The Conversion of Catechol and Protocatechuete to β-Ketoadipate by Pseudomonas putida

IV. REGULATION*

L. N. ORNSTON‡

From the Department of Bacteriology and Immunology, University of California, Berkeley, California 94720

SUMMARY

Study of the regulation of the syntheses of enzymes of the catechol and protocatechuate pathways in Pseudomonas putida has shown that two groups of enzymes are subject to coordinate control. cis,cis-Muconate-lactonizing enzyme and muconolactone isomerase, which are uniquely associated with the catechol pathway, constitute the first coordinate block of enzymes. The synthesis of these enzymes, as well as that of catechol oxygenase (which is regulated independently), seems to be induced by cis,cis-muconate.

The second coordinate block of enzymes comprises β-carboxy-cis,cis-muconate-lactonizing enzyme and γ-carboxy-β-muconate decarboxylase, which are uniquely associated with the protocatechuate pathway, and β-ketoadipate enol-lactone hydrolase, which is functional in both the protocatechuate and the catechol pathways. This group of enzymes seems to be induced by β-ketoadipate or β-keto-adipyl coenzyme A.

Moraxella lwoffi, which degrades protocatechuate and catechol through identical step-reactions, regulates the synthesis of the enzymes mediating these conversions by a different mechanism.

The reactions proceed through two series of chemically analogous intermediates, metabolic convergence occurring with the formation of the immediate precursor of β-ketoadipate, β-ketoadipate enol-lactone. Two specific sets of enzymes mediate the respective conversions of catechol and protocatechuate to this lactone; a common enzyme, β-ketoadipate enol-lactone hydrolase, converts the lactone to β-ketoadipate.

Specific assays have been developed for all of the enzymes operative in the conversions of catechol and protocatechuate to β-ketoadipate (2, 3). Without exception these enzymes are inducible, so that the existence of these specific assays has made possible a detailed study of the regulatory mechanisms controlling their synthesis, described in the present paper. The regulation of the synthesis of some of these enzymes by other species of bacteria that possess the ability to convert benzoate and p-hydroxybenzoate to β-ketoadipate has also been examined, and the results will be compared with those obtained for P. putida.

EXPERIMENTAL PROCEDURE

Biological Materials—P. putida A.3.12 (ATCC 12633) was the organism used for most of the experiments to be reported. Mutants of this strain were prepared by methods described below, except for mutant A202, which was obtained from Dr. J. Mandelstam. P. putida Cl-A (ATCC 17452), a naturally occurring strain which possesses all enzymes necessary for utilization of p-hydroxybenzoate except β-carboxy-cis,cis-muconate-lactonizing enzyme, was obtained from Dr. I. C. Gunsalus. Other bacterial strains used were Moraxella lwoffi (Vibrio 01) ATCC 11171; Pseudomonas multivorans ATCC 17759; Pseudomonas aeruginosa ATCC 17503; and Hydrogenomonas eutropha ATCC 17697.

The composition of media and the conditions of growth are described elsewhere (1).

Isolation and Characterization of Mutants—Two general classes of mutants were isolated. The first class has lost the ability to synthesize one or more of the enzymes operative in the catechol and protocatechuate pathways. Such mutants will be termed blocked mutants. The second class has acquired the ability (not possessed by the wild type) to grow exponentially at the expense of cis,cis-muconate. Such mutants will be termed permeability mutants, because the mutational change apparently involves an alteration of the cell membrane which permits the dicarboxylic acid to enter the cell.

Blocked mutants of P. putida A.3.12 were obtained by treat
Influence of various growth substrates on levels of enzymes of catechol and protocatechuate pathways in extracts from wild type P. putida A.3.12

Cultures were grown at the expense of 20 mM acetate, 20 mM succinate, 10 mM p-hydroxybenzoate, 10 mM benzoate, or 10 mM β-ketoadipate. The values given for cells grown at the expense of β-ketoadipate were obtained from a single culture. Other values represent the average of determinations on extracts prepared from at least three different cultures grown on the indicated substrate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Physiological role</th>
<th>Catechol pathway</th>
<th>p-Hydroxybenzoate</th>
<th>Benzoate</th>
<th>β-Ketoadipate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol oxygenase</td>
<td></td>
<td>&lt;0.0002</td>
<td>0.005 (0.005-0.006)</td>
<td>1.09 (0.99-1.29)</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>Cis,cis-Muconate-lactonizing enzyme</td>
<td></td>
<td>&lt;0.0002</td>
<td>&lt;0.0002</td>
<td>0.30 (0.38-0.40)</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>Muconolactone isomerase</td>
<td></td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
<td>2.20 (2.03-2.23)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Protocatechuate oxygenase</td>
<td></td>
<td>0.004</td>
<td>2.03 (2.42-2.85)</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>β-Carboxy-cis,cis-muconate-lactonizing enzyme</td>
<td></td>
<td>0.02</td>
<td>0.95 (0.89-1.03)</td>
<td>0.68 (0.64-0.75)</td>
<td>0.80</td>
</tr>
<tr>
<td>γ-Carboxymuconolactone decarboxylase</td>
<td></td>
<td>0.05</td>
<td>2.82 (2.60-3.07)</td>
<td>2.02 (1.85-2.22)</td>
<td>2.48</td>
</tr>
<tr>
<td>β-Ketoadipate enol-lactone hydrolase</td>
<td>Both pathways</td>
<td>0.03</td>
<td>1.49 (1.33-1.62)</td>
<td>1.63 (0.89-1.16)</td>
<td>1.58</td>
</tr>
</tbody>
</table>

* Numbers in parentheses represent range of values observed.

Permeability mutants were obtained without mutagenesis by spreading about 10⁶ wild type cells of P. putida A.3.12 on plates of basal agar medium containing 10 mM cis,cis-muconate. Two or three colonies appeared on most plates after several days. One colony was picked from each plate and purified by restreaking on a plate of the same medium. The mutant stocks were maintained on slants of cis,cis-muconate agar in order to counterselect possible revertants; however, they could be transferred several times on 1% yeast agar without losing the ability to grow at the expense of cis,cis-muconate.

Enzyme Assays—Cell-free extracts were prepared and enzyme assays were performed by procedures described previously (1-3). Enzyme levels in crude extracts are always expressed as units per mg of protein, a unit of enzyme being defined as the amount necessary to cause disappearance of 1.0 nmole of substrate per min under the conditions of assay. Protein concentrations were determined with biuret reagent.

RESULTS

Patterns of Induction of Enzymes of Catechol and Protocatechuate Pathways in Wild Type P. putida A.3.12—Although P. putida A.3.12 grows well at the expense of benzoate or p-hydroxybenzoate, with generation times at 30° of 50 and 75 min, respectively, few of the subsequent intermediates can support growth of the wild type. Some of the intermediates are too unstable chemically to serve as growth substrates (e.g. β-carboxy-cis,cis-muconate, γ-carboxymuconolactone). Others are toxic (e.g. catechol), or do not readily enter the cell (e.g. cis,cis-muconate). However, β-ketoadipate can support slow growth of the wild type (with a generation time of 180 min at 30°), and is sufficiently stable chemically to be used in growth experiments. Accordingly, inductive patterns with respect to the enzymes of the catechol and protocatechuate pathways were determined on extracts of the wild type after growth at the expense of benzoate, p-hydroxybenzoate, and β-ketoadipate. Basal levels of all these enzymes were determined on extracts of cells grown with acetate and with succinate, which support generation times at 30° of 200 and 45 min, respectively. The results are shown in Table I.

The basal levels of activity after growth with succinate and
The first enzyme common to both pathways — ketoadipate enol-lactone hydrolase — were all present at very low but detectable levels in acetate- and succinate-grown cells. Growth at the expense of p-hydroxybenzoate elicits high levels of activity of the four enzymes required for the conversion of protocatechuate to β-ketoadipate. The activity of protocatechuate oxygenase increases 650-fold over the basal level; the activities of the other three enzymes, about 50-fold. There is no measurable induction of cis,cis-muconate-lactonizing enzyme or muconolactone isomerase; the slightly increased level of catechol oxygenase activity may be attributed to nonspecific catalysis by protocatechuate oxygenase (2). Accordingly, the inductive response resulting from growth with p-hydroxybenzoate is physiologically specific.

Growth at the expense of benzoate elicits high levels of activity of the four enzymes required for the conversion of catechol to β-ketoadipate. The activity of catechol oxygenase increases at least 5000-fold over the basal level, the activity of cis,cis-muconate-lactonizing enzyme at least 2000-fold, and the activity of muconolactone isomerase at least 100-fold. The activity of β-ketoadipate enol-lactone hydrolase increases about 35-fold. However, the inductive response to benzoate is physiologically nonspecific, since levels of two enzymes uniquely operative in the protocatechuate pathway, β-carboxyl-trans-cis,cis-muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase, likewise increase about 35-fold. The levels of activity of the three latter enzymes in benzoate-grown cells are characteristically somewhat lower than in p-hydroxybenzoate-grown cells (Table I). It should be noted that the ability of extracts of benzoate-grown cells to attack β-carboxyl-trans-cis,cis-muconate and γ-carboxymuconolactone cannot be attributed to nonspecific catalysis by cis,cis-muconate-lactonizing enzyme and muconolactone isomerase, respectively, since it has been shown that purified preparations of these two enzymes have no activity on the carboxylated analogues of their natural substrates (3). Hence, it is evident that an intermediate produced in the course of benzoate metabolism can induce synthesis of two enzymes uniquely operative in the protocatechuate pathway. Protocatechuate oxygenase, the third enzyme unique to the protocatechuate pathway, is possibly induced to a very slight extent by growth with benzoate; its activity increases 5-fold over the basal level. This small increase of specific activity might simply reflect the ability of catechol oxygenase to attack protocatechuate at a very low rate.

Although β-ketoadipate is the eventual common product of the action of the enzymes of the catechol and protocatechuate pathways, cells grown at its expense contain high levels of activity of three of these enzymes: β-carboxyl-trans-cis,cis-muconate-lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase. Their levels are comparable to the levels resulting from growth at the expense of p-hydroxybenzoate. The other enzymes of the catechol and protocatechuate pathways are not measurably induced by growth at the expense of β-ketoadipate.

**β-Carboxyl-trans-cis,cis-muconate Coordinate Block** — The absolute specific activities of β-carboxyl-trans-cis,cis-muconate-lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase are different in extracts of cells grown with benzoate, p-hydroxybenzoate, and β-ketoadipate, but their relative specific activities remain closely comparable (Table I). This fact suggested that their syntheses might be coordinate. All the enzymes of the catechol and protocatechuate pathway are subject to catabolite repression by glucose and succinate which permitted a more rigorous test of their presumptive coordinateness.

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**Fig. 1. Coordinate regulation of the syntheses of β-carboxyl-trans-cis,cis-muconate-lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase.** Enzyme levels were determined in extracts of wild type P. putida which had been grown for at least seven generations in mineral medium containing the following carbon sources: 1, 10 mM p-hydroxybenzoate; 2, 10 mM benzoate; 3, 10 mM p-hydroxybenzoate and 10 mM glucose; 4, 10 mM benzoate and 10 mM glucose; 5, 10 mM benzoate and 10 mM succinate; 6, 10 mM benzoate and 30 mM succinate; 7, 10 mM benzoate and 30 mM glucose; 8, 10 mM p-hydroxybenzoate and 10 mM succinate; 9, 10 mM succinate; 10, 10 mM glucose.
ordinate nature. A series of cultures was grown with varying ratios of a specific inducer (either benzoate or p-hydroxybenzoate) and a compound which causes catabolite repression (either glucose or succinate). Extracts from each culture were assayed for four enzymes of the catechol and protocatechuate pathways. The results (Fig. 1) show that strict proportionality is maintained between the levels of β-carboxy-cis,cis-muconate-lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase over a wide range of specific activity. These enzymes therefore constitute a coordinate block. There is, in contrast, no proportionality between the levels of β-ketoadipate enol-lactone hydrolase and of catechol oxygenase. Catechol oxygenase is not induced by growth with p-hydroxybenzoate, and is far more sensitive to catabolite repression than the enzymes of the β-carboxy-cis,cis-muconate block.

cis,cis-Muconate Block—Experiments analogous to those described in the preceding section showed (Fig. 2) that two enzymes unique to the catechol pathway, cis,cis-muconate-lactonizing enzyme and muconolactone isomerase, likewise constitute a coordinate block, termed the cis,cis-muconate block. As shown in Fig. 2, the synthesis of these two enzymes is regulated independently of that of catechol oxygenase, the third enzyme unique to the catechol pathway. Whereas synthesis of enzymes of the cis,cis-muconate block is more sensitive to succinate (45% average repression) than to glucose (14% average repression), synthesis of catechol oxygenase is affected to an almost equal extent by both these compounds (about 75% average repression).

The experiments with the wild type of *P. putida* A3.12 described above reveal in broad outline the control mechanisms governing the synthesis of the enzymes of the catechol and protocatechuate pathways. The synthesis of catechol oxygenase and protocatechuate oxygenase, the first enzymes of the catechol and protocatechuate pathways, respectively, are each individually controlled. The second and third enzymes unique to the catechol pathway, cis,cis-muconate-lactonizing enzyme and muconolactone isomerase, constitute a coordinate block. The second and third enzymes unique to the protocatechuate pathway, β-carboxy-cis,muconate-lactonizing enzyme and γ-carboxymuconolactone decarboxylase, together with β-ketoadipate enol-lactone hydrolase, the first enzyme common to both pathways, constitute a second coordinate block. However, these experiments do not reveal the specific inducers operative in the four separately controlled inducible events. A clearer insight into this question was obtained through experiments with mutants, described in the following sections.

**Inductive Properties of cis,cis-Muconate**—Succinate-grown cells of the wild type of *P. putida* cannot grow at all with cis,cis-muconate; benzoate-grown cells grow arithmetically with it. However, spontaneous mutants that are capable of exponential growth with cis,cis-muconate can be obtained by selection (see "Experimental Procedure"). One such mutant, CM3A, was studied in detail. After growth with benzoate, its enzymic constitution is indistinguishable from that of the wild type (Table II). However, benzoate-grown cells of CM3A consume oxygen rapidly when furnished with cis,cis-muconate, whereas benzoate-grown wild type cells do not (Fig. 3). These facts indicate that CM3A is a mutant with altered permeability properties, which permit cis,cis-muconate to enter the cell more readily than it can enter the cell of the wild type.

When mutant CM3A is grown at the expense of cis,cis-muconate, all the enzymes of the catechol pathway are induced, to-

![Graph showing coordinate regulation of enzyme synthesis](http://www.jbc.org)

**Fig. 2.** Coordinate regulation of the synthesis of cis,cis-muconate-lactonizing enzyme and muconolactone isomerase. Enzyme levels were determined in extracts of wild type *P. putida* which had been grown at least seven generations in mineral medium containing the following carbon sources: 1, 10 mM benzoate; 2, 10 mM benzoate; 3, 10 mM benzoate and 10 mM glucose; 4, 10 mM benzoate and 10 mM glucose; 5, 10 mM benzoate and 5 mM glucose; 6, 5 mM benzoate and 5 mM glucose; 7, 5 mM benzoate and 10 mM glucose; 8, 5 mM benzoate and 20 mM glucose; 9, 10 mM benzoate and 20 mM glucose; 10, 10 mM benzoate and 5 mM succinate; 11, 10 mM benzoate and 10 mM succinate; 12, 10 mM benzoate and 20 mM succinate; 13, 5 mM benzoate and 5 mM succinate; 14, 5 mM benzoate and 10 mM succinate; 15, 5 mM benzoate and 20 mM succinate.

**Table II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activities in extracts from cells grown with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Benzoate</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Catechol oxygenase</td>
<td>0.94</td>
</tr>
<tr>
<td>cis,cis-Muconate-lactonizing enzyme</td>
<td>0.39</td>
</tr>
<tr>
<td>Muconolactone isomerase</td>
<td>2.60</td>
</tr>
<tr>
<td>β-Carboxy-cis,cis-muconate-lactonizing enzyme</td>
<td>0.69</td>
</tr>
<tr>
<td>γ-Carboxymuconolactone decarboxylase</td>
<td>2.74</td>
</tr>
<tr>
<td>β-Ketoadipate enol-lactone hydrolase</td>
<td>1.44</td>
</tr>
</tbody>
</table>
Catcchol and Protocatechuate Pathways in P. putida. IV

Table III
Levels of enzymes of catechol pathway in extracts of wild type P. putida A.3.12 and of benzoate-negative mutants, grown at expense of 10 mm glucose with benzoate (10 mm) as inducer

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Wild type</th>
<th>DLM8</th>
<th>NG22</th>
<th>NG4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol oxygenase</td>
<td>0.31</td>
<td>&lt;0.002</td>
<td>0.26</td>
<td>0.15</td>
</tr>
<tr>
<td>cis,cis-Muconate-lactonizing enzyme</td>
<td>0.14</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Muconolactone isomerase</td>
<td>1.19</td>
<td>0.03</td>
<td>1.07</td>
<td>2.69</td>
</tr>
<tr>
<td>3-Ketoadipate enol-lactone hydrolase</td>
<td>0.33</td>
<td>0.00</td>
<td>0.08</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Fig. 3. Rates of oxidation of cis,cis-muconate and benzoate by wild type P. putida and permeability mutant CM3A. Cells were grown at the expense of 10 mm benzoate, washed with 0.05 m disodium potassium phosphate buffer, pH 7.0, and 6 x 10^8 cells were suspended in flasks containing 100 mmoles of sodium phosphate buffer, pH 7.0, in a final volume of 1.6 ml. The center well contained 0.2 ml of 10% NaOH. Substrate (10 mumoles in 0.2 ml) was added from a side arm at 0 min, and the rate of oxygen consumption at 30° was followed in a Gilson differential respirometer.
double mutation. This pleiotropic effect further supports the conclusion that cis,cis-muconate-lactonizing enzyme and muconolactone isomerase share regulatory linkage.

Induction in Strains with Blocks in Protocatechuate Pathway—
Nearly every wild type strain of *P. putida* can grow at the expense of both benzoate and p-hydroxybenzoate; however, an extensive nutritional survey of this species (6) revealed one strain, *P. putida* CI-A (ATCC 17452), which could grow at the expense of benzoate, but not of p-hydroxybenzoate. A mutant of *P. putida* A.3.12 with the same nutritional phenotype (strain A202) was also available. Both these strains, when grown with benzoate, synthesize normal levels of γ-carboxymuconolactone decarboxylase and β-ketoadipate enol-lactone hydrolase, but do not synthesize any detectable β-carboxy-cis,cis-muconate-lactonizing enzyme (Table IV). Their inductive responses to early members of the protocatechuate pathway are somewhat difficult to determine, since both p-hydroxybenzoate and protocatechuate are growth-inhibitory. However, both strains can grow slowly in a medium containing 10 mM glucose and 5 mM protocatechuate. Table V shows their inductive patterns after growth for three generations in this medium, compared to the inductive pattern of the wild type of *P. putida* A.3.12 grown under the same conditions. It is evident that both *P. putida* CI-A and mutant A202 can synthesize appreciable amounts of protocatechuate oxygenase; but the enzymes of the β-carboxy-cis,cis-muconate block are not induced. The growth-inhibitory effects of p-hydroxybenzoate and protocatechuate can probably be interpreted as the result of an intracellular accumulation of endogenously generated β-carboxy-cis,cis-muconate. This experiment shows that β-carboxy-cis,cis-muconate is not an inducer of the enzymes of the β-carboxy-cis,cis-muconate block, even though it is the primary substrate for this block of enzymes.

**Table IV**
Levels of enzymes of β-carboxy-cis,cis-muconate coordinate block in extracts of several strains of *P. putida* after growth at expense of 10 mM benzoate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Wild type</th>
<th>Mutant A202</th>
<th>Wild type</th>
<th>Mutant A202</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Carboxy-cis,cis-muconate-lactonizing enzyme</td>
<td>0.68</td>
<td>&lt;0.008</td>
<td>0.68</td>
<td>&lt;0.008</td>
</tr>
<tr>
<td>γ-Carboxymuconolactone decarboxylase</td>
<td>2.02</td>
<td>1.15</td>
<td>1.87</td>
<td></td>
</tr>
<tr>
<td>β-Ketoadipate enol-lactone hydrolase</td>
<td>1.03</td>
<td>0.78</td>
<td>0.91</td>
<td></td>
</tr>
</tbody>
</table>

**Table V**
Enzyme levels in extracts of several strains of *P. putida* after growth on 10 mM glucose in presence of β-ketoadipate

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Protocatechuate oxygenase</td>
<td>0.88</td>
</tr>
<tr>
<td>β-Carboxy-cis,cis-muconate-lactonizing enzyme</td>
<td>0.23</td>
</tr>
<tr>
<td>γ-Carboxymuconolactone decarboxylase</td>
<td>0.72</td>
</tr>
<tr>
<td>β-Ketoadipate enol-lactone hydrolase</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Inductive Patterns of Mutant A14—One of the induced mutants isolated from *P. putida* A.3.12, mutant A14, is unable to grow at the expense of either benzoate or p-hydroxybenzoate, although it can grow, with a generation time similar to that of the wild type, at the expense of β-ketoadipate. Growth of this mutant on β-ketoadipate results in full induction of β-carboxy-cis,cis-muconate-lactonizing enzyme and induction of γ-carboxymuconolactone decarboxylase to a level of about 10% of that characteristic of the wild type; no activity of enol-lactone hydrolase is detectable (Table VI). Mutant strain A14 has therefore undergone a pleiotropic mutation, in which the activity of one enzyme
shown elsewhere that growth of mutant A14 in the presence of muconate block under these conditions (Table VII). It has been shown that there is no induction of any enzymes of the muconate block at levels comparable to those of the wild type; but grown in the presence of benzoate, mutant A14 synthesizes the two enzymes of the muconate coordinate block, although these are substantially induced in wild type cells grown under the same conditions (Table VIII). However, it should be noted that mutant A14 has the normal basal level of β-carboxy-cis,cis-muconate-lactonizing enzyme, and is therefore capable, following induction with protocatechuate, of a slow endogenous synthesis of γ-carboxymuconolactone. The absence of induction of either β-carboxy-cis,cis-muconate-lactonizing enzyme or γ-carboxymuconolactone decarboxylase under these circumstances therefore indicates that γ-carboxymuconolactone is also unable to serve as an inducer for the enzymes of the β-carboxy-cis,cis-muconate coordinate block.

Several independent revertants of mutant A14, selected for the ability to grow at the expense of benzoate, all regained simultaneously the ability to grow at the expense of p-hydroxybenzoate. After growth with benzoate, these revertants contained γ-carboxymuconolactone decarboxylase at the normal wild type level. However, the level of β-ketoadipate enol-lactone hydrolase was in each case substantially lower than that characteristic of the wild type (Table IX). In the revertants, this enzyme is much less stable than the wild type enzyme, and its rapid loss of activity during extraction and assay probably explains the relatively low values shown in Table IX.

The experiments with mutants described in this and preceding sections indicate that none of the substrates for the enzymes of the β-carboxy-cis,cis-muconate coordinate block possesses inductive function. Since growth at the expense of β-ketoadipate elicits full induction of the block (Table I), the inducer is evidently normally produced through the action of the enzymes of the block. Since these enzymes are not induced in either succinate- or acetate-grown cells (Table I), either β-ketoadipate or β-ketoadipyl-CoA must be the intermediate which elicits induction.

**Preliminary Observations on Synthesis of Enzymes of Protocatechuate Pathway in Other Species of Bacteria**—Since many different bacteria can metabolize benzoate and p-hydroxybenzoate through β-ketoadipate, the enzymes of the catechol and protocatechuate pathways provide valuable material for the comparative analysis of regulatory mechanisms. We have examined the synthesis of β-carboxy-cis,cis-muconate-lactonizing enzyme, γ-carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase by four other species, in order to determine whether the coordinate control observed in *P. putida* is characteristic of bacteria.
flagellated bacterium and its DNA has a far different nutritional spectrum. M. wofii, similar to that of pseudomonads might be caused by endogenous function for \( \beta \)-ketoadipate cannot, however, be eliminated by growth with benzoate or p-hydroxybenzoate-grown cells. Alternatively, \( \beta \)-ketoadipate enol-lactone hydrolase is the exclusively metabolite common to the catechol, protocatechuate, and adipate pathways is \( \beta \)-ketoadipyl-CoA. Accordingly, the results shown in Tables X and XI suggest that \( \beta \)-ketoadipyl-CoA elicits the synthesis of the \( \beta \)-carboxy-cis,cis-muconate block of enzymes, and is possibly the sole inducer of them. An inductive function for \( \beta \)-ketoadipate cannot, however, be excluded; induction of the \( \beta \)-carboxy-cis,cis-muconate block in adipate-grown pseudomonads might be based on endogenous formation of \( \beta \)-ketoadipate, through hydrolysis of the thiol ester. The two other species examined, H. eutropha and M. lwolfi, are not closely related to P. aeruginosa, P. multivorans, and P. putida. H. eutropha, although possessing a DNA base composition similar to that of P. putida (7), is peritrichously flagellated, and has an entirely different nutritional spectrum. M. lwolfi is a non-flagellated bacterium and its DNA has a far lower content of guanine plus cytosine than either the pseudomonads or H. eutropha (7). As shown in Tables XII and XIII, the syntheses of \( \beta \)-carboxy-cis,cis-muconate-lactonizing enzyme, \( \gamma \)-carboxy-muconolactone decarboxylase, and \( \beta \)-ketoadipate enol-lactone hydrolase are not coordinately controlled in H. eutropha and M. lwolfi. All three enzymes are induced in these species as a result of growth with p-hydroxybenzoate, but only \( \beta \)-ketoadipate enol-lactone hydrolase activity is induced to a significant extent by growth with benzoate. Although the biochemistry of the catechol and protocatechuate pathways in M. lwolfi is identical with that in P. putida (1), these data show that the regulatory control of these pathways is entirely different in the two species.

### TABLE XII

**Influence of growth substrate on enzyme levels in H. eutropha**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Growth substrate</th>
<th>Succinate</th>
<th>Benzoate</th>
<th>( p )-Hydroxybenzoate</th>
<th>Adipate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )-Carboxy-cis,cis-muconate</td>
<td>0.005</td>
<td>0.002</td>
<td>0.61</td>
<td>&lt;0.002</td>
<td></td>
</tr>
<tr>
<td>( \beta )-Carboxy-cis,cis-muconate</td>
<td>0.01</td>
<td>0.01</td>
<td>1.34</td>
<td>&lt;0.005</td>
<td></td>
</tr>
<tr>
<td>( \beta )-Ketoadipate enol-lactone hydrolase</td>
<td>0.02</td>
<td>1.38</td>
<td>0.68</td>
<td>&lt;0.005</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE XIII

**Influence of growth substrate on enzyme levels in M. lwolfi**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Growth substrate</th>
<th>Succinate</th>
<th>Benzoate</th>
<th>( p )-Hydroxybenzoate</th>
<th>Adipate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )-Carboxy-cis,cis-muconate</td>
<td>0.04</td>
<td>0.15</td>
<td>1.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \beta )-Ketoadipate enol-lactone hydrolase</td>
<td>0.02</td>
<td>0.05</td>
<td>0.72</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Two other species of the genus *Pseudomonas*, *P. aeruginosa* and *P. multivorans*, were studied. Both differ from *P. putida* in many phenotypic respects (6), and the molar percentage of guanine plus cytosine in their DNA is also significantly different (7). As shown in Tables X and XI, \( \beta \)-carboxy-cis,cis-muconate-lactonizing enzyme, \( \gamma \)-carboxy-muconolactone decarboxylase, and \( \beta \)-ketoadipate enol-lactone hydrolase are induced to comparable levels in both species by growth at the expense of either benzoate or p-hydroxybenzoate. Both basal and induced levels of these enzymes are similar to those observed in *P. putida*. It is therefore probable that their synthesis is coordinately controlled in all three species, and elicited by the same metabolite inducer. *P. aeruginosa* and *P. multivorans* can grow on adipate, but *P. putida* cannot. As shown in Tables X and XI, adipate-grown cells of these two species contain levels of \( \beta \)-carboxy-cis,cis-muconate-lactonizing enzyme, \( \gamma \)-carboxy-muconolactone decarboxylase, and \( \beta \)-ketoadipate enol-lactone hydrolase comparable to those in benzoate or p-hydroxybenzoate-grown cells. Although the pathway of adipate dissimilation has not been determined, the only likely metabolite common to the catechol, protocatechuate, and adipate pathways is \( \beta \)-ketoadipyl-CoA. Accordingly, the results shown in Tables X and XI suggest that \( \beta \)-ketoadipyl-CoA elicits the synthesis of the \( \beta \)-carboxy-cis,cis-muconate block of enzymes, and is possibly the sole inducer of them. An inductive function for \( \beta \)-ketoadipate cannot, however, be excluded; induction of the \( \beta \)-carboxy-cis,cis-muconate block in adipate-grown pseudomonads might be induced by endogenous formation of \( \beta \)-ketoadipate, through hydrolysis of the thiol ester. The two other species examined, *H. eutropha* and *M. lwolfi*, are not closely related to *P. aeruginosa*, *P. multivorans*, and *P. putida*. *H. eutropha*, although possessing a DNA base composition similar to that of *P. putida* (7), is peritrichously flagellated, and has an entirely different nutritional spectrum. *M. lwolfi* is a non-flagellated bacterium and its DNA has a far lower content of guanine plus cytosine than either the pseudomonads or *H. eutropha* (7). As shown in Tables XII and XIII, the syntheses of \( \beta \)-carboxy-cis,cis-muconate-lactonizing enzyme, \( \gamma \)-carboxy-muconolactone decarboxylase, and \( \beta \)-ketoadipate enol-lactone hydrolase are not coordinately controlled in *H. eutropha* and *M. lwolfi*. All three enzymes are induced in these species as a result of growth with p-hydroxybenzoate, but only \( \beta \)-ketoadipate enol-lactone hydrolase activity is induced to a significant extent by growth with benzoate. Although the biochemistry of the catechol and protocatechuate pathways in *M. lwolfi* is identical with that in *P. putida* (1), these data show that the regulatory control of these pathways is entirely different in the two species.

### DISCUSSION

Conclusions concerning the regulation of the synthesis of enzymes of the catechol and protocatechuate pathways in *P. putida* are summarized in Fig. 4. These findings provide some insight into the sequence of inductive events that takes place when uninduced cells are exposed to these diphenolic compounds or form them endogenously from metabolic precursors.

Two successive inductive events permit the synthesis of the four enzymes that convert protocatechuate to \( \beta \)-ketoadipate; induction of protocatechuate oxygenase, and induction of the three enzymes of the \( \beta \)-carboxy-cis,cis-muconate coordinate block. The inducer of protocatechuate oxygenase has not been identified; it appears to be either the substrate or one of two subsequent intermediates in the protocatechuate pathway. This can be inferred from the fact that the synthesis of protocatechuate oxygenase is elicited by growth with protocatechuate, but not by growth with benzoate. The enzymes of the \( \beta \)-carboxy-cis,cis-muconate block, which convert \( \beta \)-carboxy-cis,cis-muconate to \( \beta \)-ketoadipate, are present in uninduced cells at about 2% of fully induced levels. Consequently, the product of protocatechuate oxygenase, \( \beta \)-carboxy-cis,cis-muconate, can be converted to \( \beta \)-ketoadipate without induction. Since \( \beta \)-ketoadipate or \( \beta \)-ketoadipyl-CoA elicits the synthesis of this coordinate block, \( \beta \)-ketoadipate formation triggers induction. The regulation of the synthesis of the enzymes that convert \( \beta \)-ketoadipate to succinate and acetyl-CoA has not been studied.

The synthesis of the enzymes that convert catechol to \( \beta \)-ketoadipate requires three separately controlled inductive events. The synthesis of catechol oxygenase is elicited by its product, cis,cis-muconate. There is no experimental evidence which excludes catechol itself as an inducer, but it seems improbable that a diphenolic compound and a dicarboxylic acid could act interchangeably as inducers. If cis,cis-muconate is in fact the sole inducer, the extremely low levels of catechol oxygenase in uninduced cells (less than 0.05% of the fully induced level) must be sufficient to permit an effective endogenous generation of inducer.

1. D. Davis, personal communication.
cis, cis-Muconate also elicits coordinate synthesis of the two enzymes that catalyze its conversion to \( \beta \)-ketoadipate enol-lactone. Thus, a single intermediate plays a dual inductive role in the catechol pathway; it accelerates the rate of synthesis of the enzyme that produces it, as well as the rates of synthesis of the enzymes that decompose it.

The metabolic product of the cis, cis-muconate coordinate block, \( \beta \)-ketoadipate enol-lactone, lies at the point of metabolic convergence with the protocatechuate pathway. Induction of the first enzyme common to the two pathways, \( \beta \)-ketoadipate enol-lactone hydrolase, can take place only after its substrate has been converted to \( \beta \)-ketoadipate, since this enzyme is a member of the \( \beta \)-carboxy-cis, cis-muconate coordinate block. The other two enzymes of the block, although they have no function in the catechol pathway, are induced in cells metabolizing catechol or one of its precursors.

Control mechanisms in two metabolic sequences convergent to catechol (Fig. 5) have also been studied in fluorescent pseudomonads (8-10). The syntheses of the five enzymes that convert \( \alpha \)-mandelate to benzoate are coordinate; either mandelate or the product of the second enzyme in the sequence, benzoylformate, can act as the inducer (8). A sequential inductive step occurs at the levels of benzoate (9). The syntheses of the three enzymes that convert \( \beta \)-tryptophan to anthranilate are induced by L-kynurenine, the product of the second enzyme of the pathway. The first and second enzymes of the \( \beta \)-tryptophan pathway (present at low levels in uninduced cells) mediate synthesis of the inducer when \( \beta \)-tryptophan is furnished exogenously. L-Kynurenine (like cis, cis-muconate) has a double inductive role; it acts as an inducer for the coordinate synthesis of the two enzymes that form it, as well as for synthesis of the enzyme that converts it to anthranilate and L-alanine. The enzyme that converts anthranilate to catechol is induced sequentially (10).

It is evident that many of the enzymes operative in the dissimilation of \( \alpha \)-mandelate and \( \beta \)-tryptophan pathways by fluorescent pseudomonads are coordinately induced. Synthesis of such coordinate blocks of enzymes requires a single inductive event. It is also clear that enzymes the synthesis of which is governed independently may be induced by the same metabolite. Hence two inductive events may be triggered simultaneously. At certain points in these catabolic pathways a sequential inductive step, defined as a shift in the nature of the inducer, occurs (Fig. 5).

Sequential inductive steps can occur at sites where a compound that is an intermediate in a catabolic pathway can also serve as
primary growth substrate; benzoate and anthranilate are examples of such compounds. Benzoate elicits the synthesis of the enzymes requisite for its utilization without inducing the enzymes that catalyze its formation from mandelate. Similarly, anthranilate induces only enzymes associated with its dissimilation. Sequential steps also occur at sites where intermediates enter a catabolic sequence from a convergent metabolic pathway. Catechol, for example, may be produced endogenously by the degradation of either benzoate or anthranilate. Sequential induction at the level of catechol permits the synthesis of the enzymes that degrade it without the synthesis of the enzymes that form it from the aromatic acids. Thus, sequential induction usually permits the utilization of a wide variety of carbon sources with minimal synthesis of nonfunctional enzymes. In this light, it is interesting to note that a sequential inductive step does not occur at the site of convergence of the catechol and protocatechuate pathways in P. putida. In order to metabolize further $\beta$-ketoadipate enol-lactone produced through the catechol pathway, cells must synthesize (as a consequence of regulatory linkage) two enzymes uniquely associated with the protocatechuate pathway.

One explanation for this bizarre regulatory mechanism is that $\beta$-carboxy-cis,cis-muconate, $\gamma$-carboxymuconolactone, and $\beta$-ketoadipate enol-lactone do not possess chemical structures which permit them to function as inducers. If this were the case, the synthesis of the enzymes that mediate their dissimilation would have to be induced either by a metabolic precursor or by a metabolic product. In P. putida, the enzymes are induced by their product, $\beta$-ketoadipate (or $\beta$-ketoacidyl-CoA). In contrast, the syntheses of $\beta$-carboxy-cis,cis-muconate-lactonizing enzyme and $\gamma$-carboxymuconolactone decarboxylase are not induced by these compounds in M. luofii. The syntheses of all four of the enzymes that convert protocatechuate to $\beta$-ketoadipate are coordinately regulated in M. luofii; protocatechuate is the probable inducer of the coordinate block. This control system does not provide a mechanism for the synthesis of $\beta$-ketoadipate enol-lactone hydrolase by cells utilizing catechol precursors. This difficulty is circumvented in M. luofii by the synthesis of an isofunctional enzyme, which is specifically induced by growth with benzoate. Hence, the control mechanisms in M. luofii permit strict economy of induced enzyme synthesis, but require two structural genes governing synthesis of isofunctional enzymes.

Pseudomonads are not the only bacteria in which some enzymes can be induced by their products rather than by their substrates. L-Histidine is degraded to L-glutamate by four enzymes in Aerobacter aerogenes; these enzymes are induced by the product of the first enzyme of the pathway, urocanase (11, 12). Since only two of the four enzymes are subject to coordinate control, this intermediate triggers three simultaneous inductive events (13). Two cases of product induction have been reported in Escherichia coli: $\alpha$-glycerophosphate induces glycerokinase (14); and $\beta$-galactosidase is induced only after it catalyzes the transformation of lactose to another $\beta$-galactoside (15). The occurrence of product induction in diverse bacterial systems indicates that it may have selective value.

Palleroni and Stanier (10) proposed that product induction of the enzymes of the L-tryptophan pathway by L-kynurenine prevents induction of the catabolic enzymes by endogenously synthesized L-tryptophan. This would be the case if the $K_m$ of the first enzyme of the catabolic pathway, L-tryptophan pyrrolase, were substantially higher than both the internal pool size of L-tryptophan and the $K_m$ of tryptophanyl ribonucleic acid synthetase. Schlesinger, Scoto, and Magasanik (12) concluded that the hypothesis of Palleroni and Stanier might provide a satisfactory explanation of their own observations on the regulatory mechanisms governing the synthesis of the enzymes that degrade L-histidine in A. aerogenes. The $K_m$ of histidinyl ribonucleic acid synthetase is far lower than the $K_m$ of histidine ammonia-lyase in this organism. Hence, the enzymes that degrade L-histidine are not synthesized in the absence of a relatively high concentration of exogenously supplied L-histidine (12).

The hypothesis of Palleroni and Stanier does not provide an interpretation for the other cases of product induction that have been reported. Catechol, for example, does not play a known biologically significant role in P. putida. There is no reason to believe that catechol is produced endogenously in the absence of its metabolic precursors. Some other factor must promote selective pressure in favor of product induction of catechol oxygenase. One such factor might be the economy of protein synthesis that results from the specific induction of enzymes that degrade compounds with structures related to catechol. Product induction of catechol oxygenase decreases the probability that the synthesis of this enzyme would be stimulated by chemical analogues of catechol because both catechol and cis,cis-muconate, widely disparate in chemical properties, must interact with biological systems of high specificity before increased synthesis of this enzyme is initiated. Even those diphenolic compounds that are cleaved by catechol oxygenase cannot elicit the synthesis of the enzyme unless their products are dicarboxylic acids with structures closely related to cis,cis-muconate.

Coordinate regulation in the catechol and protocatechuate pathways cannot yet be explored on the genetic level, since no mechanism of recombination has been discovered in P. putida. The enzymes of the cis,cis-muconate and $\beta$-carboxy-cis,cis-muconate coordinate blocks may be under the control of two complex operons; but the only available evidence bearing on this question, the properties of mutants, is difficult to interpret. The revertible mutant NG4 (Table III), which has lost simultane-
ously the ability to synthesize both enzymes of the cis,cis-muconate coordinate block, could be the consequence of a polarity mutation in one structural gene of a complex operon (16), but it is also explainable as the result of a mutation in a regulatory gene governing the functions of two separate operons (17). Mutant A14, which is impaired in the synthesis of enzymes of the $\beta$-carboxy-cis,cis-muconate coordinate block (Table V) provides stronger evidence for control by a complex operon. This revertible mutant makes no $\beta$-ketoacidipate enol-lactone hydrolase, but synthesizes $\gamma$-carboxymuconolactone decarboxylase at a low rate and $\beta$-carboxy-cis,cis-muconate-lactonizing enzyme at a normal rate. A pleiotropic effect of this nature cannot be explained by mutation in a regulatory gene, but is fully compatible with the assumption of a polarity mutation in one structural gene of a complex operon (18, 19). The properties of revertants (Table IX) point to the structural gene for $\beta$-ketoacidipate enol-lactone hydrolase as the site of the mutation: in all these revertants, the capacity to synthesize both affected enzymes is restored; but $\beta$-ketoacidipate enol-lactone hydrolase is far less stable than in the wild type. The reversion can therefore be interpreted as the consequence of a further amino acid substitution at (or

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1 J. L. Cánoves, personal communication.
near) the site originally changed by mutation in the β-ketoadipate enol-lactone hydrolase of the wild type.

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