. Microbiol.* **13**, 361.
- Weimberg, R. (1959). *J. biol. Chem.* **234**, 727.