The Bacterial Degradation of Catechol

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1. Two strains of *Pseudomonas* were grown with phenol and used to prepare cell extracts that metabolized catechol with the transient formation of 2-hydroxymuconic semialdehyde. 2. One of these preparations catalysed the conversion of 1 mol. of catechol into 1 mol. each of formate and 4-hydroxy-2-oxovalerate. 3. A method for the determination of 4-hydroxy-2-oxovalerate is described, together with some properties of this compound and its 2,4-dinitrophenylhydrazone. 4. Another partially purified cell extract converted 1 mol. of 4-hydroxy-2-oxovalerate, formed enzymically from catechol, into 1 mol. each of acetaldehyde and pyruvate. This aldolase had a pH optimum of about 8.8, was stimulated by Mg^{2+} ions and appeared to attack only one enantiomer of synthetic 4-hydroxy-2-oxovalerate.

Catechol is a reaction intermediate in the bacterial metabolism of phenol, benzoic acid, anthranilic acid and other compounds (for a review see Dagley, Evans & Ribbons, 1960). In some species of *Pseudomonas* the benzene nucleus of catechol is cleaved by a pyrocatechase to give *cis-cis*-muconic acid (Evans, Smith, Linstead & Elvidge, 1951; Hayaishi, Katagiri & Rothberg, 1957; Nakagawa, Inoue & Takeda, 1963; Taniuchi, Kojima, Nakazawa & Hayaishi, 1964), whereas in other species catechol is oxidized to 2-hydroxymuconic semialdehyde by an enzyme that was designated 'catechol 2,3-oxygenase' by Dagley *et al.* (1960) and 'metapyrocatechase' by Kojima, Itada & Hayaishi (1961). When the oxidation of catechol by a 2,3-oxygenase was first reported Dagley & Stopher (1959) found that cell-free extracts of a *Pseudomonas* converted catechol into pyruvate with no evolution of carbon dioxide and no consumption of oxygen in addition to that required by the ring-fission reaction. Nishizuka, Ichiyama, Nakamura & Hayaishi (1962), on the other hand, showed that another *Pseudomonas* converted the product of ring-fission, 2-hydroxymuconic semialdehyde, into acetate and pyruvate by a reaction sequence that involved two oxidative steps and a decarboxylation of 4-oxalocrotonate (5-oxohex-2-ene-1,6-dioate). In the present work we have confirmed the observations of Dagley & Stopher (1959) and have shown that 2-hydroxymuconic semialdehyde is metabolized to formate, acetaldehyde and pyruvate by a reaction sequence in which 4-oxalocrotonate does not appear as an intermediate. Preliminary reports of this work have been published

(Dagley & Gibson, 1964; Dagley, Chapman, Gibson & Wood, 1964).

MATERIALS AND METHODS

Maintenance and growth of organisms. The two strains used were motile Gram-negative rods having characteristics of *Pseudomonas* species. *Pseudomonas* (L) was isolated from garden soil in Leeds by elective culture in *o*-cresol medium, *Pseudomonas* (U) from the mud of a stream in Urbana by use of a *m*-cresol medium. Stock cultures of both organisms were maintained on nutrient-agar slopes sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C.2). *Pseudomonas* (L) was subcultured monthly, but, owing to a tendency of cultures to lose viability, *Pseudomonas* (U) was subcultured every 2 weeks.

For experimental use cells were grown with forced aeration at 30° in media adjusted with NaOH to pH 7.2 and containing (per l.): KH_2PO_4 , 2 g.; $(NH_4)_2SO_4$, 1 g.; $MgSO_4 \cdot 7H_2O$, 0.4 g. For *Pseudomonas* (U) this medium was completed by addition of 0.6% of phenol and 0.3% of Lab-Lemco (Oxoid Division of Oxo Ltd., London). A shake culture (500 ml. in a 2l. conical flask) was grown overnight to the stationary phase and used as inoculum for batches of 10–20l. For *Pseudomonas* (L), 0.3% of phenol was added to the medium since higher concentrations inhibited growth; also, the inoculation procedure differed insofar as 30 ml. of a shake culture was first grown in medium containing 0.3% of phenol and 0.4% of Lab-Lemco. No further additions of Lab-Lemco were made at later stages of growth when the 30 ml. culture was used to inoculate 1l. of medium (0.3% of phenol), which was used in turn as inoculum for 10l. of the same medium. Foaming during the growth of large crops was minimized by the addition (2 ml./l.) of a 1% emulsion of Silicone MS Antifoam A (Hopkin and Williams Ltd., Chadwell Heath, Essex). Cells were harvested in the late

exponential phase of growth, washed once by suspension in phosphate buffer (2 g. of KH_2PO_4 /l. brought to pH 7.5 with NaOH), and then centrifuged at 4300g for 10 min. at 3°.

Preparation of cell extracts. Freshly grown cells were disintegrated by exposure for 4 min. at 0° to the output of an MSE-Mullard ultrasonic disintegrator at an average frequency of 20 kcyc./sec. For this purpose the harvested wet cells were resuspended in phosphate buffer, 2 ml. of buffer being used for each 1 g. of cell paste. In some experiments the bacterial press of Hughes (1951) was employed and the disrupted cells were resuspended in three times their (wet) weight of phosphate buffer: no difference in the properties of the extracts was observed when this method was used. After clarification of extracts by centrifuging at 26000g_{av} for 50 min., protein concentrations were determined by the method of Sols (1947): such extracts usually contained 25–30 mg. of protein/ml. Gas exchanges catalysed by cell extracts were measured in Warburg respirometers at 30°: for those involving uptake of O_2 , centre wells contained strips of Whatman no. 542 filter paper and 0.2 ml. of 20% (w/v) KOH; for CO_2 evolution, 0.2 ml. of either 5 N-HCl or 6% (w/v) perchloric acid was tipped from the centre well to stop the reaction and expel all CO_2 from solution. Perchloric acid was preferred as a protein precipitant when solutions were to be examined spectroscopically.

Partial purification of cell extracts. Crude cell extracts were treated with 0.2 vol. of 2% (w/v) protamine sulphate, the precipitate was removed by centrifuging and the supernatant solution was brought to 70% saturation by the addition of a saturated neutralized solution of $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitate (60 mg. of protein) was centrifuged and dissolved in 2 ml. of phosphate buffer, pH 7.5 (KH_2PO_4 ; 2 g./l.), and the solution was applied to a column (20 cm. $\times$ 2 cm.) of Sephadex G-25 (100–270 mesh). The same buffer was used to elute the extract, which was then free from $(\text{NH}_4)_2\text{SO}_4$. Such extracts (15–20 mg. of protein/ml.) converted catechol into 4-hydroxy-2-oxovalerate, which was not further metabolized. Alternatively, the pellet obtained after treatment with $(\text{NH}_4)_2\text{SO}_4$ was dissolved in phosphate buffer to give a protein concentration of 15–20 mg./ml. and dialysed against three changes of phosphate buffer for 3 hr. with stirring. After this treatment, extracts of *Pseudomonas* (U) converted 4-hydroxy-2-oxovalerate into pyruvate, which was not metabolized further.

Chromatography and electrophoresis. When a compound was identified by chromatography its R_F was always referred to that of authentic material run on the same chromatogram. For 2,4-dinitrophenylhydrazones of oxo acids, two methods were used, namely, one-dimensional ascending systems on Whatman no. 4 papers and thin-layer chromatography. The solvents used for paper chromatography were: *A*, butan-1-ol-ethanol- $(\text{NH}_4)_2\text{CO}_3$ buffer (40:11:19, by vol.) (Dagley, Fewster & Happold, 1952); *B*, butan-1-ol-ethanol-water (7:1:2, by vol.) (Smith & Smith, 1960); *C*, 2-methylbutan-2-ol-ethanol-water (5:1:4, by vol.; upper layer) (Altmann, Crook & Datta, 1951). In these solvents R_F values for the 2,4-dinitrophenylhydrazones of pyruvate were: solvent *A*, 0.67, 0.81; solvent *B*, 0.52, 0.66; solvent *C*, 0.59, 0.79. For the derivative of 4-hydroxy-2-oxovalerate these values were: solvent *A*, 0.70, 0.83; solvent *B*, 0.54, 0.68; solvent *C*, 0.66, 0.80. The two spots for each derivative in a particular solvent are presumably those for stereoisomers. Glass plates (20 cm. $\times$ 20 cm.) for thin-layer chromatography were first coated to a thickness of 0.01 in. with Kieselgel G (E. Merck

A.-G., Darmstadt, Germany) and then activated at 100° for 30 min. This type of chromatography proved to be particularly useful for the identification of 4-hydroxy-2-oxovalerate since its 2,4-dinitrophenylhydrazone when held at 100° for 3 min. with 2 N-HCl is largely converted into the derivative of 4-methyl-2-oxobutyrolactone with a marked change in R_F values for the systems used. The solvents were: *D*, benzene-ethyl formate-propionic acid (18:1:1, by vol.); *E*, light petroleum (b.p. 60–90°)-ethyl formate-propionic acid (70:30:15.4, by vol.) (Ronkainen, 1963). The R_F values of 2,4-dinitrophenylhydrazones in solvents *D* and *E* were as follows: pyruvate (solvent *D*), 0.20; (solvent *E*), 0.24; 4-hydroxy-2-oxovalerate (solvent *D*), 0.22; (solvent *E*), 0.03, 0.14; 4-methyl-2-oxobutyrolactone (solvent *D*), 0.40; (solvent *E*), 0.32, 0.45.

Two methods were also used to identify 2,4-dinitrophenylhydrazones of neutral carbonyl compounds, namely reverse-phase chromatography on paper (Horner & Kirmse, 1955; Asatoor, 1962) and thin-layer chromatography with, as solvent, diethyl ether-*n*-hexane (1:3, v/v). In this solvent R_F values for the 2,4-dinitrophenylhydrazones of the compounds stated were: formaldehyde, 0.18; acetaldehyde, 0.22; propionaldehyde, 0.35; butyraldehyde, 0.44; acetone, 0.28.

Catechol and 4-methyl-2-oxobutyrolactone were detected by ascending chromatography (Whatman no. 1 paper). In the solvent benzene-methanol-acetic acid (45:8:4, by vol.) (Petrowitz, 1962) R_F values for catechol and 4-methyl-2-oxobutyrolactone were respectively 0.55 and 0.67; in chloroform-methanol-water-formic acid (100:20:19:0.8, by vol.; organic phase) (Reio, 1958) they were respectively 0.62 and 0.85. Compounds were located by first spraying with a 2% (w/v) solution of 2,6-dibromoquinone-4-chloroimide in ethanol followed by heating at 100° for 5 min., when red-brown spots were given with catechol and green spots with 4-methyl-2-oxobutyrolactone.

High-voltage paper electrophoresis (Gross, 1958) of organic acids was carried out with 0.1 M- $(\text{NH}_4)_2\text{CO}_3$ at pH 8.9 by using 5000 v for 40 min., and compounds were located by spraying with bromocresol purple.

Formate was identified and determined by partition chromatography on ether-washed Celite 535 (Johns-Manville Co. Ltd., London, S.W.1), as described by Swim & Krampitz (1954). Fractions (8 ml.) were collected, 2 ml. of CO_2 -free water was added to each and the formic acid was titrated with 11.6 M-NaOH with phenol red as indicator. The identity of formate in reaction mixtures was confirmed by the colorimetric method of Grant (1948). Formic acid was isolated by steam-distillation or by continuous ether extraction: the distillate was neutralized with NaOH and then evaporated to a suitable small volume; or the ether extract was shaken with sufficient NaOH solution for re-extraction. The formate in solution (0.5 ml.; about 50 μg .) was reduced to formaldehyde by 80 mg. of magnesium foil to which was added 0.5 ml. of conc. HCl dropwise with shaking. When incubated at 100° for 30 min. with 0.18% 4,5-dihydroxynaphthalene-2,7-disulphonic acid (chromotropic acid) a pink compound was formed, having a characteristic absorption spectrum in the region 350–650 m μ .

Materials. Catechol, sodium pyruvate and acetaldehyde were from British Drug Houses Ltd., Poole, Dorset. Catechol was purified by sublimation, and acetaldehyde by distillation, before use. 4-Methyl-2-oxobutyrolactone was prepared according to the method of Rossi & Schinz (1948).

Solutions of 4-hydroxy-2-oxovalerate were prepared by mild alkaline hydrolysis of 4-methyl-2-oxobutylolactone. The lactone (11.4 mg.; 100 μ moles) was dissolved in 9.9 ml. of distilled water, 0.1 ml. of *n*-NaOH was added and hydrolysis allowed to proceed to completion at room temperature (14 hr.). Solutions were stable at -18° . [14 C]Formate was from The Radiochemical Centre, Amersham, Bucks. Sephadex G-25 (100–270 mesh) was from Pharmacia, Uppsala, Sweden. Samples of 4-oxalocrotonic acid were kindly given by Dr Y. Nishizuka and Dr P. J. Chapman. Alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC1.1.1.1) was from L. Light and Co. Ltd., Colnbrook, Bucks.; it contained no detectable lactate dehydrogenase (L-lactate-NAD oxidoreductase, EC1.1.1.27). Lactate dehydrogenase was from British Drug Houses Ltd. and contained no alcohol dehydrogenase. Both enzymes were diluted to 10 mg. of protein/ml. before use.

Chemical and enzymic analyses. Acetaldehyde was determined by the method of Barker & Summerson (1941) as modified by Hullin & Noble (1953); pyruvate was determined by the method of Friedemann & Haugen (1943). The determination of 4-hydroxy-2-oxovalerate was based on its ready lactonization in acid solution to give 4-methyl-2-oxobutylolactone, which absorbs strongly at 230 $m\mu$ (Fig. 3). Solutions of 4-hydroxy-2-oxovalerate (0–1 μ mole) in 3.0 ml. of 0.83 *N*-HCl were held at 100° for 3 min., cooled in ice and extinctions read at 230 $m\mu$ with 0.83 *N*-HCl as a blank. A linear calibration curve was given for solutions of synthetic 4-hydroxy-2-oxovalerate over this range of concentration, lactonization of 70% of the acid being consistently observed.

Assay of radioactive formate. Samples (0.05 ml.) of each fraction recovered were taken with a calibrated micro-pipette. Each was applied to a lens-paper disk, gummed to an aluminium planchet, dried for 10 min. at 12 cm. from a light bulb (100 w) and counted for a minimum of 10000 counts with a thin-window gas-flow counter (Nuclear-Chicago Corp.). Samples so prepared were 'infinitely thin'.

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

RESULTS

Conversion of catechol into formate and 4-hydroxy-2-oxovalerate

Identification and determination of formate. Cell extracts of *Pseudomonas* (L) grown with phenol were treated with protamine sulphate and ammonium sulphate and passed down a column of Sephadex G-25. When 1 ml. of this treated extract was incubated in a Warburg flask with 3 ml. of a reaction mixture containing 20 μ moles of catechol and 130 μ moles of phosphate buffer, pH 7.5, 20 μ moles of oxygen were taken up in 15 min. and a compound having the spectrum of 2-hydroxymuconic semialdehyde (Dagley *et al.* 1960) was formed and then disappeared. Five such reaction mixtures, containing a total of 100 μ moles of catechol, were incubated for an additional time of 25 min. (total time of incubation, 40 min.), during which there was no further uptake of oxygen and no evolution of carbon dioxide. Flask contents were pooled, 2.5 ml. of

5 *N*-sulphuric acid was added and the precipitate was removed by centrifuging. After the addition of 0.3 ml. of [14 C]formate (1.25 μ moles; 6 μ c, giving 15×10^5 counts/min. on planchets) the solution was extracted continuously with ether for 48 hr. and acids were then re-extracted by titration with sufficient 11.6 *M*-sodium hydroxide to give an aqueous solution alkaline to phenol red. This was concentrated to 1 ml. and chromatographed on Celite (Swim & Krampitz, 1954); each emerging fraction was titrated and the radioactivity assayed. The elution patterns of titrated acid and added [14 C]formate were identical (Fig. 1); 90 μ moles of formic acid as determined by titration were eluted and 83% (12.4×10^5 counts/min.) of the added radioactive material was recovered. Experiments with the synthetic compound showed that 4-hydroxy-2-oxovalerate was not eluted from the column in this experiment.

A solution obtained by extracting enzymic products with ether and re-extracting into alkali was treated with magnesium and acid and then with chromotropic acid. The pink compound produced gave a spectrum in the region 350–650 $m\mu$ identical with that obtained when authentic formate was

Fig. 1. Chromatography on Celite. Catechol was oxidized by a Sephadex-treated extract of *Pseudomonas* (L), [14 C]formate was added, the reaction mixture was extracted with ether and applied to the column, and the eluted fractions were titrated and assayed for radioactivity. O, Titrated acid; ●, [14 C]formate.

taken through the same procedure, with peaks at 382, 485 and 580 $m\mu$.

In another experiment, the products of oxidation of 10 μ moles of catechol by Sephadex-treated extract of *Pseudomonas* (L) were taken through ether into dilute ammonia and freeze-dried overnight. The dried sample was dissolved in 0.2 ml. of water and 0.02 ml. was taken for examination by high-voltage paper electrophoresis at 5000 v for 40 min.; 0.005 μ C of [14 C]formate was also applied to the chromatogram alongside this sample. Compounds were marked after spraying with bromocresol purple, the chromatogram was sprayed again with 0.5 M-potassium dihydrogen phosphate and then radioautographed. Three reaction products, and no others, were discernible: one spot at 44.3 cm. towards the anode corresponded exactly with the radioactivity of added [14 C]formate; the other two spots corresponded to 4-hydroxy-2-oxovalerate (at 45.7 cm.) and its lactone (at 29.4 cm.).

Metabolism of formate. When cell extracts were

Fig. 2. Oxidation of catechol and formate by cell-free preparations of *Pseudomonas* (L). Catechol (10 μ moles) was oxidized by soluble enzymes (O) and, at the time shown by the arrow on curve A, a 'particulate fraction' from the cells was added. Uptakes of oxygen catalysed by this fraction are shown by curve B for 10 μ moles of formate and by curve C for 10 μ moles of acetate, pyruvate, 4-hydroxy-2-oxovalerate or catechol.

prepared, a pellet of disrupted cell components remained when the clear supernatant was removed. About 2 g. (wet wt.) of the upper layer of the pellet was washed four times by centrifuging with phosphate buffer, pH 7.5, and resuspended in 10 ml. of the same buffer. This particulate fraction oxidized formate to completion (5 μ moles of oxygen/10 μ moles of formate; Fig. 2, curve B), but no oxygen uptake was detected with acetate, pyruvate, 4-hydroxy-2-oxovalerate or catechol as substrate. Catechol (10 μ moles) was incubated with a Sephadex-treated cell extract of *Pseudomonas* (L) until consumption of oxygen had ceased and the yellow colour due to 2-hydroxymuconic semialdehyde had disappeared; a particulate suspension was then tipped from a second side arm, when a further reaction occurred that ceased when 67 μ l. (3 μ moles) of oxygen had been taken up (Fig. 2, curve A). This experiment was repeated in another flask that contained hydrochloric acid instead of potassium hydroxide in the centre well, and 6 μ moles of carbon dioxide were released when acid was tipped at the end of the second reaction. Since there was no evolution of carbon dioxide when catechol was oxidized by cell extracts free from particulate matter, this experiment confirmed previous observations that formate is enzymically produced from catechol. However, the amount of formate determined was 60% of that expected, in contrast with the value obtained by isolation and chromatography, which was about theoretical. This discrepancy was also shown when acid production was followed by means of an automatic titrator (for a description, see Ribbons & Evans, 1962): from 10 μ moles of catechol, maintained at pH 6.9 by additions of sodium hydroxide during the course of the reaction, 16 μ equiv. of H^+ was formed. Since it has been shown in other experiments that Sephadex-treated extracts of *Pseudomonas* (L) convert 1 mol. of catechol into 1 mol. of the monobasic 4-hydroxy-2-oxovalerate, these findings also indicate a yield of formate 40% lower than that expected on the assumption that 1 mol. of catechol gives rise to 1 mol. of formate. In these experiments formate was not isolated but was directly determined in the presence of the cell extracts that had catalysed its formation.

Identification and determination of 4-hydroxy-2-oxovalerate. Experiments with high-voltage paper electrophoresis, described above, showed that catechol was oxidized to formate and 4-hydroxy-2-oxovalerate by Sephadex-treated extracts of *Pseudomonas* (L). The following properties of synthetic 4-hydroxy-2-oxovalerate and its lactone, 4-methyl-2-oxobutylrolactone, were also used to study the material formed enzymically. The spectrum of the lactone (Fig. 3) showed λ_{max} 228 $m\mu$ in acid (ϵ 5200) and 262 $m\mu$ in alkaline solution (ϵ 4800); the free acid showed end-absorption between 200 and 280

Fig. 3. Spectra of 0.2 mm 4-methyl-2-oxobutylolactone in 0.3 N-HCl (○) and in 0.3 N-NaOH (●).

mμ. On standing in 0.83 N-hydrochloric acid at 4° the free acid was converted overnight into a mixture containing 70% of the lactone, as shown by the increase in extinction at 228 mμ: an equilibrium appeared to be reached since the yield of lactone was not increased on standing for longer periods. At 100°, lactonization to give an equilibrium mixture occurred in 3 min.; this was the basis for the determination of 4-hydroxy-2-oxovalerate described in the Materials and Methods section. The 2,4-dinitrophenylhydrazone of 4-hydroxy-2-oxovalerate is also readily lactonized, as was shown by varying the time of incubation of the acid with 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid at 30° in the procedure of Friedemann & Haugen (1943) for the determination of oxo acids. From Table 1, the concentration of the derivative of 4-hydroxy-2-oxovalerate reached a maximum after incubation with reagent for 3 min., but after 50 min. the extinction due to hydrazones that could be extracted from ethyl acetate into sodium carbonate had decreased to 40% of the value after 3 min. Thin-layer chromatography (solvents *D* and *E*) of the hydrazones passing into ethyl acetate at the first extraction of the procedure of Friedemann & Haugen (1943) showed that at 3 min. the only hydrazone present was that of 4-hydroxy-2-oxovalerate, but at 50 min. there were also strong spots due to the derivative of 4-methyl-2-oxobutylolactone. This derivative has no carboxyl group and is extracted with difficulty from ethyl acetate into 10% (w/v) sodium carbonate: thus, in the experiment of Table 1 when the lactone was incubated with reagent for 50 min., the extinction of an alkaline solution of the extracted derivative was only 15% of that obtained when the free acid was incubated with reagent for 3 min. and taken through the same procedure. Thin-layer chromatography confirmed that when the ethyl acetate solution from the first extraction was shaken with 10%

Table 1. Lactonization of the 2,4-dinitrophenylhydrazone of 4-hydroxy-2-oxovalerate

Solutions contained, in 3 ml., 1 μmole of either 4-hydroxy-2-oxovalerate or 4-methyl-2-oxobutylolactone and were incubated at 30° with 1 ml. of reagent (2,4-dinitrophenylhydrazine in 2 N-HCl) for different times. After incubation, 2,4-dinitrophenylhydrazones were extracted into 8 ml. of ethyl acetate and then into 6 ml. of 10% (w/v) Na₂CO₃. To 5 ml. of this solution was added an equal volume of 1.5 N-NaOH to produce a red colour; the extinction was measured at 450 mμ.

Time of incubation with reagent (min.)	<i>E</i> ₄₅₀ of extracted derivatives of	
	4-Hydroxy-2-oxo- valerate	4-Methyl-2-oxo- butylolactone
1	0.93	0.07
3	1.15	0.11
6	0.97	0.14
10	0.86	0.15
15	0.76	0.16
25	0.60	0.17
35	0.52	0.17
50	0.46	0.17

(w/v) sodium carbonate, most of the 2,4-dinitrophenylhydrazone of the lactone remained in the ethyl acetate solution. The derivative of 4-hydroxy-2-oxovalerate appeared to lactonize more readily than did the acid itself; thus, when incubated at 30° with 2 N-hydrochloric acid, 60% of the acid lactonized in 150 min., whereas the changes in extinction in Table 1 (from 1.15 to 0.46) indicate that with the 2,4-dinitrophenylhydrazone this proportion lactonized in less than 50 min.

The identity of 4-hydroxy-2-oxovalerate formed by enzymic oxidation of catechol was also confirmed from the infrared-absorption spectrum of the 2,4-dinitrophenylhydrazone of the lactone of this compound. To a reaction mixture that contained, in 30 ml., 2 m-moles of phosphate buffer, pH 7.5, and a Sephadex-treated extract (150 mg. of protein) of *Pseudomonas* (L), were added 100 μmoles of catechol. The mixture was stirred mechanically at 22° and additions of the solution of catechol were made in portions of 20 μmoles after the disappearance of the yellow colour of the ring-fission product from each previous addition. When all the catechol had been added, the reaction mixture was stirred for a further 20 min., 15 ml. of 5 N-hydrochloric acid was added and the precipitate was removed by centrifuging. The solution was then held at 100° for 5 min. to convert 4-hydroxy-2-oxovalerate into its lactone, 25 ml. of 0.1% 2,4-dinitrophenylhydrazone in 2 N-hydrochloric acid was added and the yellow precipitate was collected on the centrifuge and dissolved in ethyl acetate. This solution was shaken with 10% (w/v) sodium carbonate to remove 4-hydroxy-2-

oxovalerate, applied as a band to the base of a layer (0.02 in. thick) of Kieselgel G and chromatographed with solvent *E*; 4-methyl-2-oxobutyrolactone 2,4-dinitrophenylhydrazone was then removed from the plate and eluted with ethyl acetate. When this solvent was evaporated, 6 mg. of yellow crystals, m.p. 168–171°, was obtained. 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°); and, in particular, a strong band was given in both cases at 5.7 μ in the region where γ -lactones show characteristic absorption (Bellamy, 1958).

Sephadex-treated extracts of *Pseudomonas* (L) were used for quantitative studies of the conversion of catechol into 4-hydroxy-2-oxovalerate. In three separate experiments, 10 μ moles of catechol were incubated in Warburg flasks with cell extract (12–15 mg. of protein) until uptake of oxygen ceased and no yellow colour due to ring-fission product persisted. In each case protein was precipitated with perchloric acid, a portion of the acid solution was heated and the spectrum shown to be that of a solution of 4-methyl-2-oxobutyrolactone (Fig. 2). From the extinctions at 230 m μ the amounts of 4-hydroxy-2-oxovalerate were calculated to be 9.5, 10.0 and 10.0 μ moles respectively.

Conversion of catechol into pyruvate and acetaldehyde

Pseudomonas (U) appeared to metabolize catechol by the same reactions as those described for *Pseudomonas* (L); thus Sephadex-treated extracts also accumulated 4-hydroxy-2-oxovalerate and formate from catechol. However, since crude extracts of *Pseudomonas* (U) converted 4-hydroxy-2-oxovalerate into pyruvate much more rapidly than did extracts of *Pseudomonas* (L), the former organism was used to study this conversion. Extracts of *Pseudomonas* (U) were treated successively with protamine sulphate and ammonium sulphate, and after dialysis they attacked 4-hydroxy-2-oxovalerate but not pyruvate; they are referred to below as 'dialysed extracts'. When such a cell extract was incubated with catechol, pyruvate and acetaldehyde were formed and the yield of acetaldehyde, but not that of pyruvate, was greatly decreased in the presence of NADH. When alcohol dehydrogenase and NADH were both present, no acetaldehyde was detected (Table 2). Similarly, no pyruvate accumulated when lactate dehydrogenase was added with NADH; instead, when an ether extract of the reaction products was chromatographed in phenol-formic acid-water (500:13:167, w/v/v) (Kornberg, 1958) a brown spot with the same R_f as authentic lactic acid appeared on spraying with aniline-xylose

Table 2. *Effect of additions of NADH, lactate dehydrogenase and alcohol dehydrogenase on the conversion of catechol into pyruvate and acetaldehyde*

Warburg flasks contained, in 3 ml. of 0.1 M-phosphate buffer, pH 7.5, 5.0 μ moles of catechol and dialysed extract (26 mg. of protein) of *Pseudomonas* (U). Lactate dehydrogenase (2 mg. of protein), alcohol dehydrogenase (2 mg. of protein) and NADH (50 μ moles) were added as indicated; reaction mixtures were incubated at 30° for 1 hr., during which time 5.0 μ moles of oxygen were consumed in each flask. To each mixture 0.5 ml. of 6% (w/v) perchloric acid was then added, and the amounts of acetaldehyde and pyruvate present in the centrifuged solutions were determined.

Addition	Acetaldehyde (μ moles)	Pyruvate (μ moles)
None	2.0	4.3
NADH	0.37	4.0
Lactate dehydrogenase + NADH	0.25	0.00
Alcohol dehydrogenase + NADH	0.00	4.10

reagent (Nordmann & Nordmann, 1960). When pyruvate accumulated its identity was confirmed by forming the 2,4-dinitrophenylhydrazone, which was extracted into ethyl acetate, then into 10% (w/v) sodium carbonate and finally into ethyl acetate after acidification: paper chromatography in solvents *A* and *B*, and thin-layer chromatography in solvents *D* and *E*, gave spots with R_f values identical with those of pyruvate 2,4-dinitrophenylhydrazone applied to the same chromatograms. The identity of acetaldehyde was confirmed by an experiment in which a solution of catechol (100 μ moles) was added dropwise to 20 ml. of 0.1 M-potassium dihydrogen phosphate, pH 7.5, and dialysed extract (260 mg. of protein) of *Pseudomonas* (U) contained in a closed flask fitted with a magnetic stirrer. A stream of air that had first passed through 0.1% 2,4-dinitrophenylhydrazine in 2 N-hydrochloric acid was blown through the reaction vessel and thence into a second tube containing the same solution of 2,4-dinitrophenylhydrazine, where a yellow precipitate was collected (yield, 10 mg.), m.p. 147° unchanged by admixture with an authentic sample of acetaldehyde 2,4-dinitrophenylhydrazone; the infrared-absorption spectrum and that of the authentic compound showed all absorption bands common to both. The compound isolated from the enzymic reaction also behaved as acetaldehyde 2,4-dinitrophenylhydrazone in reverse-phase chromatography on paper (Horner & Kirmse, 1955; Asatoor, 1962) and in thin-layer chromatography.

When acetaldehyde is formed by reactions that require incubation at 30° for about 1 hr. losses may occur owing to volatility or to metabolism of the compound by the cell extract used. Sodium pyrosulphite (metabisulphite) was therefore added to

trap acetaldehyde when it was desired to study the quantitative conversion of catechol into acetaldehyde and pyruvate. Each one of a series of Warburg flasks contained, in 3 ml., 5.0 μ moles of catechol, 170 μ moles of phosphate buffer, pH 7.5, 10 μ moles of sodium pyrosulphite and dialysed extract (13 mg. of protein) of *Pseudomonas* (U). Uptake of oxygen was measured, and at various intervals flasks were removed, their contents discharged into 0.5 ml. of ice-cold 6% (w/v) perchloric acid, precipitates were removed by centrifuging and each solution was diluted with water to 5.0 ml. Acetaldehyde was determined in 0.2 ml. samples and the remaining solution was held at 100° for 3 min. to convert 4-hydroxy-2-oxovalerate into its lactone, which was determined in the solution, after cooling, by measurement of the extinction at 230 m μ and reference to a calibration curve. The amount of pyruvate in this solution was determined for 1.0 ml. samples by a modification of the method of Friedemann & Haugen (1943): the time of incubation with 0.1% 2,4-dinitrophenylhydrazine at 30° was decreased to 5 min. and a correction was applied for the lactonized 4-hydroxy-2-oxovalerate present; thus a solution that contained 0.5 μ mole of pyruvate and 0.5 μ mole of lactonized 4-hydroxy-2-oxovalerate gave E_{450} 0.66, of which 0.03 was contributed by the hydrazone of the lactonized acid. The amount of catechol that remained at a given time was also calculated from the uptake of oxygen at that time. Catechol disappeared and 4-hydroxy-2-oxovalerate was formed during the first 10 min., reaching a maximal concentration between 10 and 15 min. (Fig. 4). As 4-hydroxy-2-oxovalerate disappeared, pyruvate and

acetaldehyde were formed in equimolar amounts, and at 41 min. about 2.5 μ moles of each compound were present. Between 15 and 45 min., 2.2 μ moles of 4-hydroxy-2-oxovalerate were metabolized to give 2.2 μ moles each of pyruvate and acetaldehyde.

The conversion of catechol into acetaldehyde and pyruvate was stimulated by Mg^{2+} ions. Thus, when

Table 3. *Effect of pH on the conversion of 4-hydroxy-2-oxovalerate into pyruvate*

Reaction mixtures contained dialysed extract (7.5 mg. of protein) of *Pseudomonas* (U) in 0.1 M-tris brought to various pH values with HCl. Each was incubated with 5.0 μ moles of synthetic 4-hydroxy-2-oxovalerate at 30° for 30 min., reactions were stopped with perchloric acid, and pyruvate present in the cooled centrifuged solutions was determined after they had been heated at 100° for 3 min. to convert unchanged substrate into its lactone. Control reactions from which cell extract was omitted showed that non-enzymic conversion of 4-hydroxy-2-oxovalerate did not occur under these conditions.

pH	Pyruvate (μ moles)
7.0	1.00
7.3	1.15
7.5	1.34
8.0	1.48
8.4	1.83
8.8	1.93
9.2	1.90
9.5	1.57

Table 4. *Enzymic conversion of synthetic 4-hydroxy-2-oxovalerate into acetaldehyde and pyruvate*

A series of Warburg flasks contained, in 3 ml. of 0.1 M-tris buffer, pH 8.5, 10 μ moles of sodium pyrosulphite, 5.0 μ moles of synthetic 4-hydroxy-2-oxovalerate, 5.0 μ moles of $MgSO_4$ and dialysed extract (13 mg. of protein) of *Pseudomonas* (U). Reactions at 30° were stopped at various times by additions of perchloric acid from centre wells and the mixtures were cooled in ice and centrifuged at 0°. Acetaldehyde was determined in 0.3 ml. portions; the remainder was heated at 100° for 3 min. and pyruvate was determined after cooling. In control flasks for which substrate was omitted or the cell extract was boiled, neither pyruvate nor acetaldehyde was detected.

Time (min.)	Pyruvate (μ moles)	Acetaldehyde (μ moles)
1	0.15	0.13
3	0.35	0.25
5	0.64	0.55
10	1.20	1.10
15	1.75	1.50
20	2.30	1.90
30	2.40	1.94
35	2.40	1.89
40	2.40	1.85

Fig. 4. Conversion of catechol into 4-hydroxy-2-oxovalerate, pyruvate and acetaldehyde by a cell-free extract of *Pseudomonas* (U). ○, Catechol; ●, 4-hydroxy-2-oxovalerate; □, pyruvate; Δ, acetaldehyde.

5.0 μ moles of catechol were incubated with dialysed cell extract (26mg. of protein) for 45min., the amount of pyruvate formed was increased from 3.3 to 4.8 μ moles by the addition of 5.0 μ moles of magnesium sulphate.

Metabolism of synthetic 4-hydroxy-2-oxovalerate. A stimulation by Mg^{2+} ions, similar to that observed for catechol degradation, was also shown for the conversion of synthetic 4-hydroxy-2-oxovalerate into pyruvate. The pH optimum for this conversion was about 8.8 (Table 3). Measurements of pyruvate formed from synthetic 4-hydroxy-2-oxovalerate, which were made at pH 8.5 after the addition of Mg^{2+} ions, showed that only half the substrate, presumably one enantiomer, was metabolized (Table 4). The concentration of pyruvate increased linearly for 20 min. and ceased when 2.4 μ moles had accumulated from 5.0 μ moles of substrate; measurements of the extinction at 230 m μ made after lactonization showed that 2.5 μ moles of 4-hydroxy-2-oxovalerate remained. In this experiment, acetaldehyde was formed but the amounts determined were less than those for pyruvate, probably because acetaldehyde is volatile and at pH 8.5 is less effectively trapped by sodium pyrosulphite than it is at pH 7.5.

DISCUSSION

Sephadex-treated extracts of both species of *Pseudomonas* converted 1.0 μ mole of catechol into 1.0 μ mole of 4-hydroxy-2-oxovalerate with a transitory formation of 2-hydroxymuconic semialdehyde and an uptake of 1.0 μ mole of oxygen; no carbon dioxide was evolved and the other reaction product was identified as formate by several methods. When the formate was separated from the reaction mixture by acidification, extraction and chromatography, about 1.0 μ mole of formate was given from 1.0 μ mole of catechol: thus, in the experiment of Fig. 1, 83% of added labelled formate was recovered when taken through this extraction procedure, and 90 μ moles of formate were obtained from the metabolism of 100 μ moles of catechol to which had been added 1.25 μ moles of [^{14}C]formate. However, when formate was determined directly in reaction mixtures, without prior acidification and extraction, yields of 0.6 μ mole from 1.0 μ mole of catechol were obtained; this was the case when the amount of formate was calculated from the acid produced as the reaction proceeded, or when formate was determined by

addition of a particulate cellular fraction to the reaction mixture (Fig. 2). It appears that some of the formate may remain bound to certain components of the cell extract such as the enzymes that catalyse its formation; bound formate would be liberated only when these components were precipitated by acid.

Catechol was metabolized to 4-hydroxy-2-oxovalerate and then to acetaldehyde and pyruvate by dialysed extracts of *Pseudomonas* (U) that had been treated with protamine sulphate and ammonium sulphate, each μ mole of enzymically formed 4-hydroxy-2-oxovalerate giving rise to 1 μ mole each of acetaldehyde and pyruvate (Fig. 4). However, only one-half of a sample of synthetic 4-hydroxy-2-oxovalerate was attacked by these cell extracts and gave 0.5 μ mole of pyruvate/1.0 μ mole of substrate, suggesting that only one isomer was metabolized: C-4 of this compound is asymmetric. The reaction is of the same type as that described by Shannon & Marcus (1962) for the cleavage of 4-carboxy-4-hydroxy-2-oxovalerate (4-hydroxy-4-methyl-2-oxoglutarate), which is catalysed by an aldolase isolated from germinating peanuts: both enzymes are stimulated by Mg^{2+} ions and show pH optima above 8.5; however, the aldolase for 4-hydroxy-2-oxovalerate showed no requirement for a thiol compound, unlike the aldolase studied by Shannon & Marcus (1962).

Our results are summarized in Scheme 1, which differs in several respects from that proposed by Nishizuka *et al.* (1962) for the degradation of catechol by another species of *Pseudomonas*. The cell extracts used by Nishizuka *et al.* (1962) catalysed a slow uptake of oxygen in addition to that required for ring-fission, 2-hydroxymuconic semialdehyde being oxidized to 4-oxalocrotonate, which was then decarboxylated to give 4-hydroxy-2-oxovalerate. Cell extracts used in the present work consumed no oxygen in addition to that required to open the benzene nucleus of catechol, no carbon dioxide was evolved when pyruvate was formed from catechol, and Sephadex-treated extracts that converted catechol into 4-hydroxy-2-oxovalerate did not decarboxylate 4-oxalocrotonate. However, since untreated extracts of *Pseudomonas* (L) decarboxylate 4-oxalocrotonate readily, it is probable that the compound is a metabolite of this organism although it is not an obligatory intermediate in the metabolism of catechol by cell extracts: thus the reactions described by Nishizuka *et al.* (1962) might be cata-

Scheme 1.

lysed in *Pseudomonas* (L) by enzymes bound to cellular structures that would not be solubilized by the methods we used.

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