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Synthesis of the Enzymes of the Mandelate Pathway by *Pseudomonas putida*

I. Synthesis of Enzymes by the Wild Type

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ABSTRACT

HEGEMAN, G. D. (University of California, Berkeley). Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of enzymes by the wild type. J. Bacteriol. **91**:1140–1154. 1966.—The control of synthesis of the five enzymes responsible for the conversion of D(−)-mandelate to benzoate by *Pseudomonas putida* was investigated. The first three compounds occurring in the pathway, D(−)-mandelate, L(+)-mandelate, and benzoylformate, are equipotent inducers of all five enzymes. A nonmetabolizable inducer, phenoxyacetate, also induces synthesis of these enzymes; but, unlike the metabolizable inducer-substrates, it does not elicit synthesis of enzymes that mediate steps in the pathway beyond benzoate. Under conditions of semigratuity, DL-mandelate elicits immediate synthesis at a steady rate of the first two enzymes of the pathway, but two enzymes which act below the level of benzoate are synthesized only after a considerable lag. Succinate and asparagine do not significantly repress the synthesis of the enzymes responsible for mandelate oxidation.

The remarkable variety of organic compounds that can serve as sole sources of carbon and energy for fluorescent pseudomonads was demonstrated by the nutritional studies of den Dooren de Jong (Thesis, Technische Hogeschool te Delft, Delft, The Netherlands, 1926). Biochemical investigations on this group of bacteria, begun some 20 years later, revealed that the attack on many organic substrates occurs through specific metabolic pathways of considerable biochemical complexity, and is typically mediated by inducible enzyme systems. The mechanisms for the oxidation of aromatic substrates have been subjected to particularly detailed biochemical analysis. Figure 1 shows the convergent pathways operative in the oxidation of L-tryptophan, D-mandelate, and *p*-hydroxybenzoate. A total of 20 specific enzymes, all inducible, are required to convert the benzenoid nucleus of these three primary substrates to succinate and acetylcoenzyme A (CoA).

Stanier (18), Suda, Hayaishi, and Oda (21), and Stanier and Sleeper (20) explored by manometric methods the patterns of induction elicited by compounds that are members of these pathways. In general, exposure to any given inducer-substrate induced cells for the immediate oxidation of later intermediates in that specific pathway; but if the inducer-substrate in question was a metabolic intermediate, it did not induce oxidation of earlier compounds in the reaction sequence. Cross-induction of sequences in parallel, convergent pathways was not observed. Such findings led Stanier (18), Suda, Hayaishi, and Oda (21), and Karlsson and Barker (12) to propose independently an explanation of the mechanism of these complex inductions, which is now known as the hypothesis of sequential induction. This hypothesis proposes that each inducer-substrate in such pathways triggers the specific synthesis of the enzyme responsible for its conversion to the next intermediate of the metabolic pathway; induction thus occurs in stepwise fashion, being metabolically (and presumably also temporally) sequential.

The specific evidence for sequentiality in any one of these pathways was relatively fragmentary, but the hypothesis was widely accepted at the time, mainly because there was no obvious counter-hypothesis. Since then, many examples of coordinate inductions have been discovered in other bacteria. Complete inductive coordinateness in the pathways of aromatic oxidation by fluorescent pseudomonads can be definitely eliminated on the basis of existing data. However, the

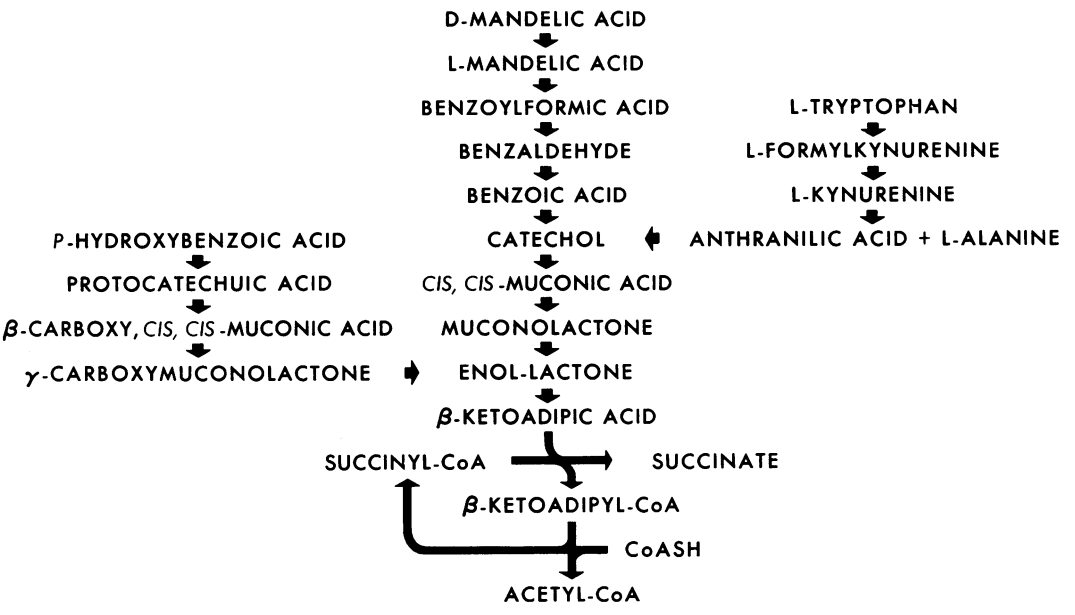


FIG. 1. *Convergent pathways for the oxidation of three aromatic compounds by Pseudomonas putida.*

possibility of a coordinate induction of small groups of enzymes, interspersed with sequential inductions, is by no means excluded. Indeed, the recent analysis by Palleroni and Stanier (16) of the synthesis of the early enzymes in the tryptophan pathway by *Pseudomonas fluorescens* Tr-23 showed the synthesis of tryptophan pyrrolase and formylkynurenine formamidase to be coordinate.

The work reported here was undertaken to determine the mechanism for control of synthesis of the enzymes of the mandelate pathway in *P. putida* A.3.12. The enzymology of this reaction sequence has been studied in some detail (8, 11, 15). It is a sequence of considerable complexity, a total of 12 specific inducible enzymes being required to convert D-mandelate to succinate and acetyl-CoA (Fig. 2). The first five enzymes, required to convert D-mandelate to benzoate, will be termed the *mandelate group*; those which convert catechol to β-ketoadipate will be termed the *catechol group*.

MATERIALS AND METHODS

Organism and methods of cultivation. P. putida, strain A.3.12 (ATCC 12633), was used as biological material. This strain has been previously designated as *P. fluorescens* in the studies of Stanier and others (8, 14, 15, 17–20).

All the media to be described below were prepared in a mineral base which contained (per liter): 200 mg of nitrilotriacetic acid, 580 mg of $MgSO_4 \cdot 7H_2O$, 67 mg of $CaCl_2 \cdot 2H_2O$, 0.2 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 2.0 mg of $FeSO_4 \cdot 7H_2O$, 1 ml of Hutner's "metals 44" (3), 1.0 g of $(NH_4)_2SO_4$, and 0.025 mole each of KH_2PO_4 and Na_2HPO_4. This provided all the known trace elements, nitrogen, sulfur, and phosphorus. The final *p*H was 6.85. In the kinetic experiments to be described, the concentration of phosphate buffer was doubled. For determination of viable count, checks on purity, and routine maintenance, 1.0% (w/v) yeast extract was used as carbon and energy source. Asparagine $\cdot \frac{1}{2}H_2O$ or Na_2-succinate $\cdot 6H_2O$ was used as the carbon and energy source to grow uninduced cells (i.e., cells not induced for the enzymes of the mandelate pathway). The normal concentration of these substances was 0.4% (w/v), but 1.0% (w/v) asparagine was used in the kinetic experiments where it was necessary to obtain dense populations. Aromatic acids (mandelic, benzoylformic, and most compounds screened for ability to act as nonmetabolizable inducers) were added to the basal medium as the sodium salts at a concentration of 0.01 M (about 0.2%, w/v) unless otherwise stated. Carbon and energy sources or inducers were sterilized separately and added to the culture aseptically before inoculation. Solid media were prepared by the addition of 1.0% (w/v) agar (Oxoid Ionagar No. 2) to the liquid medium. To prevent discoloration, the agar and mineral solutions were autoclaved separately, each at double strength, and were mixed before dispensing.

Cultures were incubated at 30 C on a rotary shaker. Growth was measured turbidimetrically, either in a spectrophotometer (Beckman model DU) at 680 mμ or in a colorimeter (Klett-Summerson) fitted with a no. 66 filter. Cultures were appropriately diluted for measurement if preliminary readings fell beyond the range of proportionality. Dry weight and cell count were indirectly determined by means of

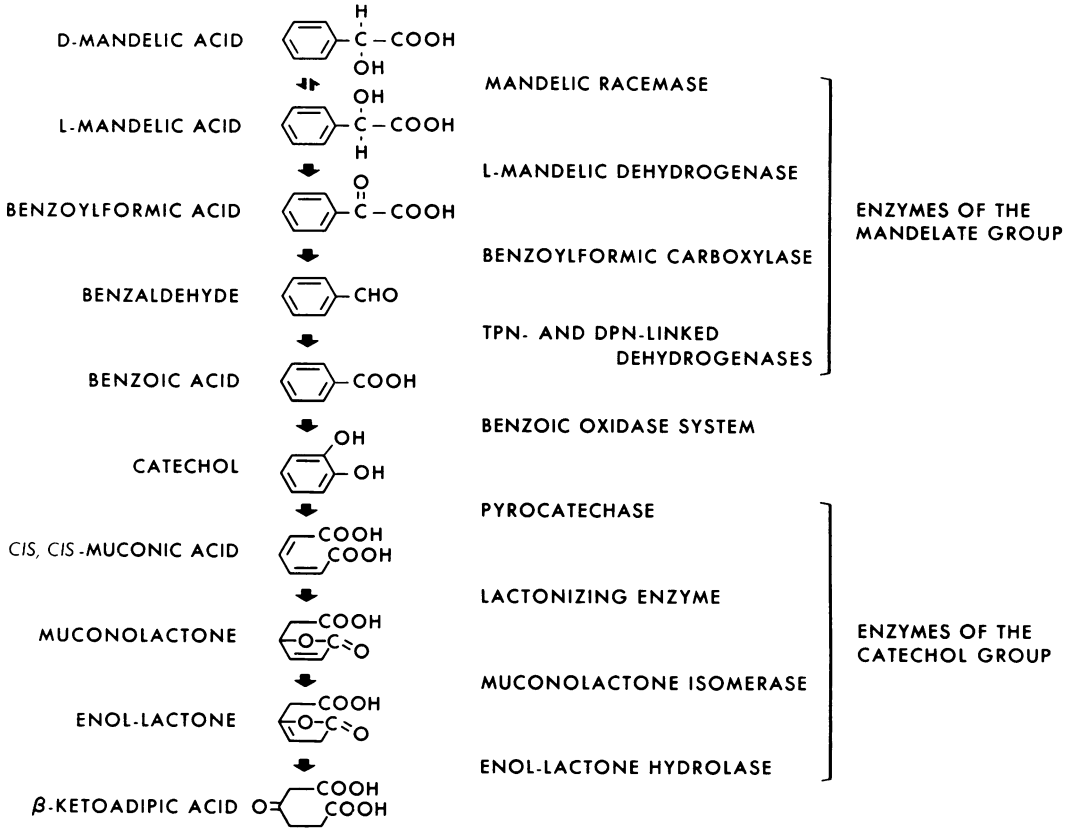


FIG. 2. *The mandelate pathway.*

previously prepared calibration curves relating these quantities to turbidity.

Enzymological methods and extraction. Cells were always harvested from exponentially growing cultures. Exploratory experiments showed that exhaustion of the carbon and energy source by a culture, even in the presence of adequate inducer, was followed by a rapid decline in the level of several inducible activities. Cells from cultures of small volume were harvested in a refrigerated centrifuge operated at 5,000 × *g* for 10 min. The pellets were washed in chilled 0.05 M phosphate buffer (Na_2HPO_4-KH_2PO_4, *p*H 6.8) and recentrifuged. The cells were then either immediately extracted, or stored as a paste at −40 C. In kinetic studies on induction, samples of cultures were poured over sufficient quantities of flake ice made from distilled water to lower the temperature of the suspension to 0 C almost immediately. The cells were subsequently harvested as described above. Large batches of cells, required for the purification of enzymes, were harvested in an unrefrigerated air-driven Sharples centrifuge and stored, without washing, in the frozen state.

Cells were resuspended for extraction in 0.05 M phosphate buffer (*p*H 6.8) at a density of 20 g of wet, packed cells in 100 ml of buffer, and extracts were prepared by sonic oscillation. With small samples of cells, a 20-kc, 60-w probe-type oscillator (Measuring and Scientific Equipment Co., Ltd., London, England) was used. Larger quantities of cells were extracted with 9- and 10-kc oscillators (Raytheon Manufacturing Co., Waltham, Mass.). Conditions were standardized by determining the time of exposure necessary to attain a 95% decrease in absorbancy of treated suspensions at 680 mμ. The volume of suspensions treated ranged from 2 to 50 ml. All extractions were carried out on samples jacketed at 0 C. No special precautions were taken to provide a reducing or inert atmosphere during extraction, since exploratory experiments showed that none of the enzymes studied was measurably sensitive to the presence of air.

After sonic treatment, the extract was centrifuged in the cold for 10 min at 5,000 × *g* to remove unbroken cells. A small portion of the supernatant fluid was set aside for protein determinations, and the rest was subjected to centrifugation at 38,000 rev/min for 60 min in the 40 head of a Spinco Model L preparative ultracentrifuge (ca. 100,000 × *g*). This treatment sediments the particulate components of the crude extract which contain reduced pyridine nucleotide oxidase, cytochromes, and L(+)-mandelate dehydrogenase (8). After decantation of the soluble fraction, the pellet and the walls of the tube were rinsed with chilled 0.05 M phosphate buffer (*p*H 6.8), and the

pellet was resuspended by syringe in a small amount of the same buffer. This suspension will be termed the *particle fraction.*

All enzyme preparations were assayed as soon as possible, usually within 24 hr after extraction, and were kept in closed tubes in ice until that time. Most of the soluble mandelate enzymes were quite stable, and could be frozen routinely during purification. However, L(+)-mandelate dehydrogenase, present in the particle fraction, was markedly inactivated by freezing. It was found that addition of 40% (v/v) ethylene glycol to particle preparations would permit storage at −40 C with no measurable loss of L(+)-mandelate dehydrogenase activity for several months.

In crude extracts, as well as the soluble and particle fractions derived from them, protein was determined by the biuret method (22), modified according to Pardee (*personal communication*). When the total amount of protein was small, the procedure of Lowry et al. (13) was employed. Bovine serum albumin, fraction V (Armour Pharmaceutical Co., Kankakee, Ill.), dried over silica gel, was used as a standard for both methods.

An essential precondition for the studies which were envisaged was the development of a series of rapid and sensitive spectrophotometric assays for as many of the enzymes of the mandelate pathway as possible. Such assays had already been developed for the benzaldehyde dehydrogenases (8) and the enzyme that lactonizes *cis-cis* muconate (17). In the course of this work, we developed spectrophotometric assays for mandelate racemase, L(+)-mandelate dehydrogenase, benzoylformate decarboxylase, and pyrocatechase. We also used the specific assays of Ornston and Stanier (15) for the two enzymes that convert (+)-muconolactone to β-ketoadipate. All five enzymes which function in the conversion of D(−)-mandelate to benzoate (the mandelate group) and all enzymes mediating the conversion of catechol to β-ketoadipate (the catechol group) were assayed spectrophotometrically. The enzyme system that converts benzoate to catechol does not lend itself to simple assay methods and is highly unstable (11). Accordingly, this enzyme was not studied. Also excluded from study were the enzymes that convert β-ketoadipate to acetyl and succinyl CoA.

All assays were performed on the soluble fraction (100,000 × *g* supernatant) except that for the L(+)-mandelate dehydrogenase, which is exclusively found in the particle fraction. Reactions were conducted in 1-cm path-length silica cuvettes containing a total liquid volume of 3 ml. Measurements were made in recording spectrophotometers (Gilford model 2000 or Cary model 14). The reaction temperature was 23 to 25 C in all cases.

In extracts of cells cultivated under the same conditions, the reproducibility of specific activities determined for most of the enzymes studied was about ±30%. The fluctuations are attributable in part to errors of measurement associated with the determinations of activity and protein, and in part to the variable degrees of enzyme inactivation which occurred during preparation, handling, and storage of extracts.

Spectrophotometric assays. L(+)-mandelate dehydrogenase [no International Union of Biochemistry Enzyme Commission number; systematic name: L(+)-mandelate: (acceptor) oxidoreductase]. The method of assay was suggested to us by W. C. Schuster. It is dependent on the reduction of an acceptor dye, 2,6-dichlorophenol-indophenol, to the leuco form concomitant with the oxidation of L(+)-mandelate to benzoylformate. The reaction was measured at 600 mμ, near the maximal absorbancy of the oxidized form of the dye. In addition to enzyme, the reaction mixture contained 200 μmoles of phosphate buffer (*p*H 7.0), 25 μmoles of sodium DL-mandelate, and 0.4 μmole of 2,6-dichlorophenol-indophenol. A reference cuvette contained all these components except the substrate. The typical curve for dye reduction as a function of time is sigmoid in form; the rate was estimated from the intermediate linear portion of the curve. The addition of 0.001 M NaCN increased the duration of linearity, but not the reaction rate. Cyanide was not used routinely. The reaction rate was proportional to enzyme concentration over a wide range, except at extremes of concentration. With a value of 20.6×10^6 for the molar absorbancy coefficient of the dye at 600 mμ and *p*H 7.0 (1), a decrease of 6.72 absorbancy units corresponds to the oxidation of 1 μmole of L(+)-mandelate to benzoylformate.

Mandelate racemase (I.U.B.E.C. No. 5.1.2.2, mandelate racemase). The assay depends upon the observation that preparations of washed particle fraction from mandelate-induced cells will oxidize only the L(+) isomer of mandelate, and that the D(−) isomer does not interfere. Accordingly, racemase activity may be determined by measuring the rate of dye reduction in a system containing D(−)-mandelate as substrate, together with an excess of washed particle fraction. The reaction mixture contained, in addition to enzyme, 200 μmoles of phosphate buffer (*p*H 7.0), 25 μmoles of D(−)-mandelate (Mann Research Laboratories, New York, N. Y.), a sufficient quantity of washed particle fraction to oxidize a saturating quantity of L(+)-mandelate at 20 times the maximal racemase rate measured, and 0.4 μmole of Na_2-2,6-dichlorophenol-indophenol. A reaction mixture complete except for substrate served as reference. Preparations of washed particle fraction were assayed for racemase in a separate control; any contaminating activity was subtracted from that measured. The D(−)-mandelate used as substrate was freed from contaminating L(+) isomer by treatment with washed particle fraction. As in the assay for L-mandelate dehydrogenase, to which the racemase reaction was coupled, a decrease of 6.72 absorbancy units at 600 mμ under the conditions of the assay corresponds to the conversion of 1 μmole of mandelate from the D(−) to the L(+) isomer.

Benzoylformate decarboxylase (E.C. No. 4.1.1.7, benzoylformate carboxy-lyase). The assay exploits the fact that the substrate, benzoylformate (Aldrich Chemical Co., Inc., Milwaukee, Wis.), has a molecular extinction coefficient of 81 at 334 mμ and *p*H 6.0, while benzaldehyde and subsequent products have negligible absorbancy at this wavelength. The reaction mixture contained, in addition to enzyme, 200

μmoles of phosphate buffer (*p*H 6.0), 25 μmoles of Na-benzoylformate, and 50 μg of thiamine pyrophosphate chloride. The reference cuvette contained no substrate. The course of the reaction was followed at 334 mμ. A decrease in absorbancy of 0.0272 units corresponds to the decarboxylation of 1 μmole of substrate.

Nicotinamide adenine dinucleotide (NAD)-benzaldehyde dehydrogenase (E.C. No. 1.2.1.6, benzaldehyde: NAD oxidoreductase) and *NAD phosphate (NADP)-benzaldehyde dehydrogenase* (E.C. No. 1.2.1.7, benzaldehyde: NADP oxidoreductase). The assay procedures for these two enzymes are presented together here, since the reactions are identical in all respects except for their specificity with respect to the pyridine dinucleotides. The procedures were modified from Gunsalus et al. (8). The assays measure the benzaldehyde-dependent reduction of NAD or NADP, followed at 340 mμ. Each cuvette contained 200 μmoles of tris(hydroxymethyl)aminomethane·HCl (Tris-HCl) at *p*H 8.0, 3 μmoles of redistilled benzaldehyde (stored under nitrogen), and 2.5 μmoles of the appropriate dinucleotide in the oxidized form (Sigma Chemical Co., St. Louis, Mo.). Substrate was omitted from the reference cell. An increase in absorbancy at 340 mμ of 2.07 units corresponds to the oxidation of 1 μmole of substrate to benzoate.

Pyrocatechase (E.C. No. 1.99.2.2, catechol 1,2-oxygenase). The assay was based on the observation of Sistrom and Stanier (17) that the subsequent enzyme of the pathway (the lactonizing enzyme) is completely inhibited by disodium ethylenediaminetetraacetate (EDTA). *Cis-cis* muconate, the product of pyrocatechase, will therefore accumulate in the presence of EDTA. Pyrocatechase itself is not sensitive to EDTA at the concentrations employed. The reaction was measured at 260 mμ, at which wavelength catechol absorbs weakly and *cis-cis* muconate absorbs strongly. The reaction mixture contained, besides enzyme, 4.0 μmoles of EDTA, 0.3 μmole of catechol, and 200 μmoles of phosphate buffer (*p*H 7.0). The conversion of 1 μmole of catechol to *cis-cis* muconate causes an increase in absorbancy at 260 mμ of 5.60 units.

Lactonizing enzyme [E.C. No. 5.5.1.1, (+)-4-carboxymethyl-4-hydroxyisocrotonolactone lyase (decyclizing)]. The assay used was essentially that of Sistrom and Stanier (17), with slight modification. The disappearance of *cis-cis* muconate determined at 260 mμ was used as a measure of reaction velocity, since the reaction products absorb only negligibly at this wavelength. The reaction mixture contained 200 μmoles of Tris-HCl (*p*H 8.0) and 0.3 μmole of *cis-cis* muconate, sodium salt, and 3.0 μmoles of $MnCl_2$. The disappearance of 1 μmole of *cis-cis* muconate was equivalent to a decrease of 5.75 absorbancy units under the conditions of the assay.

Delactonizing enzyme [E.C. No. 3.1.1.16, (+)-4-carboxymethyl-4-hydroxyisocrotonolactonase]. This enzyme system, first described by Sistrom and Stanier (17), was recently found to comprise two physically separable activities—muconolactone isomerase and enol-lactone hydrolase (15). Although the intermediate (the β-ketoadipate enol-lactone) has been crystallized, the two enzymes were assayed by coupling reactions, i.e., adding the complementary enzyme in excess and measuring the overall reaction according to Sistrom and Stanier (17).

Cultures of *P. putida* A.3.12 grown with *p*-hydroxybenzoate contain a high (induced) level of enol-lactone hydrolase, but an undetectable level of muconolactone isomerase. Accordingly, the muconolactone isomerase assay system contained excess enol-lactone hydrolase in the form of a rate-saturating quantity of extract prepared from wild-type *P. putida* A.3.12 grown on *p*-hydroxybenzoate. In addition, the system contained a measured limiting quantity of the extract to be assayed, 3.0 μmoles of the DL-lactone, and 100 μmoles of Tris-HCl (*p*H 8.0). A decrease of 0.476 absorbancy units at 230 mμ corresponds to the isomerization of 1 μmole of (+)-muconolactone.

The enol-lactone hydrolase was measured by the use of a mutant of strain A.3.12, incapable of producing detectable enol-lactone hydrolase, even when grown in the presence of compounds which serve to induce this enzyme in the wild type. Extracts of benzoate-induced cells of this mutant were added to the reaction mixtures in sufficient quantity to provide a rate-saturating concentration of enol-lactone. Known quantities of enol-lactone hydrolase could thus be assayed in the overall reaction described by Sistrom and Stanier (17). The conditions were otherwise the same as those described above for the assay of muconolactone isomerase.

Radiochemical assays. Assays for mandelate racemase, L(+)-mandelate dehydrogenase, and benzoylformate decarboxylase were devised to extend the sensitivity of measurement beyond that of the photometric assays. The compositions of the reaction mixtures for these radiochemical modifications were as described for the spectrophotometric assays, but the total reaction volumes were reduced to 0.25 ml. The substrates for the assays were D(−)- and DL-mandelate-carboxyl-C^{14} and benzoylformate-carboxyl-C^{14} of known specific activity. The reactions were carried out in 16 × 150 mm test tubes fitted with gas-tight serum caps, from which depended 1 × 3 cm strips of Whatman no. 1 filter paper wet with 25 μliters of 10 N NaOH. The reaction mixture was prepared separately; 0.25-ml portions were dispensed into a series of tubes, capped, and incubated at 25 C in a reciprocating water bath. Reactions were stopped at intervals by injecting through the cap a quantity of 5 N H_4SO_4 sufficient to bring the measured *p*H of the reactions to 0.5. The tubes were incubated for 30 min after addition of the acid, to ensure complete entrapment of CO_2 on the filter paper strips. The stoppers were then removed, and the papers were placed directly in scintillation-counter sample vials. The samples were counted as described below. Controls were prepared by omitting substrates and by omitting one or more enzymes. When several hours of incubation were necessary, a drop of toluene was added to the reaction mixture as a preservative. All activities were proportional to the amount of enzyme added within the limits of measuring error.

In the benzoylformate decarboxylase assay, the

rate of release of labeled CO_2 from the carboxyl-carbon of the substrate was taken directly as a measure of activity. From a knowledge of the specific activity of the labeled substrate and the efficiency of counting the trapped CO_2, the rate of the reaction could be calculated in millimicromoles of substrate decomposed per unit time.

Extracts of mutants, which lacked the enzymatic function to be measured, were prepared from cultures grown in the presence of inducer, and were added to the reaction mixtures when mandelate racemase and L(+)-mandelate dehydrogenase assays were performed on extracts of uninduced cells of the wild type. These extracts served as genetically resolved biological reagents and provided, in excess, the otherwise deficient activities of the mandelate group enzymes linking the measured reaction to the release of CO_2 from the carboxyl-carbon of benzoylformate. Mandelate racemase and L(+)-mandelate dehydrogenase activities were calculated as described above for the benzoylformate decarboxylase.

Chemicals and reagents. Organic compounds screened for the ability to act as nonmetabolizable inducers were obtained from a wide variety of commercial sources, or synthesized according to standard methods available in the literature. If these compounds failed to melt within a two-degree range which included the reported value, they were recrystallized from appropriate solvents until a satisfactory melting point was obtained.

Cis-cis muconic acid and DL-γ-carboxy-Δ^{α}-butenolide (DL-4-carboxymethyl-4-hydroxyisocrotonolactone) were synthesized according to Elvidge et al. (6).

DL-Mandelic acid-carboxyl-C^{14} was synthesized according to Fieser and Fieser (7) with the following modification: the potassium bisulfite addition product of benzaldehyde was isolated, purified, and added in excess to a small amount of sodium-C^{14}-cyanide. This improved the radiochemical yield and specific activity, since the formation of mandelonitrile has an equilibrium constant near unity. Benzoylformic acid carboxyl-C^{14} and D(−)-mandelic acid-carboxyl-C^{14} were prepared by treating DL-mandelate-carboxyl-C^{14} with thoroughly washed particle fraction prepared from cells of a mutant strain of *P. putida* A.3.12 unable to form racemase. This treatment converts the L(+)-mandelate to benzoylformate and leaves the D(−) isomer unchanged. The acids were extracted with ether from the acidified mixture and were separated by descending preparative chromatography on paper (Whatman 3MM) with the solvent of Williams and Kirby (23). The acids were located by radioautography (Kodak No-Screen X-ray film) and eluted with 0.1 M Na_2HPO_4. Identity and chemical purity were determined by R_F and ultraviolet-absorption spectra. Radiochemical purity was established for all three acids by ascending paper chromatography (Whatman no. 1) with ethyl alcohol-acetic acid-water (8:1:1), and by ascending chromatography on the same paper in the solvent of Williams and Kirby (23). The chromatograms were examined in a gas-flow scanner (Nuclear-Chicago Actigraph) fitted with a scaler and strip chart recorder. Activities were determined in a thin-window gas-flow counter (Nuclear-Chicago) at infinite thinness, or in a refrigerated scintillation spectrometer (Packard Tricarb) with the use of the dioxane-base scintillator of Bray (2). Counting efficiency was determined by use of a polystyrene-C^{14} standard or C^{14}-toluene of known activity. All samples were counted to a variance of less than 1%.

RESULTS AND DISCUSSION

Enzymatic constitution and inducers of the wild type of P. putida A.3.12—levels of enzymes of the mandelate pathway in induced and uninduced cells. A striking fact that emerged from early studies on the metabolism of aromatic acids by *P. putida* A.3.12 (18) was the apparently complete inability of cells grown in either yeast extract or mineral-asparagine medium to attack immediately the aromatic compounds upon which they could grow. The explanation then offered was that the necessary enzymes were inducible, and this conclusion was confirmed for some of the enzymes of the aromatic pathways by direct measurement of activity in cell-free extracts prepared from cells after growth in the presence or absence of inducer-substrates (8). However, none of these early studies accurately established either the absolute levels of activity of the mandelate pathway enzymes in fully induced cells, or their possible maximal basal levels in uninduced cells. This was, accordingly, the first aspect of the problem which had to be examined. Cells of *P. putida* A.3.12 were harvested during exponential growth, extracts were prepared, and the enzymes of the mandelate pathway were assayed individually by spectrophotometric means. The growth substrates employed as sole carbon sources were the two isomers of mandelate, the racemic mixture, benzoylformate, benzoate, and asparagine, the last-named being used to ascertain the basal levels of the enzymes in uninduced cells.

The racemic mixture and the individual isomers of mandelate are all equally effective as inducers (Table 1). In cells which have been grown on the L(+) isomer, the racemase is functionally superfluous. Thus, the L(+) isomer does serve to induce formation of racemase, an apparent exception to the principle of "sequential induction" as originally stated (4). An even more striking exception is the induction to full levels of both mandelate racemase and L(+)-mandelate dehydrogenase by benzoylformate. It could be argued, however, that benzoylformate may be reduced within the cell, giving rise to one or both isomers of mandelate, which in turn may act as the real inducers of these two enzymes. This interpretation can be ruled out on the basis of experiments described in a later paper (9). The ability of benzaldehyde to act as an inducer was

TABLE 1. *Levels of mandelate pathway enzymes found in extracts of wild-type Pseudomonas putida A.3.12 grown with the isomers of mandelate and benzoylformate as sole carbon and energy sources*[a]

Enzyme	Carbon and energy sources			
	DL-Mandelate[b]	L(+)-Mandelate[c]	D(−)-Mandelate[c]	Benzoylformate[b]
Mandelate racemase	400[d] (301–485)	412 —	364 —	337 (268–377)
L(+)-Mandelate dehydrogenase	522 (486–566)	710 —	668 —	552 (471–627)
Benzoylformate decarboxylase	603 (525–711)	625 —	585 —	595 (593–600)
NAD-benzaldehyde dehydrogenase	265 (243–287)	240 —	320 —	312 (234–351)
NADP-benzaldehyde dehydrogenase	152 (146–158)	140 —	160 —	178 (146–232)
Pyrocatechase	288 (271–305)	350 —	225 —	391 (272–310)
Lactonizing enzyme	485 (420–550)	530 —	420 —	443 (387–500)
Muconolactone isomerase	2700 (2420–2950)	— —	— —	2700 —
Enol-lactone hydrolase	1350 (1180–1520)	— —	— —	1265 —

[a] All activities are expressed as millimicromoles of substrate converted per minute per milligram of protein.
[b] Three extracts were assayed.
[c] One extract was assayed.
[d] Average values are given. Numbers in parentheses indicate range.

not investigated because of the toxicity of the compound and its tendency to undergo a rapid spontaneous oxidation in air.

As shown in Table 2, extracts of asparagine-grown (uninduced) cells contain levels of the nine enzymes of the mandelate pathway less than or equal to the reliable limits of the spectrophotometric assays. Benzoate-grown cells contain undetectable quantities of the five enzymes of the mandelate group, but have elevated levels of the catechol group activities. This finding is in accord with the earlier conclusions of Stanier (18), based upon manometric analysis.

Basal levels by radiochemical techniques. To establish the true magnitude of the response of the cell to inducer, it was necessary to determine the basal rate of synthesis of at least some of the mandelate group enzymes in the absence of inducer. Since noninduced cells have levels of activity of mandelate pathway enzymes so low that they are undetectable by usual spectrophotometric procedures, more refined measurements of the mandelate racemase, L(+)-mandelate dehydrogenase, and benzoylformate decarboxylase were performed on extracts of noninduced cells by use of radiochemical techniques (Table 3). Since the conditions of both spectrophotometric and radiochemical assays are identical, the values obtained are directly comparable. Extracts were prepared in the usual manner from cells grown in liquid asparagine-basal medium from an inoculum transferred several times on yeast extract-mineral agar.

Since specific activities reflect differential rates of synthesis, a comparison of the specific activities of Table 3 (noninduced) with those of Table 1 (induced) permits the calculation that growth of the wild type of *P. putida* A.3.12 in the presence of 0.01 M DL-mandelate increases the rates of synthesis of mandelate racemase, 2,000-fold, of L(+)-mandelate dehydrogenase 2,300-fold, and of benzoylformate decarboxylase at least 430-fold.

Observations on the absence of metabolite repression. Experiments on the kinetics of induction and the response of the wild type to nonmetabolizable inducers entailed the use of asparagine or succinate as principal or accessory carbon and energy sources. If asparagine or succinate repressed the synthesis of the enzymes of the mandelate pathway to any extent, the interpretation of many of these experiments would be

TABLE 2. *Levels of mandelate pathway enzymes found in extracts of wild-type Pseudomonas putida A.3.12 grown with asparagine and benzoate as sole carbon and energy sources*[a]

Enzyme	Cells grown on asparagine[b]	Per cent of level in mandelate-grown cells	Cells grown on benzoate[c]	Per cent of level in mandelate-grown cells
Mandelate racemase	≦0.15[d]	0.038	≦0.15	0.038
L(+)-Mandelate dehydrogenase	≦0.3	0.058	≦0.3	0.058
Benzoylformate decarboxylase	≦2.0	0.33	≦2.0	0.33
NAD-benzaldehyde dehydrogenase	≦0.5	0.21	≦0.5	0.21
NADP-benzaldehyde dehydrogenase	≦0.5	0.33	≦0.5	0.33
Pyrocatechase	≦0.05	0.17	360 (242–460)	125
Lactonizing enzyme	≦0.01	0.02	430 (371–490)	90
Muconolactone isomerase	≦10.0	0.37	2,360[e]	88
Enol-lactone hydrolase	≦10.0	0.74	1,190[e]	89

[a] All activities are expressed as millimicromoles of substrate converted per minute per milligram of protein.
[b] Four extracts were assayed.
[c] Two extracts were assayed.
[d] Average values are given. Numbers in parentheses indicate range.
[e] One extract was assayed.

difficult or impossible. Consequently, it was essential to determine whether any such repression existed, and, if so, what its magnitude might be.

Asparagine-grown cells were used to inoculate a series of five flasks of basal medium containing, as carbon and energy sources, asparagine alone, asparagine and DL-mandelate in a molar ratio of 3:1, 1:1, and 1:3, and DL-mandelate alone. The entire course of growth was followed turbidimetrically. In each case, the time at which mandelate began to disappear from the medium was determined by spectrophotometric analysis of samples of the culture supernatant fluid. The generation times of mandelate- and asparagine-grown cells differ only very slightly: 57 min for the former, 52 min for the latter (Fig. 3). In flasks furnished with mixtures of the two substrates, the observed generation times lay between those established for the two substrates alone, and in no case was a diauxic lag observed. Furthermore, the time at which mandelate began to disappear was essentially the same for all cultures which contained this compound. An analogous experiment in which succinate replaced asparagine yielded the same results. These facts all point very strongly to the absence of any marked repression of the enzymes of the mandelate pathway by asparagine or succinate. More direct evidence with respect to one such enzmye was obtained by harvesting samples of cells from each mandelate-containing culture during the course of exponential growth and prior to the exhaustion of

TABLE 3. *Specific activities of some mandelate group enzymes in asparagine-grown (noninduced) cells of wild-type Pseudomonas putida A.3.12 determined by radiochemical procedures*

Enzyme	Specific activity*	Per cent of fully induced wild-type cells†
Mandelate racemase	0.156	0.052
L(+)-Mandelate dehydrogenase	0.172	0.043
Benzoylformate decarboxylase	1.44	0.210

* Expressed as millimicromoles of substrate converted per minute per milligram of protein.
† Values taken as per cent of the mean activities for DL-mandelate-grown cells presented in Table 1.

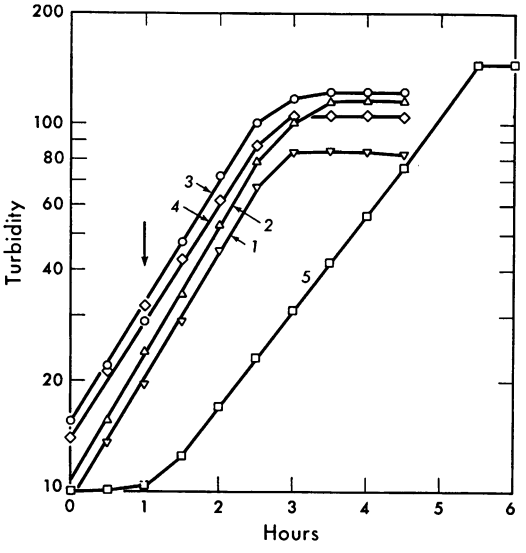


FIG. 3. *Course of growth of the five cultures described in the text: (1) 5 × 10⁻³ M asparagine; (2) 2.5 × 10⁻³ M asparagine and 2.5 × 10⁻³ M DL-mandelate; (3) 1.66 × 10⁻³ M asparagine and 3.33 × 10⁻³ M DL-mandelate; (4) 3.33 × 10⁻³ M asparagine and 1.66 × 10⁻³ M DL-mandelate; (5) 5 × 10⁻³ M DL-mandelate, as carbon and energy sources in basal medium, respectively. The arrow indicates the time at which mandelate begins to disappear from the cultures.*

mandelate, and determining the specific activity of benzoylformate decarboxylase on extracts prepared therefrom. As shown in Table 4, cells of these four cultures contain essentially identical (±20%) specific activities of benzoylformate decarboxylase.

These results, and those of the mixed-substrate growth experiment, indicate that the repressive effects of accessory carbon sources on synthesis of the mandelate pathway enzymes observed by Mandelstam and Jacoby (14) could be neglected in interpreting the results reported here.

Molecular specificity of induction of L(+)-mandelate dehydrogenase. A number of analogues of mandelic acid and benzoylformic acid were collected and screened for the ability to induce early enzymes of the mandelate pathway. The large number of determinations involved were performed by making use of the qualitative assay procedure for L(+)-mandelate dehydrogenase (screening test) described in connection with the isolation of constitutive mutants (10). The wild type of *P. putida* A.3.12 was grown as patches on plates of mineral-succinate agar to which the analogues had been added. After growth had occurred, the patches were tested for L(+)-mandelate dehydrogenase activity. Analogues which gave indication of positive results in this test were

TABLE 4. *Specific activity of benzoylformate decarboxylase in extracts of cells grown in the presence of mixtures of asparagine and DL-mandelate*

Carbon and energy source	Specific activity of benzoylformate decarboxylase*
Asparagine alone	≦2
Asparagine and DL-mandelate, 1:1	630
Asparagine and DL-mandelate, 1:2	570
Asparagine and DL-mandelate, 2:1	623
DL-Mandelate alone	585

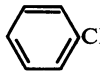
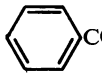
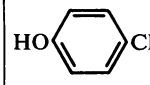
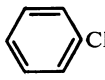
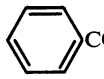
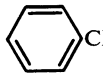
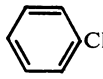
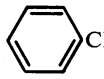
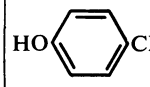
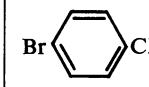
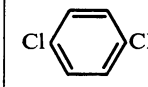
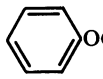
* Expressed as millimicromoles of substrate converted per minute per milligram of protein.

subsequently retested for inductive function by a more precise method. Each such compound was added to a culture in succinate-basal medium, the cells were harvested after growth had occurred, extracts were prepared, and the levels of L(+)-mandelate dehydrogenase were measured by spectrophotometric assay.

Any compounds which proved to be effective as inducers under these conditions were then carefully examined for their ability to be metabolized. Criteria for nonmetabolizability included: (i) lack of stimulation of oxygen uptake upon addition to suspensions of cells grown in the presence of the compound; (ii) lack of qualitative or quantitative change of ultraviolet spectra of culture supernatant fluids after prolonged cultivation in the presence of the compound; and in some cases (iii) quantitative re-extraction and spectrophotometric purity determination after addition to liquid cultures and prolonged incubation of the compound in the presence of mandelate-grown cells.

Among the compounds which proved to be inducers (Table 5), D(−)- and L(+)-mandelate, benzoylformate, and DL-*p*-hydroxymandelate are known to be metabolized through the action of the enzymes of the mandelate group (8). DL-Phenyl-1,2-ethanediol and phenylglyoxal are probably precursors of mandelate and metabolized via the mandelate pathway, since they support the growth of *P. putida* A.3.12, and cells grown at the expense of DL-phenyl-1,2-ethanediol have the fully induced complement of mandelate pathway activities. DL-Mandelamide, methyl and ethyl DL-mandelate, and ethyl *m*-hydroxy-DL-mandelate will all support growth. The first three of these compounds probably give rise to DL-mandelate through hydrolysis; the mode of metabolism of ethyl *m*-hydroxy-DL-mandelate is unknown, but probably also entails hydrolysis. *p*-Chloro and *p*-bromo-DL-mandelate do not sup-

TABLE 5. *Compounds effective as inducers*

Name of compound	Structural formula	Metabolized by enzymes of the mandelate group	Utilized for growth	Metabolized without growth
D- and L-mandelate		+	+	ND*
Benzoylformate		+	+	ND
p-Hydroxy DL-mandelate		+	+	ND
DL-Phenyl 1,2-ethanediol		+	+	ND
Phenylglyoxal		+	+	ND
DL-Mandelamide		+	+	ND
Methyl-DL-mandelate		+	+	ND
Ethyl-DL-mandelate		+	+	ND
Ethyl *meta* hydroxy DL-mandelate		+	±	+
p-Chloro-DL-mandelate		+	−	+
DL-*p*-bromo-mandelate		+	−	+
Thiophenyl acetate		−	−	−(spectra)
Phenoxy-acetate		−	−	−(spectra, extraction and O_2 uptake)

* Not determined.

port growth. Oxygen consumption by induced cells of *P. putida* A.3.12 at the expense of these two compounds indicates that they are metabolized via the mandelate group enzymes to the level of benzoate. The inability of these halogen-substituted analogues to support growth probably reflects the unacceptability of the corresponding benzoic acids as substrates for the benzoate oxidase system (11).

Neither thiophenylacetate nor phenoxyacetate are metabolized, but they function as inducers. Thiophenylacetate was toxic and seemed a poorer inducer than phenoxyacetate at equivalent concentrations. Both these compounds, it should be noted, have an electronegative center in approximately the same position as that which is created by the carbonyl or hydroxyl functions of benzoylformate and mandelate, respectively. They also share the intact benzene ring and carboxyl function with these compounds.

The majority of the noninducing compounds did not support growth (Table 6), but some ex-

TABLE 6. *Compounds ineffective as inducers*

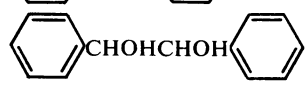
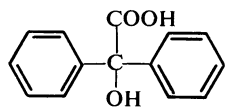
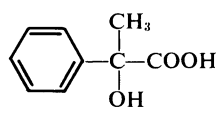
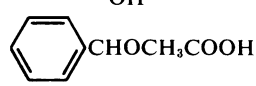
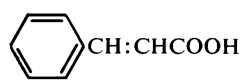
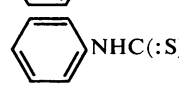
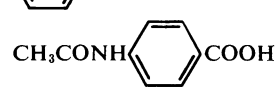
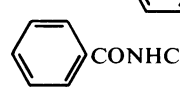
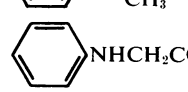
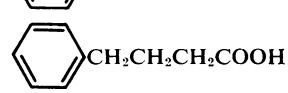
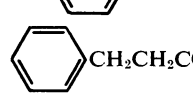
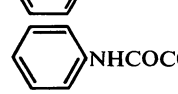
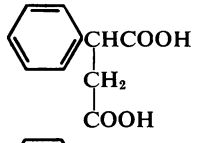
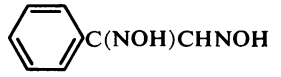
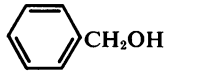
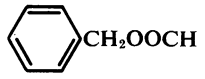
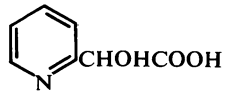
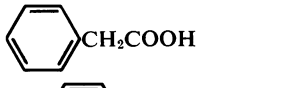
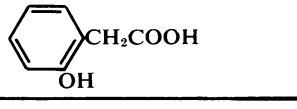
Name of compound	Structural formula	Utilization for growth
Benzil		–
Benzoin		–
Meso dihydro benzoin		–
DL-Benzilate		–
DL-Atrolactate		–
DL-1-Methoxy phenylacetate		–
Trans cinnamate		–
Phenyl thiourea		– (toxic)
N-phenyl maleamate		– (toxic)
p-Acetimidobenzoate		–
Hippurate		+
Benzoyl-β-alanine		+
N-benzyl DL-alanine		±
N-phenyl glycine		±
1-Phenyl butyrate		+
p-Hydroxyphenyl-1-butyrate		+
1-Phenyl, -3-ketobutyrate		+
Oxanilate		–

TABLE 6.—*Continued*

Name of compound	Structural formula	Utilization for growth
2-Phenyl succinate		±
Phenylglyoxal dioxime		–
Benzyl alcohol		+
Benzylformate		ND*
DL-1-Methyl benzyl alcohol		– (toxic)
Acetophenone		– (toxic)
2-Hydroxyacetophenone		– (toxic)
Pyridine-2-DL-glycollate		ND
Thiophene-2-DL-glycollate		– (toxic)
DL-phenylalanine		+
3-Phenylpyruvate		+
3-Phenyl-DL-lactate		+
Phenylacetate		+
p-Hydroxyphenylacetate		ND
o-Hydroxyphenylacetate		ND

* Not determined.

ceptions should be noted. Hippurate and benzoyl-β-alanine are presumably hydrolyzed to yield benzoate and the corresponding amino acids, all capable of supporting growth of *P. putida* A.3.12. Benzyl alcohol is also probably metabolized through benzoate and β-ketoadipate. Cells of *P. putida* A.3.12 grown on benzyl alcohol have fully induced levels of the enzymes of the catechol group, but undetectable levels of the mandelate group enzymes. The mechanism of the conversion

of benzyl alcohol to benzoate is unknown. The remaining metabolizable aromatic compounds which do not serve as inducers are presumably converted to aliphatic intermediates via the homogentisate pathway. Phenylbutyrate, *p*-hydroxyphenylbutyrate, and phenyl-3-ketobutyrate probably undergo oxidation to *p*-hydroxyphenylacetate and phenylacetate. DL-Phenylalanine and 3-phenyl-DL-lactate probably undergo deamination or oxidation to yield 3-phenylpyruvate, and subsequently phenylacetate (5). Most aromatic compounds which were metabolized through phenylacetate and homogentisate shared the peculiar property of inducing a low level of the enzymes of the mandelate group in cells grown on solid media, although they failed to do so in liquid cultures. The phenomenon may explain the finding of Stanier that cells grown on phenylacetate plates can immediately oxidize DL-mandelate to the level of benzoate (19).

Substitution of the aromatic ring of the inducer molecule seems to have little effect on function (*p*-chloro, *p*-bromo, *m*- and *p*-hydroxymandelate all induce). On the other hand, the pyridine and thiophene analogues of mandelate which were tested do not induce. Apparently the nature of the aromatic ring is important although substitution of the ring does not abolish function. The requirement for an intact carboxyl group is hard to assess from these data, since nearly all analogues tested in which this moiety alone was modified probably gave rise to mandelate through hydrolytic or oxidative attack (ethyl and methyl esters of DL-mandelate, mandelamide, DL-phenyl-1,2-ethanediol, and phenylglyoxal) or were otherwise metabolized (benzyl alcohol). However, compounds analogous to mandelate or benzoylformate but with the carboxyl group replaced by methyl or hydroxymethyl functions did not induce (DL-1-methyl benzyl alcohol, acetophenone, and 2-hydroxyacetophenone). There seems to be a stringent requirement for an electronegative function in or on the side chain of the inducing molecule. This may be a carbonyl or hydroxyl group (benzoylformate or mandelate) or even thioether or oxygen ether function (phenylthioacetate and phenoxyacetate). The presence of an ether oxygen not linked to the benzene ring, as in 1-methoxy phenylacetate, will not permit induction; however, compounds with methyl or larger groups projecting from the side chain (DL-atrolactate and DL-benzilate) do not induce either. The steric configuration of the oxygen-bearing function is apparently not important, since D(−)-mandelate and DL-mandelate are essentially equipotent inducers, as will be shown later (9).

The length and proximity of the groups upon the side chain are probably not too critical, since 3-phenyl-DL-lactic acid and 3-phenyl pyruvate are capable of causing some derepression. 3-Keto-1-phenylbutyrate does not induce, indicating that one but not two methylene groups may be interposed between the aromatic nucleus and the oxygen-bearing carbon of the side chain before inducer function is abolished. Since the three compounds upon which this argument is based are all metabolized, any conclusion on this point is tenuous.

In summary, the molecular specificity for the induction of the early mandelate enzymes is quite high. Although 36 structural analogues of mandelate and benzoylformate not metabolized via mandelate or benzoylformate were tested, only one, phenoxyacetate (Table 5), gave fair derepression in liquid medium.

Enzymes evoked by phenoxyacetate. Strain A.3.12 was grown in basal-asparagine medium containing 0.01 M sodium phenoxyacetate; the cells were harvested and extracted, and the extracts were assayed for the enzymes of the mandelate pathway. The five enzymes of the mandelate group are all induced, and within the limits of experimental error they are coordinate (Table 7). On an equimolar basis, phenoxyacetate was only 10 to 20% as effective as the natural metabolizable inducers. Pyrocatechase and the lactonizing enzyme are not induced. Thus, it seems that the biosynthesis of the mandelate pathway enzymes is coordinate in part, the first five member-enzymes of the pathway constituting a regulatory unit.

Kinetics of induction. Investigation of the regulation of synthesis of the enzymes for tryptophan oxidation in *P. fluorescens* Tr-23 by Palleroni and Stanier (16) showed that enzymes synthesized in response to a common inducer may be recognized and distinguished from separately controlled enzymes in the same pathway on the basis of the kinetics of their synthesis. Accordingly, a study of the kinetics of synthesis of the mandelate pathway enzymes in *P. putida* A.3.12 was undertaken.

DL-Mandelate was added to a culture growing exponentially in asparagine-basal medium, to provide a final concentration of 0.01 M. Samples were removed at 20-min intervals, and the cells were harvested and extracted. Each extract was assayed for mandelate racemase and L(+)-mandelate dehydrogenase in the mandelate group, and for lactonizing enzyme and muconolactone isomerase in the catechol group. The specific activities of the four enzymes are plotted against time elapsed after addition of inducer in Fig. 4.

The two enzymes of the mandelate group appear almost immediately, but measurable synthesis of members of the catechol group occurs only 40 to 60 min after the addition of mandelate to the culture. The mandelate group enzymes

TABLE 7. *Activities of mandelate pathway enzymes in extracts of cells grown in the presence of 0.01* M *phenoxyacetate**

Enzyme	Specific activity†	Per cent of mandelate-grown wild-type cells‡
Mandelate racemase	45 ± 10.5	11.2 ± 3.3
L(+)-Mandelate dehydrogenase	102 ± 25	20.0 ± 6.0
Benzoylformate decarboxylate	67 ± 20	12.8 ± 3.8
NAD-benzaldehyde dehydrogenase	56 ± 17	21.0 ± 6.3
NADP-benzaldehyde dehydrogenase	32 ± 10	21.0 ± 6.3
Pyrocatechase	≦0.05 (undetectable)	0.016
Lactonizing enzyme	≦0.01 (undetectable)	0.018

* The mean of values from three separate experiments.

† Expressed as millimicromoles of substrate converted per minute per milligram of protein.

‡ The specific activities as per cent of the mean values of those for extracts of DL-mandelate-grown cells presented in Table 1.

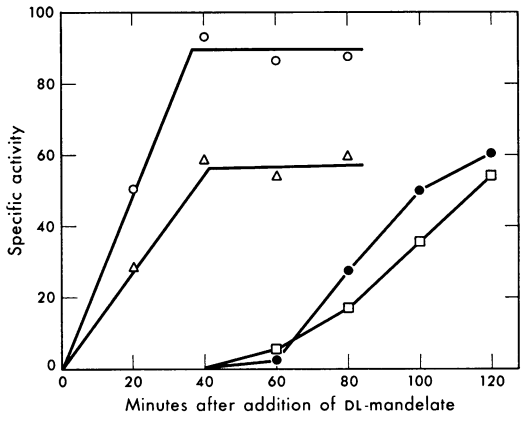


FIG. 4. *Time course of appearance of four enzymes of the mandelate pathway after addition of* DL-*mandelate to a culture of Pseudomonas putida strain A.3.12 growing in asparagine-basal medium. Specific activities are expressed as millimicromoles per minute per milligram of extract protein except for muconolactone isomerase where the units are 10^{-4} μmoles per min per mg of protein. Symbols:* □, *muconolactone isomerase;* ○, *mandelic racemase;* △, L-*mandelic dehydrogenase;* ●, *lactonizing enzyme.*

reach a nearly constant specific activity in the culture at 40 min; the two enzymes of the catechol group continue to increase in activity for 2 hr after the addition of mandelate.

The absence of autocatalytic kinetics in the appearance of the mandelate group activities implies that an inducible concentrating permease for mandelate is not present in *P. putida* A.3.12.

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