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The Metabolic Divergence in the *meta* Cleavage of Catechols by *Pseudomonas putida* NCIB 10015

Physiological Significance and Evolutionary Implications

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1. A reinvestigation of the catabolic pathway(s) used by *Pseudomonas putida* NCIB 10015 (Dagley's strain) for the degradation of phenol and the cresols has proved the existence of a metabolic divergence after *meta* cleavage of the catechols formed by hydroxylation of the primary substrates. The ring-fission products of catechol and 4-methylcatechol are shown to be simultaneously catabolized by two different enzymic activities, an NAD^+-dependent dehydrogenase and a cofactor-independent hydrolase. The metabolizing activities of both ring-fission products in extracts of cells grown on phenol and the cresols (*o*-, *m*- and *p*-cresol) were found to be nonspecific; thermal inactivation of extracts of phenol-grown cells has shown that this nonspecificity is attributable to only one enzyme expressing each activity and that the two activities are located on separate proteins.

2. Extracts of cells grown on all four substrates contain high induced levels of the *meta* cleavage suite of enzymes functional in the dissimilation of catechol, including both the 4-oxalocrotonate branch (NAD^+-dependent 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonate tautomerase and 4-oxalocrotonate decarboxylase) and the hydrolytic branch (2-hydroxymuconic semialdehyde hydrolase).

3. The hydroxylase, oxygenase, dehydrogenase and hydrolase activities are shown to be nonspecific and can also act upon the methyl derivatives of their respective substrates. A constant pattern of specificity was found for these enzymes, independent of the monophenolic substrate used for growth.

4. From studies with a mutant lacking phenol hydroxylase, the entire suite of *meta* cleavage enzymes are shown to be coincidently induced from the top by the primary substrate (phenol or the cresols).

5. The evolutionary and physiological implications of the divergent pathway are discussed.

Two different pathways have been reported in *Pseudomonas* species for the dissimilation of 2-hydroxymuconic semialdehyde after *meta* cleavage of catechol. Nishizuka *et al.* [1] demonstrated an NAD^+-dependent dehydrogenation of 2-hydroxymuconic semialdehyde to 4-oxalocrotonate (2-oxohex-4-ene-1,6-dioate), whereas Dagley and Gibson [2], and Bayly and Dagley [3] showed that with their strain a hydrolyic fission of the ring-cleavage product occurs, yielding formate and 2-oxopent-4-enoic acid. The catabolic scheme proposed by Dagley and his co-workers [2,3] has subsequently been widely accepted as the general pathway of catechol *meta* cleavage in bacteria [4–7].

We have recently shown that both hydrolase and dehydrogenase activities coexist in crude extracts of benzoate-grown *Azotobacter* species [8,9] and of a naphthalene-grown pseudomonad [10] for the degradation of 2-hydroxymuconic semialdehyde. The enzymic reactions shown in the scheme of Fig. 1 have been demonstrated to operate in these organisms for the oxidation of catechol *via meta* cleavage [9; unpublished results of Catterall, Sala-Trepat and Williams]. The branch involving the hydrolase is identical to the pathway proposed by Dagley and his collaborators [2,3], and that involving the 2-hydroxymuconic semialdehyde dehydrogenase (the 4-oxalocrotonate branch) bears similarities to that

Abbreviation. NADase, NAD glycohydrolase.

Enzymes. Catechol 1,2-oxygenase or catechol: oxygen 1,2-oxidoreductase (EC 1.13.1.1); catechol 2,3-oxygenase or catechol: oxygen 2,3-oxidoreductase (EC 1.13.1.2); NAD nucleosidase or NAD glycohydrolase (EC 3.2.2.6); acetate kinase or ATP: acetate phosphotransferase (EC 2.7.2.1).

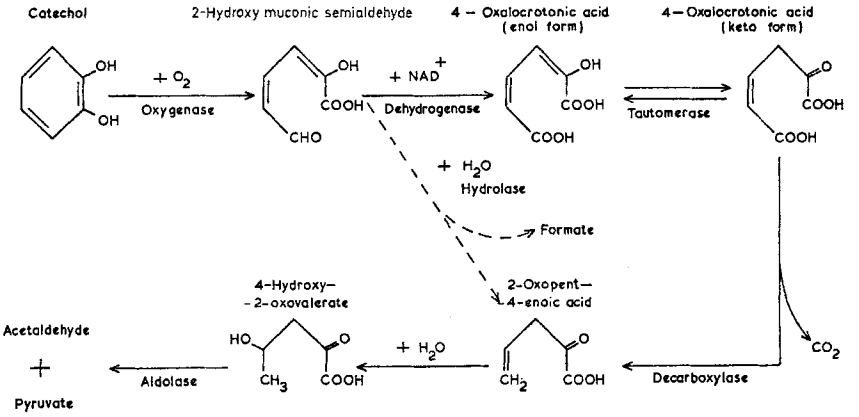

Fig. 1. *The* meta *cleavage pathway for the oxidation of catechol by* Azotobacter *species and a naphthalene-grown pseudomonad*

proposed by Nishizuka *et al.* [1]. In *Azotobacter* species the 4-oxalocrotonate branch was, however, found to be the only one of physiological significance for the metabolism of catechol [9]. A comparison of the levels of activity and induction of the hydrolase and dehydrogenase activities in the naphthalene-grown pseudomonad suggested that the 4-oxalocrotonate branch could also be the more important of the two for the dissimilation of 2-hydroxymuconic semialdehyde in this strain [10].

In view of these results it was considered of interest to reinvestigate the *meta* cleavage pathway of catechol in the original organism, a *Pseudomonas putida* strain, with which Dagley and Gibson [2], and Bayly and Dagley [3] had elucidated their metabolic map. We present here results which establish the coexistence of the two catabolic branches (Fig. 1) for the metabolism of the *meta* cleavage product of catechol in this strain, but indicate that the divergent metabolic steps fulfill different physiological functions as they appear to be involved in the degradation of different catechols. The apparent redundancy of these two metabolic branches under certain growth conditions is explained in terms of the coincident inductive control and the evolutionary origin of the pathway. Preliminary reports of part of these results have been made [12, 13].

MATERIAL AND METHODS

Organism and Growth Conditions

Most of the experiments described in this paper were carried out with the original *Pseudomonas* U strain of Dagley and associates [2, 3, 14], obtained from the National Collection of Industrial Bacteria (Aberdeen, Scotland) where it is kept under the designation NCIB 10015. It must be said that this organism has been misquoted in several publications [3, 5, 12, 13] as NCIB 10105. This strain classified as *Pseudomonas putida* (biotype A) by Stanier *et al.* [15] has also been used by Feist and Hegeman [5] in their work on the regulation of tangential pathways.

A mutant (5P) derived from this strain, which lacks phenol hydroxylase activity was kindly provided by Dr G. D. Hegeman (Department of Bacteriology and Immunology, University of California, Berkeley, California).

Cells were grown in a synthetic mineral medium containing 1.0 g/l $(NH_4)_2SO_4$, 5.0 g/l KH_2PO_4, 0.1 g/l $MgSO_4 \cdot 7H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 0.5 g/l nitrilotriacetic acid, and 1 ml/l of the stock salt solution of Bauchop and Elsden [16]. The pH was adjusted to pH 7.0 with 5 N NaOH. The organic carbon and energy sources were added to the mineral basal medium at the following concentrations: phenol and cresols, 4 mM; benzoate 5 mM and succinate 11 mM. Large scale cultures for preparation of enzyme extracts were incubated at 30 °C under forced aeration in 10-l Pyrex bottles. Growth was estimated turbidimetrically with a Unicam SP 800 spectrophotometer and bacterial cell mass was determined from a standard curve relating absorbance readings at 610 nm to dry weight.

Oxygen-Uptake Experiments with Whole Cells

Exponentially growing cells were harvested by centrifugation at 15000 × *g* for 10 min at 4 °C, washed twice in 100 mM phosphate buffer, pH 7.5, and resuspended in the same buffer. The suspensions were adjusted to give an absorbance reading of approximately 0.50 at 610 nm in the Unicam SP spectrophotometer. The respiratory capability of these cell suspensions at the expense of different substrates was determined by measuring the rate of oxygen uptake with a Clark oxygen electrode (Rank Bros., Bottisham, Cambridge, England) at 30 °C connected to a 10 mV Vitatron recorder; 1.8 ml of cell suspension were placed in the reaction vessel after vigorous

aeration. At zero time, 0.2 ml of substrate solution (equivalent to 5 μmol substrate) was introduced into the reaction chamber by means of a calibrated syringe. At 30 °C the O_2 concentration in the air-saturated cell suspension was taken to be 0.26 mM.

Preparation of Cell-Free Extracts

Crude extracts were prepared as described previously [9] except that centrifugation was carried out at 150000 × *g* for 60 min.

NADase Treatment

NADase (NAD glycohydrolase) was prepared from cultures of *Pseudomonas fluorescens* KB1 as described by Sala-Trepat and Evans [9]. To destroy the endogeneous NAD^+, crude extracts from *Pseudomonas putida* NCIB 10015 were incubated at room temperature with an identical volume of NADase preparation for 60 min. The mixtures were then kept at 0 °C until enzyme assays were performed. The activities of the ring-fission product-metabolizing enzymes in extracts to which phosphate buffer, pH 7.5, was added instead of NADase preparation did not differ significantly from those found in untreated extracts.

Preparation of Solutions of the Ring-Fission Products of Catechols

Solutions of these compounds were obtained using heat-treated extracts (55 °C for 10 min) of phenol-grown cells of *Pseudomonas putida* NCIB 10015 as described elsewhere [9].

Enzyme Assays

All assays were performed at room temperature using silica cuvettes (1-cm path) in a Unicam SP 800 recording spectrophotometer.

The following enzymes were assayed by published methods: catechol 2,3-oxygenase, ring-fission product-metabolizing enzymes, 4-oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase, 4-hydroxy-2-oxovalerate aldolase [9] and catechol 1,2-oxygenase [17].

2-Hydroxymuconic semialdehyde hydrolase activity was determined by calculating the rate of disappearance of 2-hydroxymuconic semialdehyde from the rate of decrease in absorbance at 375 nm in reaction mixtures containing NADase-treated extracts. The reaction mixture contained 0.1 μmol 2-hydroxymuconic semialdehyde, 250 μmol of phosphate buffer, pH 7.5 and NADase-treated extracts in a volume of 3 ml.

2-Hydroxymuconic semialdehyde dehydrogenase was determined by subtracting the hydrolase activity from the rate of disappearance of 2-hydroxymuconic semialdehyde produced by the same but untreated cell extracts in the presence of added NAD^+. The assay mixture contained 0.1 μmol 2-hydroxymuconic semialdehyde, 1.0 μmol NAD^+, 250 μmol phosphate buffer, pH 7.5, and cell extracts in a volume of 3 ml.

With the exception of the 4-oxalocrotonate tautomerase activity, the unit of enzyme activity is defined as the amount of enzyme necessary to convert one μmol substrate into product in 1 min under the assay conditions indicated. The unit of tautomerase activity is defined as the amount of enzyme necessary to cause an initial decrease of 1.0 absorbance units per minute under the prescribed assay conditions.

Chemical Measurements

4-Oxalocrotonate was determined in the reaction mixtures as previously described [9]. Acetate was measured by the acetokinase method of Rose [18]. Proteins were estimated by the biuret method [19] using bovine serum albumin as standard.

Chemicals and Enzymatic Reagents

4-Oxalocrotonic acid was synthesized by the method of Lapworth [20] and 4-methyl-2-oxobutyrolactone by the method of Rossi and Schinz [21]. The lactone was hydrolysed to yield 4-hydroxy-2-oxovalerate by the method of Dagley and Gibson [2]. All other chemicals were obtained from commercial sources.

RESULTS

Role of the Catechol meta-*Cleavage Pathway in* Pseudomonas putida *NCIB 10015*

The results shown in Table 1 concerning the induction of a catechol 1,2-oxygenase in cells of *Pseudomonas putida* NCIB 10015 after growth on benzoate and of a catechol *meta*-cleaving oxygenase after growth on phenol or any cresol (*o*, *m*- and *p*-cresol) confirm the conclusion drawn by Feist and Hegeman [5] that the *meta* pathway serves in this

Table 1. *Induction of different catechol oxygenases in cells of* Pseudomonas putida *NCIB 10015 grown with phenol, with cresols and with benzoate*
Enzyme activities were determined in cell-free extracts as described in Material and Methods and are expressed in units per mg protein

Growth substrate		Specific activity of	
		Catechol 1,2-oxygenase	Catechol 2,3-oxygenase
		U/mg protein	
Benzoate	5 mM	0.50	0.0015
Phenol	4 mM	<0.01	0.99
o-Cresol	4 mM	<0.01	0.96
m-Cresol	4 mM	<0.01	0.85
p-Cresol	4 mM	<0.01	0.68
Succinate	11 mM	<0.01	0.001

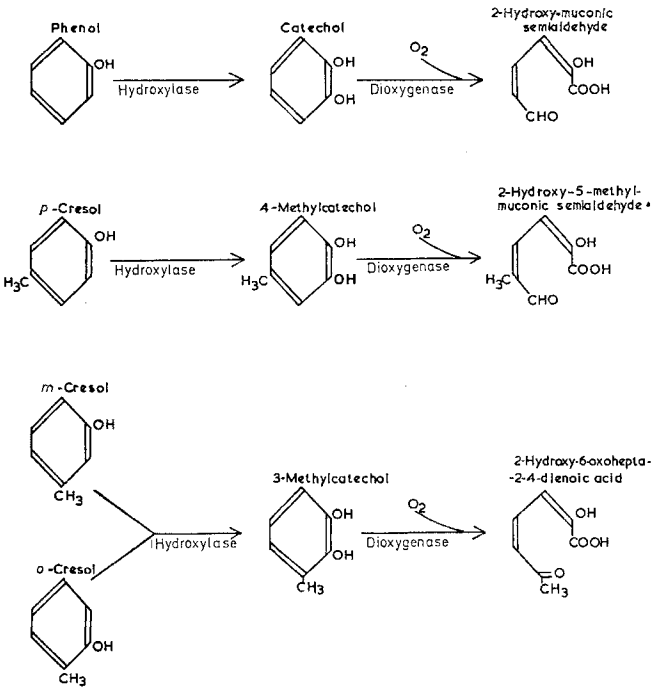

Fig. 2. *Primary steps in the oxidation of phenol and cresols by* Pseudomonas putida *NCIB 10015*

strain as a general mechanism for the dissimilation of alkyl derivatives of catechol derived from substituted phenolic compounds. These authors showed that the *ortho* route (3-oxoadipate pathway) is more specific and serves primarily in the catabolism of nonphenolic precursors of catechol and catechol itself.

Spectrophotometric examinations of the yellow culture supernatants from *P. putida* NCIB 10015 cells grown with phenol and *p*-cresol evidenced that 2-hydroxymuconic semialdehyde (λ_{max} 375 nm at pH 7.5 and 12.0, and 230 nm at pH 2.5) accumulated in the phenol cultures and 2-hydroxy-5-methylmuconic semialdehyde (λ_{max} 382 nm at pH 7.5 and 12.0, and 320 nm at pH 2.5) in the *p*-cresol ones. When washed cell suspensions (6 mg dry weight/ml) of the same organism grown with *o*- and *m*-cresol were incubated at 30 °C with continuous shaking with their growth substrates (5 μmol/ml) accumulation of a yellow compound with identical spectral characteristics to those of the ring-fission product of 3-methylcatechol [14], namely 2-hydroxy-6-oxohepta-2,4-dienoic acid, was invariably observed. These observations are in agreement with the results of similar experiments reported by Bayly *et al.* [14] and support the metabolic scheme shown in Fig. 2 for the dissimilation of phenol and cresols by this strain.

Oxidation of Different Substrates by Whole-Cells Suspensions of Pseudomonas putida *NCIB 10015 Grown on Various Compounds*

As can be seen in Fig. 2 the first step in the metabolism of monophenolic substrates by *P. putida* NCIB 10015 is a hydroxylation to yield catechol or a methyl-substituted catechol. As found by other workers [5, 6] this hydroxylase activity could not be demonstrated in cell-free extracts and consequently was determined in washed suspensions of whole cells by oxygen uptake. The relative rates of oxygen uptake by the whole cells grown on phenol or the cresols at the expense of these compounds are similar and independent of the growth substrate (Table 2). In each case *o*-cresol is metabolized most rapidly followed by phenol, *p*-cresol and *m*-cresol. Because these results were obtained with whole cells they can be affected by permeability factors and by other oxygen-consuming reactions after the first oxygenative step. However the highest rate of respiration obtained with the catechols indicate that, disregarding permeability factors, the limiting step in the consumption of oxygen on the monophenolic substrates is their hydroxylation. It is therefore reasonable to assume that the relative rates of respiration with phenol and the cresols afford a measure of hydroxylase activity in each of the cells. The low Q_{O_2} values of succinate-grown cells with the phenols show that the hydroxylase activity is induced by growth upon the monophenolic substrates. Furthermore the constant pattern of specificity found for this hydroxylase activity irrespective of the growth substrate suggests that only one nonspecific hydroxylase is induced for the metabolism of all these phenolic compounds.

The meta *Cleavage of Catechols by Cell-Free Extracts*

Crude extracts of *P. putida* NCIB 10015 grown on phenol or the cresols oxidized catechol and the methylcatechols at high rates with the accumulation of yellow compounds. The catechol-oxidising enzyme(s) was assayed in these extracts spectrophotometrically by the appearance of the yellow ring-cleavage products. The results shown in Table 3 demonstrate that the substrate specificity of the *meta*-cleaving activity is almost identical in crude extracts of cells obtained after growth on the different monophenolic substrates. The same relative values were obtained using cell-free extracts previously heated to 55 °C for 10 min; this treatment inactivates the enzymes that degrade the muconic semialdehydes.

These results indicate that a single nonspecific oxygenase is induced after growth on the different monophenols. The metabolism of phenols and the cresols by *P. putida* NCIB 10015 appears therefore

Table 2. *Rates of oxygen uptake at the expense of various compounds by washed-cell suspensions of* Pseudomonas putida *NCIB1005*
Oxygen consumption was measured at 30 °C using a Clark oxygen electrode as described in Material and Methods. The rates of oxygen uptake are expressed as Q_{O_2} (O_2 consumed in µl/h per mg dry weight), corrected for endogenous respiration (Q_{O_2} 11—17). Figures in brackets represent relative values for cells grown on a particular substrate (uptake with *o*-cresol = 100). The rate of oxygen uptake in the presence of 3-methylcatechol decreased very rapidly during the reaction and Q_{O_2} values for this substrate were calculated from the initial rate. n.d. = not determined

Growth substrate	Rate of oxygen uptake with							
	Phenol	*o*-Cresol	*m*-Cresol	*p*-Cresol	Catechol	4-Methyl-catechol	3-Methyl-catechol	Succinate
	µl $O_2 \times h^{-1} \times mg^{-1}$							
Phenol	93 (73)	127 (100)	56 (44)	64 (50)	284	652	238	5.3
o-Cresol	72 (71)	101 (100)	48 (47)	61 (60)	231	477	222	5.3
m-Cresol	125 (68)	183 (100)	98 (53)	103 (56)	270	509	228	8
p-Cresol	43 (77)	186 (100)	117 (62)	133 (71)	302	488	n.d.	11
Succinate	8	9	9	11	11	n.d.	n.d.	260

Table 3. *Substrate specificity of the* meta-*cleaving oxygenase activity in crude extracts of* Pseudomonas putida *NCIB 10015 cells grown on phenol and cresols*
Enzyme activity was determined spectrophotometrically by measuring the increase in absorbance at 375 nm, 382 nm and 388 nm due to the formation of 2-hydroxymuconic semialdehyde, 2-hydroxy-5-methylmuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoic acid from catechol, 4-methylcatechol and 3-methylcatechol, respectively. Assay cuvettes contained in a total volume of 3 ml: 250 µmol phosphate buffer, pH 7.5, 0.2 µmol of the corresponding catechol and extract. An increase in absorbance at 375, 382 and 388 nm of 1.2, 1.05 and 0.56 absorbance units corresponds to the appearance of 0.1 µmol of 2-hydroxymuconic semialdehyde, 2-hydroxy-5-methylmuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoic acid, respectively, under these conditions. Figures in brackets represent specific activities (µmol product formed $\times$ $min^{-1} \times$ mg $protein^{-1}$)

Growth substrate	Relative rates of *meta*-cleavage		
	Catechol	4-Methyl-catechol	3-Methyl-catechol
	%	% (µmol $\times$ min^{-1} $\times$ mg^{-1})	%
Phenol 4 mM	28	100 (3.5)	42
o-Cresol 4 mM	30	100 (3.2)	50
m-Cresol 4 mM	32	100 (2.62)	48
p-Cresol 4 mM	28	100 (2.38)	49

to be initiated by the same enzymes (hydroxylase and oxygenase) which show low specificity towards the methyl derivatives of their substrates.

The Metabolism of the meta-*Cleavage Products by Cell-Free Extracts*

As shown in Table 4 incubation of 2-hydroxymuconic semialdehyde with extracts of phenol-grown cells produced results qualitatively similar to those found in *Azotobacter* [8, 9] and the naphthalene-grown pseudomonad [10]: the rate of its disappearance was stimulated upon addition of NAD^+ and partially reduced (by about 35%) by preincubating the extracts with a NADase preparation. The results concerning 4-oxalocrotonate formation in the presence of EDTA (to inhibit the 4-oxalocrotonate-degrading activity, see [9]) correlated with this in that it was formed in virtually stoichiometric quantities in the NAD^+-supplemented extracts and even in crude extracts 35% of the 2-hydroxymuconic semialdehyde was converted to it. Only when NADase-treated extracts were used was there no 4-oxalocrotonate formation. We therefore assume that in

Table 4. *Rates of 2-hydroxymuconic semialdehyde degradation and stoichiometry of 4-oxalocrotonate and NADH formation by cell-free extracts from* Pseudomonas putida *NCIB 10015 grown on phenol*
Rates of 2-hydroxymuconic semialdehyde degradation were measured by following the decrease in absorbance at 375 nm in reaction mixtures containing 250 µmol phosphate buffer, pH 7.5, 0.1 (or 0.2) µmol 2-hydroxymuconic semialdehyde and extract in a total volume of 3 ml. NAD^+ (1.0 µmol) was added where indicated. NADase-treated extracts were prepared as indicated in Material and Methods. The formation of NADH and 4-oxalocrotonate was estimated as described previously and expressed as nmol per nmol 2-hydroxymuconic semialdehyde metabolized [9]. All the figures given in this table represent mean values of several determinations

Type of extract	Additions to the reaction mixture	Rate of degradation	NADH formed	4-oxalocrotonate formed
		nmol $\times$ $min^{-1} \times mg^{-1}$	nmol/nmol	
Crude	None	24.5	—	0.35
	NAD^+	248	0.94	0.92
NADase-treated	None	16	—	0

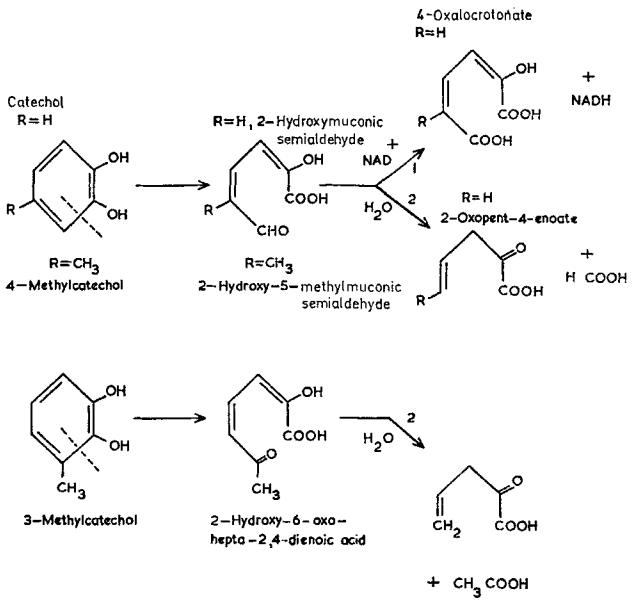

Fig. 3. *Reaction schemes showing the enzymatic steps responsible for the degradation of the ring-fission products of catechols by extracts of* Pseudomonas putida *NCIB 10015*

NADase-treated extracts the 2-hydroxymuconic semialdehyde is degraded by the hydrolase activity reported by Dagley and Gibson [2] and Bayly and Dagley [3] which produces 2-oxopent-4-enoic acid and formate (Fig. 3); we have not been able to measure formate production quantitatively under these conditions by the method used previously [8, 10] but have detected it qualitatively and have also shown the stoichiometric formation of acetate by the action of the same extracts upon 2-hydroxy-6-oxohepta-2,4-dienoic acid, the ring-cleavage product of 3-methylcatechol (see Fig. 3).

In the presence of NAD^+ the virtually stoichiometric formation of 4-oxalocrotonate and NADH (Table 4), which accumulated since levels of NADH oxidase are very low under the assay conditions, indicates the action of a 2-hydroxymuconic semialdehyde dehydrogenase (Fig. 3).

The formation of 4-oxalocrotonate (0.35 nmol per nmol 2-hydroxymuconic semialdehyde metabolized) in the reaction mixtures where crude extracts were used and the reduction (by about 35%) of 2-hydroxymuconic-semialdehyde-degrading activity on NADase treatment reflect the presence in crude extracts of a certain amount of endogenous NAD^+ which in conjunction with the low levels of NADH oxidase activity found in these extracts appear to be sufficient for the action of the dehydrogenase. These results indicate that the degradation of 2-hydroxymuconic semialdehyde by crude extracts is simultaneously channelled through two different ways, one involving the dehydrogenase which is responsible for the

Table 5. *Rates of 2-hydroxy-5-methylmuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoic acid degradation by extracts from* Pseudomonas putida *NCIB 10015 grown on phenol*
Rates of 2-hydroxy-5-methylmuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoic acid degradation were measured by following the decrease in absorbance at 382 nm and 388 nm, respectively, in reaction mixtures containing 250 µmol phosphate buffer, pH 7.5, 0.2 µmol substrate (2-hydroxy-5-methylmuconic semialdehyde or 2-hydroxy-6-oxohepta-2,4-dienoic acid) and extracts in a total volume of 3 ml. NAD^+ (1.0 µmol) was added where indicated

Type of extract	Additions to the reaction mixture	Rates of degradation	
		2-Hydroxy-5-methylmuconic semialdehyde	2-Hydroxy-6-oxohepta-2,4-dienoic acid
		nmol × min^{-1} × mg protein^{-1}	
Crude	None	19	350
	NAD^+	260	350
NADase-treated	None	7.2	350

metabolism of about 35% of the ring-cleavage product and the other involving the hydrolase of Dagley and associates [2, 3, 14]. In assaying for the hydrolase activity we have therefore used extracts preincubated with NADase to destroy the endogenous NAD^+; as an assay for the dehydrogenase we have taken the rate of substrate disappearance in NAD^+-supplemented extracts from which the rate of identical NADase-treated extracts has been subtracted.

Extracts of phenol-grown cells were nonspecific in their action and also metabolized 2-hydroxy-5-methylmuconic semialdehyde and 2-hydroxy-6-oxohepta-2,4-dienoic acid, the ring-fission products of 4-methylcatechol and 3-methylcatechol, respectively (Table 5). The pattern of metabolism of 2-hydroxy-5-methylmuconic semialdehyde was similar to that of 2-hydroxymuconic semialdehyde indicating both hydrolase and NAD^+-dependent dehydrogenase activities. However, the rate of 2-hydroxy-6-oxohepta-2,4-dienoic acid degradation was unaffected by addition of NAD^+ or on NADase treatment, showing no NAD^+-dependent activity; this is not unexpected since the *meta* cleavage product of 3-methylcatechol possesses no oxidisable aldehyde group as do the other ring-fission products (see Fig. 3).

When these results are quantitatively compared to those obtained with *Azotobacter* strains [8, 9] and with the naphthalene-grown pseudomonad [10] it is evident that the hydrolase activity is much higher in the *P. putida* organism.

The Demonstration of the Existence of Only Two Different and Nonspecific Enzymes Responsible for the Degradation of the Ring-Cleavage Products of Catechols in Pseudomonas putida *NCIB 10015*

Similar results to those described for extracts from phenol-grown cells were obtained when ex-

Table 6. *Substrate specificity of the hydrolase and dehydrogenase activities metabolizing the* meta-*cleavage products of catechol derivatives in extracts of* Pseudomonas putida *NCIB 10015*

Enzyme activities were estimated as described under Material and Methods using the substrates indicated below. Decrease of absorbance was monitored at 375 nm for 2-hydroxymuconic semialdehyde, 382 nm for 2-hydroxy-5-methylmuconic semialdehyde and 388 nm for 2-hydroxy-6-oxohepta-2,4-dienoic acid

Growth substrate	Relative activities					
	Dehydrogenase activity against			Hydrolase activity against		
	2-Hydroxymuconic semialdehyde	2-Hydroxy-5-methylmuconic semialdehyde	2-Hydroxy-6-oxohepta-2,4-dienoic acid	2-Hydroxymuconic semialdehyde	2-Hydroxy-5-methylmuconic semialdehyde	2-Hydroxy-6-oxopepta-2,4-dienoic acid
	%	%	%	%	%	%
Phenol	100	118	0	4.5	2	100
o-Cresol	100	113	0	6.0	3	100
m-Cresol	100	118	0	7	3.5	100
p-Cresol	100	128	0	3	1.5	100

tracts from cells grown on any of the cresols were used. In Table 6 are shown the relative activities of the hydrolase and dehydrogenase towards the ring-cleavage products of catechol, 4-methylcatechol and 3-methylcatechol in extracts from cells grown upon the different phenolic substrates. The same pattern of specificity is observed independent of the growth substrate; the NAD^+-dependent dehydrogenase activity metabolized 2-hydroxymuconic semialdehyde and the ring-fission product of 4-methylcatechol at approximately the same high rate but as expected could not act upon the ring-fission product of 3-methylcatechol; conversely, the hydrolase activity was very active on the latter product but attacked 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde at very low relative rates. As indicated for the hydroxylase and oxygenase activities this constant pattern of specificity irrespective of the growth substrate suggests that only a single hydrolase and a single dehydrogenase are induced after growth on the different monophenols.

The results shown in Fig. 4 support this conclusion. The rates on thermal inactivation of the hydrolase activity on all three ring-fission products and of the dehydrogenase activity upon 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde indicate that the two activities in phenol-grown cells are due entirely to two nonspecific enzymes and not to a number of different enzymic entities acting specifically on each of the products. It also indicates that the hydrolase and dehydrogenase activities are located on separate protein moieties which is not surprising but which was impossible to show in the original work with *Azotobacter* [9] because of the much lower levels of the hydrolase activity.

Further Enzymic Steps in the Metabolism of the Ring-Fission Products

Using the assays reported previously [9] we were able to show the presence of 4-oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase and 4-hydroxy-2-oxovalerate aldolase activities in extracts of cells grown on all the monophenolic substrates (Table 7). All the enzymes measured are inducible since they are either undetectable or present at very low levels in succinate-grown cells (Table 7). Furthermore, all are induced to very similar levels independent of the monophenolic compound utilized as growth substrate.

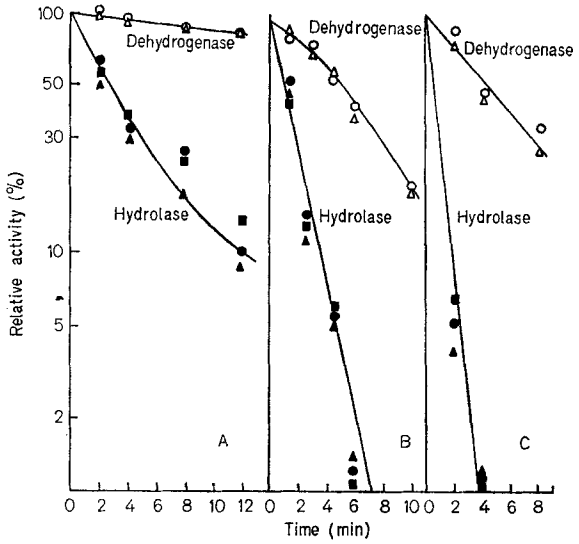

Fig. 4. *The kinetics of thermal inactivation of the dehydrogenase and hydrolase activities involved in the metabolism of the ring-fission products of catechols by extracts of* Pseudomonas putida *NCIB 10015*. Samples (2 ml) of crude extracts were heated at (A) 43 °C, (B) 45 °C, (C) 47 °C for various times, 50 sec being allowed for thermal equilibration. The extracts were then chilled in ice and assayed for activity after removing the precipitated protein by centrifugation. The filled symbols correspond to values of the hydrolase activity towards the different substrates; the NAD^+-dependent semialdehyde dehydrogenase data are represented by the open symbols. Substrates are indicated as follows: (●, ○) 2-hydroxymuconic semialdehyde; (▲, △) 2-hydroxy-5-methylmuconic semialdehyde; and (■) 2-hydroxy-6-oxohepta-2,4-dienoic acid

Table 7. *Specific activities of the enzymes of the* meta*-cleavage pathway of catechol in cell-free extracts of* Pseudomonas putida *NCIB 10015 grown on different substrates*
Enzyme activities were estimated as described under Material and Methods

Growth substrate	Enzyme activities					
	Catechol 2,3-oxygenase	2-Hydroxymuconic semialdehyde dehydrogenase	2-Hydroxymuconic semialdehyde hydrolase	4-Oxalocrotonate tautomerase	4-Oxalocrotonate decarboxylase	4-Hydroxy-2-oxovalerate aldolase
	U/mg protein					
Phenol	0.99	0.23	0.016	85	2.27	0.025
o-Cresol	0.96	0.16	0.019	81	2.77	0.019
m-Cresol	0.85	0.11	0.020	50	1.41	0.036
p-Cresol	0.68	0.12	0.011	55	1.23	0.015
Succinate	0.001	0.003	<0.0002	0.8	<0.02	0.005

Table 8. *Specific activities of enzymes of the* meta*-cleavage pathway in extracts of* Pseudomonas putida *NCIB 10015 wild type and of a mutant (5P) unable to synthesize phenol hydroxylase, grown on various substrates*
Specific activities are expressed in units per mg protein in crude extracts. Activities for the phenol hydroxylase were determined in whole cells as described in Table 2 and are given as Q_{O_2}

P. putida	Substrate	Specific activity of						
		Phenol hydroxylase	Catechol 2,3-oxygenase	2-Hydroxy-muconic semialdehyde dehydrogenase	2-Hydroxy-muconic semialdehyde hydrolase	4-Oxalo-crotonate tautomerase	4-Oxalo-crotonate decarboxylase	4-Hydroxy-2-oxovalerate aldolase
		$\mu l\ O_2 \times h^{-1} \times mg^{-1}$				U/mg		
Wild type	11 mM succinate	3	0.001	0.003	<0.0002	0.8	<0.02	0.005
	11 mM succinate + 4 mM phenol	18	0.30	0.03	0.002	38	0.41	0.015
Mutant (5P)	11 mM succinate	1	0.0026	0.007	<0.0002	0.9	<0.02	0.004
	11 mM succinate + 4 mM phenol	0.8	0.42	0.04	0.002	40	0.38	0.015

It has not proved possible to demonstrate conclusively the presence of 2-oxopent-4-enoate in the metabolism of 4-oxalocrotonate by this strain as with *Azotobacter* [9] since it does not appear to accumulate even transiently, possibly because of the higher levels of the hydratase destroying it. Dagley (personal communication) has studied the latter activity in cell-free extracts of this strain and found it to be present at extraordinarily high levels.

The substrate specificities of the 4-oxalocrotonate tautomerase, 4-oxalocrotonate decarboxylase and 4-hydroxy-2-oxovalerate aldolase have not yet been studied in detail in extracts from cells grown with the different monophenols, but preliminary evidence indicates that they can also attack the corresponding methyl-substituents derived from 2-hydroxy-5-methylmuconic semialdehyde (unpublished observations).

Induction Properties of a Mutant Unable to Synthesize the Hydroxylase

Using a blocked mutant derived from Dagley's organism, Feist and Hegeman [5] have shown that the enzymes of the metabolic pathway of Dagley and associates (hydrolytic branch of Fig. 1) are induced from the top by the primary substrate. It was of special interest to investigate whether the same pattern of induction applies for the enzymes of the 4-oxalocrotonate branch. The data presented in Table 8 obtained with the same mutant which lacks the hydroxylase activity show that this is the case. The 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonate tautomerase and 4-oxalocrotonate decarboxylase together with the oxygenase, hydrolase and aldolase are induced at approximately the same levels found in the wild type by growth of the mutant in the presence of phenol. Since, as is shown in Table 8, the mutant is unable to oxidize phenol (and the cresols) it must be concluded that in this strain phenol itself is the inducer of the whole sequence of enzymes shown in Fig. 1.

DISCUSSION

The results presented here support the reaction scheme shown in Fig. 5 for the metabolism of the *meta* cleavage products of catechols after hydroxylation of monophenolic substrates by *Pseudomonas putida* NCIB 10015. As was found in a naphthalene-

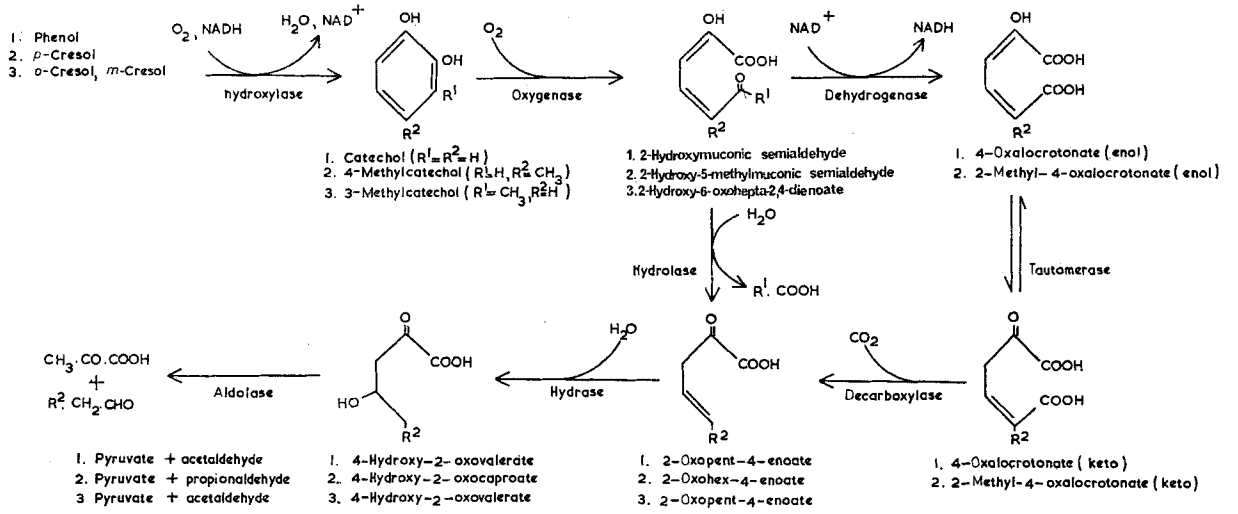

Fig. 5. *The metabolic pathway used by* Pseudomonas putida *NCIB 10015 for the oxidation of monophenolic substrates*

grown pseudomonad [10] the degradation of catechol (and 4-methylcatechol) also proceed by two coexistent pathways in the organism studied here (original strain of Dagley). These pathways diverge after ring-cleavage as the ring-fission product, 2-hydroxymuconic semialdehyde (or 2-hydroxy-5-methylmuconic semialdehyde), is simultaneously catabolized by both an NAD^+-dependent dehydrogenase and a co-factor-independent hydrolase, and they converge again at the level of 2-oxopent-4-enoic acid (or 2-oxohex-4-enoic acid) the product of the hydrolase activity.

The specificity patterns of the ring-fission product-metabolizing enzymes (hydrolase and dehydrogenase) suggest that the two branches of the pathway are involved in the metabolism of different catechols. The hydrolytic activity is particularly active upon the ring-fission product of 3-methylcatechol and only slightly active upon those of catechol and 4-methylcatechol, whereas the specificity of the dehydrogenase shows a reverse pattern being completely inactive upon the ring-fission product of 3-methylcatechol and virtually equally active upon 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde. It therefore appears that the enzymes of the 4-oxalocrotonate branch of the pathway are functional in the metabolism of catechol, 4-methylcatechol and their metabolic precursors (phenol or *p*-cresol) and that the physiological role of the hydrolytic activity is essentially limited to the dissimilation of 3-methylcatechol and its metabolic precursors, namely *o*- and *m*-cresol.

The constant pattern of specificity observed for the hydroxylase, oxygenase, hydrolase and dehydrogenase activities in cells grown on the different monophenolic substrates suggests that growth of *Pseudomonas putida* NCIB 10015 upon phenol or the cresols induces a single set of nonspecific enzymes which can catalyze the conversion of any one of the primary substrates into central intermediary metabolites. Furthermore, the results obtained with the mutant unable to synthesize the hydroxylase have shown that the whole set of enzymes including both branches of the pathway are subjected to coincident inductive control by the primary substrates (phenol or cresols). Whether this induction is coordinate or not is being studied at present in our laboratory.

The nonspecificity of induction and action of the enzymes up ot the ring-fission step has positive advantages for the cell since it provides economy of information (genetic material) and protein synthesis.

The coexistence of the two branches for the metabolism of catechol and 4-methylcatechol is explained in terms of the nonspecificity of the hydrolase activity functional in the degradation of 3-methylcatechol, and of the coincident inductive control of all the enzymes of the pathway exerted by any one of the monophenolic substrates. The existence of this metabolic divergence is, however, somewhat puzzling since it has the physiologically awkward consequences for the cell that synthesis of nonfunctional proteins (the enzymes of the 4-oxalocrotonate branch) occurs under certain conditions of induction (by growth on *o*- or *m*-cresol). The positive advantages of synthesising a single sequence of inducible enzymes in response to a number of potential substrates are partially counterbalanced by the biosynthetic burden imposed by the presence of a metabolic divergence at the level of the ring-fission products. It would seem more advantageous for the cell to have developed at this point a single enzyme (the hydrolase) with a broader specificity which could have allowed a

rapid degradation of all the *meta* cleavage products. However, the particular characteristics of the pathway as it is found in *Pseudomonas putida* NCIB 10015 suggest an attractive theory as to its evolutionary origin which might account for this apparent redundancy. If the 4-oxalocrotonate pathway became functional in the metabolism of phenol and *para*-substituted derivatives, the cell could not metabolize *o*- or *m*-substituted phenols owing to the fact that the ring-fission product of 3-methylcatechol is not a substrate for the NAD^+-dependent semialdehyde dehydrogenase. However, modification of an already existent hydrolase in the cell to give a product further down the pathway already present would be an economical way of making more substrates available for growth with minimal genetic alteration. The metabolic pattern found in *Azotobacter* strains [9] which are unable to grow at the expense of *o*- or *m*-cresol and lack an induicible 2-hydroxymuconic semialdehyde (or 2-hydroxy-6-oxohepta-2,4-dienoic acid) hydrolase, lends support to this theory.

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