Co-metabolism of Methyl- and Chloro-Substituted Catechols by an *Achromobacter* sp. Possessing a New *meta*-Cleaving Oxygenase

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Co-metabolism of 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol by an *Achromobacter* sp. was shown to result in the accumulation of 2-hydroxy-3-methylmuconic semialdehyde, 4-chloro-2-hydroxymuconic semialdehyde and 3,5-dichloro-2-hydroxymuconic semialdehyde respectively. Formation of these products indicated that cleavage of the aromatic nucleus of the substituted catechols was accomplished by a new *meta*-cleaving enzyme, catechol 1,6-oxygenase. This enzyme was equally active on both chloro- and methyl-substituted catechols.

Dagley & Stopher (1959) described a dioxygenase that incorporated two atoms of oxygen across a double bond between one hydroxylated carbon atom and an adjacent unsubstituted carbon atom in catechol. The action of this catechol 2,3-oxygenase resulted in the formation of 2-hydroxymuconic semialdehyde. Bayly & Dagley (1969) studied the action of catechol 2,3-oxygenase from a *Pseudomonas* sp. on catechol, 3-methylcatechol and 4-methylcatechol. The oxidation products were identified as 2-hydroxymuconic semialdehyde, 2-hydroxy-6-oxohepta-2,4-dienoic acid and 2-hydroxy-5-methylmuconic semialdehyde respectively.

Although methyl-substitution on the aromatic nucleus of catechols does not appear to interfere with the action of catechol 2,3-oxygenase, reports on the oxidation of halogenated catechols indicate that *meta*-cleavage of the aromatic nucleus of these compounds does not occur readily.

This paper reports on the co-metabolism of 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol by a new *meta*-cleaving oxygenase of an *Achromobacter* sp. Co-metabolism describes the phenomenon by which a micro-organism oxidizes a compound without being able to utilize the carbon or energy derived from this oxidation to sustain growth. The products resulting from co-metabolism of these compounds indicate that fission of the aromatic nucleus is catalysed by a catechol 1,6-oxygenase, rather than the previously reported *meta*-cleaving enzyme, catechol 2,3-oxygenase. Part of this work has already been published (Horvath, Duxbury & Alexander, 1970).

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MATERIALS AND METHODS

Organism. The isolated organism used in this study was a Gram-negative, non-sporulating, colourless, rod-shaped organism that was motile via peritrichous flagella. It was identified as an *Achromobacter* sp. on the basis of its biochemical characteristics (Breed, Murray & Smith, 1957). The organism was maintained on a benzoate-salts agar as described by Horvath & Alexander (1970a). Co-metabolism studies used resting cells suspended in phosphate buffer, pH 7.2 (Gomori, 1955). The methyl- and chloro-catechols were used at concentrations of 0.1-1.0 mg/ml.

Qualitative and quantitative determinations. Oxygen uptake by resting cells of the *Achromobacter* sp. during the co-metabolism of 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol was determined by standard manometric techniques (Umbreit, Burris & Stauffer, 1964).

U.v. spectral analyses were performed with a Beckman DBG recording spectrophotometer. I.r. spectra were examined by using KBr macro-pellets in a Beckman IR 10 spectrophotometer. Mass spectra were obtained with a Perkin-Elmer model 270 mass spectrometer.

The oxidation products were determined by titration of the acids with mm-NaOH to a phenolphthalein endpoint.

The presence of a free aldehyde moiety was confirmed by the ability of the compound to reduce Tollen's reagent (English, 1961).

Isolation of oxidation products. The co-metabolized catechol supernatants (pH 7.2) were extracted three times with equal volumes of diethyl ether to remove the unoxidized catechols. The aqueous phase was acidified with m-HCl and extracted twice with ether. The ether phases were combined, dried over anhydrous Na₂SO₄ and evaporated to dryness. The residue, which consisted of the substituted muconic semialdehyde, was very unstable for both of the chlorinated products.

The semialdehyde was oxidized to a dicarboxylic acid by adding solid Ag₂O to the supernatant before extraction

with ether. Oxidation was allowed to proceed for 24 h at 25°C. The solution was filtered and the filtrate was extracted as described above.

Phenylhydrazone derivatives were prepared by the addition of 2 mM-phenylhydrazine hydrochloride to solutions of the semialdehyde in water acidified with M-HCl.

Picolinate derivatives of the 3,5-dichlorocatechol oxidation products were prepared by incubating the product in a solution of 0.1 M-NH₄Cl in 0.5 M-HCl at 30°C for 1 week. The derivative was isolated by continuous ether extraction of the above solution for 8 h.

Chemicals. Sources of chemicals were as follows. 3-Methylcatechol and 4-chlorocatechol, Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.); 3,5-dichlorocatechol, K & K Laboratories (Plainview, N.Y., U.S.A.); phenylhydrazine, Eastman Organic Chemicals (Rochester, N.Y., U.S.A.); anhydrous Na₂SO₄, Allied Chemical Corp. (Morristown, N.J., U.S.A.); anhydrous diethyl ether, Fisher Scientific Company (Fair Lawn, N.J., U.S.A.); Ag₂O, Mallinckrodt Chemical Works (New York, N.Y., U.S.A.).

RESULTS

An *Achromobacter* sp. capable of co-metabolizing methyl- and chloro-catechols, but not of utilizing

these compounds as sole carbon and energy sources for growth, was isolated by an enrichment-culture technique as described by Horvath & Alexander (1970a). This organism oxidized 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol at the same rate and with the consumption of 1 mol of oxygen/mol of substrate co-metabolized. The oxidation of these compounds occurred without an adaptive lag period when the cells used were previously grown in a benzoate-salts medium.

The product resulting from the oxidation of 3,5-dichlorocatechol exhibited u.v. spectra in acidic and basic solutions that indicated the occurrence of keto-enol tautomerism. The oxidation products of 3-methylcatechol and 4-chlorocatechol showed similar u.v. properties.

The i.r. spectra of these products, shown in Fig. 1, indicated that they were substituted 2-hydroxy-muconic semialdehydes. Oxidation of the aromatic nucleus of the substituted catechols therefore appeared to be catalysed by a *meta*-cleaving enzyme, resulting in the corresponding substituted muconic semialdehydes. The products reduced

Fig. 1. I.r. spectra of products resulting from the co-metabolism of (a) 3,5-dichlorocatechol, (b) 3-methylcatechol, and (c) 4-chlorocatechol.

Tollen's reagent, providing further evidence for a semialdehyde structure.

A mass spectrum of the 3-methylcatechol oxidation product had a parent-ion peak at m/e 156 and a fragmentation pattern that established its identity as 2-hydroxy-3-methylmuconic semialdehyde.

The 3,5-dichlorocatechol oxidation product was unstable in pure form and 4h after isolation no longer reduced Tollen's reagent. A mass spectrum of the semialdehyde could not be obtained because of this instability. However, a mass spectrum of the product after decomposition showed a parent-ion peak at m/e 226. The 3:2:1 ratio in intensity of the P, P+2 and P+4 peaks established the presence of two chlorine atoms in the product and also in the fragments represented at m/e 209, 192 and 164. These peaks also established the presence

of two carboxyl groups on the molecule. The fragments represented by peaks at m/e 122 and 116 allowed location of the double bond in the molecule and identified the position of the chlorine substituents. The mass spectrum obtained is shown in Fig. 2(a). The semialdehyde formed by co-metabolism of 3,5-dichlorocatechol was oxidized with silver oxide before isolation. The oxidized product no longer reduced Tollen's reagent and had a mass spectrum identical with that of the auto-oxidized product (Fig. 2b).

A phenylhydrazone derivative of the semialdehyde was prepared before isolation of the compound. The mass spectrum of this derivative showed a parent-ion peak at m/e 300. The fragmentation pattern obtained confirmed the identity of the product of 3,5-dichlorocatechol oxidation as 3,5-dichloro-2-hydroxymuconic semialdehyde.

Fig. 2. Mass spectra of the product resulting from co-metabolism of 3,5-dichlorocatechol: (a) after decomposition in air; (b) after oxidation with Ag_2O to the dicarboxylic acid. An ionizing voltage of 70 eV and an inlet temperature of 90°C were used.

Fig. 3. I.r. spectrum of a picolinate derivative of the product resulting from co-metabolism of 3,5-dichlorocatechol.

Fig. 4. Accumulation of 2-hydroxy-3-methylmuconic semialdehyde (○), 4-chloro-2-hydroxymuconic semialdehyde (△) and 3,5-dichloro-2-hydroxymuconic semialdehyde (□) resulting from the co-metabolism of 3-methylcatechol, 4-chlorocatechol and 3,5-dichlorocatechol respectively.

In addition, a picolinate derivative of this product was prepared. This derivative melted at 153°C and had the i.r. spectrum shown in Fig. 3. This spectrum further supported identification of the compound as 3,5-dichloropicolinic acid. The parent-ion peak at m/e 191 and the fragmentation

pattern in the mass spectrum established the identity of this compound as 3,5-dichloropicolinic acid.

The product produced by cleavage of 4-chlorocatechol reduced Tollen's reagent, exhibited u.v. properties similar to those of 3,5-dichloro-2-hydroxymuconic semialdehyde and had an i.r. spectrum identical with that of the latter compound. Although its mass spectrum could not be obtained, owing to its instability, it was tentatively identified as 4-chloro-2-hydroxymuconic semialdehyde from the similarity of its properties to those of 3,5-dichloro-2-hydroxymuconic semialdehyde.

The substituted catechols were converted into the corresponding substituted 2-hydroxymuconic semialdehydes in stoichiometric amounts (Fig. 4). The accumulation of these products in the culture supernatant indicated that further metabolism of the compounds did not occur.

DISCUSSION

Co-metabolism of the herbicide, 2,3,6-trichlorobenzoate, by a *Brevibacterium* sp. was shown to result in an accumulation of 3,5-dichlorocatechol in the medium (Horvath, 1970). Further oxidation of this compound by the *Brevibacterium* sp. did not occur. However, an *Achromobacter* sp. was isolated that was capable of oxidizing 3,5-dichlorocatechol.

The oxidation of 3,5-dichlorocatechol by this *Achromobacter* sp. resulted in a marked colour

change in the medium. The disappearance of colour on acidification and its reappearance in basic solution indicated a *meta*-cleavage of the benzene nucleus. The u.v. spectra of the product of co-metabolism were very similar to the spectra reported by Bayly & Dagley (1969) for 2-hydroxymuconic semialdehyde. In addition, the i.r. spectrum of this product (Fig. 1) was very similar to that reported by Kojima, Itada & Hayaishi (1961) for 2-hydroxymuconic semialdehyde and added further support for the muconic semialdehyde structure. The spectrum obtained in this work and that published by Kojima *et al.* (1961) both exhibited strong bands at 1710 and 1620 cm^{-1} indicating a semialdehyde structure. The C-H stretching bands at 2920 and 2840 cm^{-1} are characteristic of aldehydes and were present on both i.r. spectra. The strong OH absorption band seen at 1140 cm^{-1} in Fig. 1 was also prominent in the previously published spectrum. The remaining bands, with one exception, are also seen in the spectrum published by Kojima *et al.* (1961). The significant difference between the two spectra is a band at 700 cm^{-1} (Fig. 1) which is not present in the i.r. spectrum reported previously. This band indicated the presence of a C-Cl structure in the compound under study. The mass-spectral results obtained for this compound confirmed its identity as 3,5-dichloro-2-hydroxymuconic semialdehyde. The identification of this oxidation product indicated the action of a new *meta*-cleaving enzyme, catechol 1,6-oxygenase.

To confirm the catechol 1,6-oxygenase cleavage of the aromatic ring, the action of the *Achromobacter* sp. on 3-methylcatechol was studied. The i.r. spectrum of the product resulting from the co-metabolism of 3-methylcatechol (Fig. 1) was identical with that of 3,5-dichloro-2-hydroxymuconic semialdehyde except for the C-Cl band at 700 cm^{-1} (Colthup, Daly & Wiberley, 1964). The reduction of Tollen's reagent by this compound further suggested the presence of a free aldehyde structure. The mass spectrum of the oxidation product established its identity as 2-hydroxy-3-methylmuconic semialdehyde.

Bayly & Dagley (1969) showed that 3-methylcatechol, oxidized by catechol 2,3-oxygenase, gives 2-hydroxy-6-oxohepta-2,4-dienoic acid. The 2-hydroxy-3-methylmuconic semialdehyde isolated and identified in this work could only result from a 1,6-cleavage of the benzene ring. This 1,6-cleavage is shown as reaction 2 in Scheme 1 and is compared with the previously reported 2,3-cleavage, shown as reaction 1.

This cleavage mechanism was also shown to occur with 4-chlorocatechol, a product resulting from the co-metabolism of *m*-chlorobenzoate by an *Arthrobacter* sp. (Horvath & Alexander, 1970a). Co-metabolism of this catechol yielded a product with

	R ¹	R ²	R ³
3-Methylcatechol	CH ₃	H	H
4-Chlorocatechol	H	Cl	H
3,5-Dichlorocatechol	Cl	H	Cl

Scheme 1. Comparison of the products resulting from cleavage of the aromatic nucleus of substituted catechols by: (1) catechol 2,3-oxygenase; (2) catechol 1,6-oxygenase.

u.v. and i.r. properties similar to those of 3,5-dichloro-2-hydroxymuconic semialdehyde. Although a mass spectrum of this product could not be obtained, it was tentatively identified as 4-chloro-2-hydroxymuconic semialdehyde.

The cause of co-metabolism of the substituted catechols is not yet clear. The 1,6-cleavage of the benzene ring may result in a product for which the organism possesses no enzyme. Specificity of the enzyme that metabolizes 2-hydroxymuconic semialdehyde for an unsubstituted muconic semialdehyde would also cause the phenomenon of co-metabolism. Enzyme specificity has been shown to be the cause of co-metabolism of *m*-chlorobenzoate (Horvath & Alexander, 1970a) and of 2,3,6-trichlorobenzoate (Horvath, 1970). The possibility of a toxic environment resulting from the production of the substituted muconic semialdehydes must also be considered, although this seems less likely to be the cause of co-metabolism than either of the above explanations, owing to the high conversion of the catechols into the muconic semialdehydes.

The phenomenon of co-metabolism has been proposed as a technique for the accumulation of biochemical intermediates (Horvath & Alexander, 1970b). The demonstration of a new catechol 1,6-oxygenase by use of co-metabolism studies

indicates its possible importance as a technique for the study of microbial enzyme specificity and action.

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