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Regulation of Pathways Degrading Aromatic Substrates in *Pseudomonas putida*

ENZYMIC RESPONSE TO BINARY MIXTURES OF SUBSTRATES

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1. Induction constants (K_{ind}) and repression constants (K_{rep}), which are a measure of the affinity of the inducers or repressors for the induction systems, were measured for mandelate, benzoate and *p*-hydroxybenzoate in *Pseudomonas putida*. 2. From these results, the enzymic response of the organism to media containing pairs of these substrates was predicted. Nitrogen-limited chemostats, operated at high growth rates, were used to investigate these predictions in cells grown first on one aromatic substrate with the second added later. 3. In general, the values of K_{ind} and K_{rep} predicted quite accurately the response to substrate mixtures. Thus, in the presence of mandelate and either benzoate or *p*-hydroxybenzoate, the enzymes of mandelate metabolism were repressed almost completely, and the bacteria were fully induced for the alternative substrate (benzoate or *p*-hydroxybenzoate), which was preferentially utilized for growth. When benzoate and *p*-hydroxybenzoate were the two substrates in the mixture, the enzymes for metabolism of the latter were strongly repressed and growth took place mainly on benzoate. 4. The enzymic response to mixed substrates did not result in the metabolism of the better growth substrate, but in the substrate requiring the synthesis of fewer enzymes. Thus benzoate is used in preference to mandelate although the latter supports a faster growth rate. It is nevertheless considered that, with our present knowledge of the natural habitat of the organism, it is impossible to decide whether protein economy or growth rate was the factor determining the evolution of this control system.

Pseudomonas putida can utilize many aromatic substrates as sole carbon and energy sources (den Dooren de Jong, 1926; Stanier *et al.*, 1966). Mandelate, benzoate and *p*-hydroxybenzoate are metabolized to succinate and acetate by parallel pathways that converge at β-oxoadipate enol lactone (Scheme 1) (Ornston & Stanier, 1964). The induction of the enzymes of the pathways is complex (Scheme 1) (Stanier *et al.*, 1965; Hegeman, 1966*a*,*b*,*c*; Ornston, 1966; Hosokawa, 1970). Mandelate racemase (E1), mandelate dehydrogenase (E2), benzoylformate decarboxylase (E3) and benzaldeyhde dehydrogenase (E4) convert DL-mandelate into benzoate and form a co-ordinate group induced by DL-mandelate. Further metabolism of benzoate, via catechol, requires the sequential induction of benzoate oxidase (E5) and pyrocatechase (E6). Metabolism of *p*-hydroxybenzoate involves the induction of *p*-hydroxybenzoate hydroxylase (E7) and protocatechuate oxygenase (E8). Each induction group, or regulon (Maas & Clark, 1964), is subject to repression by its immediate end-product and by the products of succeeding regulons (Mandelstam & Jacoby, 1965; Stevenson & Mandelstam, 1965) (Scheme 2). In each regulon repression is additive and can be reversed, at least in part, at higher inducer concentrations (Mandelstam & Jacoby, 1965).

This pattern of regulation implies that the enzymic response of the organism to mixtures of aromatic substrates should be selective. Although in this system the mechanisms of induction and repression are not known in detail, it can be assumed that for induction to occur the inducer must interact with another molecule, possibly an apo-repressor. If we also assume that this interaction has the form of an adsorption isotherm, an induction constant (K_{ind}) can be defined as the inducer concentration that supports half the maximum induction rate. Similarly, the repressor can also be assumed to interact at some site and this interaction will also have a half-saturation value, the repression constant (K_{rep}). If these constants are known for the components of a binary substrate mixture, and if the affinities they measure are indeed the factors that determine enzyme induction, it should be possible to predict the

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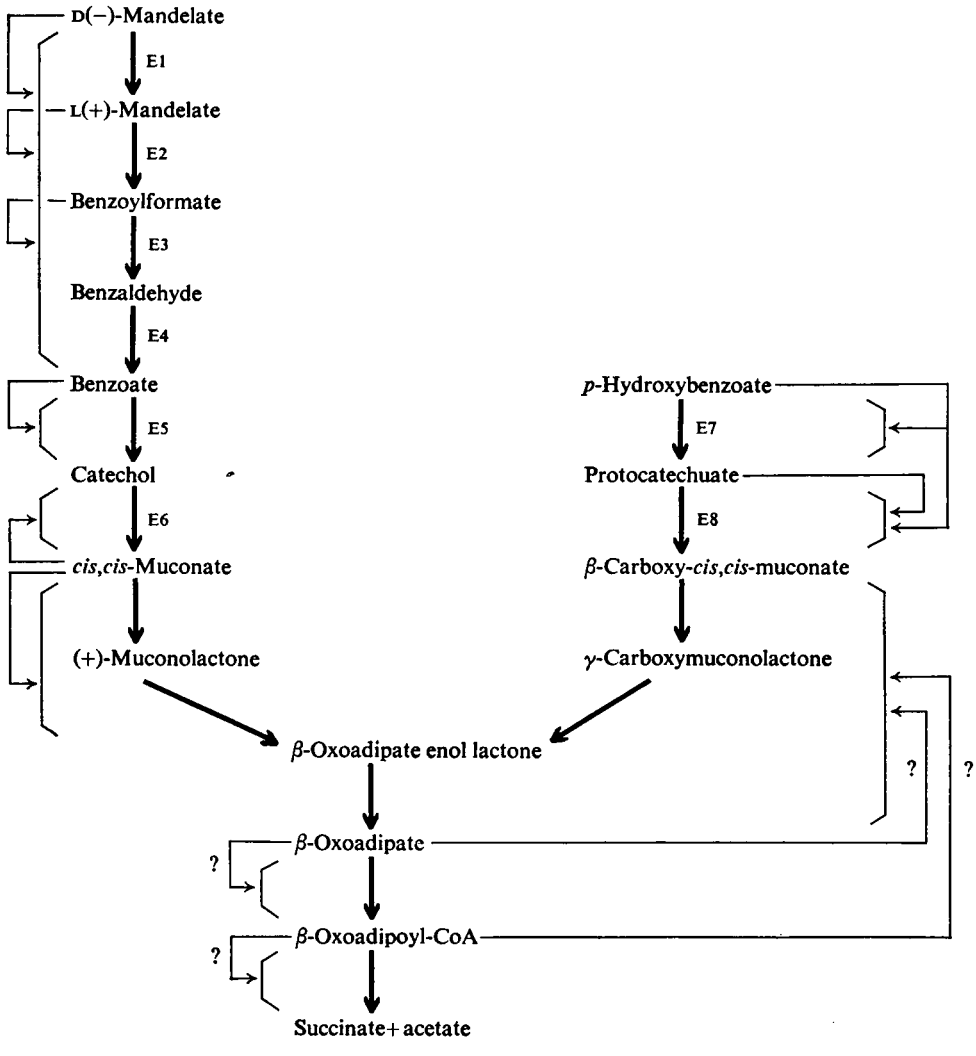


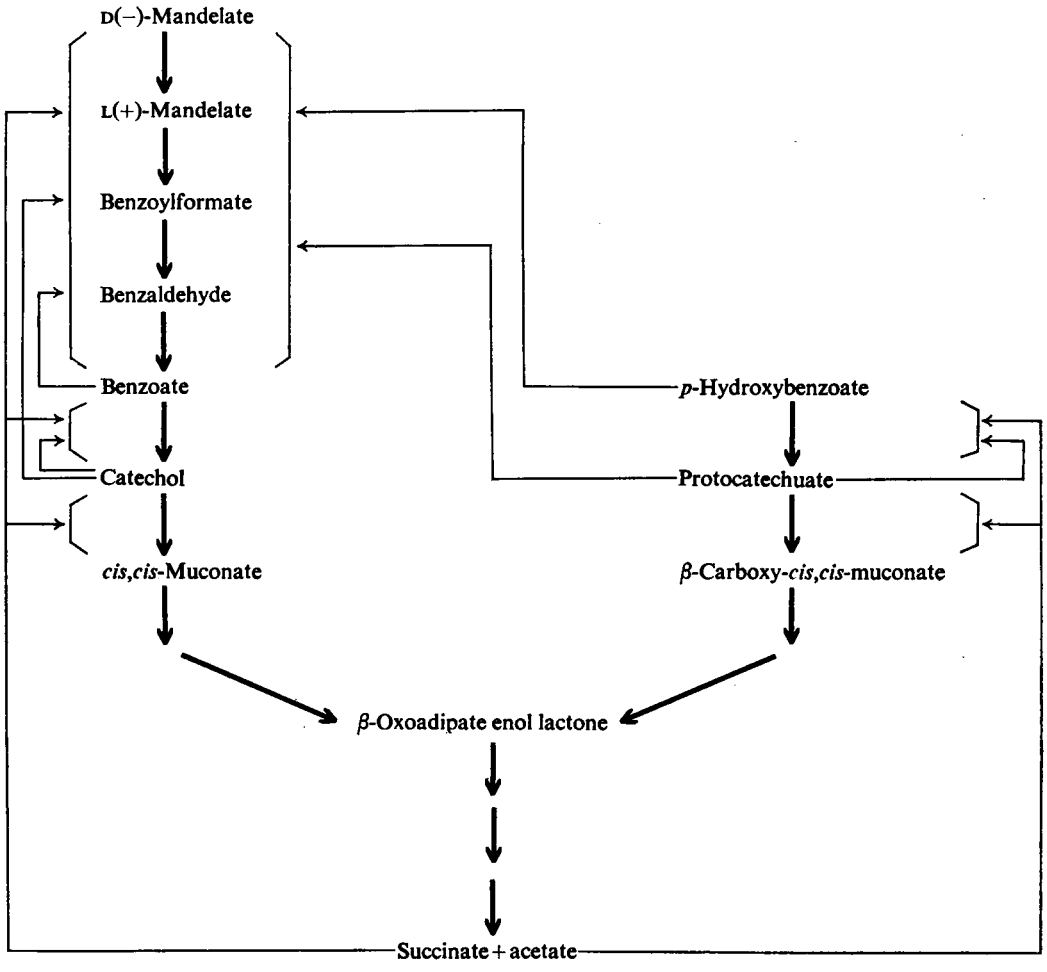
Scheme 1. *Degradation of aromatic substrates by Pseudomonas putida*

Degradation is indicated by bold arrows, induction of the enzymes is indicated by the light arrows and the induction groups (regulons) by the brackets.

enzymic response of the organism to such a medium.

This paper has two parts. The first deals with the measurement of induction and repression constants for the aromatic system and the predictions that can be made from them. The second describes the enzymic response of the organism to media each containing two aromatic carbon sources. The three substrates chosen, mandelate, benzoate and *p*-hydroxybenzoate, were used in pairs and this allowed us to make predictions about substrates that enter different pathways or that enter the same pathway but at different levels. By studying the change in enzymic composition of cells grown first on one aromatic substrate and then presented with another alternative substrate, it was possible to see whether or not the past history of the cells influenced the response, i.e. whether the 'resident' enzyme pathway had a selective advantage over a 'competing' pathway.

Primarily, this study was concerned with the enzymic response of cells presented with alternative substrates. When both enzyme systems are predicted to be present, the relative rates of substrate utilization



Scheme 2. *'Multi-sensitive' repression of the enzymes concerned in the degradation of aromatic substrates in Pseudomonas putida*

The regulons are indicated by brackets and the light arrows show which of the intermediates of the pathways exert repressor effects on those regulons (after Stevenson & Mandelstam, 1965). Bold arrows show the degradation pathways.

cannot be predicted without additional kinetic data. Only where one of the enzyme systems is predicted to be completely repressed can the overall substrate utilization be inferred from the K_{Ind} and K_{rep} values.

Methods

Organism and mutants

Pseudomonas putida biotype A, strain 90 (Stanier *et al.*, 1966), A.T.C.C. 12633, previously known as *P. putida* A3.12 (Ornston & Stanier, 1966), was used. Mutants defective in one or more of the enzymes of the aromatic pathways were those isolated from the parent strain by Mandelstam & Jacoby (1965) and Stevenson & Mandelstam (1965). No growth (for at least 24h) was observed on the relevant substrate and the activity of the defective enzyme was undetectable. The mutants used in this study are designated *md*$^-$ (mandelate dehydrogenase negative), *benz*$^-$ (benzoate oxidase negative) and *p-hydroxybenz*$^-$ (*p*-hydroxybenzoate hydroxylase negative).

Maintenance of organism. The parent and mutant strains were kept at room temperature on nutrient–agar slopes, subcultured monthly, and were checked periodically for their microscopic appearance and

their growth on aromatic compounds. Every 3–4 months, freeze-dried samples were resuscitated in nutrient broth and used to prepare fresh slopes.

Growth of organism. The mineral-salts medium of Mandelstam & Jacoby (1965) was used in conical flasks filled to no more than one-fifth of their capacity and shaken vigorously at 30°C. Glucose was used at 0.5% (w/v); the concentrations of the other substrates are indicated in the text. All carbon sources were autoclaved, except for *p*-hydroxybenzoate, which was sterilized by passage through a sterile membrane filter (Oxoid; 4cm diam.). Cells were harvested by centrifugation (3000*g* for 20min) and then washed in potassium phosphate buffer (0.1M, pH7).

Design and operation of the chemostat

A simple chemostat was used, consisting of an inverted cone-shaped growth vessel (500ml) in a water bath. Filtered moist warmed air (1 litre/min) was used to mix and aerate the contents (100ml) of the growth vessel. A peristaltic pump supplied sterile medium from a reservoir (5 litres) through silicone-rubber tubing at a constant rate that could be set at any value up to 2ml/min. A siphon maintained the contents of the growth vessel constant.

The medium used in the chemostat was a modified version of the mineral-salts medium in which the nitrogen was made growth-limiting. Ammonium sulphate was decreased to 0.2 or 0.25g/litre, NaCl (1.0g/litre) replaced the NH_4Cl, and the sulphate was made up with Na_2SO_4 (0.8g/litre). Silicone emulsion was added (0.1ml/litre) to control foaming during growth. Nitrogen limitation was confirmed by testing the effluent medium with Nessler's reagent and by observing, at the end of the experiment, a rapid increase in culture density when $(NH_4)_2SO_4$ was added directly to the growth vessel. The dilution rate (0.45–0.6vol./h) was set so that the doubling time was close to the maximum for the carbon source being used. When enzymic adaptation to media containing two aromatic substrates was investigated, wild-type cells were first grown to steady state on one of the substrates (10mM). The relevant enzyme was assayed and the substrate concentration in the vessel determined. The medium feed was then changed to a reservoir containing the same carbon source together with the alternative (also at 10mM). Enzyme and substrate measurements were then made during the transition period until the new steady state had been reached.

Induction in growing cultures

Unless otherwise stated, cultures in exponential growth in glucose-containing minimal medium were treated with inducer and samples were removed periodically for the assay of the induced enzyme. Chloramphenicol (50μg/ml) was added to stop further enzyme synthesis and the cells were harvested and washed. Growth was followed by measuring the E_{600}, which was related to bacterial dry weight by using a calibration curve. Rates of induction were calculated as units of enzyme activity/mg dry wt. of bacterial mass synthesized.

Enzyme assays

All enzyme activities were calculated on the basis of one unit = 1nmol of substrate (or O_2) consumed/min.

L(+)-*Mandelate dehydrogenase.* This was assayed spectrophotometrically in extracts of sonicated cells with 2,6-dichlorophenolindophenol as electron acceptor (Hegeman, 1966*a*).

Benzoylformate decarboxylase (*EC* 4.1.1.7; *benzoylformate carboxy-lyase*). This was assayed by measuring manometrically the decarboxylation of benzoylformate in toluene-treated cells in the presence of thiamin pyrophosphate (Mandelstam & Jacoby, 1965). The carbon dioxide production was corrected for retention by the buffer (pH6).

Benzoate oxidase. The usual procedures for breaking cells destroy most or all of this activity (Sleeper *et al.*, 1950; Mandelstam & Jacoby, 1965). Therefore oxygen uptake accompanying the oxidation of benzoate by whole cells was measured manometrically whenever appreciable amounts of enzyme were expected. Measurement of the enzyme in samples with low specific enzyme activities required a more sensitive method based on the release of $^{14}CO_2$ from [*carboxy*-^{14}C]benzoic acid. Washed cells from the induction medium were resuspended in fresh potassium phosphate buffer (0.1M, pH7, 10ml) containing chloramphenicol (50μg/ml). Portions (2.5ml) were incubated at 30°C with shaking in sealed double-side-arm manometer flasks. After 10min for equilibration, labelled benzoate (0.5ml, 6μmol, 0.02μCi) was tipped in from one side arm. The centre well contained NaOH (0.2ml, 2M). After 20, 40, or 60min incubation, H_2SO_4 (0.5ml, 3.5M) was tipped in from the other side arm and the incubation continued for a further 30min to allow time for the absorption of $^{14}CO_2$. The centre well was then rinsed out with 0.8ml of water and the radioactivity determined (see below). Samples of the original benzoate were also counted for radioactivity and controls incubated to measure the activity of uninduced cells.

p-Hydroxybenzoate hydroxylase. This was assayed by measuring the rate of disappearance of *p*-hydroxybenzoate from buffered cell suspensions (Stevenson & Mandelstam, 1965).

Assay of substrates in culture media

Mandelate. This was assayed by coupling its oxidation by L(+)-mandelate dehydrogenase to potassium ferricyanide. Washed particles containing mandelate

dehydrogenase were prepared by the method of Hegeman (1966*a*) from cells grown on mandelate. Samples (1.0ml) containing the mandelate to be assayed were filtered to remove the cells (Oxoid filter, 2cm diam.) and the filtrate was assayed at 30°C in cuvettes (1ml, 1cm light-path) containing $K_3Fe(CN)_6$ (0.01M, 0.1ml), KCN (0.01M, 0.1ml), washed particle preparation, filtrate and 0.1M-potassium phosphate buffer (to 1.0ml). Sufficient particle preparation was added to oxidize the mandelate completely within 10–15min. The decrease in E_{420} was followed in a Cary 14 recording spectrophotometer. The assay was standardized with known amounts of DL-mandelate.

This method proved satisfactory for samples from cultures grown on mandelate or on mandelate+*p*-hydroxybenzoate. Those grown on benzoate+mandelate, however, contained a compound (not benzoate itself) that reduced ferricyanide in the presence of the particle preparation and made it impossible to assay mandelate. Mandelate in these growth media was determined as follows. [*carboxy*-^{14}C]Mandelate was added to the growth medium (0.001–0.002μCi/ml). Its metabolism released C-1 (as $^{14}CO_2$) so that the radioactivity in the medium was then a measure of the mandelate left. Samples (1.0ml) were acidified with conc. H_2SO_4 to release the $^{14}CO_2$, neutralized after 30min at room temperature with NaOH (10M) and counted for radioactivity.

Benzoate. This was conveniently measured radiochemically by a method that was identical with that for mandelate except that [*carboxy*-^{14}C]benzoate replaced the [^{14}C]mandelate.

p-Hydroxybenzoate. The colorimetric method described by Stevenson & Mandelstam (1965) was used.

Measurement of radioactivity

All counting was carried out in a liquid-scintillation counter (Beckman L200 or Wallac Decem-NTL^{314}). The scintillation fluid contained butyl-PBD (0.7%, w/v) and naphthalene (8%, w/v) in toluene–2-methoxyethanol (3:2, v/v). Aqueous samples (0.4ml) were mixed with 10ml of scintillant in glass vials and a minimum of 1000 counts was collected (standard error about 3%).

Reagents

All reagents except those listed below were supplied by British Drug Houses Ltd. (Poole, Dorset, U.K.) and were AnalaR grade where available. DL-Mandelic acid was used throughout. Sigma Chemical Co. (St. Louis, Mo., U.S.A.) supplied Antifoam B emulsion (10% silicone) and thiamin pyrophosphate. Benzoic acid (sodium salt) was made by May and Baker Ltd. (Dagenham, Essex, U.K.). Chloramphenicol was purchased from Parke Davis Ltd. (Greenford, Middx., U.K.). Butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] was a product of CIBA (A.R.L.) Ltd. (Duxford, Cambs., U.K.). Benzoylformate was synthesized by the method of Oakwood & Weisgerber (1955). Radioactive chemicals were from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of DL-[*carboxy*-^{14}*C*]*mandelic acid*

Hegeman's (1966*a*) modification of the method of Fieser (1957) was used. The purity of the product was checked spectrophotometrically and by paper chromatography in four different solvent systems. The radioactive product and authentic mandelate behaved identically. At least 99% of the label in the preparation was released by washed cells induced by growth on mandelate, but not by uninduced cells.

Measurement of growth rates on various substrates

A broth culture of wild-type cells was used to provide small inocula for a series of flasks containing minimal medium and a single carbon source (10mM). The flasks were vigorously aerated at 30°C and the doubling times determined. The values are in Table 1.

Results

Measurement of induction constants for the benzoate, p-hydroxybenzoate and mandelate regulons

Preliminary experiments showed that glucose was a weak repressor of the mandelate regulon in both growing and in non-growing (nitrogen-starved) cells (Mandelstam & Jacoby, 1965), and was thus a suitable carbon source for investigating induction in the aromatic pathways. Additional experiments with the chemostat confirmed this finding and showed that the presence of glucose made little difference to the steady-state induction of any of the three regulons in

Table 1. *Growth rates of Pseudomonas putida*

Wild-type cells were grown from a small inoculum in minimal medium containing the relevant substrate (10mM) as sole source of carbon and energy. Growth was followed at 600nm.

Substrate	Doubling time (min)
Succinate	42
Glucose	70–80
Mandelate	45–50
Benzoate	48–55
p-Hydroxybenzoate	60–70

cells growing on an aromatic substrate (Higgins, 1971).

The dependence of induction rate on inducer concentration was therefore investigated by measuring initial rates of enzyme synthesis in cells growing exponentially in glucose-containing minimal medium and induced over a range of inducer concentrations.

K_{ind} for benzoate oxidase. Because benzoate is a very efficient inducer, low concentrations (<0.5mM) had to be used for sub-maximum rates of induction.

These low inducer concentrations could not be maintained constant for long, and culture samples suitable for initial-rate measurements had too low a specific enzyme activity for assay by the whole cell oxidation method. The assay based on the decarboxylation of [*carboxy*-^{14}C]benzoate was used.

Wild-type cells, growing exponentially in glucose-containing minimal medium, were induced (100μg dry wt./ml) with benzoate (0.125–0.5mM). Before the enzyme appeared, there was a lag, the length of which varied somewhat between experiments but which did not depend on the inducer concentration. The results (Fig. 1) were analysed by the method of Lineweaver & Burk (1934). The double-reciprocal plot was linear and the K_{ind} value was about 0.5mM.

K_{ind} for p-hydroxybenzoate hydroxylase. After the addition of *p*-hydroxybenzoate to wild-type cells (200μg dry wt./ml) growing exponentially in glucose-containing minimal medium, there was a lag of about 15min before enzyme synthesis started. This has also been observed by Hosokawa (1970). *p*-Hydroxybenzoate was found to be a very efficient inducer and its metabolism made it very difficult to maintain the low inducer concentrations constant long enough to measure initial rates of enzyme synthesis. Satisfactory induction rates with below 0.01mM inducer concentrations could not be obtained. At 0.02mM, induction still seemed to be at a maximum (Fig. 2) and, although a value for K_{ind} could not be obtained, it was clear that the K_{ind} was below 0.01mM. The use of inducer analogues to avoid this problem of inducer metabolism was rejected, since the analogue and the natural inducer might have very different affinities for the induction system.

K_{ind} for the mandelate regulon. Problems associated with inducer metabolism can be avoided in this case by using a mutant blocked at mandelate dehydrogenase (*md*$^-$). DL-Mandelate is not metabolized but still serves as an inducer of the remaining enzymes of the co-ordinate group (Scheme 1). Of these, benzoylformate decarboxylase was selected as an easily measurable 'indicator' enzyme for the regulon.

Induction was followed in the usual way, and at all concentrations of inducer a linear rate of synthesis was established (Fig. 3). At concentrations above 0.75mM, the induction system appeared to be saturated. The rates at lower concentrations were dependent on the inducer concentration, but the results could not be analysed by the method of Lineweaver & Burk (1934). The double-reciprocal plot was non-linear and had a positive rather than a negative intercept. However, inspection of the rates

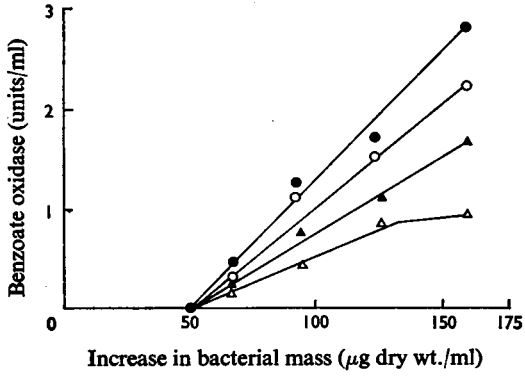


Fig. 1. *Induction of benzoate oxidase: effect of benzoate concentration*

Wild-type cells were induced with benzoate at 0.5 (●), 0.3 (○), 0.2 (▲) and 0.125mM (△). Samples were removed at intervals and assayed for benzoate oxidase (decarboxylation of $C_6H_5{}^{14}CO_2H$). The inducer was added when the culture density was 100μg dry wt./ml and there was usually a lag of 20–40min before induced enzyme synthesis began.

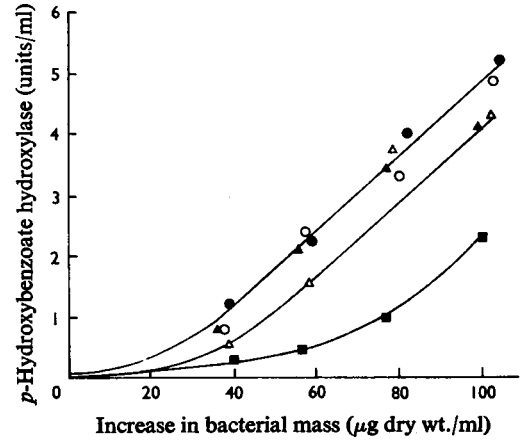


Fig. 2. *Induction of p-hydroxybenzoate hydroxylase: effect of p-hydroxybenzoate concentration*

Wild-type cells (200μg dry wt./ml) were induced with *p*-hydroxybenzoate at 0.2 (●), 0.1 (○), 0.05 (▲), 0.02 (△) and 0.01mM (■), and samples were taken for the assay of *p*-hydroxybenzoate hydroxylase.

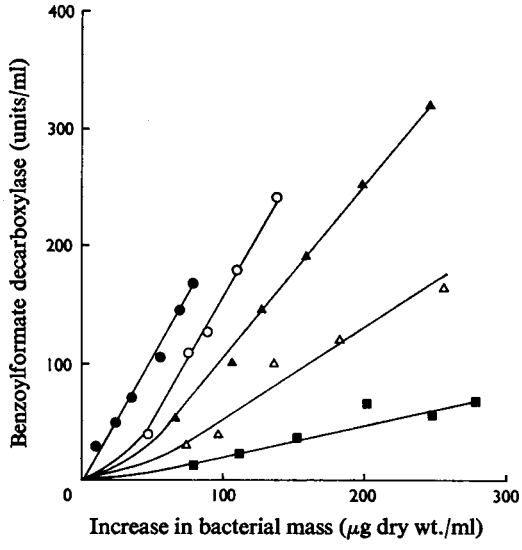


Fig. 3. *Induction of the mandelate regulon: effect of mandelate concentration*

A *md*⁻ mutant was induced with mandelate at 2.0 (●), 1.0 (○), 0.75 (▲), 0.5 (△) and 0.25 mM (■), and samples were taken for the assay of the 'indicator' enzyme, benzoylformate decarboxylase. Because there was a lag in the enzyme synthesis at lower concentrations, the inducer was added to the exponentially growing cultures at different cell densities (25–125 μg dry wt./ml), so that all the cultures were at about the same density during the phase of active enzyme synthesis.

(Fig. 3) suggested that the inducer concentration giving half the maximum rate of induction was about 0.5 mM. The results of Stanier *et al.* (1965) give a K_{ind} value of about 0.2 mM.

The same experiment (Fig. 3) also showed that, at concentrations below 2.0 mM, a delay occurred between addition of the inducer and the synthesis of the enzymes. This lag was not evident at saturating concentrations of inducer. The possible significance of this lag will be discussed in the following paper (Higgins & Mandelstam, 1972).

The results for the induction of the three regulons are given in Table 2.

Measurement of repression constants

In predicting the enzymic adaptation of *P. putida* to media containing pairs of aromatic substrates, it is important to know the qualitative and quantitative effects each can have on the induction of the regulons for the other substrates. It is already known that

Table 2. *Summary of the induction constants (K_{ind}) and the inducer concentrations for maximum induction in the aromatic system of Pseudomonas putida*

The initial rate of synthesis of the inducible enzyme was measured in cells growing on glucose and induced with a range of inducer concentrations. The K_{ind} was calculated as described in the text.

Inducer	Concn. for maximum induction (mM)	K_{ind} (mM)
Mandelate	1.0–2.0	≈0.5
Benzoate	0.5–1.0	≈0.5
p-Hydroxybenzoate	≈0.02	<0.01

benzoate and *p*-hydroxybenzoate repress the mandelate regulon (see Scheme 2). This section describes experiments to measure constants for these repressor effects. Although the mechanism of this repression, like that of induction, need not concern us at present, account must be taken of the observation that 'escape' could occur from the repression normally exerted by succinate on the induction by mandelate, and that succinate-adapted cells showed little repression (Stevenson & Mandelstam, 1965). Such transient repression will be of significance in predicting enzymic adaptation to mixed substrates, since the steady-state enzymic response may well differ from that observed in short-term experiments. In this study, repression was studied with a view to determining whether such transient repression occurs, as well as measuring the K_{rep}.

In the following experiments, inducer and repressor were added simultaneously to cells growing exponentially on glucose. The induced enzyme was measured and the rate compared with that in a control culture without repressor. Where possible, suitable mutants were used to provide conditions of gratuity with respect to the repressor. Under these conditions, not only was the repressor concentration maintained constant but its conversion into an intermediate with a different repressor action was prevented.

Repression of the mandelate regulon by p-hydroxybenzoate. A *p-hydroxybenz*⁻ mutant was induced with mandelate (1.5 mM) and *p*-hydroxybenzoate was added at concentrations up to 0.06 mM. Even the lowest concentration (0.015 mM) was sufficient to depress the rate of synthesis of benzoylformate decarboxylase (Fig. 4*a*). The repressor did not alter the time at which enzyme synthesis started. At a higher concentration (5 mM), mandelate partly reversed the repression (Fig. 4*b*). Analysis of the results by the method of Dixon (1953) showed that the K_{rep} was about 0.02 mM.

Repression of p-hydroxybenzoate hydroxylase by

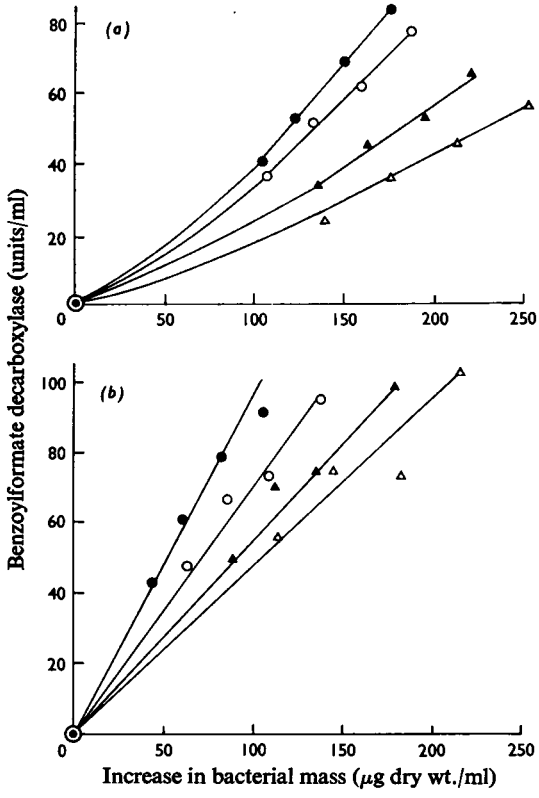


Fig. 4. *Effect of p-hydroxybenzoate on the induction of the mandelate regulon*

A *p-hydroxyybenz*$^-$ mutant was induced with mandelate at 1.5mM (*a*) or 5.0mM (*b*). *p*-Hydroxybenzoate was added at the same time at 0 (●), 0.015 (○), 0.03 (▲) or 0.06mM (△). Culture densities (μg dry wt./ml) at the time of induction were 60 (*a*) and 90 (*b*). Samples were taken at intervals and assayed for benzoylformate decarboxylase.

benzoate. The effect of benzoate on the induction of *p*-hydroxybenzoate hydroxylase by *p*-hydroxybenzoate (1.0mM) was followed in a *benz*$^-$ mutant. A moderate repressor effect of benzoate (2.0mM) was observed, which was partly reversed by increasing the inducer concentration over the range 1.0–10.0mM (Fig. 5). The results were analysed by the method of Hunter & Downs (1945) and gave a value for K_{rep} of about 1.0mM. Benzoate did not affect the time-course of the induction.

Repression of p-hydroxybenzoate hydroxylase by mandelate. Mandelate (2.0mM) had no effect on the rate of synthesis of *p*-hydroxybenzoate hydroxylase in a *md*$^-$ mutant induced with *p*-hydroxybenzoate (1.0mM). Even when the mandelate concentration was

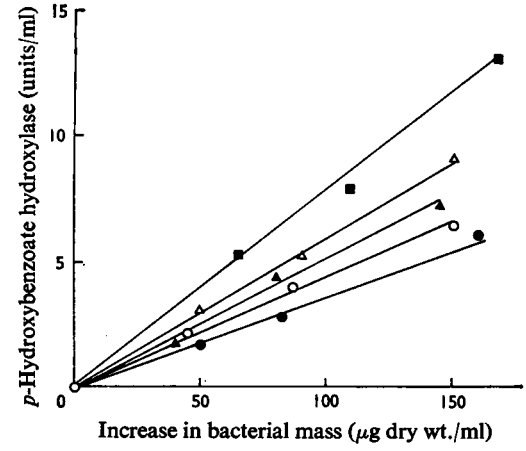


Fig. 5. *Effect of benzoate on the induction of p-hydroxybenzoate hydroxylase*

A *benz*$^-$ mutant was induced with *p*-hydroxybenzoate at 1.0 (●), 2.0 (○), 5.0 (▲) and 10.0mM (△) in the presence of 2.0mM-benzoate. A control flask received only 1.0mM-*p*-hydroxybenzoate (■). Inducer and repressor were both added when the culture density was 100μg dry wt./ml. Samples were taken at intervals and assayed for *p*-hydroxybenzoate hydroxylase.

raised to 10mM there was no repression and the time-course of the induction was unaffected.

Repression of benzoate oxidase by mandelate. The above experiment was repeated with benzoate (1.0mM) as inducer. Again mandelate (2.0mM) exerted no repressor effect and failed to alter the time-course of the induction. Increasing the mandelate concentration to 5.0mM was also without effect on the kinetics of induction. This was to be expected, since the utilization of mandelate in wild-type cells would, in any case, require induction of benzoate oxidase.

Repression of benzoate oxidase by p-hydroxybenzoate. *p*-Hydroxybenzoate (2.0 or 10.0mM) did not appear to repress the synthesis of benzoate oxidase in a *p-hydroxybenz*$^-$ mutant induced with benzoate (1.0mM).

Repression of the mandelate regulon by benzoate. A double mutant (*md*$^-$, *benz*$^-$) was induced with mandelate (2.5mM), and benzoate (0.05–1.0mM) was added at the same time. In the presence of benzoate, the final rate of synthesis of the enzymes of the mandelate regulon was unaffected, but the repressor caused a considerable delay to occur between the addition of the inducer and the onset of enzyme synthesis (Fig. 6). Tests showed that the escape from repression was not due to benzoate metabolism. At higher concentrations of benzoate (5–10mM) the final rate of enzyme synthesis in the period after the

severe transient repression was always less than in the control culture, indicating that a degree of permanent repression was present (Higgins, 1971). The kinetics of repression were such that no K_{rep} value could be calculated, but it was found that the effect of benzoate was very much less in cells fully or partly induced for the mandelate regulon (Higgins, 1971).

The results on repression in the aromatic pathways are summarized in Table 3.

Prediction of enzymic response to mixed aromatic substrates

Enzymic adaptation to mixed aromatic substrates could be investigated by transferring bacteria previously grown on glucose to media containing more than one aromatic substrate and observing the induction of the enzymes. Under these conditions, however, the cells would be initially unable to grow, the adaptation would be slow and the kinetics of induction complex. In a growing culture, induction would be more rapid and the kinetics simpler. Hence in this study it was convenient to start with cells growing on one aromatic carbon source and to add the second later. The order in which the two substrates were presented could then be reversed to determine whether this affected the steady state.

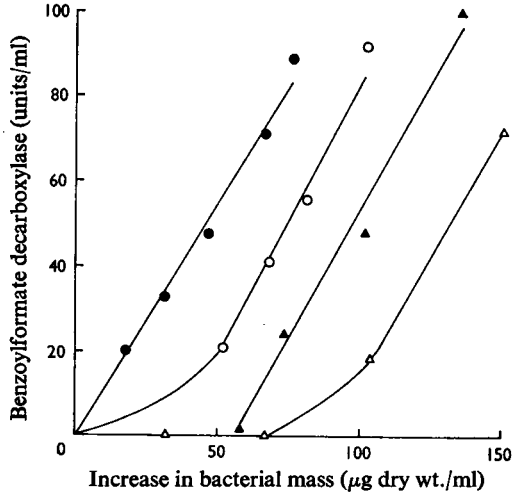


Fig. 6. *Effect of benzoate on the induction of the mandelate regulon*

A double mutant (*md*$^-$, *benz*$^-$) was induced with mandelate (2.5mM). At the same time, benzoate was added at 0 (●), 0.05 (○), 0.2 (▲) or 1.0mM (△). Samples were taken at intervals and assayed for benzoylformate decarboxylase. Because there was a lag in the enzyme synthesis, the inducer was added to exponentially growing cultures at different cell densities (30–80μg dry wt./ml), so that all the cultures were at similar densities during the phase of active enzyme synthesis.

It was convenient to do this type of experiment in a nitrogen-limited chemostat, which made it possible to grow cells for long periods in the presence of constant, but easily varied, concentrations of substrates. However, Mandelstam (1962) has shown that cultures of *Escherichia coli*, grown at low growth rates in nitrogen-limited chemostats, experienced intensified catabolite repression of the lactose operon. Hence, even though low growth rates were not used in this study, it was first necessary to rule out the possibility that this intensified repression would occur in the aromatic system and invalidate the predictions that we wished to make based on the results from batch cultures. The results of two experiments supported the notion that nitrogen limitation does not in fact introduce factors invalidating a direct comparison of batch and continuous culture methods.

First, the specific activities of the enzymes were examined in cells growing in batch and continuous cultures. The specific activity of the relevant enzyme was determined at steady state in wild-type cells growing in a chemostat supplied with one of the aromatic substrates (10mM) as sole carbon source. By growing cells from small inocula to high density (about 500μg dry wt./ml) in flasks containing minimal medium and aromatic inducers (10mM), the steady-state specific activities of the enzymes could be determined for batch culture. The results of a number of experiments of this type showed that nitrogen limitation did not repress these enzymes. For instance, values for benzoylformate decarboxylase in the chemostat were 993 units/mg (variation 835–1100) and 1093 units/mg (variation 770–1270) in batch cultures.

Secondly, the effect of inducer concentration on the

Table 3. *Summary of the repression data in the aromatic pathways of Pseudomonas putida*

Repressor	Regulon ...	Mandelate	Benzoate	*p*-Hydroxybenzoate
Mandelate		—	No repression	No repression
Benzoate		Moderate repression (complex kinetics)	—	Moderate repression (K_{rep} = 1.0mM)
p-Hydroxybenzoate		Strong repression (K_{rep} = 0.02mM)	No repression	—

induction of the mandelate regulon was examined in the chemostat and compared with the results of a similar investigation in batch culture. Benzoylformate decarboxylase is stable in growing cells (S. J. Higgins & J. Mandelstam, unpublished work) so that its specific activity in cells grown to steady state in a chemostat should be a direct measure of its rate of induction in those cells. A *md*$^-$ mutant was grown on glucose in the chemostat with increasing concentrations of mandelate. After each increment in the mandelate concentration, the activity of benzoylformate decarboxylase was measured at steady state. The variation in the specific activity of this enzyme over the mandelate concentration range 0–10mM is shown in Fig. 7. Full induction occurred at 5mM, and a Lineweaver & Burk (1934) plot of the results gave a straight line indicating 0.7mM as the K_{ind}. These values are similar to, but not identical with, the values for the mandelate regulon obtained from batch cultures (Table 2). It will be apparent (see below) that the predictions that can be made are so clear-cut that they would be unaffected by this amount of variation in the constants.

Predictions based on the values of K_{ind} and K_{rep}. We are now able to consider what the change in enzymic composition of wild-type cells is likely to be after the changeover from growth in a medium containing one substrate to one containing two aromatic substrates. With the three substrates, mandelate, benzoate and *p*-hydroxybenzoate, there are three possible binary mixtures and each may be preceded by growth on either component as sole carbon source, making six experimental situations in all. These will now be considered.

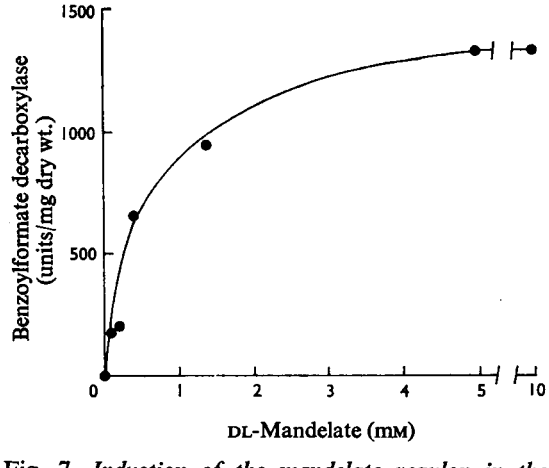


Fig. 7. *Induction of the mandelate regulon in the chemostat: effect of mandelate concentration*

A *md*$^-$ mutant was grown to steady state in a nitrogen-limited chemostat on glucose. Increasing concentrations of mandelate were added and the steady-state specific activity of benzoylformate decarboxylase was measured after each addition. Steady state was assumed to be reached within 10 generations after each increase in concentration. The cell density was 350μg dry wt./ml and the doubling time 90min.

(1) Mandelate
(2) *p*-Hydroxybenzoate
→ Mandelate + *p*-hydroxybenzoate

p-Hydroxybenzoate hydroxylase should be fully induced at 0.02mM whereas the mandelate regulon requires an inducer concentration 50–100 times higher. Although mandelate has no repressor effect on the induction of *p*-hydroxybenzoate hydroxylase, *p*-hydroxybenzoate is a potent repressor of the mandelate regulon (K_{rep} = 0.02mM). Thus cells already growing on mandelate should cease to synthesize the enzymes of the mandelate regulon immediately *p*-hydroxybenzoate is added at a concentration substantially in excess of the K_{rep} (say >0.1mM). At the same time *p*-hydroxybenzoate hydroxylase should be immediately induced at the maximum rate. A transition period should then ensue in which the mandelate enzymes are diluted out by growth and the specific activity of *p*-hydroxybenzoate hydroxylase rises towards the value associated with fully induced cells. On the other hand, cells growing on *p*-hydroxybenzoate should fail to synthesize any mandelate enzymes when mandelate is added at any reasonable concentration.

(3) Benzoate
(4) Mandelate
→ Benzoate + mandelate

Both regulons should be fully induced at inducer concentrations above about 1.0mM, so that repressor effects should be the factors determining the enzymic adaptation to a mixture of the substrates. Mandelate is not a repressor of benzoate oxidase, and the repressor effect of benzoate on the mandelate regulon is complex. First, benzoate exerts a severe transient repression in cells not adapted to mandelate. In mandelate-grown cells this repression is much less. Secondly, at substrate concentrations there is also a residual permanent repression. Hence, cells that are already growing on benzoate should not synthesize mandelate enzymes for some time after mandelate is added. Eventually, synthesis should start at a repressed rate and at steady state the specific enzyme activity of the mandelate regulon should be less than in fully induced cells. Benzoate oxidase should remain fully induced. On the other hand, cells already growing on mandelate should experience no immediate severe transient repression of the mandelate regulon after benzoate is added, but in the long term the

specific activity of the mandelate regulon should be decreased. Benzoate oxidase is, of course, sequentially induced in cells growing on mandelate, but its state of induction presumably depends on the intracellular concentration of benzoate derived from mandelate. This may well be submaximal and the benzoate regulon may not therefore be fully induced in such cells. The addition of benzoate to cells growing on mandelate might or might not be expected to result in a further increase in the specific activity of benzoate oxidase.

(5) *p*-Hydroxybenzoate
(6) Benzoate → *p*-Hydroxybenzoate + benzoate

In the absence of repressors, *p*-hydroxybenzoate hydroxylase should be fully induced by inducer concentrations exceeding 0.02mM, whereas benzoate oxidase should require inducer concentrations 25–50 times higher. Thus, with these substrates at 5–10mM, both induction systems should be saturated and the amounts of the enzymes formed would be determined by the repressor effects. Now *p*-hydroxybenzoate has no repressor effect on benzoate oxidase, whereas benzoate is a moderate repressor of *p*-hydroxybenzoate hydroxylase ($K_{rep} = 1.0$mM). Cells growing in the presence of both substrates would in the long run be expected to be fully induced for benzoate oxidase and partly induced for *p*-hydroxybenzoate hydroxylase. In the transition period after the addition of benzoate to cells growing on *p*-hydroxybenzoate, the specific activity of *p*-hydroxybenzoate hydroxylase should decrease and that of benzoate oxidase should increase from its basal value. Conversely, in cells first grown on benzoate, the addition of *p*-hydroxybenzoate should not affect benzoate oxidase but the hydroxylase should be immediately induced at a sub-maximal rate and increase to a steady-state value substantially less than that found in fully induced cells.

Thus in each of the mixed-substrate media it is predicted that the enzyme for one substrate, the 'preferred' substrate, should be fully induced, whereas those for the other, 'less preferred', substrate should be only partly induced at steady state. The extent of the induction of this second enzyme system should depend on the ratio of the concentrations of the two substrates. The particular enzyme system present at the time of changeover to the two-substrate medium should not influence the eventual steady state. The major predictions discussed above are summarized in Scheme 3, and it should be stressed that they refer to the enzymic response of the cells and not to the utilization of the substrates. In the absence of further kinetic data, the relative rates of substrate utilization can only be predicted when one enzyme system is likely to be completely or almost completely repressed.

Second substrate (added to first)	First substrate: Mandelate	First substrate: Benzoate	First substrate: *p*-Hydroxybenzoate
Mandelate		Benzoate oxidase: Remains fully induced Mandelate enzymes: Rise to partially induced value (after initial lag)	*p*-Hydroxybenzoate hydroxylase: Remains fully induced Mandelate enzymes: Almost totally repressed
Benzoate	Mandelate enzymes: Falls to partially induced value Benzoate oxidase: Remains induced (may or may not increase slightly)		*p*-Hydroxybenzoate hydroxylase: Falls to lower value Benzoate oxidase: Rises to fully induced value
p-Hydroxybenzoate	Mandelate enzymes: Almost totally repressed *p*-Hydroxybenzoate hydroxylase: Rises to fully induced value	Benzoate oxidase: Remains fully induced *p*-Hydroxybenzoate hydroxylase: Rises to partially induced value	

Scheme 3. *Enzymic adaptation to mixed aromatic substrates: summary of the major predictions*

The predictions are based on the results in Tables 2 and 3. Wild-type cells were considered to be grown on one aromatic substrate and then to receive an additional aromatic carbon source. Both were assumed to be present at concentrations sufficient for full induction in the absence of repressors. The expected changes in specific activities of the inducible enzymes involved after the changeover to the binary mixture are indicated in the scheme.

Such is the case with the mandelate regulon in cells growing in the presence of *p*-hydroxybenzoate and mandelate; they should not use mandelate.

Experimental investigation of enzymic response to binary mixtures of aromatic substrates

(1) *Mandelate → mandelate+p-hydroxybenzoate.* After the changeover from growth on mandelate to growth in the presence of mandelate+*p*-hydroxybenzoate, the concentration of *p*-hydroxybenzoate (Fig. 8*a*) rapidly exceeded the K_{ind} for *p*-hydroxybenzoate hydroxylase and the K_{rep} for benzoylformate decarboxylase. The former was induced and the latter repressed (Fig. 8*b*). Steady state, reached after about seven generations, was characterized by full induction of *p*-hydroxybenzoate hydroxylase and virtually complete (92%) repression of the mandelate regulon. The steady-state concentration of mandelate in the growth vessel reflected this change in the induction of the mandelate regulon; its utilization decreased by 95% and the concentration of mandelate in the vessel was 9.8 mM. The concentration of *p*-hydroxybenzoate at steady state was 4.8 mM.

Thus, at steady state, the cells were adapted to growth almost entirely at the expense of *p*-hydroxybenzoate, and they reached this steady state after a transition period during which both enzyme systems were present and both substrates were used.

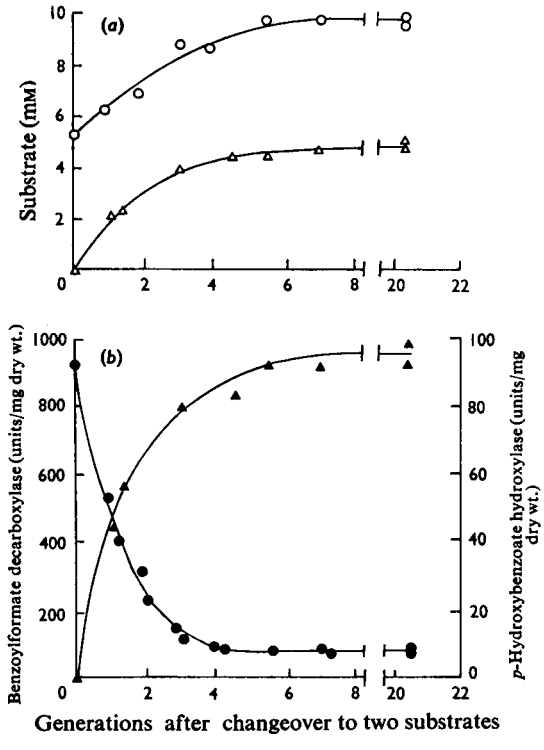


Fig. 8. *Enzymic response to mixed aromatic substrates: (1) mandelate → mandelate+p-hydroxybenzoate*

Wild-type cells were grown in a nitrogen-limited chemostat with mandelate (10 mM) as sole carbon source at a doubling time of 75 min. The specific activity of benzoylformate decarboxylase (●) and the mandelate concentration in the vessel (○) were determined at steady state. *p*-Hydroxybenzoate (10 mM) was then added to the inflowing medium and the specific activities of benzoylformate decarboxylase and *p*-hydroxybenzoate hydroxylase (▲) and the mandelate and *p*-hydroxybenzoate (△) concentrations were measured at intervals until the new steady state had been reached. The cell density was constant at 300 μg dry wt./ml. (*a*) Substrate concentrations in the growth vessel; (*b*) enzyme specific activities.

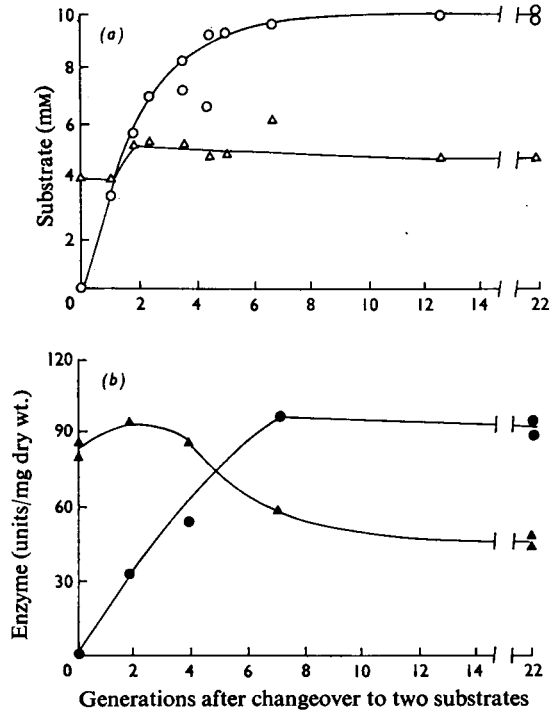


Fig. 9. *Enzymic response to mixed aromatic substrates: (2) p-hydroxybenzoate → mandelate+p-hydroxybenzoate*

Details are the same as for Fig. 8 except that the order of addition of the substrates was reversed, i.e. *p*-hydroxybenzoate (10 mM) was the first substrate and mandelate (10 mM) was added later. (*a*) Substrate concentrations in the growth vessel; (*b*) enzyme specific activities.

(2) *p-Hydroxybenzoate → mandelate+p-hydroxybenzoate.* The mandelate regulon was only poorly induced after switchover from growth on *p*-hydroxybenzoate to growth in the presence of mandelate+*p*-hydroxybenzoate (Fig. 9*b*). The specific activity of benzoylformate decarboxylase at steady state was less than 10% of that expected for full induction (cf. Fig. 8). Not surprisingly, the mandelate concentration (9.8mM) was close to the inflowing concentration (Fig. 9*a*). This was the result predicted (Scheme 3). Rather surprisingly, however, the specific activity of *p*-hydroxybenzoate hydroxylase decreased by about 45% after the changeover, although the utilization of *p*-hydroxybenzoate decreased by only 15%. This decrease in the induction of *p*-hydroxybenzoate hydroxylase had not been predicted, but was reproducibly observed whenever the changeover was made from *p*-hydroxybenzoate to the mixed substrates. When mandelate was the first substrate, the decrease was not seen.

With this exception, the enzymic response of *P. putida* to a medium containing both *p*-hydroxybenzoate and mandelate was as predicted from the K_{ind} and K_{rep} values.

(3) *Benzoate → benzoate+mandelate.* The spectrophotometric assay for mandelate used in the previous experiments could not be used in this instance (see the Methods section). Both mandelate and benzoate had to be measured by radioactivity, which required two chemostats to be run in parallel, identical in all respects except that in one mandelate was labelled and in the other benzoate. The concentrations of both substrates could then be followed.

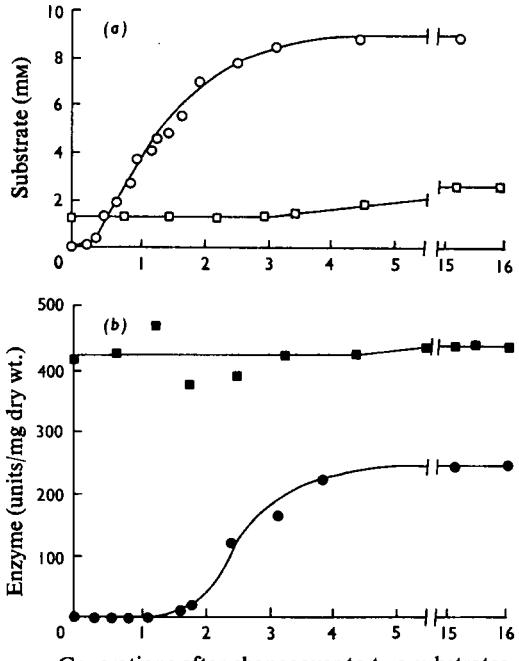


Fig. 10. *Enzymic response to mixed aromatic substrates:* (3) *benzoate → benzoate+mandelate*

Wild-type cells were grown in a nitrogen-limited chemostat with benzoate (10mM) as sole carbon source at a doubling time of 95min. The specific activity of benzoate oxidase (■) was measured at steady state and then mandelate (also at 10mM) was added to the inflowing medium. The changes in the specific activities of benzoate oxidase and benzoylformate decarboxylase (●) were measured while the new steady state was being established. The concentrations of benzoate (□) and mandelate (○) in the growth vessel were determined by running two parallel chemostats under identical conditions, with either benzoate or mandelate labelled with ^{14}C (see the text). The cell density in both chemostats was 380μg dry wt./ml. (*a*) Substrate concentrations in the growth vessel; (*b*) enzyme specific activities.

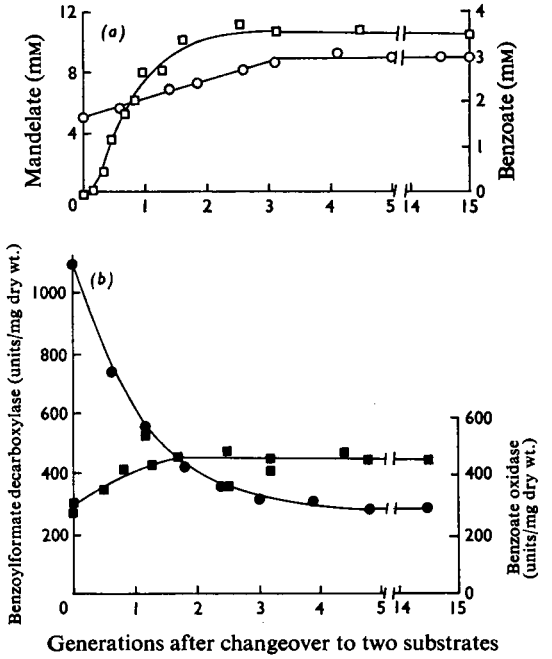


Fig. 11. *Enzymic response to mixed aromatic substrates:* (4) *mandelate → benzoate+mandelate*

Details are the same as for Fig. 10 except that the order of addition of the substrates was reversed. Mandelate (10mM) was the first substrate and benzoate (10mM) was added later. (*a*) Substrate concentrations in the growth vessel; (*b*) enzyme specific activities.

After the changeover to the mixed-substrate medium, benzoate oxidase was unaffected (Fig. 10*b*). A delay was seen (1.5 generations) before the mandelate enzymes began to be synthesized, even though the mandelate concentration (Fig. 10*a*) had exceeded the K_{ind} within about half a generation. As expected, these enzymes were repressed (70–75%) at steady state and the cells grew at the expense of both compounds, with benzoate the predominating growth substrate. The steady-state concentration of benzoate was 2.5mM and that of mandelate 9.0mM.

(4) *Mandelate → benzoate+mandelate.* In the reverse experiment, the specific activity of benzoate oxidase was slightly increased after the changeover. The mandelate regulon, as measured by benzoylformate decarboxylase, was repressed and its specific activity was decreased during growth to 25% of its starting value (Fig. 11*b*). The mandelate utilization reflected this, and the mandelate concentration rose from 5.0 to 9.0mM (Fig. 11*a*). The benzoate concentration at steady state was 3.5mM.

In the experiments with these two substrates, the results were as predicted in Scheme 3 and the steady state was not affected by the order of addition of the substrates.

(5) *p-Hydroxybenzoate → benzoate+p-hydroxybenzoate.* About one generation after the changeover to the mixed substrates, there was a decrease in the specific activity of *p*-hydroxybenzoate hydroxylase (Fig. 12*b*). The delay can be accounted for by the

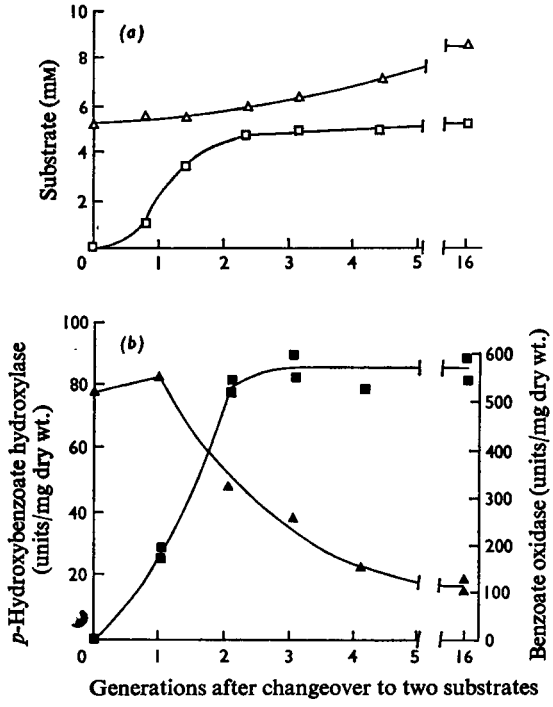


Fig. 12. *Enzymic response to mixed aromatic substrates:* (5) *p-hydroxybenzoate → p-hydroxybenzoate +benzoate*

Wild-type cells were grown in a nitrogen-limited chemostat with *p*-hydroxybenzoate (10mM) as sole carbon source at a doubling time of 87 min. The specific activity of *p*-hydroxybenzoate hydroxylase (▲) and the *p*-hydroxybenzoate concentration in the growth vessel (△) were determined at steady state. Benzoate (10mM) was then added to the inflowing medium and the specific activities of *p*-hydroxybenzoate hydroxylase and benzoate oxidase (■) and the concentrations of *p*-hydroxybenzoate and benzoate (□) were measured at intervals until the new steady state had been reached. The cell density was constant at 360μg dry wt./ml. (*a*) Substrate concentrations in the growth vessel; (*b*) enzyme specific activities.

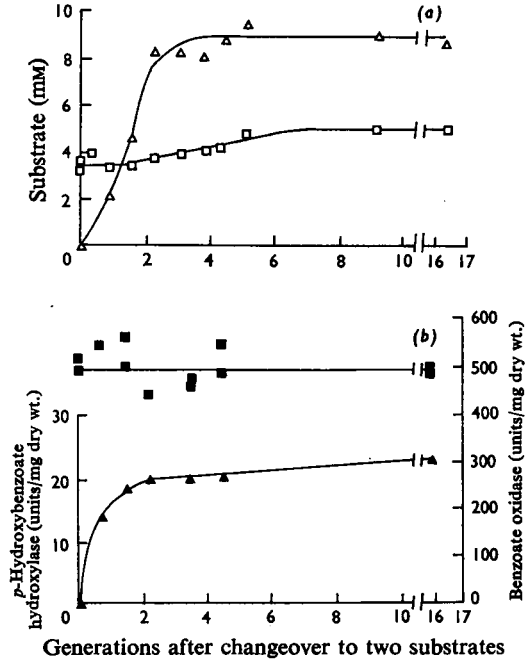


Fig. 13. *Enzymic response to mixed aromatic substrates:* (6) *benzoate → p-hydroxybenzoate+benzoate*

Details are the same as for Fig. 12 except that the order of addition of the substrates was reversed, i.e. benzoate (10mM) was the first substrate and *p*-hydroxybenzoate (10mM) was added later. (*a*) Substrate concentrations in the growth vessel; (*b*) enzyme specific activities.

fact that the benzoate took about this time to build up a significantly repressing concentration (Fig. 12*a*). At steady state, the enzyme was about 75% repressed and, as a result, the *p*-hydroxybenzoate concentration rose from 5.2 to 8.6mM.

On the other hand, benzoate oxidase was rapidly induced after the benzoate concentration reached an inducing value. At steady state, the benzoate concentration was 5.5mM and it is apparent that this substrate is being used predominantly.

(6) *Benzoate → p-hydroxybenzoate+benzoate.* The reverse experiment resulted in partial induction of *p*-hydroxybenzoate hydroxylase (25–35% at steady state), whereas the benzoate regulon remained fully induced (Fig. 13*b*). The utilization of *p*-hydroxybenzoate reflected the extent of induction of its enzyme. At steady state the utilization of benzoate was again predominant (Fig. 13*a*).

The results of this and the previous experiment were very much as predicted (Scheme 3).

Further tests of the predictions about enzymic adaptation to mixed aromatic substrates

Since in this system the inducers and repressors interact and repression can be at least partly reversed at high inducer concentrations, it was predicted that the extent of induction of the enzyme for the less preferred substrate would depend on the concentration ratio of the two substrates in the mixture. The enzyme for the preferred substrate should remain fully induced at all ratios (unless, of course, the concentration of its own substrate fell below that necessary for full induction). The effect of wider variation in the substrate ratios on the steady-state enzyme activities was tested for each of the three binary-substrate media. The results are presented in Table 4 and show that it was indeed possible to increase the induction of the enzymes for the less preferred substrate by greatly increasing its relative concentration. Conversely, its repression could be intensified by decreasing this concentration ratio. In no case did the 'dominant' enzyme become repressed.

Discussion

Predictions about the enzymic adaption of *P. putida* to binary mixtures of aromatic substrates based on K_{ind} and K_{rep} values were found to be fairly reliable, so it appears that these constants are indeed what determine the adaptation. It seems reasonable to expect that a knowledge of the constants for other

Table 4. *Effect of steady-state substrate concentration ratio on the enzymic response of cells to binary mixtures of aromatic substrates*

Wild-type cells were grown in nitrogen-limited chemostats (doubling time 70–80min) on one or two aromatic substrates. The specific activity of the enzyme(s) and the substrate concentration(s) were measured. The substrate concentrations in the feed are given in parentheses.

Mandelate (mM)	*p*-Hydroxybenzoate (mM)	Steady-state substrate ratio (mandelate/*p*-hydroxybenzoate)	Benzoylformate decarboxylase activity (units/mg)	*p*-Hydroxybenzoate hydroxylase activity (units/mg)
4.9 (10)	—	(∞)	1020	—
17.0 (20)	2.1 (6)	8.10	250	88
9.8 (10)	4.8 (10)	2.04	80	94
0.91 (1)	14.1 (20)	0.05	<5	95

Benzoate (mM)	Mandelate (mM)	Steady-state substrate ratio (mandelate/benzoate)	Benzoylformate decarboxylase activity (units/mg)	Benzoate oxidase activity (units/mg)
—	14.5 (20)	(∞)	935	330
1.2 (7.5)	18.9 (20)	16.0	466	520
12.6 (20)	0.93 (1)	0.74	<5	497

p-Hydroxybenzoate (mM)	Benzoate (mM)	Steady-state substrate ratio (*p*-hydroxybenzoate/benzoate)	*p*-Hydroxybenzoate hydroxylase activity (units/mg)	Benzoate oxidase activity (units/mg)
4.5 (10)	—	(∞)	88	—
16.75 (20)	4.8 (8.33)	3.5	53.5	500
2.8 (2.9)	17.0 (25)	0.17	5.0	480

substrates would, at least in principle, enable one to predict the behaviour of the organism in more complex mixtures of substrates.

The permeability of *P. putida* to aromatic substrates is complex (Higgins & Mandelstam, 1972), so that the constants may in reality refer to the entry systems for the substrates and will not therefore be simply related to the intracellular concentrations of the inducers or repressors. However, we have been concerned with the response of the whole cell to the medium and therefore the apparent K_{ind} and K_{rep} were what was of importance. True values for K_{ind} and K_{rep} would have been misleading if they had been over-ridden by permeability effects.

It was predicted that the growth history of the culture should have no effect on the steady-state enzymic response of the cells to mixed substrates. This was in general substantiated except for cells growing in the presence of mandelate and *p*-hydroxybenzoate. Here the order of presentation of the substrates altered somewhat the steady-state enzyme values. We have no explanation for this departure from the predicted behaviour.

It is somewhat difficult to offer an unequivocal explanation for the enzymic behaviour of the organism towards mixtures of aromatic substrates. Pardee (1961) has suggested that an ability to grow fast is of immense selective advantage to micro-organisms. Certainly in the laboratory, economy of protein synthesis and strict control of enzyme activity, both of which would be expected to improve the overall growth efficiency of the cell, have been shown to be of selective advantage (Roeptke *et al.*, 1944; Novick, 1961; Zamenhof & Eichhorn, 1967; Baich & Johnson, 1968). Thus one would expect that when bacteria are presented with a choice of substrates, the better growth substrate would be used preferentially, as has been observed with *Pseudomonas oxalaticus* (Blackmore & Quayle, 1968). However, from this study it is clear that growth rate does not seem to be the factor involved in the adaptation of *P. putida* to mixtures of aromatic substrates. For instance, mandelate can support a higher growth rate than can *p*-hydroxybenzoate, yet the latter was used by cells growing in the presence of these two substrates together. Economy of protein synthesis may be the determining factor in the choice of the substrates. The metabolism of benzoate requires less protein synthesis than does the metabolism of mandelate, which is higher up the same pathway. When mandelate and benzoate were the competing substrates, benzoate was used preferentially. Without detailed investigation of the molecular concentrations of the enzymes of the pathways it is not possible to say whether the choice exhibited in the other binary mixtures is also determined by economy of protein synthesis.

However, it has to be remembered that in the laboratory the growth conditions used were very far removed from those found in the natural environment of this group of organisms, the soil. To extrapolate from one set of conditions to the other and to decide on this basis what the determining factor had been in the evolution of the regulatory system seems unjustifiable in our present state of knowledge.

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