Oxoenoic Acids as Metabolites in the Bacterial Degradation of Catechols

By R. C. BAYLY AND S. DAGLEY

Department of Microbiology, Monash University Medical School, Alfred Hospital, Prahran, Vic., Australia,
and Department of Biochemistry, University of Minnesota, St Paul, Minn. 55101, U.S.A.

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1. Partially purified extracts of a *Pseudomonas* converted the *meta* ring-fission product of 4-methylcatechol into a compound having spectroscopic and chemical properties consistent with its being 2-oxohex-4-enoic acid. 2. Catechol and 3-methylcatechol were both metabolized to a compound that appeared to be 2-oxopent-4-enoic acid. 3. Solutions of norvaline and norleucine were prepared from these metabolites. 4. A reaction scheme is presented for the conversion of catechols into hydroxyoxo acids after *meta* ring-fission.

Certain species of *Pseudomonas* are able to utilize phenol and cresols (Bayly, Dagley & Gibson, 1966), arylsulphonates (Cain & Farr, 1968) and other aromatic compounds as sources of carbon for growth by converting them into catechols. After *meta* fission of the benzene nucleus by a dioxygenase, at least two reactions occur that involve the participation of water: one reaction releases a carboxylic acid by hydrolytic fission, and in another water is added across an ethylenic bond to give a hydroxyoxo acid (Dagley & Gibson, 1965; Bayly *et al.* 1966). Thus the ring-fission products from catechol and 3-methylcatechol both give 4-hydroxy-2-oxovalerate (IV, Scheme 1) and that from 4-methylcatechol gives 4-hydroxy-2-oxohexanoate (VII); an aldolase then converts each hydroxyoxo acid into an aldehyde and pyruvate. In the present work we sought to identify reaction intermediates formed when ring-fission compounds (I), (II) and (V) are converted into hydroxyoxo acids, with the object of determining the time-sequence of the reactions in which the two molecules of water participate. Apart from the appearance of an unidentified oxo acid when 2-hydroxy-5-methylmuconic semialdehyde (V) was metabolized (Bayly *et al.* 1966), no reaction intermediates have hitherto been detected during these conversions.

Coulter & Talalay (1967, 1968) demonstrated that cell-free extracts of steroid-induced *Pseudomonas testosteroni* converted synthetic 2-oxo-*cis*-hex-4-enoic acid into 4-hydroxy-2-oxohexanoic acid (VII). From studies of the materials that accumulate from compounds (I), (II) and (V) by the action of partially purified extracts of a fluorescent *Pseudomonas* we conclude that 2-oxohex-4-enoate (VI) and 2-oxopent-4-enoate (III) are reaction intermediates when 4-methylcatechol and

either 3-methylcatechol or catechol are metabolized respectively.

MATERIALS AND METHODS

Partial purification of cell extracts. The organism used was *Pseudomonas* N.C.I.B. 10105 (*Pseudomonas* U). Dagley & Gibson (1965) have described its maintenance and growth with phenol, the carbon source used throughout this work, and also the preparation of crude cell-free extracts. These were partially purified as follows. To the crude cell extract at 2° was added 0.2 vol. of 2% (w/v) protamine sulphate with stirring. The precipitate was removed by centrifuging at 26000g_{av.} for 20 min. The supernatant solution, at 2°, was brought to 30% saturation by the addition, with stirring, of solid (NH₄)₂SO₄, and the precipitate was removed by centrifuging and discarded. The solution was brought to 40% saturation by further addition of (NH₄)₂SO₄, and the precipitate was collected on the centrifuge, dissolved in phosphate buffer (KH₂PO₄, 2g./l.), pH 7.4, and then dialysed against three changes of the same buffer for 4–5 hr. The extract was now applied to a column (40 cm. × 2.5 cm.) of Sephadex G-200 that had been equilibrated at 4° with phosphate buffer (KH₂PO₄, 2g./l.), pH 7.4; it was eluted with the same buffer at 4° and 3 ml. fractions were collected. The *E*₂₈₀ values of fractions were measured and their ability to metabolize the ring-fission product from 4-methylcatechol was also tested by observing the rate of decrease in *E*₃₈₂ when each fraction was added to a solution of the compound. Protein was eluted in fractions 15–45, but enzymic activity was present only in fractions 28–35. Fractions 30–34, which had similar activities, were pooled and are referred to as 'partially purified extract'. Activity was retained for at least 2 days at 0°.

Sephadex-treated extract. This was prepared by passing a crude cell extract, (NH₄)₂SO₄ fraction, through Sephadex G-25 (Dagley & Gibson, 1965). It catalysed the rapid conversions of ring-fission compounds into their hydroxyoxo acids, which have negligible light-absorption at the wavelengths used.

Preparation of solutions of the products of ring-fission of catechols. To 40 μ moles of the catechol in 20 ml. of phosphate buffer (KH_2PO_4 , 20 g./l.), pH 7.4, was added 2.5 ml. of a heat-treated extract of this *Pseudomonas* (Dagley & Gibson, 1965). The mixture was incubated in air in a 100 ml. conical flask with shaking at 30° for 15 min., and then 2.5 ml. of 10% (w/v) HClO_4 was added and the precipitate removed by centrifuging. The solution was extracted with ether (3 $\times$ 10 ml.). The ether layer was removed and shaken with phosphate buffer (KH_2PO_4 , 20 g./l.), pH 7.4 (3 $\times$ 3 ml.), and ether retained in the buffer was then removed by bubbling with N_2 . The solution of ring-fission compound was made up to 10 ml. with the same buffer, the extinction of a suitably diluted sample was read at λ_{max} . for the particular compound and the concentration of the solution was calculated from the recorded values of ϵ (Bayly *et al.* 1966). Solutions were prepared on the day of use and were kept in ice. They were unstable and could not be stored, even when frozen.

Chromatography. Thin-layer chromatography of 2,4-dinitrophenylhydrazones of oxo acids and their lactones was carried out on Kieselgel G with solvents A and B of Bayly *et al.* (1966). Whenever a compound was identified by chromatography its R_F was referred to that of authentic material run on the same chromatogram. Amino acids were chromatographed on Whatman no. 1 paper in the following solvent systems: C, butan-1-ol-acetic acid-water (15:1:5, by vol.); D, 2-methylpropan-1-ol-formic acid-water (19:2:1, by vol.) (Hassall & Greenberg, 1963). Chromatograms were dried at 50°, sprayed with 2% (w/v) ninhydrin in acetone and heated at 60° for 10 min. The R_F values of norvaline were: in solvent C, 0.43; in solvent D, 0.50; and of norleucine: in solvent C, 0.60; in solvent D, 0.65. 4-Methyl-2-oxobutyrolactone and 4-ethyl-2-oxobutyrolactone were chromatographed on Kieselgel G and detected as before (Bayly *et al.* 1966).

Materials. The purification of catechols, the synthesis of 4-methyl-2-oxobutyrolactone and 4-ethyl-2-oxobutyrolactone and the preparation of solutions of 4-hydroxy-2-oxovalerate and 4-hydroxy-2-oxohexanoate were as described by Bayly *et al.* (1966). DL-Norvaline and DL-norleucine were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

RESULTS

Metabolism of ring-fission products by partially purified extracts. Solutions were prepared of each ring-fission product (I, II and V) that contained 0.05 μ mole of substrate/ml. of phosphate buffer, pH 7.4. To 3 ml. of each of the solutions of compounds (I) and (V) was added, at 23°, 1 ml. of partially purified extract (0.6 mg. of protein); the extract added to compound (II) was diluted tenfold since this compound is attacked much more rapidly than the other two ring-fission products. The decrease in extinction as each compound disappeared was then observed at its wavelength of maximum light-absorption (Bayly *et al.* 1966) and extinctions due to products were measured in the range 265–274 μ m until there were no further increases. To each mixture was then added 0.5 ml.

of 5N-hydrochloric acid, protein was removed by centrifuging and spectra were recorded in the range 220–320 μ m at pH 2.0, 7.4 and 11.0. The reaction products showed light-absorption maxima (Table 1) at different wavelengths from those of the substrates metabolized. The products formed from compounds (I) and (II) gave identical spectra; that for the product from compound (V) was different and showed peaks at the same wavelengths as those reported for 2-oxo-*cis*-hex-4-enoic acid by Coulter & Talalay (1967, 1968). Extinctions for the two products were similar, but reliable determinations were hindered by the fact that partially purified extracts still contained some of the later enzymes of the degradative sequence. The enzymic synthesis and degradation of these metabolites was demonstrated by measuring changes of extinction as described above, for reactions performed in duplicate. To the second reaction of each pair was added 'Sephadex-treated extract' (0.4 mg. of protein) when the concentration of the reaction product had reached a maximum.

Fig. 1 shows that partially purified extracts catalysed the disappearance of the ring-fission compound (V) from 4-methylcatechol with simultaneous accumulation of material with λ_{max} . 274 μ m (pH 7.4); this was rapidly removed when Sephadex-treated extract was added. Results of experiments with as substrates the ring-fission compounds (I and II) from catechol and 3-methylcatechol differed only in one detail, namely that the accumulating compound, with λ_{max} . 265 μ m (pH 7.4), was removed by addition of Sephadex-treated extract in each case about twice as fast as that from 4-methylcatechol.

Conversion of metabolites into lactones. Coulter & Talalay (1967) showed that 2-oxohex-4-enoic acid (VI) and 4-hydroxy-2-oxohexanoic acid (VII) were converted by dilute mineral acid into the same compound, 4-ethyl-2-oxobutyrolactone. The first reaction appears to be an isomerization and the second involves elimination of water. Each ring-fission compound (25 μ moles) was incubated at 30° with 20 ml. of phosphate buffer, pH 7.4, containing 20 mg. of extract protein until the concentration of reaction product reached a maximum. At this time 2.5 ml. of 5N-hydrochloric acid was added, the mixture was cooled to 2° and protein was removed by centrifuging at 2°. The solution was then heated at 100° for 15 min., cooled to 2° and divided into three portions.

The first portion was used to examine u.v. spectra, which were consistent in each case with lactone formation: the 4-methyl and 4-ethyl derivatives of 2-oxobutyrolactone both have λ_{max} . 228 μ m at pH 2, shifted to λ_{max} . 260 μ m at pH 11 (Bayly *et al.* 1966). The second portion of each of the three solutions was extracted overnight with

ether, the ethereal solution dried over anhydrous sodium sulphate and the ether removed under vacuum. The residue was dissolved in ethanol and

Table 1. *Spectroscopic properties of compounds formed by enzymic degradation of ring-fission products of catechols*

Catechol, 3-methylcatechol and 4-methylcatechol were converted by heat-treated extracts into ring-fission products, namely 2-hydroxymuconic semialdehyde (I), 2-hydroxy-6-oxohepta-2,4-dienoic acid (II) and 2-hydroxy-5-methylmuconic semialdehyde (V). Roman numerals refer to their positions in metabolic Scheme 1. These compounds were then incubated with a partially purified extract and the spectra of reaction products were recorded in the range 220–320 m μ .

Substrate metabolized... pH	$\lambda_{\max.}$ (m μ) of reaction product		
	(I)	(II)	(V)
2.0	270	270	281
7.4	265	265	274
11.0	305	305	307

Fig. 1. Accumulation of a metabolite from 2-hydroxy-5-methylmuconic semialdehyde and its degradation by a Sephadex-treated extract. A solution of 2-hydroxy-5-methylmuconic semialdehyde in 0.15M-phosphate buffer, pH 7.4, was prepared enzymically from 4-methylcatechol. To this solution in a cuvette containing 0.15 μ mole of substrate in a final volume of 3 ml. was added partially purified extract (0.6 mg. of protein) and extinctions were measured: O, disappearance of 2-hydroxy-5-methylmuconic semialdehyde (E_{382}); Δ , appearance of metabolite (E_{274}). Sephadex-treated extract, which metabolizes 2-hydroxy-5-methylmuconic semialdehyde to 4-hydroxy-2-oxohexanoate, was added as shown by the arrow: $\square$, disappearance of metabolite (E_{274}). In a duplicate experiment no addition was made at this point and E_{274} is shown by the broken line.

chromatographed on Kieselgel G in acetic acid-chloroform (1:10, v/v). When chromatograms were sprayed to detect lactones, spots with the R_F of 4-methyl-2-oxobutyrolactone were given by the degradation products from compounds (I) and (II), whereas the degradation product from compound (V) gave rise to 4-ethyl-2-oxobutyrolactone. The identities of the lactones were confirmed by incubating each third portion of the solutions for 10 min. at 30° with 0.1% 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid. The resulting 2,4-dinitrophenylhydrazones were extracted into ethyl acetate, the solution was shaken successively with 10% (w/v) sodium carbonate and water and was then dried over anhydrous sodium sulphate. The solution was evaporated to a small volume and chromatographed on Kieselgel G in solvents A and B. The 2,4-dinitrophenylhydrazones derived from compounds (I) and (II) were identified as the derivative of 4-methyl-2-oxobutyrolactone, and that from compound (V) was identified as the derivative of 4-ethyl-2-oxobutyrolactone.

Identification of oxoenoic acids by their conversion into amino acids. Ring-fission compounds (25 μ -moles of each) were again metabolized by the partially purified extract, protein was precipitated with 5N-hydrochloric acid and removed, and 2,4-dinitrophenylhydrazones, prepared by incubation with the reagent for 10 min. at 30°, were extracted into ethyl acetate and separated into two fractions. Acidic 2,4-dinitrophenylhydrazones were removed

Table 2. *Chromatography of acidic 2,4-dinitrophenylhydrazones of compounds formed by enzymic degradation of ring-fission products of catechols*

Solutions of ring-fission products (designated I, II and V as in Table 1) were prepared from catechol, 3-methylcatechol and 4-methylcatechol respectively by the action of heat-treated extracts. The solutions were incubated with partially purified extracts and the products of these reactions were converted into 2,4-dinitrophenylhydrazones and extracted into ethyl acetate, 10% (w/v) sodium carbonate and again into ethyl acetate. The derivatives were applied to Kieselgel G and their chromatographic behaviour in solvents A and B (see the text) was compared with that of derivatives of related hydroxyoxo acids and their lactones.

Solvent...	R_F	
	A	B
2,4-Dinitrophenylhydrazone of		
4-Methyl-2-oxobutyrolactone	0.26	0.36, 0.46
4-Ethyl-2-oxobutyrolactone	0.34	0.46, 0.63
4-Hydroxy-2-oxovalerate	0.06	0.13, 0.26
4-Hydroxy-2-oxohexanoate	0.08	0.16, 0.33
Metabolite from compound (I)	0.17	0.45
Metabolite from compound (II)	0.17	0.45
Metabolite from compound (V)	0.22	0.48, 0.52

from 4-methylcatechol. Catechol, like 3-methylcatechol, was metabolized to a reaction intermediate that furnished norvaline, in accordance with the observation that formate is released when 2-hydroxymuconic semialdehyde is degraded (Dagley & Gibson, 1965).

In principle, the conversion of ring-fission compounds (I) and (II) into 4-hydroxy-2-oxovalerate (IV), or of compound (V) into 4-hydroxy-2-oxohexanoate (VII), might take place by one of two types of reaction sequence. Hydrolytic fission of the carboxylic acid, formate or acetate, might occur first, as in Scheme 1; alternatively, water might first be added to an ethylenic bond, so that the hydrolytic fission would give rise to formate or acetate plus the hydroxyoxo acid (IV) or (VII). The present work establishes that the second sequence suggested does not occur. Thus, if water were added first, the precursor of compound (IV) would contain six carbon atoms and that of compound (VII) would contain seven; whereas their conversion into norvaline and norleucine showed that they contained five and six carbon atoms respectively. The chemical structures of these postulated intermediates (III and VI) are not conclusively proved, but the fact that they are oxoenoic acids is shown by their enzymic conversion into hydroxyoxo acids; moreover their 2,4-dinitrophenylhydrazones gave norvaline or norleucine on reduction, whereas the derivatives of compounds

(IV) and (VII) gave a different pair of amino acids with much smaller R_F values in solvents *C* and *D*. The assigned structure of compound (VI) is strengthened by the observation that the compound showed light-absorption maxima identical with those reported for synthetic 2-oxohex-4-enoic acid (Coulter & Talalay, 1967, 1968), and is affected in the same manner by changes in pH. Like the synthetic material, compound (VI) was converted into 4-ethyl-2-oxobutyrolactone when heated with dilute mineral acid; compound (III) was also lactonized by this treatment to give 4-methyl-2-oxobutyrolactone.

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REFERENCES

- Bayly, R. C., Dagley, S. & Gibson, D. T. (1966). *Biochem. J.* **101**, 293.
Cain, R. B. & Farr, D. R. (1968). *Biochem. J.* **106**, 859.
Coulter, A. W. & Talalay, P. (1967). *Biochem. biophys. Res. Commun.* **29**, 413.
Coulter, A. W. & Talalay, P. (1968). *J. biol. Chem.* **243**, 3238.
Dagley, S. & Gibson, D. T. (1965). *Biochem. J.* **95**, 466.
Hassall, H. & Greenberg, D. M. (1963). *J. biol. Chem.* **238**, 1423.