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# The Metabolism of Protocatechuate by *Pseudomonas testosteroni*

By S. DAGLEY, P. J. GEARY AND J. M. WOOD
*Department of Biochemistry, University of Leeds, and Department of Biochemistry, University of Minnesota, St Paul, Minn.* 55101, *U.S.A.*

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1. Protocatechuate 4,5-oxygenase, purified 21-fold from extracts of *Pseudomonas testosteroni*, was examined in the ultracentrifuge and assigned a mol.wt. of about 140000. 2. When diluted, the enzyme rapidly lost activity during catalysis. Inactivation was partially prevented by L-cysteine. 3. With a saturating concentration of protocatechuate (1·36mM), $K_m$ for oxygen was 0·303mM. This value is greater than the concentration of oxygen in water saturated with air at 20°. 4. Cell extracts converted protocatechuate into γ-carboxy-γ-hydroxy-α-oxovalerate, which was isolated as its lactone. 5. γ-Carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase activity was stimulated by $Mg^{2+}$ ions and mercaptoethanol. Cells grown with *p*-hydroxybenzoate as carbon source contained higher concentrations of this enzyme than those grown with succinate.

In their taxonomic study of the aerobic pseudomonads, Stanier, Palleroni & Doudoroff (1966) found that the simple aromatic acid most extensively utilized by these organisms as a growth substrate is *p*-hydroxybenzoate. This compound is first oxidized to protocatechuate (3,4-dihydroxybenzoate) and the benzene nucleus is then opened in one of two ways: either by '*ortho* cleavage' to give β-carboxy-*cis*-*cis*-muconic acid, or by '*meta* cleavage' to give γ-carboxy-α-hydroxy-*cis*-*cis*-muconic semialdehyde (Dagley, Evans & Ribbons, 1960). The mechanism of cleavage of protocatechuate is a taxonomically significant character, since all the strains of a given species of *Pseudomonas* that were able to perform this reaction did so by the same mechanism. Moreover, the *ortho* cleavage of protocatechuate was characteristic of the entire fluorescent group whereas *meta* cleavage was confined to the non-fluorescent organisms, *Pseudomonas acidovorans* and *Pseudomonas testosteroni* (Stanier *et al.* 1966). These observations stimulated interest in the properties of the two protocatechuate dioxygenases. Whereas protocatechuate 3,4-oxygenase has been crystallized (Hayaishi, 1966), protocatechuate 4,5-oxygenase has been described as an extremely unstable enzyme, the activity of which is most difficult to maintain (Wheelis, Palleroni & Stanier, 1967). We have studied some of the factors that determine the activity of this enzyme and have obtained preparations that appear to be essentially pure when examined in the analytical ultracentrifuge or by disk electrophoresis. Previously, investigations were confined to crude cell extracts that exhibited maximal activity at pH 7·0–7·5 when supplemented with $Fe^{2+}$ ions (Dagley & Patel, 1957; Ribbons & Evans, 1962; Cain, 1962). We also report experiments that support a pathway for the conversion of protocatechuate into formate and pyruvate proposed by Dagley, Chapman, Gibson & Wood (1964).

## MATERIALS AND METHODS

*Maintenance and growth of organism.* The non-fluorescent *Pseudomonas* N.C.I.B. 8893 was grown under forced aeration at 30° with *p*-hydroxybenzoic acid (1g./l.) as the sole source of carbon in the mineral salts medium of Dagley & Patel (1957). These authors isolated the organism, now classified as *Pseudomonas testosteroni* (Stanier *et al.* 1966), by elective culture in a *p*-cresol medium. Stock cultures were maintained on nutrient–agar slopes sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) and were subcultured monthly.

*Preparation of cell extracts.* Suspensions of washed cells were broken, as described by Dagley & Gibson (1965), either by sonic treatment in an MSE disintegrator or by crushing without abrasive in the bacterial press of Hughes (1951). Crushed cells, 24g. wet wt., were taken up in 48ml. of phosphate buffer, pH 7·3, containing 5mg. each of ribonuclease and deoxyribonuclease (Koch–Light Laboratories Ltd., Colnbrook, Bucks.). After clarification by centrifuging at 26000$g_{av.}$, the extract was brought to 60% saturation of $(NH_4)_2SO_4$ at 20° by addition, with stirring, of a neutralized solution of $(NH_4)_2SO_4$. The precipitate formed was collected on the centrifuge, dissolved in phosphate buffer, pH 7·3, and dialysed for 12hr. with stirring at 5° against two changes of the same buffer. The resulting solution, which contained 15mg. of protein/ml., is referred to as 'dialysed extract'. Extracts of cells broken in the MSE disintegrator were treated with protamine sulphate

(Bayly, Dagley & Gibson, 1966) to remove nucleic acids and brought to 70% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in phosphate buffer (60mg. of protein/ml.) and applied to a column (30cm. × 2cm.) of Sephadex G-25 (fine grade; 20–80 $\mu$). When eluted with the same buffer, the extract was free of $(NH_4)_2SO_4$ and contained 20–30mg. of protein/ml. This preparation is referred to as 'Sephadex-treated extract'.

All phosphate buffers contained 3·3g. of $KH_2PO_4$/l. adjusted to pH 7·3 with NaOH. Other buffers, except those used specifically for disk electrophoresis, contained 0·1 M-tris brought to the stated pH with HCl.

*Assay and purification of protocatechuate 4,5-oxygenase.* At pH 7·3 the ring-fission product from protocatechuate is bright yellow and absorbs strongly at 410m$\mu$. The colour provides a sensitive, qualitative method of detecting the presence of the enzyme in a fraction of a cell extract; but uncertainties are introduced into spectrophotometric assays by the rapid loss of activity, only partially reversed by cysteine (Fig. 3), that the enzyme suffers on dilution. Accordingly, although more expensive in enzyme, respirometry was made the basis of the assay procedure. For each determination it was necessary to use an amount of enzyme that catalysed a linear uptake of about 50 $\mu$l. of $O_2$ in a reaction time of 5min. This rate was well below the maximum imposed by diffusion of $O_2$ to the enzyme, and a stoicheiometric relationship of 1 $\mu$mole of $O_2$/$\mu$mole of protocatechuate was shown to obtain during the course of a reaction under these controlled conditions. The main compartment of each Warburg flask contained: 0·2ml. of 15mM-L-cysteine; 1·5ml. of phosphate buffer; 0·5ml. of enzyme; 0·1ml. of 33mM-$FeSO_4,7H_2O$ (added last). The centre well contained 0·2ml. of 20% (w/v) KOH and the gas phase was air. After equilibration at 30°, 0·5ml. of 10mM-protocatechuic acid in phosphate buffer was added from the side arm. One unit of activity was defined as the amount, in mg. of protein, that oxidized 1 $\mu$mole of protocatechuate/min. The protein contents of extracts containing 10–20mg./ml. were determined by the method of Sols (1947) and those with 2–10mg./ml. by a modified biuret method (Layne, 1957).

For further purification, a dialysed extract was prepared as described except that, when cell debris had been removed, the extract was centrifuged further for 2·75hr. at 48000rev./min. in a Spinco model L preparative centrifuge. The enzyme was then adsorbed on DEAE-cellulose (Cellex-D; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Preliminary experiments showed that the enzyme was inactivated by passage down a column of this material and a batch treatment was therefore adopted. To 25ml. of the dialysed extract were added 2·5ml. of 33mM-$FeSO_4,7H_2O$ and then 20g. of DEAE-cellulose suspended in 150ml. of phosphate buffer. The slurry was allowed to stand for 5min. at 0° and was then filtered at the pump. The DEAE-cellulose was resuspended in 150ml. of phosphate buffer containing 1% (w/v) $(NH_4)_2SO_4$, thoroughly mixed and filtered, and the eluate was discarded. After resuspension in 150ml. of phosphate buffer containing 3% (w/v) $(NH_4)_2SO_4$ and filtration, the filtrate was brought to 70% saturation with $(NH_4)_2SO_4$ and the precipitate collected on the centrifuge. The pellet was dissolved in 5ml. of buffer and the solution was centrifuged to remove insoluble material. At this stage the protein sedimented in the ultracentrifuge as a single large peak preceded by a small 'shoulder'. This faster-sedimenting component was removed by passage through Sephadex. The enzyme (4ml.) was applied to the top of a column (52cm. × 2·3cm.) of Sephadex G-200 and was eluted with 0·1 M-$KH_2PO_4$ buffer, pH 7·3, maintained at a constant head of 10cm. and a flow rate of 8–10ml./hr. The profile of eluted protein in fractions of 4·5ml., collected at 3–5° in the cold-room, showed two peaks of maximum protein content, one being present in tubes 10–20 and the other in tubes 22–36. Protocatechuate 4,5-oxygenase was shown qualitatively to be present in the second series of tubes and absent from the first. The contents of tubes 24–34 inclusive were pooled, protein was precipitated by adding solid $(NH_4)_2SO_4$ to give 70% saturation and the precipitate was collected on the centrifuge and dissolved in 5ml. of phosphate buffer (3·3g. of $KH_2PO_4$/l., pH 7·3). Insoluble material was removed by centrifuging and the supernatant solution was stored, without dialysis, at −22°. The solution was yellow and showed some activity towards protocatechuate without added $Fe^{2+}$ ions, although the addition of $Fe^{2+}$ caused a stimulation of $O_2$ uptake of about 60%. $Fe^{2+}$ ions were added before the enzyme was adsorbed on DEAE-cellulose, so that a proportion of these ions remained bound to the protein through subsequent operations. The steps in the purification, which was 21-fold, are summarized in Table 1; the purified protein sedimented as a single peak in the ultracentrifuge.

*Sedimentation and diffusion.* Sedimentation-velocity experiments were performed in a Spinco model E ultracentrifuge. The diffusion characteristics of the protein were examined by using a synthetic-boundary cell in the ultracentrifuge. After the boundary had been set up at a low speed (12000rev./min.) the protein was observed to diffuse evenly over an interval of 56min., and the area–height method was used to calculate the diffusion coefficient (Schachman, 1957), a correction being applied for the time taken to form the boundary.

*Measurement of oxygen utilized.* The progress of purification of the enzyme was followed by means of Warburg respirometry, and an oxygen electrode was used for investigating the properties of the purified enzyme. The apparatus, from Rank Bros., Bottisham, Cambs., consisted of a water-jacketed, air-tight Perspex vessel with a reaction volume of 6·5ml. The contents, at 20°, were stirred by means of a rotating magnet, and concentrations of $O_2$ were measured by a Clark oxygen electrode fitted with a microammeter (range 0–5 $\mu$A) and recorder. The instrument was calibrated by measuring the electrode current, first for a known concentration of $O_2$, and then after adding dithionite to exhaust $O_2$, when a small residual reading was given. A linear relationship obtained between concentrations and currents. The solubility of $O_2$ in water exposed to water-saturated air was taken as 9·17p.p.m. at 20° (Wasser, 1962), equivalent to 0·287mM-oxygen. Certain experiments were performed with a similar appratus at the 'Shell' Research Laboratories, Sittingbourne, Kent, where the vessel had a reaction volume of 5ml.

*Disk electrophoresis.* After purification, the enzyme was examined by electrophoresis in polyacrylamide gels with the apparatus described by Davis (1964). The buffers and gels were those of Williams & Reisfeld (1964). The sample of protein was applied in a large-pore gel at pH 5·5, the small-pore gel provided a running pH of 7·5, and tris–barbitone buffer, pH 7·0, was used in both reservoirs.

Electrophoresis was performed at room temperature (about 20°) with a constant current of 4 mA/tube at 100–300 v. The lower reservoir was positive, and the running time was about 40 min. Gels were removed from their tubes, protein was stained by immersion for 1 hr. in 1% (w/v) Amido Black in 7% (v/v) acetic acid, and excess of dye was then removed by immersion for 48 hr. in 7% (v/v) acetic acid with several changes of solvent. The materials used in disk electrophoresis were from Kodak Ltd. (Kirkby, Liverpool).

*Chromatography and paper electrophoresis.* Paper chromatography of DNP-hydrazones was conducted as described by Dagley & Gibson (1965). Their solvent systems *A* and *C* are so designated in this work; and we also used that of Shannon & Marcus (1962), referred to here as solvent *B*: butan-1-ol–ethanol–aq. 0·5 N-$NH_3$ soln. (7:1:2, by vol.). High-voltage paper electrophoresis (Dagley & Gibson, 1965) was used to identify formic acid.

*Materials.* Pyruvic aldol, prepared from triple-distilled pyruvic acid (De Jong, 1901), was a generous gift from Dr A. Marcus. It was used to prepare solutions of γ-carboxy-γ-hydroxy-α-oxovalerate by mild hydrolysis with N-KOH (Shannon & Marcus, 1962). Lactate dehydrogenase (L-lactate–NAD oxidoreductase, EC 1.1.1.27) was used for the spectrophotometric determination of pyruvate and was obtained from British Drug Houses Ltd., Poole, Dorset.

*Infrared-absorption spectra.* Samples (3 mg.) of DNP-hydrazones were mulled in Nujol and their spectra were recorded by a Unicam SP. 200 spectrometer.

## RESULTS

### *Properties of protocatechuate 4,5-oxygenase*

*Molecular weight.* A schlieren diagram of the sedimenting enzyme is shown in Fig. 1. The sedimentation coefficient (*S*) and diffusion coefficient (*D*) for a solution of 8·4 mg. of protein/ml. in phosphate buffer at 20° were $5{\cdot}22 \times 10^{-13}$ sec. and $3{\cdot}39 \times 10^{-7}$ cm.$^2$ sec.$^{-1}$ respectively. The molecular weight was calculated to be approx. 140 000, assuming a partial specific volume of 0·73.

*Homogeneity.* Although the preparation, purified as described, appeared to be homogeneous in the ultracentrifuge, disk electrophoresis revealed five bands (Fig. 2*a*), and further purification was attempted. The enzyme solution was brought to 33% saturation with ammonium sulphate, the precipitate collected on the centrifuge and the supernatant solution brought to 50% saturation to give a second precipitate. A third fraction was precipitated at 65% saturation. All three precipitates were enzymically active when dissolved

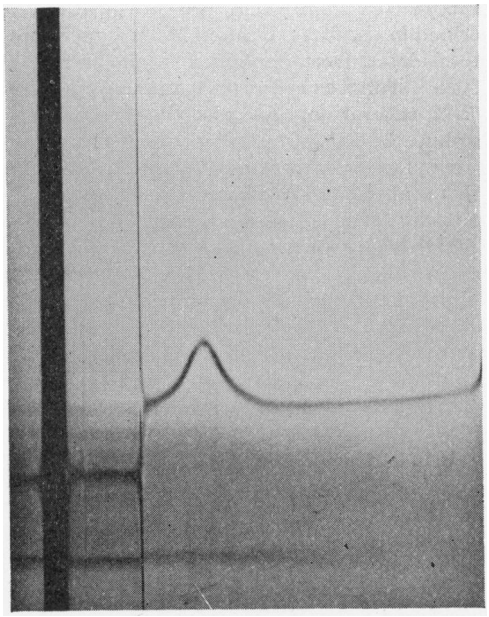


Fig. 1. Ultracentrifuge pattern for protocatechuate 4,5-oxygenase. The concentration was 8·4 mg. of protein/ml. in 24 mM-potassium phosphate buffer, pH 7·3. The photograph was taken 24 min. after 59 780 rev./min. had been reached at 20°. Sedimentation was from left to right.

Table 1. *Summary of purification of protocatechuate 4,5-oxygenase*

One unit of enzyme is the amount that catalyses the oxidation of 1 μmole of protocatechuate/min. at 30° and pH 7·3.

| Step | Method | Total protein (mg.) | Enzyme (total units) | Sp. activity (units/mg. of protein) | Yield (%) | Purification |
|---|---|---|---|---|---|---|
| 1 | Crude extract | 1938 | 1070 | 0·55 | 100 | 1·0 |
| 2 | Supernatant from ultracentrifuge | 1360 | 1070 | 0·79 | 100 | 1·4 |
| 3 | 0–60%-satd. ammonium sulphate precipitate, dissolved and dialysed | 816 | 775 | 0·95 | 72 | 1·7 |
| 4 | Adsorbed on DEAE-cellulose, eluted with ammonium sulphate, concentrated and dialysed | 96 | 402 | 4·2 | 39 | 7·6 |
| 5 | Pooled selected fractions from Sephadex G-200, precipitated with ammonium sulphate | 34 | 386 | 11·5 | 36 | 21 |

in phosphate buffer, but when they were submitted to electrophoresis it was found that the strongly staining lowest band (nearest the anode) was absent from the fraction collected at 33% saturation. This band therefore appeared to be due to inactive protein, and since its concentration increased with that of ammonium sulphate used in fractionation (Fig. 2*b*) the purification procedure was modified accordingly. The initial precipitation at 60% saturation was replaced by 50% saturation with ammonium sulphate, and after the preparation had been taken through the purification steps described in the Materials and Methods section, the protein eluted from Sephadex in the second peak was precipitated as before with ammonium sulphate at 70% saturation, but was then redissolved in phosphate buffer (10mg. of protein/ml.). Finally, the enzyme was precipitated with ammonium sulphate at 45% saturation. A solution of this precipitate (6mg. of protein/ml.), when diluted to give 100–200 $\mu$g. of protein in the sample gel, gave the electrophoretic pattern of Fig. 2(*c*). In contrast with previous purified preparations, which were yellow, this solution was colourless, and the lowest dense band of Fig. 2(*a*) had been eliminated. However, despite this fact, only a small increase in specific activity over that achieved by the procedure of Table 1 was found, indicating a 23-fold as against a 21-fold purification. Attempts to crystallize the enzyme were not successful. Nozaki, Kagamiyama & Hayaishi (1963) crystallized catechol 2,3-oxygenase from preparations purified 25–29-fold over crude extracts.

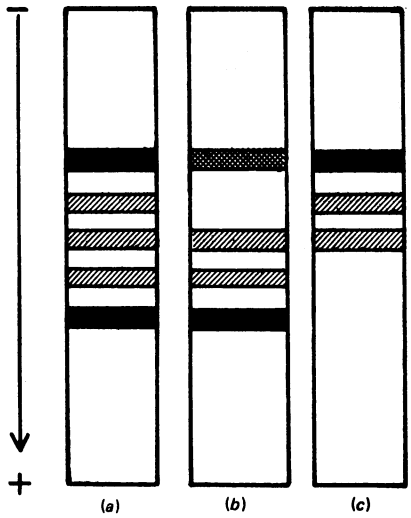



Fig. 2. Polyacrylamide-gel disk electrophoresis: (*a*) preparation of protocatechuate 4,5-oxygenase, which gave the schlieren diagram of Fig. 1; (*b*) solution of the precipitate obtained at 50–65% saturation with ammonium sulphate; (*c*) enzyme prepared by the modified procedure described in the text. The enzyme appears in the top band. Solutions (*a*) and (*b*) were yellow, solution (*c*) was colourless. The intensity of staining (in decreasing order) is indicated by ■, ▨, ▩.

*Stability of the enzyme.* Activity was rapidly lost when the enzyme was diluted, even in relatively crude preparations. Thus 0·3ml. of dialysed extract (3·7mg. of protein/ml.) catalysed the rapid consumption of 112 $\mu$l. of oxygen required for the ring-fission of 5 $\mu$moles of protocatechuate; but the reaction was barely detectable when only 0·1ml. of the same extract was used (Fig. 3). This low activity was raised in the presence of cysteine, but the enzyme ceased to function before the theoretical amount of oxygen was consumed. It appeared that at high dilution a molecule of the oxygenase could catalyse only a few ring-fissions before it became inactive, a process that was delayed by cysteine with the result that oxygen uptake increased. Inactivation during catalysis by a purified enzyme was shown by means of the oxygen analyser, which contained the following mixture: phosphate buffer, 4·8ml.; 15mM-L-cysteine, 0·1ml.; 33mM-ferrous sulphate, 0·05ml.; 0·6mg. of enzyme protein. After addition of 0·5 $\mu$mole of protocatechuate, oxygen uptake was measured until the reaction ceased, when 0·5 $\mu$mole of protocatechuate was again added; this was repeated for a third addition. Theoretical amounts of oxygen were consumed for the first two additions and the third exhausted the oxygen in the cell. The initial rates were respectively 0·380, 0·342 and 0·273 $\mu$mole of oxygen/min. The experiment was then repeated with the same reaction mixture except that the amount of enzyme protein was lowered to 0·12mg. The consumption of oxygen was theoretical for the first addition of protocatechuate but the enzyme was almost inactive by the time the third was made. The initial rates were respectively 0·091, 0·058 and 0·012 $\mu$mole of oxygen/min. When cysteine was omitted the enzyme lost activity more rapidly, but addition of cysteine did not restore activity once this had been lost during catalysis.

When purified enzyme was collected as a precipitate from ammonium sulphate it could be stored at 2–5°, or as an undialysed solution at −22°, without loss of activity for up to 6 weeks. Tubes were sealed with Parafilm but the air was not displaced by nitrogen. The solution also retained 80% of its activity when incubated for 24hr. at 20°, but it was far less stable when dialysed. Although a dialysed, purified enzyme lost no activity during 20hr. at 0°, it was completely inactive after 5hr. at 20° with or without additions of cysteine, ammonium sulphate or acetone. The last-named compound protects catechol 2,3-oxygen-

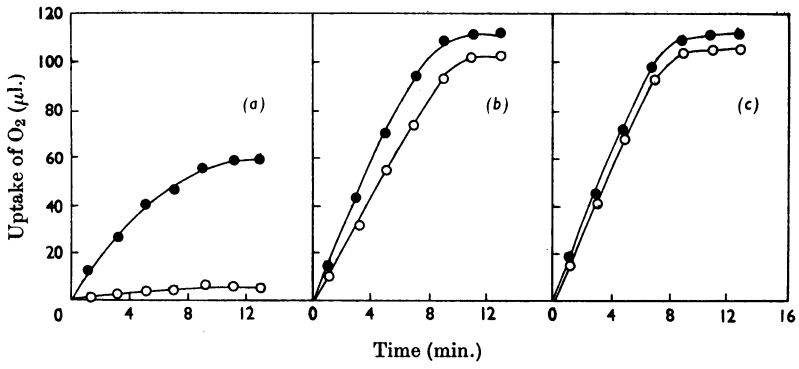



Fig. 3. Effects of dilution and addition of L-cysteine on the protocatechuate 4,5-oxygenase activities of cell extracts. The main compartment of each Warburg flask contained, in 2·8ml., 17m-moles of potassium phosphate buffer, pH 7·3, 3·3μmoles of $FeSO_4,7H_2O$ and (*a*) 0·1ml., (*b*) 0·2ml., (*c*) 0·3ml. of dialysed cell extract (3·7mg. of protein/ml.). The centre well contained a solution of KOH. After equilibration at 30°, 5μmoles of protocatechuate were added from the side arm. ○, No L-cysteine; ●, 3μmoles of L-cysteine hydrochloride.

ase against inactivation (Nozaki *et al.* 1963); this enzyme is also much more sensitive to air than our preparations of protocatechuate 4,5-oxygenase.

*Kinetic measurements.* Purified enzyme was prepared by the procedure modified as described to remove the inactive yellow component, and the relationships between initial reaction velocities and concentrations of enzyme, protocatechuate and oxygen were investigated.

In the first series the oxygen analyser contained: air-saturated phosphate buffer, 4·8ml.; 15mM-L-cysteine, 0·1ml.; 33mM-ferrous sulphate, 0·05ml.; amounts of enzyme in the range 0·06–0·54mg. of protein. Reactions were started by addition of 1μmole of protocatechuate. A plot of initial reaction rate against amount of enzyme was linear with a slope of 2·08μmoles of oxygen/min./mg. of protein. For a mol.wt. of 140000 this gives 294μmoles of oxygen/min./μmole of enzyme at 20° and at concentrations of protocatechuate and oxygen of 0·2μmole/ml. and 0·287μmole/ml. respectively.

Secondly, the same reaction mixture was used except that the amount of enzyme was constant at 0·18mg. of protein and the amounts of protocatechuate added were in the range 0·1–2·0μmoles. Initial reaction velocities varied correspondingly from 3·4 to 10·0μmoles of oxygen/min. A plot of reciprocals of velocities and substrate concentrations gave a straight line from which a $K_m$ value of 0·046mM-protocatechuate was obtained, with oxygen at normal physiological concentrations, namely 0·287mM for a solution saturated with air at 20°.

The dependence of reaction velocity on oxygen concentration with a saturating concentration of protocatechuate was investigated by preparing three samples of the same phosphate buffer. The first was saturated with air, the second was thoroughly gassed with methane, which removed air from the buffer but did not otherwise affect the enzymic reaction, and the third was saturated with oxygen. From the absorption coefficient of 0·028 (Glasstone, 1946), it can be calculated that the concentration of oxygen in the last-named buffer at 20° was 1·25mM. After oxygenation, each container was sealed with a screw cap having a rubber insert for removal of buffer by means of a syringe. A solution of the required oxygen concentration was prepared immediately before use by mixing two of the three samples of buffer in the vessel of the oxygen analyser. In these experiments ferrous sulphate was not added, to avoid non-enzymic removal of oxygen: the enzyme contained sufficient bound iron to provide a satisfactory rate of reaction. The concentration of L-cysteine throughout was 0·4mM, and that of protocatechuate was 1·36mM, providing a saturating concentration of this substrate for the enzyme. No inhibition of the enzyme was observed with 1·36mM-protocatechuate. Each reaction was started by addition of 0·3mg. of enzyme protein. Reciprocals of concentrations and initial velocities (Table 2) were plotted to give a straight line from which a $K_m$ value of 0·303mM-oxygen was obtained, with protocatechuate at a saturating concentration.

*Specificity.* The purified enzyme was specific for protocatechuate and did not attack catechol, 4-methylcatechol, 2,3-dihydroxybenzoate or vanillic acid, in which the hydroxyl group at C-3 of protocatechuate bears a methyl substituent. Stimulation of activity was observed only with $Fe^{2+}$ ions; $Mg^{2+}$

Table 2. *Effect of initial concentration of oxygen on rate of oxidation of protocatechuate*

Rates of oxidation catalysed by a purified preparation of protocatechuate 4,5-oxygenase were measured by the oxygen electrode at 20° with protocatechuate at a saturating concentration of 1·36mM.

| Concn. of $O_2$ (mM) | 1·023 | 0·868 | 0·529 | 0·369 | 0·287 | 0·08 | 0·04 | 0·01 |
|---|---|---|---|---|---|---|---|---|
| Initial velocity ($\mu$mole of $O_2$/min.) | 0·970 | 0·946 | 0·850 | 0·656 | 0·476 | 0·211 | 0·125 | 0·033 |

ions were not effective, and $Co^{2+}$, $Mn^{2+}$ and $Ni^{2+}$ ions were inhibitory.

*Metabolism of the ring-fission product of protocatechuate 4,5-oxygenase.* When protocatechuate was oxidized by fresh Sephadex-treated extracts (see the Materials and Methods section) carbonyl compounds were detected only in trace amounts, although a transitory appearance of yellow colour due to γ-carboxy-*cis*-*cis*-muconic semialdehyde was observed during the course of the reaction. Such extracts catalysed the disappearance of pyruvate without consumption of oxygen, possibly by fission to formate and acetate (Strecker, 1955), although this activity was lost when extracts were frozen and thawed. However, when reactions were run with 0·7mM-semicarbazide or 1·3mM-sodium bisulphite present, compounds accumulated that formed DNP-hydrazones; these were extracted with ethyl acetate and identified by paper chromatography in solvents *A*, *B* and *C* as the derivatives of pyruvic acid and the γ-lactone of γ-carboxy-γ-hydroxy-α-oxovaleric acid. The lactone ('pyruvic aldol') is readily identified since spots of its DNP-hydrazone turn scarlet when sprayed with ethanolic sodium hydroxide, in contrast with the brown or yellow colours given by several other derivatives. Spots were also given that ran as the DNP-hydrazones of either oxaloacetic acid or γ-carboxy-γ-hydroxy-α-oxovaleric acid, but it was not possible to decide between these two compounds solely by means of paper chromatography. From these observations it was tentatively concluded that, after ring-fission, protocatechuate gave γ-carboxy-γ-hydroxy-α-oxovalerate by reactions similar to those which follow the *meta* fission of catechol. Pyruvic aldol is readily formed non-enzymically from γ-carboxy-γ-hydroxy-α-oxovalerate under the acidic conditions used to remove protein from extracts. Dagley & Gibson (1965) showed that catechol is oxidized to γ-hydroxymuconic semialdehyde, which undergoes enzymic hydrolytic fission to formate and γ-hydroxy-α-oxovalerate, a compound that also readily lactonizes in acid.

*Isolation of pyruvic aldol.* Sephadex-treated extract was dialysed against running tap water for 2hr., when the activity of γ-carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase was found to be negligible. The fact that this preparation oxidized protocatechuate rather slowly without addition of $Fe^{2+}$ ions proved advantageous for preparative purposes, for when oxygenase activity was high, the ring-fission product tended to accumulate and inhibit its own breakdown; further, $Fe^{2+}$ ions were found to catalyse a non-enzymic disappearance of γ-carboxy-γ-hydroxy-α-oxovalerate. The extract (50ml.; 1g. of protein) was stirred magnetically at 20° during the dropwise addition of 20mM-protocatechuate. Yellow ring-fission product appeared and disappeared during the reaction, which was continued until 0·45m-mole of protocatechuate had been used and no further yellow colour was seen. At this point, when protocatechuate 4,5-oxygenase had evidently lost its activity, 10ml. of 30% (w/v) perchloric acid was added, the precipitated protein was removed by centrifuging and the supernatant solution was heated at 100° for 5min. to complete lactonization of γ-carboxy-γ-hydroxy-α-oxovalerate. The ethereal solution obtained by extracting five times with 100ml. portions of ether was evaporated under reduced pressure to leave a pale-yellow deposit, which was exposed to a stream of air for 1·5hr. to facilitate the removal of contaminating pyruvic acid and formic acid. When the solid was taken up in 5ml. of dry ether, a small residue that did not dissolve gave the ultraviolet-absorption spectrum of 2,4-lutidinic acid: this was probably formed by the non-enzymic reaction of ring-fission product with traces of ammonia. The ethereal solution was again evaporated, and the cream-coloured residue was dried over phosphorus pentoxide. Although bands in the infrared-absorption spectrum of this material coincided with those of pyruvic aldol, there was also a maximum in ultraviolet absorption at 290mμ not given by pyruvic aldol and probably due to unchanged protocatechuate. Accordingly, the isolated material was dissolved in 2N-hydrochloric acid containing 0·1% (w/v) DNP-hydrazine and allowed to stand for 3 days at 30°, when the yellow DNP-hydrazone was collected, washed with ice-cold water and crystallized from chloroform. When examined in solvents *A*, *B* and *C* this compound showed the same chromatographic behaviour as the derivative of authentic pyruvic aldol, and had the same m.p. (195° decomp.) and infrared-absorption spectrum (Found: C, 42·9; H, 3·1; N, 16·5; $C_6H_{10}O_8N_4$ requires C, 42·6; H, 2·9; N, 16·6%).

*γ-Carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase.* This enzyme converts 1 mole of substrate into 2 moles of pyruvate, the appearance of which can be followed by the decrease in extinction at 340mμ as NADH is oxidized by added lactate dehydrogenase (EC 1.1.1.28). Under the conditions chosen the rate of oxidation of NADH was proportional to the pyruvate concentration of the cell extract. Further, the Sephadex-treated extracts used in this section of the work had been frozen and thawed, and did not attack pyruvate. An absolute requirement for a thiol compound could not be demonstrated, as Shannon & Marcus (1962) found for the enzyme from germinating peanuts, but since there was a three-fold increase in rate on addition of 1·8mM-mercaptoethanol, this was included in reaction mixtures.

A requirement was shown (Fig. 4) for $Mg^{2+}$, which could be replaced less effectively by $Mn^{2+}$; $Zn^{2+}$ ions had little effect, whereas $Fe^{2+}$ and $Co^{2+}$ were inhibitory to the enzyme but catalysed non-enzymic disappearance of substrate.

The lyase catalysed neither the breakdown of γ-hydroxy-α-oxovalerate nor that of pyruvic aldol, which inhibited the reaction as its concentration was increased (Fig. 5). Maximum activity was shown at pH 9, but under these conditions the correction for non-enzymic breakdown is appreciable, and reactions were usually run at pH 8. The enzyme was induced by growth of the *Pseudomonas* with *p*-hydroxybenzoate, when cell extracts contained about four times the amount found in succinate-grown cells (Fig. 6).

When incubated at 30° with 2mg. of extract protein in 2·9ml. of 30mM-tris (pH 8) until the reaction ceased (15min.), low concentrations of substrate gave stoicheiometric yields of pyruvate: thus 0·048, 0·099 and 0·151μmole of pyruvate were formed from 0·025, 0·050 and 0·075μmole of γ-carboxy-γ-hydroxy-α-oxovalerate respectively. Since the substrate used was synthesized chemically it therefore appears that both of its enantiomers were attacked, as Shannon & Marcus (1962) also found; this contrasts with the observations made with other species of *Pseudomonas* grown with phenol, by which only one enantiomer of synthetic γ-hydroxy-α-oxovalerate (Dagley & Gibson, 1965) and one of γ-hydroxy-α-oxohexanoate (Bayly *et al.* 1966) were metabolized by cell extracts. When the concentration of the substrate was increased to 0·1mM the reaction did not reach completion in

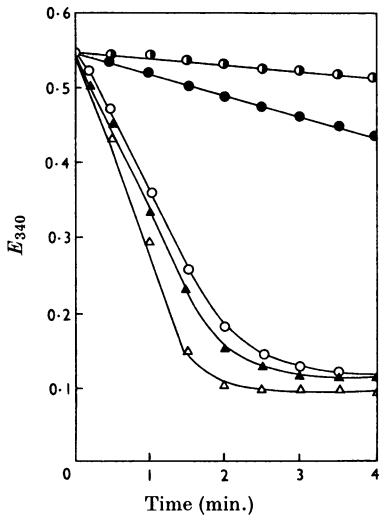



Fig. 4. Requirement of γ-carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase for $Mg^{2+}$ ions. Each cuvette contained, in 2·8ml., 0·24m-mole of tris–HCl buffer, pH 8·0, 4μmoles of γ-carboxy-γ-hydroxy-α-oxovalerate, 5μmoles of mercaptoethanol, 0·3mg. of NADH, lactate dehydrogenase (1mg. of protein) and the following additions of $MgCl_2$: △, 1·5μmoles; ▲, 1μmole; ○, 0·5μmole; ●, none. Reactions were started by adding Sephadex-treated extract (2mg. of protein). ◑, Substrate and $MgCl_2$ omitted.

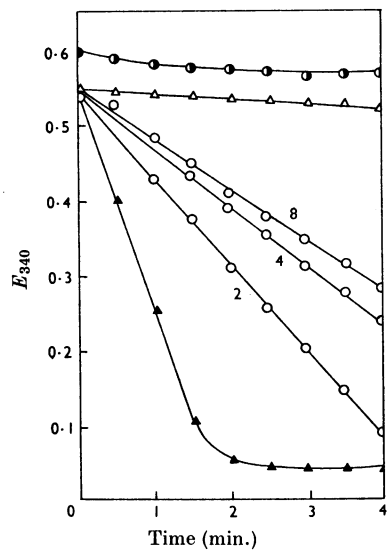



Fig. 5. Specificity of γ-carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase and its inhibition by pyruvic aldol. Cuvettes contained 1·5μmoles of $MgCl_2$ and the same amounts of tris–HCl buffer, pH 8, mercaptoethanol, NADH, lactate dehydrogenase and γ-carboxy-γ-hydroxy-α-oxovalerate (4μmoles) as in Fig. 4. Reactions were started by adding Sephadex-treated extract (2mg. of protein). ▲, No further additions; ○, additions of 2, 4 or 8μmoles of pyruvic aldol as indicated by the respective numbers; △, γ-carboxy-γ-hydroxy-α-oxovalerate replaced by 4μmoles of γ-hydroxy-α-oxovalerate; ◑, γ-carboxy-γ-hydroxy-α-oxovalerate omitted from the reaction mixture.

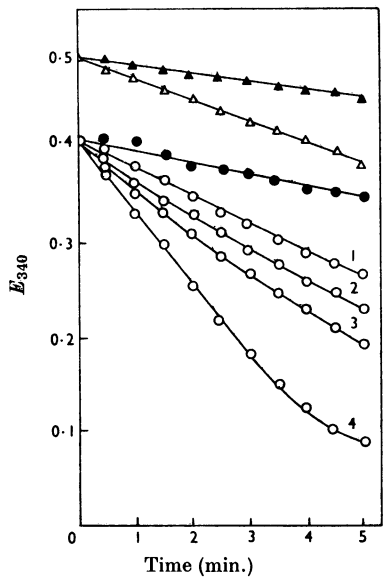



Fig. 6. Influence of the source of carbon for growth on γ - carboxy - γ - hydroxy - α - oxovalerate pyruvate - lyase activity. Sephadex-treated extracts were prepared from cells grown with either succinate or *p*-hydroxybenzoate. Cuvettes contained the same amounts of tris–HCl buffer, pH 8, mercaptoethanol, NADH, lactate dehydrogenase and $MgCl_2$ as in Fig. 5. Reactions were started by adding the appropriate extract (2mg. of protein). Succinate-grown cells: △, 4μmoles of γ-carboxy-γ-hydroxy-α-oxovalerate present; ▲, substrate omitted. *p*-Hydroxybenzoate-grown cells: ○, with additions of 1, 2, 3 and 4μmoles of γ-carboxy-γ-hydroxy-α-oxovalerate as indicated by the respective numbers; ●, substrate omitted.

20min. and proceeded more slowly as pyruvate accumulated. This may account for the fact that when Sephadex-treated extracts supplemented with 1·9mM-mercaptoethanol, 1mM-ferrous sulphate and 1mM-magnesium sulphate were incubated with protocatechuate, theoretical yields of pyruvate were approached only at low concentrations of the substrate. Thus 1μmole of protocatechuate in a reaction volume of 3ml. gave 1·7μmoles of pyruvate whereas the yield from 5μmoles of protocatechuate was 2·75μmoles of pyruvate. In both reactions 1μmole of oxygen was consumed for each 1μmole of protocatechuate oxygenated.

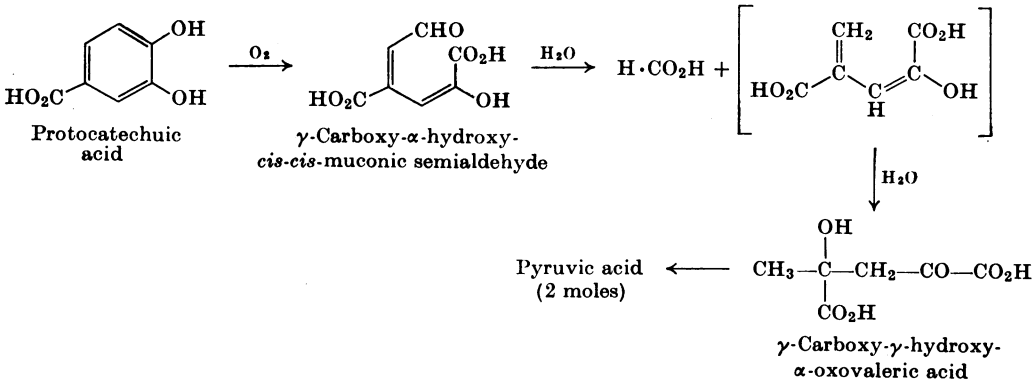
*Identification of formate.* Since protocatechuate ($C_7$) was converted into pyruvate ($C_3$) with no evolution of carbon dioxide, formate was indicated as the second product of the reaction. Accordingly, a Sephadex-treated extract was incubated for 40min. with 20μmoles of protocatechuate, and, after removal of protein by 5N-sulphuric acid, the reaction products were extracted continuously with ether for 24hr. The presence of formate in the ethereal extract was established, as described by Dagley & Gibson (1965), by means of high-voltage paper electrophoresis with [$^{14}$C]formate as a marker, and also from the pink colour, showing characteristic ultraviolet-light absorption, that was given with chromotropic acid when formate was reduced to formaldehyde with magnesium and acid. The yield of formate obtained from 5μmoles of protocatechuate by the action of a Sephadex-treated extract was determined manometrically by adding a suspension of a 'particulate fraction' prepared from disrupted *Pseudomonas* (Dagley & Gibson, 1965). This fraction oxidized formate but did not attack other possible metabolites. The amount of formate, 0·71μmole from 1μmole of protocatechuate, was less than that expected, as was also the case in the enzymic degradation of catechol (Dagley & Gibson, 1965).

## DISCUSSION

Dagley *et al.* (1964) proposed a general sequence of reactions for the bacterial metabolism of substituted catechols by *meta* fission, which, when applied to protocatechuate, gives the sequence of Scheme 1. In the present work we isolated γ-carboxy-γ-hydroxy-α-oxovalerate as its lactone (pyruvic aldol) and showed that when *Pseudomonas* was transferred from succinate as carbon source to *p*-hydroxybenzoate, the concentration of γ-carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase present in cell extracts increased fourfold. This enzyme converts 1 mole of its substrate into 2 moles of pyruvate, whereas γ-hydroxy-α-oxovalerate and γ-hydroxy-α-oxocaproate, which are formed from other catechols, give rise to 1 mole of pyruvate/mole of substrate (Dagley & Gibson, 1965; Dagley, Chapman & Gibson, 1965; Bayly *et al.* 1966). When protocatechuate was the substrate for Sephadex-treated extracts, yields greater than 1 mole of pyruvate were given, although a value of 2 moles of pyruvate/mole of protocatechuate, expected from Scheme 1, was approached only at low concentrations.

Although the present data accord with the general scheme of Dagley *et al.* (1964) for the metabolism of catechols by *meta* fission, at least two of the enzymes in Scheme 1 are specific for their particular substrates. Thus purified protocatechuate 4,5-oxygenase did not oxidize catechol, 4-methylcatechol or 2,3-dihydroxybenzoate, and γ-carboxy-γ-hydroxy-α-oxovalerate lyase did not degrade γ-hydroxy-α-oxovalerate.

The two enzymes for *meta* fission, catechol 2,3-oxygenase (Nozaki *et al.* 1963) and protocatechuate 4,5-oxygenase, both have a molecular weight of




Scheme 1.

about 140000. They both require $Fe^{2+}$ ions, which catechol 2,3-oxygenase binds much more tenaciously. Further, protocatechuate 4,5-oxygenase appears to be less sensitive to inactivation by oxygen, particularly in the presence of ammonium sulphate, but it is rendered unstable by dialysis. This procedure would also remove the small amount of bound iron, which might have a stabilizing effect; however, a dialysed preparation was not stabilized by subsequent additions of ferrous sulphate. The apparent instability arising from dilution may be due to other factors. The amount of protocatechuate oxidized, as well as the rate of the reaction, decreased with dilution and it appears that one molecule of enzyme can catalyse only a limited number of ring-fissions before it becomes inactive. Although this inactivation was delayed when cysteine was present, the amino acid did not restore activity when once this had been lost in the reaction. In this respect similar behaviour is shown by citrate lyase (EC 4.1.3.6), which becomes inactive during the reaction, so that the amount of citrate decomposed is proportional to the amount of enzyme (Dagley & Dawes, 1955; Bowen & Rogers, 1963; Tate & Datta, 1965). However, proportionality between extent of reaction and concentration of enzyme is maintained at high dilutions by citrate lyase, whereas the activity of protocatechuate 4,5-oxygenase decreased sharply when the amount of enzyme was reduced from 0·2ml. to 0·1ml. in the experiment shown in Fig. 3.

For the purified enzyme, our $K_m$ value for protocatechuate (0·046mM) is similar to that obtained by Cain (1962) for a cruder preparation (0·045mM). These values were determined at the oxygen concentration of a solution saturated with air, and this does not provide an excess of oxygen at higher concentrations of protocatechuate. When the concentration of protocatechuate was sufficient to saturate the enzyme, $K_m$ for oxygen was 0·303mM, whereas a saturated aqueous solution of air contains 0·287mM-oxygen. Evidently this enzyme operates at a concentration of oxygen far below the optimum when *Pseudomonas* is grown with forced aeration. Ogasawara, Gander & Henderson (1966) found a $K_m$ value of 0·31mM-oxygen for 3-hydroxyanthranilate oxygenase from ox kidney.

The reactions by which γ-carboxy-α-hydroxymuconic semialdehyde is converted into formate and γ-carboxy-γ-hydroxy-α-oxovalerate are formulated in Scheme 1 by analogy with those proposed for the metabolism of γ-hydroxymuconic semialdehyde by Bayly *et al.* (1966). Hegeman (1967) has reported an alternative pathway in *Rhodopseudomonas palustris* by which γ-carboxy-α-hydroxymuconic semialdehyde first undergoes a NADP-dependent oxidation, so that carbon dioxide is released instead of formate.

Extracts of *Pseudomonas* contained a γ-carboxy-γ-hydroxy-α-oxovalerate pyruvate-lyase with properties similar to those of an enzyme found in the cotyledons of germinating peanuts that catalyses the same reaction. The enzyme from peanuts was purified by Shannon & Marcus (1962); it showed an absolute requirement for $Mg^{2+}$ ions and a thiol compound, and was designated γ-hydroxy-γ-methyl-α-oxoglutarate aldolase. The enzyme from *Pseudomonas* was strongly stimulated by $Mg^{2+}$ ions and mercaptoethanol and, like the enzyme from peanuts, was inhibited by the lactone (pyruvic aldol) of its substrate.

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