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Role of Catechol and the Methylcatechols as Inducers of Aromatic Metabolism in *Pseudomonas putida*

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Pseudomonas putida NCIB 10015 metabolizes phenol and the cresols (methylphenols) by the *meta* pathway and metabolizes benzoate by the *ortho* pathway. Growth on catechol, an intermediate in the metabolism of both phenol and benzoate, induces both *ortho* and *meta* pathways; growth on 3- or 4-methylcatechols, intermediates in the metabolism of the cresols, induces only the *meta* pathway to a very limited degree. Addition of catechol at a growth-limiting rate induces virtually no *meta* pathway enzymes, but high levels of *ortho* pathway enzymes. The role of catechol and the methylcatechols as inducers is discussed. A method is described for assaying low levels of catechol 1,2-oxygenase in the presence of high levels of catechol 2,3-oxygenase and vice versa.

Pseudomonas putida NCIB 10015 (strain U of Dagley and Gibson [1]; ATCC 17514) metabolizes phenol by the divergent *meta* (or α-keto-acid) pathway (3, 10), but metabolizes benzoate by the *ortho* (or β-ketoadipate) pathway (4) in spite of a common intermediate, catechol (Fig. 1). The regulation of these tangential pathways in this strain has been studied by Feist and Hegeman (4); whereas phenol is the substrate inducer of the entire suite of *meta* enzymes (4, 10), the primary inducer of the *ortho* pathway proper is the product of the first enzyme of the pathway, *cis,cis*-muconate (4, 8). This pattern of regulation ensures that if an inducer of the *meta* pathway is present, it is preferentially expressed, as in the case of phenol, and only in the absence of an inducer of the *meta* pathway, as in the case of benzoate, can catechol accumulate and leak through to *cis,cis*-muconate, thus inducing the *ortho* pathway.

Cresols (methylphenols) also induce the *meta* pathway (10) and are metabolized via 3- or 4-methylcatechol and the corresponding methyl derivatives.

MATERIALS AND METHODS

The organism was maintained, grown, and harvested, and cell-free extracts were prepared as has been described (10). Growth on catechol and the methylcatechols was by the same methods, except that the liquid media contained no $FeSO_4$ and the stock salt solution (1) was added to only 0.5 ml/liter.

The following enzymes were assayed by published methods: catechol 2,3-oxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, 4-oxalocrotonate decarboxylase, 4-hydroxy-2-oxovalerate aldolase (11), and 2-oxopent-4-enoate hydratase (2).

2-Hydroxymuconic semialdehyde hydrolase was assayed by measuring the rate of loss of absorbance at 388 nm due to the disappearance of 2-hydroxy-6-oxohepta-2,4-dienoate prepared from 3-methylcatechol (11). The assay mixture contained 0.1 μmol of substrate, 280 μmol of sodium phosphate buffer, pH 7.5, and cell-free extract in a volume of 3 ml. The disappearance of 0.1 μmol of substrate produces a decrease in absorbance of 0.5. This activity will therefore be referred to as 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase in the tables: because of its specificity the values for its specific activity are about 20 times those published for 2-hydroxymuconic semialdehyde as substrate (10).

4-Oxalocrotonate tautomerase assays were similar to those published (11), except that the rate of decrease of absorbance of both test and blank solutions at 295 nm was measured as the tangent to the curve at an absorbance of 1.0; this gives more reproducibility, and values for the specific activity are about 0.3 times those published previously (10).

Catechol 1,2-oxygenase was assayed by the method of Hegeman (5) in a modified reaction mixture containing 280 μmol of phosphate buffer, pH 7.5, 0.2 μmol of catechol, 100 μmol of ethylenediaminetetraacetic acid, and cell-free extract in a volume of 3 ml.

Cis,cis-muconate lactonizing enzyme was assayed by the method of Ornston (9) in a modified reaction mixture containing 0.33 μmol of *cis,cis*-muconate, 5 μmol of $MnCl_2$, 250 μmol of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, and cell-free extract in 3 ml.

Oxygen uptake measurements were made in an oxygen electrode, and cells were prepared and the experiments carried out as previously described (10).

Heat treatment of cell-free extracts was carried out at 55 C for various times as has been described (11).

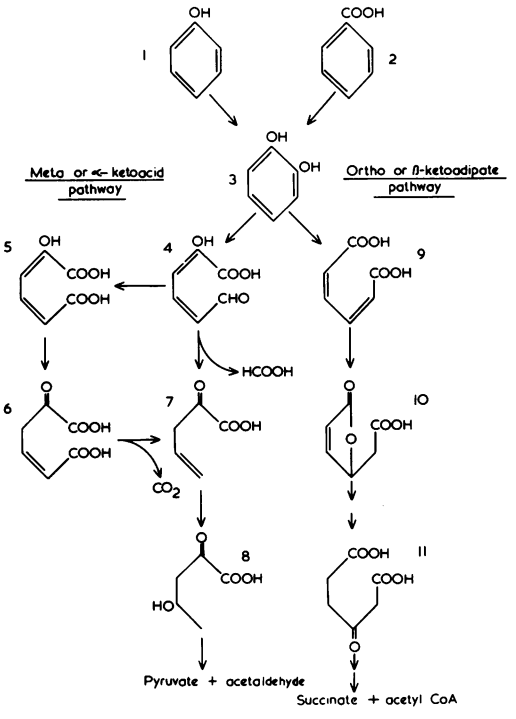

FIG. 1. *The metabolism of phenol and benzoate by Pseudomonas putida NCIB 10015. The compounds are: 1, phenol; 2, benzoate; 3, catechol; 4, 2-hydroxymuconic semialdehyde; 5, 4-oxalocrotonate (enol); 6, 4-oxalocrotonate (keto); 7, 2-oxo-4-pentenoate; 8, 4-hydroxy-2-oxovalerate; 9, cis, cis-muconate; 10, muconolactone; 11, β-ketoadipate. The enzymes in the text catalyse the following conversions: 1 → 3, phenol hydroxylase; 3 → 4, catechol 2, 3-oxygenase; 4 → 7, 2-hydroxymuconic semialdehyde hydrolase; 4 → 5, 2-hydroxymuconic semialdehyde dehydrogenase; 5 → 6, 4-oxalocrotonate tautomerase; 6 → 7, 4-oxalocrotonate decarboxylase; 7 → 8, 2-oxo-4-pentenoate hydratase; 7 → (pyruvate + acetaldehyde), 4-hydroxy-2-oxovalerate aldolase; 2 → 3, benzoate oxidase; 3 → 9, catechol 1,2-oxygenase; 9 → 10, cis, cis-muconate lactonizing enzyme. The cresols are metabolized by the meta pathway via 3- or 4-methyl catechol, and the corresponding methyl analogues of the compounds shown.*

The extracts were immediately chilled in ice and assayed for catechol 2,3-oxygenase after removal of precipitated protein by centrifugation.

Oxidation of catechol 2,3-oxygenase activity was performed by incubating cell-free extracts (0.2 ml) with 0.6 ml of 100 mM H_2O_2 and 120 μmol of phosphate buffer, pH 7.5, in a total volume of 3 ml. Catechol 1,2-oxygenase was then assayed immediately after incubation.

Catechol and the methylcatechols were purified by sublimation under vacuum.

RESULTS

Pseudomonas putida NCIB 10015 grows well on catechol on agar plates or in liquid media and slowly on 3- and 4-methylcatechols (approximate doubling times 85, 230, and 330 min, respectively, compared with benzoate and phenol, 60 and 80 min, respectively). Preliminary experiments indicated that growth on catechol elicited induction of both *ortho* and *meta* pathways as exemplified by catechol 1,2-oxygenase and catechol 2,3-oxygenase, respectively. Because the substrate for both these enzymes is catechol, quantitative estimation of one in the presence of the other is difficult, particularly when one is present in considerable excess of the other. However, catechol 1,2-oxygenase is labile to heat and catechol 2,3-oxygenase is labile to oxidation. Heat treatment at 55 C for 10 min destroyed all of the 1,2-oxygenase, leaving the 2,3-oxygenase little changed, and incubation with 30 mM H_2O_2 for 10 min destroyed virtually all of the 2,3-oxygenase, leaving the 1,2-oxygenase unchanged (Fig. 2). This treatment was used where appropriate, and the assay for the stable enzyme was performed immediately after the particular treatment (see Table 1).

Cell-free extracts of cells grown on 2.5 mM catechol contained levels of both *ortho* and *meta* pathway enzymes considerably higher than in uninduced succinate-grown cells, but less than the fully induced levels of *ortho* enzymes found in benzoate-grown cells or of *meta* enzymes found in phenol-grown cells (Table 1). Cells grown on 3- and 4-methylcatechols contained *meta* pathway enzymes induced to a slightly lower level than in catechol-grown cells, but contained no induced levels of *ortho* pathway enzymes.

The first enzymes of each pathway, the ben-

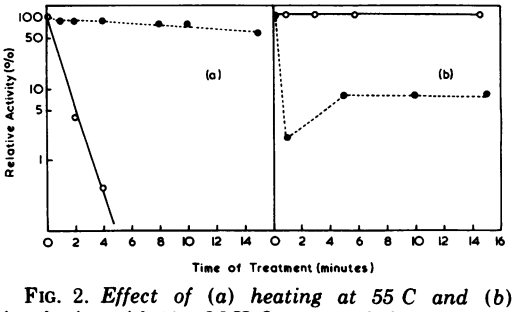

FIG. 2. *Effect of (a) heating at 55 C and (b) incubating with 30 mM H_2O_2 on catechol 1,2-oxygenase* (○) *and catechol 2,3-oxygenase* (●) *from Pseudomonas putida NCIB 10015. Catechol 1,2-oxygenase was measured in cell-free extracts of benzoate-grown cells and catechol 2,3-oxygenase in cell-free extracts of phenol-grown cells.*

TABLE 1. *Relative specific activities of enzymes of the meta and ortho pathways in cell-free extracts of Pseudomonas putida NC1B 10015*[a]

Activity	Growth substrate						
	Catechol (2.5 mM batch)	Benzoate (5 mM batch)	Phenol (2.5 mM batch)	Succinate (11 mM batch)	3-Methyl-catechol (2.5 mM batch)	4-Methyl-catechol (2.5 mM batch)	Catechol (growth limiting)[b]
Meta pathway							
Catechol 2,3-oxygenase	52	1.5[c]	100 (1.02)	0.1	29	16	1.5[c]
2-Hydroxy-6-oxohepta-2,4-dienoate hydrolase	39	1	100 (0.24)	0.6	25	12	2.6
2-Hydroxymuconic semialdehyde dehydrogenase	65	5	100 (0.12)	1.8	38	21	7
4-Oxalocrotonate tautomerase	41	0.3	100 (65.9)	0.2	20	10	2.5
4-Oxalocrotonate decarboxylase	71	1.4	100 (1.54)	0.2	25	21	2.6
2-Oxo-4-pentenoate hydratase	38	1.8	100 (3.0)	1.1	32	25	4
4-Hydroxy-2-oxovalerate aldollase	64	13	100 (0.025)	12	32	44	2.4
Ortho pathway							
Catechol 1,2-oxygenase	15[d]	100 (0.46)	ND	1.7	1.9[d]	0.5[d]	39
Cis, cis-muconate lactonizing enzyme	54	100 (0.39)	ND	1.3	1.1	0.6	42

[a] *Meta* enzymes relative to levels in phenol-grown cells and *ortho* enzymes relative to levels in benzoate-grown cells. Values in parentheses are specific activities (enzyme units per milligram of protein). ND, Not determined.

[b] Succinate grown cells were inoculated into 10 liters of sterile minimal salts medium, and catechol was added at a rate of 1.67 mmol/h over the course of 15 h by means of a peristaltic pump.

[c] Measured after heating cell-free extract at 55 C for 10 min.

[d] Measured after incubation with 30 mM H_2O_2 for 10 min.

zoate oxidase system and phenol hydroxylase, cannot be measured in cell-free systems, but qualitative estimates of their degree of induction can be made from measuring the rate of oxygen uptake stimulated in whole cells by benzoate or phenol, respectively (10). Catechol-grown cells when assayed by this method (Table 2) showed induced levels of the nonspecific phenol hydroxylase as shown by the stimulation of O_2 uptake on phenol and the cresols, though to a lesser extent than in phenol-grown cells (10). Growth on catechol did not elicit significant respiration on benzoate, in accordance with the regulation of the enzymes of the benzoate oxidase system previously reported for *P. putida* (8).

These results are in conflict with those of Feist and Hegeman (4) who failed to detect any *meta* pathway enzymes in catechol-grown cells supposedly of the same strain. In order to check whether we were still working with the same strain, we obtained a freeze-dried culture of the strain used by Feist and Hegeman directly from G. D. Hegeman, but the results both of the enzyme complement of catechol-grown cells and, more critically, of the substrate specificities of the 2,3,-oxygenase, the 2-hydroxymuconic semialdehyde dehydrogenase, and the 2-hydroxymuconic semialdehyde hydrolase (10) were identical to those found in our strain.

TABLE 2. *Oxygen uptake by catechol-grown cells of Pseudomonas putida NCIB 10015*

Substrate	Q_{o_2} (μliters of O_2 per h per mg [dry wt])
Benzoate	17
Phenol	34
o-Cresol	69
m-Cresol	52
p-Cresol	69

Measured after subtraction of endogeneous respiration (26 μliters of O_2 per h per mg [dry weight]). Values of $Q_{o_2} \leq 26$ are considered not significant.

If, instead of inoculating cells into a medium containing 2.5 mM catechol, the catechol was alternatively added at a growth-limiting rate to mineral salts medium inoculated with succinate-grown cells, the resulting enzyme constitution of the cells (last column, Table 1) showed much lower levels of *meta* pathway enzymes, but almost fully induced levels of *ortho* pathway enzymes.

DISCUSSION

The explanation of the results would appear to be fairly simple. When uninduced cells are put into catechol medium, the catechol induces the *meta* pathway enzymes, coincidently as does phenol, but not to a sufficiently high level to produce its rapid disappearance. Consequently, some of the catechol will be converted to *cis,cis*-muconate by the basal levels of 1,2-oxygenase found in uninduced cells, and because this appears to be the first inducer of the *ortho* pathway enzymes (but not of benzoate oxidase) in *P. putida* (8), the sequential induction of the *ortho* pathway is also initiated. Presumably, the catechol is then assimilated simultaneously by both pathways.

This raises the question of why, in benzoate-grown cultures, there are not comparable levels of *meta* pathway enzymes, because the sequence of events from the known regulation of *P. putida* (8) appears to be that benzoate induces the oxidase system converting benzoate to catechol, and the accumulated catechol then "leaks" to *cis,cis*-muconate which starts the sequential induction of the *ortho* pathway proper. The results in Table 1 do show some induction of the *meta* pathway in benzoate-grown cells, although no more than 15-fold in the case of the 2,3-oxygenase.

We attempted to answer the question by trying to mimic the situation occurring in cultures being induced to benzoate by adding the catechol at a growth-limiting rate by spreading its addition over 15 h. The levels of *meta* enzymes resulting are much lower than in the batch culture, and only about twice those in benzoate-grown cells, whereas the two *ortho* pathway enzymes measured are induced to comparable levels. With this enzyme complement, it is certain that virtually all of the catechol is assimilated via the *ortho* pathway.

The reason that *meta* enzymes are not induced during metabolism of benzoate is not, as has been suggested (4), that there is no inducer of the pathway during benzoate metabolism, but is a combination of the inducer which is formed, namely catechol, being a weak inducer and the fact that its intracellular concentration never rises to a sufficient level to cause induction of more than the rather insignificant activities detected. A rather similar situation appears during metabolism of benzoate by the *ortho* pathway in *Alcaligenes eutrophus* (6) when after growth on high, but not on low, concentrations of benzoate there appears to be some induction of *meta* pathway enzymes. Johnson and Stanier have proposed that only under these conditions is there accumulation of catechol and that this then induces the *meta* enzymes.

The lack of any induction of the *ortho* pathway enzymes after growth on the methylcatechols could be due to either the inability of the basal levels of catechol 1,2-oxygenase to convert them to the corresponding methylmuconic acids or, alternatively, to the inability of the methylmuconic acids, if formed, to act as primary inducers of the *ortho* pathway. We suspect the latter is the explanation, because we have strong evidence that the catechol 1,2-oxygenase of this strain is nonspecific and can oxidize both 3- and 4-methylcatechol to products which are almost certainly 2-methyl and 3-methyl *cis,cis*-muconates, respectively (unpublished data).

The induction of the *meta* pathway enzymes by catechol and the methylcatechols is probably due to the structural similarity between them and the primary and more potent inducers, phenol and the cresols. In another strain of *Pseudomonas* we have studied, *Pseudomonas arvilla* mt-2, which uses the *meta* pathway for metabolism of benzoate, which is also the substrate inducer of the pathway, catechol has much less structural analogy with benzoate, does not induce the *meta* pathway, and is therefore assimilated completely by the *ortho* pathway (7).

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