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Regulation of the Enzymes Converting L-Mandelate into Benzoate in Bacterium N.C.I.B. 8250

By A. LIVINGSTONE* and C. A. FEWSON
Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.

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L-Mandelate is oxidized to benzoate by the enzymes L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I. Conditions have been established for measuring these three enzymes as well as benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase II and catechol 1,2-oxygenase in a single cell-free extract prepared from bacterium N.C.I.B. 8250. The kinetics of induction of all these enzymes have been measured under a variety of conditions. L-Mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I appear to be co-ordinately regulated because (*a*) their differential rates of synthesis are proportional to one another under various conditions of induction and repression, (*b*) they are specifically and gratuitously induced by thiophenoxyacetate and a number of other compounds, and (*c*) mutant strains have been isolated that lack all three enzymes. Phenylglyoxylate is the primary inducer of the regulon as mutant strains lacking phenylglyoxylate carboxy-lyase form the other two enzymes in the presence of L-mandelate or phenylglyoxylate, whereas in mutant strains devoid of L-mandelate dehydrogenase activity only phenylglyoxylate induces phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I.

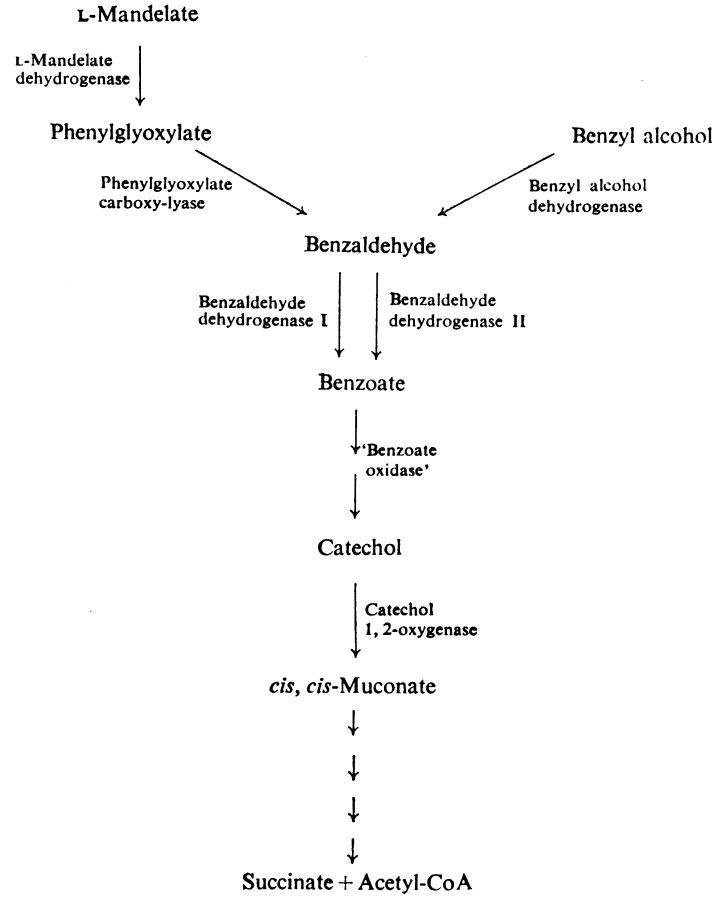
Bacterium N.C.I.B. 8250, a member of the *Acinetobacter–Moraxella* group of organisms (Véron, 1966; Fewson, 1967*a*; Baumann *et al.*, 1968), can utilize L-mandelate and benzyl alcohol as sources of carbon and energy for growth (Fewson, 1967*b*). Experiments based on the principle of simultaneous adaptation (Stanier, 1947) and on an examination of the enzymes induced under various conditions (Kennedy & Fewson, 1968*a*,*b*; Livingstone *et al.*, 1972) have indicated that these compounds are oxidized to *cis*,*cis*-muconate by the overlapping and convergent pathway shown in Scheme 1. This pathway of L-mandelate metabolism is similar to that found in other bacteria that oxidize L-mandelate (Stanier, 1948; Rosenberg & Hegeman, 1969; Ornston, 1971) but differs from that observed in the fungus *Aspergillus niger* (Jamaluddin *et al.*, 1970) where the substrate for ring cleavage is protocatechuate. Although the intermediates involved in mandelate oxidation are identical in the bacteria so far studied, the enzymes involved and the regulation of the pathways appear to show striking variations. Thus, the genetic organization and induction of the enzymes converting mandelate into benzoate are quite different in such relatively closely related species as *Pseudomonas putida* and *Pseudomonas aeruginosa* (Hegeman, 1966*a*,*b*,*c*; Chakrabarty & Gunsalus, 1970; Rosenberg, 1971; Rosenberg & Hegeman, 1969, 1971; Wheelis & Stanier, 1970). The regulation of these enzymes has not previously been studied in detail in bacterium N.C.I.B. 8250, although exploratory experiments indicated that L-mandelate dehydrogenase, phenylglyoxylate (benzoylformate) carboxy-lyase (EC 4.1.1.7) and benzaldehyde dehydrogenase I are co-ordinately controlled (Kennedy & Fewson, 1968*b*). We have investigated the regulation of the mandelate enzymes in bacterium N.C.I.B. 8250 to extend the earlier observations and compare the regulatory patterns in *Acinetobacter* and *Pseudomonas* species. Some of the results described in this paper have been presented in a preliminary form (Livingstone & Fewson, 1971).

* Present address: Beecham Research Laboratories, Clarendon Road, Worthing, Sussex, U.K.

Materials and Methods

Growth of bacterium N.C.I.B. 8250

The organism was maintained and grown in the appropriate media as described by Livingstone *et al.* (1972). Because they may be volatile or heat-labile, L-mandelate, phenylglyoxylate, benzyl alcohol, benzaldehyde, catechol and the compounds tested as inducers were dissolved in water, adjusted to pH7.0 and sterilized by filtration through Millipore filters [Millipore (U.K.) Ltd., Abbey Road, London N.W.10, U.K.; type GSWP 04700, 0.22μm] before their aseptic addition to suitable concentrations of sterile basal medium. Kinetics of enzyme induction were followed in well-aerated 10mM-glutamate–salts medium; cultures were grown for approximately one



Scheme 1. *Metabolism of* L-*mandelate and benzyl alcohol by bacterium N.C.I.B.* 8250

The oxidation of benzoate to catechol may involve the intermediate formation of 3,5-cyclohexadiene-1,2-diol-1-carboxylate (see Reiner, 1971; Reiner & Hegeman, 1971).

generation, inducer was then added and the cultures were sampled for turbidity and enzyme activity at appropriate intervals, as described by Livingstone *et al.* (1972). Enzyme complements of mutant strains were measured after 2.5h of growth in glutamate–salts medium to which appropriate concentrations of inducer had been added (Livingstone *et al.*, 1972).

Isolation of mutants

Cells were grown in 10mM-succinate–salts medium, washed and resuspended in 10mM-sodium citrate buffer, pH 6.0. They were then treated with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (Adelberg *et al.*, 1965) at a final concentration of 0.1mg/ml for 30min at 30°C. The cells were washed in citrate buffer and in some experiments enrichment of the desired types of mutant was achieved by growing the treated cells for 3h at 30°C in 5mM-L-mandelate–salts medium followed by a further 2h growth after the addition of *N*-ethyl-2-carboxy-7-chloro-4-quinolone to a final concentration of 90μM. This bactericidal agent decreased the proportion of cells able to grow on L-mandelate. Mutant strains unable to grow on L-mandelate but able to grow on benzyl alcohol or benzoate were identified and isolated in two ways: (*a*) colonies were replica-plated (Lederberg & Lederberg, 1952) on to plates containing, in order of replication, 5mM-L-mandelate–, 5mM-benzyl alcohol– or 2mM-benzoate–salts medium [solidified with 1.5% (w/v) Japanese Agar (B.D.H. Ltd., Poole, Dorset, U.K.)], and finally nutrient agar (CM3;

Oxoid Ltd., London S.E.1, U.K.) to monitor the success of replication, or (*b*) mutagen-treated cells were grown on a 'limited nutrient' salts medium solidified with 1.5% (w/v) Japanese Agar containing 5mM-L-mandelate+either 0.3mM-L-glutamate or 2mM-benzoate, followed by selection of small colonies. Mutants were isolated by all these methods and were identical with the parent strain of bacterium N.C.I.B. 8250, except for their failure to grow on L-mandelate (Fewson, 1967*a*).

Ultrasonic disruption

Washed cell suspensions were resuspended to 50mg wet wt./ml in 0.04M-tris buffer, pH8.5, and disrupted with the 13mm probe of the Dawe Soniprobe (type 1130A; Dawe Instruments Ltd., London, U.K.). The chilled suspension was treated for 3.5min at 2.5A as described by Livingstone *et al.* (1972). After centrifugation of the homogenate at 12000*g* for 25min at 4°C, the supernatant solution ('extract') was used for determination of enzyme activity. To part of the extract was added an amount of 0.2M-NaOH which had been shown to raise the pH of an equal volume of 0.04M-tris buffer, containing no protein, from 8.5 to 10.3. This treated extract was used to assay benzaldehyde dehydrogenase activity by measurement of enzyme activity before and after heating at 37°C for 2h (Livingstone *et al.*, 1972). When the total benzaldehyde dehydrogenase activity exceeded 80munits/mg of protein or when the first method gave insufficiently accurate results (see Livingstone *et al.*, 1972), a second extract was prepared by ultrasonic disruption of cells suspended in 0.08M-sodium pyrophosphate buffer, pH9.5. A time-course of inactivation at 37°C was then constructed and the results used to calculate the activities of the two benzaldehyde dehydrogenases as described by Livingstone *et al.* (1972).

For the preparation of the coupling protein used in the assay of phenylglyoxylate carboxy-lyase, cells were grown in 5mM-benzyl alcohol–salts medium, harvested, and resuspended to 50mg wet wt./ml in 0.08M-*N*-tris(hydroxymethyl)methylglycine buffer, pH9.5. Samples (20 to 25ml) were disrupted in a Rosett (1965) cell surrounded by an ice–water slurry. The power to the probe (5A) was applied for a total of 4min, but was switched off during alternate minutes to aid cooling so that the disruption took 7min in all. The homogenate was centrifuged as described above and the supernatant fluid used as the source of the coupling protein.

All extracts were assayed immediately after preparation or were stored at −60°C.

Enzyme assays

Spectrophotometric assays were conducted in 10mm path-length silica cuvettes. Measurements of extinction were made with a Unicam SP.800 Spectrophotometer (Pye Unicam Instruments Ltd., Cambridge, U.K.) connected to a Servoscribe Chart Recorder (Smiths Industries Ltd., Wembley, Middx., U.K.). Reactions were done at 27°C. Reagents, in a volume of 1ml or less, were added by means of Eppendorf pipettes (Eppendorf Marburg Micropipette; Eppendorf Gerätebau, Hamburg, West Germany). All reactions were initiated by addition of substrate, and the contents of the cuvettes were mixed with 'plumpers' (Calbiochem, Los Angeles, Calif., U.S.A.). Enzyme units are defined as μmol of substrate converted/min; specific activities are usually given as milliunits (munits) of enzyme/mg of protein. All activities were derived from initial rates under conditions where the amount of product was proportional to the concentration of enzyme and time of incubation.

L-Mandelate dehydrogenase was assayed by a modification of the method of Hegeman (1966*a*). The reaction was followed at 600nm, near the maximal extinction of the oxidized form of the dye used as electron acceptor. In addition to enzyme, the reaction mixture contained, in a total volume of 3.0ml: 200μmol of KH_2PO_4–K_2HPO_4 buffer, pH7.0; 200nmol of 2,6-dichlorophenol-indophenol; 1.5μmol of L-mandelic acid.

Phenylglyoxylate carboxy-lyase was measured by a modification of the coupled assay of Kennedy & Fewson (1968*b*). This involved determining the rate of formation of benzaldehyde by following the rate of reduction of NAD^+ in the presence of an excess of benzaldehyde dehydrogenase II. Since NADH oxidase interfered with the reduction of NAD^+ at low activities of phenylglyoxylate carboxy-lyase, the extract in which the carboxy-lyase was to be measured was heated at 37°C for 60min. This inactivated the NADH oxidase but had no significant effect on the carboxy-lyase. In addition to enzyme, the reaction mixture contained, in a total volume of 3.0ml: 200μmol of tris–HCl buffer, pH9.0; 100μg of thiamine pyrophosphate chloride (dissolved in 0.05M-tris–HCl buffer, pH9.0); 3μmol of NAD^+; 600μg of coupling protein prepared as described above; 2.5μmol of freshly prepared phenylglyxoylic acid.

Benzaldehyde dehydrogenase activities I and II were assayed by the procedures of Livingstone *et al.* (1972) which are based on the differential thermal inactivation of the two enzymes.

Benzyl alcohol dehydrogenase activity was measured by following the rate of NAD^+ reduction at 340nm in the reaction mixture modified from that of Kennedy & Fewson (1968*b*): 200μmol of sodium pyrophosphate buffer, pH9.0; 1.5μmol of NAD^+; 300nmol of benzyl alcohol; extract; total volume 3.0ml.

Catechol 1,2-oxygenase (EC 1.13.1.1) was assayed by the method of Hegeman (1966*a*).

Benzoate oxidase activity was determined in whole cells by measuring the disappearance of benzoate (Fewson *et al.*, 1970). The assay was done in 50ml Erlenmeyer flasks in a shaking water bath at 30°C. The components of the reaction mixture, contained in a volume of 15ml, were: 200μmol of tris–HCl buffer, pH7.5; 7.5μmol of benzoic acid (added as a solution adjusted to pH7.5 by the addition of 1M-NaOH); 14–20mg wet wt. of cells (added as a suspension prepared in 0.05M-tris–HCl buffer, pH7.5). The reaction was initiated by the addition of the cell suspension after all the other components had been preincubated at 30°C for 10min. Samples (2ml) were taken after 0, 10, 20, 40 and 80min, added to 3ml chilled 16.7% (w/v) perchloric acid, mixed and stood in an ice-water slurry for at least 15min. Then 0.1M-HCl (5ml) was added and the contents of the tubes were mixed. The samples were centrifuged at 4000*g* for 20min at room temperature. The supernatant solutions were decanted and their absorption spectra recorded against a water blank from 200 to 350nm. The E_{230} values were used in conjunction with a standard curve to calculate the amount of benzoate that had disappeared. This method with whole cells was used in preference to determination of the enzyme in cell-free extracts because of the poor recovery of benzoate oxidase activity in cell-free systems. The whole-cell procedure had the advantage that no chloroform extraction step (Fewson *et al.*, 1970) was necessary in the benzoate determination.

Analytical methods

Wet weights of bacteria and protein concentrations were determined as outlined by Kennedy & Fewson (1968*b*).

Materials

Amygdalin (mandelonitrile-β-gentiobioside), benzene sulphinic acid (sodium salt), benzene sulphonic acid (sodium salt), benzilic acid (diphenylglycolic acid), DL-*N*-benzoyl-α-alanine, hippuric acid (*N*-benzoylglycine), phenol (AnalaR), phenoxyacetic acid, phenylacetic acid, *N*-phenylglycine and 3-phenylpropionic acid were obtained from British Drug Houses Ltd., Poole, Dorset, U.K. 4-Chlorophenoxyacetic acid (purum), 2,4-dichlorophenoxyacetic acid (purum), 4-fluorophenoxyacetic acid (purum), 2-phenoxyethanol and 2-phenoxypropionic acid (purum) were purchased from Fluka A.-G., Buchs, Switzerland. DL-α-Aminophenylacetic acid, 4-fluoro-DL-mandelic acid, 4-hydroxyphenoxyacetic acid, 2-methoxyphenoxyacetic acid, 2-nitrophenoxyacetic acid (pure), piperonylic acid (3,4-methylenedioxybenzoic acid) and DL-tropic acid (α-phenylhydracrylic acid) were supplied by Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Atropic acid (α-phenylacrylic acid), β-benzoylpropionic acid, 2,4-dimethylphenoxyacetic acid, 3,5-dimethylphenoxyacetic acid, 3-hydroxyphenoxyacetic acid, mandelonitrile, 3-methylphenoxyacetic acid, L-β-phenyl-lactic acid, DL-2-phenyl-lactic acid and α-phenylpropionic acid were from K & K Laboratories, Plainview, N.Y., U.S.A. Benzylmalonic acid, mandelamide and phenylphosphonic acid were from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A., 2,3-dichlorophenoxyacetic acid, 3,4-dichlorophenoxyacetic acid and 3-phenoxypropionic acid from Ralph N. Emanuel Ltd., Wembley, Middx., U.K., and D(−)-mandelic acid and DL-mandelic acid-methyl ester from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. *N*-Ethyl-2-carboxy-7-chloro-4-quinolone was a gift to Dr. W. H. Holms of this Department from Pharmaceuticals Division, Imperial Chemical Industries Ltd., Alderley Park, Cheshire, U.K. All other compounds and solutions were as described by Kennedy & Fewson (1968*b*) and Livingstone *et al.* (1972).

Results

Specificity of enzyme induction

Bacterium N.C.I.B. 8250 formed all the enzymes necessary for degradation of L-mandelate to *cis,cis*-muconate when grown in the presence of L-mandelate or phenylglyoxylate (Table 1). Low amounts of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were also formed, presumably in response (Livingstone *et al.*, 1972) to benzyl alcohol and benzaldehyde which show a transitory accumulation when bacterium N.C.I.B. 8250 is grown on L-mandelate or phenylglyoxylate (C. A. Fewson, A. M. Cook & J. D. Beggs, unpublished work). Thiophenoxyacetate (1mM) induced the formation of only L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I (Table 1). This appears to be gratuitous induction as there was no detectable metabolism of thiophenoxyacetate in the growth medium and it does not support growth of this organism. Phenoxyacetate (5mM), 3-hydroxyphenoxyacetate (5mM) and 4-fluorophenoxyacetate (2.5mM) gratuitously induced the same enzymes, but to a slightly smaller extent. The specificity of induction appears to be quite strict since none of the following compounds induced significant amounts of L-mandelate dehydrogenase: atropate (0.1–2.5mM), benzilate (0.4–2mM), β-benzoylpropionate (2mM), DL-*N*-benzoyl-α-alanine (0.4–10mM), benzylmalonate (2–10mM), 4-chlorophenoxyacetate (0.4–2mM), 2,3-dichlorophenoxyacetate (0.4–10mM), 2,4-dichlorophenoxyacetate (0.4–5mM), 3,4-dichlorophenoxyacetate (0.4–1mM), 2,4-dimethylphenoxyacetate (1–10mM), 3,5-dimethylphenoxyacetate (0.4–2mM), hippurate (0.4–10mM), mandelamide (0.4–10mM), D(−)-mandelate (0.2–10mM), 2-methoxyphenoxy-

Table 1. *Activities of the mandelate pathway enzymes in bacterium N.C.I.B. 8250 after growth in the presence of different inducers*

Bacterium N.C.I.B. 8250 was grown for 150min in 10mM-glutamate–salts medium or in the same medium supplemented with 5mM-L-mandelate, 5mM-phenylglyoxylate or 1mM-thiophenoxyacetate. A 25% (v/v) unwashed 12–15h culture grown on 10mM-glutamate–salts medium was used as inoculum. The cells were harvested, washed, stored, disrupted and assayed as described in the Materials and Methods section. The values for benzoate oxidase were obtained in an experiment different from that in which the other enzyme activities were measured.

	Enzyme activity (munits/mg of protein)			
Inducer ... / Enzyme	L-Mandelate (5mM)	Phenylglyoxylate (5mM)	Thiophenoxy-acetate (1mM)	No inducer
L-Mandelate dehydrogenase	178	180	113	1.9
Phenylglyoxylate carboxy-lyase	198	272	122	1.5
Benzaldehyde dehydrogenase I	27	34	26	1.1
Benzyl alcohol dehydrogenase	18	18	5.8	4.6
Benzaldehyde dehydrogenase II	14	14	<1	2.3
Benzoate oxidase	86	n.d.*	<1	<1
Catechol 1,2-oxygenase	372	208	1.8	1.9

* n.d., not determined.

acetate (0.2–5mM), 3-methylphenoxyacetate (0.4–2mM), 2-nitrophenoxyacetate (0.4–10mM), phenol (0.2–2mM), 4-phenoxybutyrate (0.4–5mM), 2-phenoxypropionate (0.4–10mM), 3-phenoxypropionate (0.4mM), phenylacetate (0.4–10mM), L-β-phenyllactate (0.4–10mM), phenylphosphonate (0.4mM), α-phenyl-propionate (0.4–2mM), 3-phenylpropionate (0.4–10mM), piperonylate (0.4mM), and mandelonitrile, phenylglyoxal or 2-phenoxyethanol (0.4–5mM). The last three compounds depressed growth. Amygdalin, benzene sulphinate, benzene sulphonate, *N*-phenylglycine and DL-tropate led to trace amounts (<4% of fully induced) of L-mandelate dehydrogenase. DL-α-Aminophenylacetate gave high activities of the enzyme but probably only after deamination to phenylglyoxylate. Induction by the methyl ester of DL-mandelate could probably also be attributed to its metabolism to mandelate. 3- and 4-Hydroxy-DL-mandelate and 4-fluoro-DL-mandelate, which are metabolized (Kennedy & Fewson, 1968*a*), gave non-gratuitous induction of L-mandelate dehydrogenase; in these cases there was no induction of catechol 1,2-oxygenase.

Kinetics of enzyme induction

The kinetics of enzyme induction were determined by measuring the differential rates of enzyme synthesis (Monod *et al.*, 1952). An example of the results obtained is shown in Fig. 1 which covers a time-span of approximately 20min after the addition of inducer. The times of onset of maximum differential rates of synthesis of all the enzymes studied are given in Table 2. Phenylglyoxylate and benzaldehyde gave very similar results to L-mandelate and benzyl alcohol respectively.

If the induction of two enzymes is co-ordinately controlled then their differential rates of synthesis should always be proportional to one another (Jacob & Monod, 1961). The correlation between the differential rates of synthesis of L-mandelate dehydrogenase and the other enzymes of the pathway was therefore calculated for a wide range of conditions (Table 3). The results indicate that only phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I are synthesized co-ordinately with L-mandelate dehydrogenase.

Mutant strains of bacterium N.C.I.B. 8250

Isolation of mutant strains unable to grow on L-mandelate yielded three different classes, as judged by their enzyme contents and their ability to grow on phenylglyoxylate. The first of these, exemplified by strain NF 0410 (Table 4), was unable to utilize L-mandelate as a carbon and energy source but grew normally on phenylglyoxylate. Extracts of this strain grown in the presence of L-mandelate or phenylglyoxylate contained no detectable L-mandelate dehydrogenase. The second type of mutant (e.g. strain NA 36, Table 4) failed to grow on either L-mandelate or phenylglyoxylate. Subsequent analysis of cells grown on L-glutamate in the presence of these two compounds showed that they contained little phenylglyoxylate carboxy-lyase but high activities of L-mandelate dehydrogenase and benzaldehyde dehydrogenase I. Some phenylglyoxylate must,

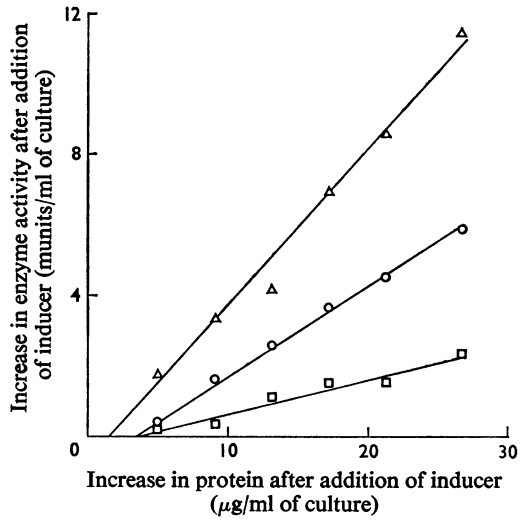


Fig. 1. *Differential plot for the induction of* L-*mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I by* L-*mandelate+benzyl alcohol*

Bacterium N.C.I.B. 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium and inoculated into 10mM-glutamate–salts medium. L-Mandelate and benzyl alcohol, each to give a final concentration of 5mM in the glutamate–salts medium, were added after approximately one generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, disrupted and assayed as described in the Materials and Methods section. Samples for determination of protein were also withdrawn from the growth flask at suitable intervals. ○, L-Mandelate dehydrogenase; △, phenylglyoxylate carboxy-lyase; □, benzaldehyde dehydrogenase I.

however, have been decarboxylated since low amounts of catechol 1,2-oxygenase were formed. The third type of blocked mutant, e.g. NF 2405, also failed to use either L-mandelate or phenylglyoxylate for growth. Extracts of this mutant contained L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I at little more than the basal activities (cf. Table 1). In this strain benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II were hyperinduced, while some catechol 1,2-oxygenase was also formed. We also isolated a mutant strain (NF 1706) that, like strain NA 36, lacked phenylglyoxylate carboxy-lyase. This strain, however, resembled NF 2405 in that it showed hyperinduction of benzyl alcohol dehydrogenase, benzaldehyde dehydrogenase II and low activities of catechol 1,2-oxygenase when grown in the presence of L-mandelate or phenylglyoxylate. These enzymes were not induced in the presence of thiophenoxyacetate (Table 4).

All the strains discussed were shown to have patterns of induction with benzaldehyde and benzyl alcohol identical with that of the parent organism. All the mutants unable to grow on L-mandelate were also unable to grow on 4-hydroxy-DL-mandelate. This confirms the view that the same relatively non-specific enzymes are responsible for the oxidation of L-mandelate and of the substituted mandelates that can support growth (Kennedy & Fewson, 1968*a*). Similar results have been obtained with mutants of *P. putida* lacking L-mandelate dehydrogenase (Stevenson & Mandelstam, 1965).

Discussion

Enzyme assays

The procedures for determination of enzyme activities were modified from those used by Kennedy & Fewson (1968*b*) so that usually all the enzyme activities can be measured in one extract. This simplifies experiments on induction, saves time, materials and effort and gives more reliable results. With benzaldehyde dehydrogenase a second extract has to be prepared in pyrophosphate buffer if the total activity is high (more than 80munits/mg of protein) or if benzaldehyde dehydrogenase II activity greatly exceeds benzaldehyde dehydrogenase I activity, because in these circumstances the benzaldehyde dehydrogenase II activity remaining after 2h of incubation at 37°C causes an overestimate of benzaldehyde dehydrogenase I activity (see Livingstone *et al.*, 1972). Usually, however, this is not necessary. In the present procedure, tris has to be used at pH10.3, outside its buffering range. This is satisfactory as the system was chosen empirically to give a measurement of the relative activities of the two benzaldehyde dehydrogenases (Livingstone, 1970; Livingstone *et al.*, 1972). For some enzymes the activity measured is less than the maximum possible because of the compromise conditions used for extraction; however, in all cases the activity detected is an accurate proportional representation of the total activity in the cell (Livingstone, 1970). The cell-free system so far developed for 'benzoate oxidase' in this organism (Fewson *et al.*, 1970) represents too low a proportion of the whole-cell activity to be used in experiments on enzyme induction, but recent work on this system in other bacteria (Reiner, 1971; Reiner & Hegeman, 1971) should allow the development of a sensitive and accurate cell-free assay.

Table 2. *Times of onset of maximum differential rates of enzyme synthesis after addition of inducers*

Bacterium N.C.I.B. 8250 was grown in 10mM-glutamate–salts medium, harvested, washed, resuspended in basal medium and inoculated into 10mM-glutamate–salts medium. The inducer was added after approximately one doubling of cell population under conditions of vigorous aeration at 30°C (Harvey *et al.*, 1968). Samples were withdrawn from the growth flask, harvested, washed, stored, disrupted and assayed as described in the Materials and Methods section. The time of onset of the maximum differential rate of enzyme synthesis was calculated as the intercept on the time-axis of the straight line representing the maximum rate of enzyme synthesis in a graph of (Δ enzyme concentration) against time. The values in parentheses represent the number of experiments.

	Time of onset of maximum rate of enzyme synthesis after addition of inducer (min)	
Inducer ...	L-Mandelate (5mM)	Benzyl alcohol (5mM)
Enzyme		
L-Mandelate dehydrogenase	5 (3)	No induction
Phenylglyoxylate carboxy-lyase	1 (2)	No induction
Benzaldehyde dehydrogenase I	5 (3)	No induction
Benzyl alcohol dehydrogenase	7 (3)	8 (3)
Benzaldehyde dehydrogenase II	8 (3)	8 (3)
Benzoate oxidase	29 (1)	24 (1)
Catechol 1,2-oxygenase	25 (2)	18 (2)

Table 3. *Correlation between the differential rates of synthesis of* L-*mandelate dehydrogenase and the other enzymes of the mandelate pathway*

In a series of independent experiments similar to that described in Fig. 1, the mandelate pathway enzymes of bacterium N.C.I.B. 8250 were induced by 5mM-L-mandelate (3)*, 5mM-phenylglyoxylate (2), 1mM-thiophenoxyacetate, 5mM-L-mandelate+5mM-benzyl alcohol, 5mM-L-mandelate + 5mM-acetate + 5mM-succinate, 5mM-benzyl alcohol (2), 5mM-benzyl alcohol + 5mM-acetate + 5mM-succinate, 1mM-benzaldehyde, 1mM-benzoate and 1mM-catechol. In each experiment the differential plot was drawn for each enzyme, and from the gradient of the graph the differential rate of enzyme synthesis was calculated. The correlation coefficients were then calculated for each individual correlation between the differential rates of synthesis of L-mandelate dehydrogenase and the other enzymes of the mandelate pathway.

Enzyme	Correlation coefficient
Phenylglyoxylate carboxy-lyase	0.98
Benzaldehyde dehydrogenase I	0.98
Benzyl alcohol dehydrogenase	−0.17
Benzaldehyde dehydrogenase II	−0.22
Benzoate oxidase	−0.27
Catechol 1,2-oxygenase	−0.39

* The values in parentheses represent the number of times the experiment was done.

Regulation of enzymes converting L-*mandelate into benzoate*

The co-ordinate control of L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase I is indicated by: (*a*) the correlation between the differential rates of synthesis of these enzymes under a variety of conditions including repression and gratuitous induction (Table 3), (*b*) the gratuitous synthesis of these and no other enzymes by thiophenoxyacetate (Table 1), phenoxyacetate, 4-fluorophenoxyacetate and 3-hydroxyphenoxyacetate, (*c*) the properties of blocked mutants, particularly strain NF 2405 (Table 4) which may be a regulatory mutant.

Calculation of times of onset of maximum differential rates of enzyme synthesis after addition of inducer (Table 2) may give slight overestimates if the induction shows a significant period of acceleration in rate. In fact we did not generally observe appreciable acceleration phases of enzyme induction. Presumably this was because we deliberately chose relatively high concentrations of inducers. Induction at less than saturating concentrations of inducers is, however, of considerable interest and Higgins & Mandelstam (1972) have shown that it is possible to obtain estimates of the 'K_{ind}' for at least some of the mandelate enzymes of *P. putida*. There is not such a clear-cut difference in the times of appearance of the enzymes as Hegeman (1966*a*) found for some enzymes of the mandelate pathway of *P. putida*,

Table 4. *Activities of mandelate pathway enzymes in mutant strains of bacterium N.C.I.B. 8250 after growth in the presence of various potential inducers*

Mutant strains were grown for 2.5h in 10mM-glutamate–salts medium supplemented with L-mandelate, phenylglyoxylate or thiophenoxyacetate. A 25% (w/v) unwashed 12–14h culture grown on 10mM-glutamate–salts medium was used as inoculum. The cells were harvested, washed, stored, disrupted and assayed as described in the Materials and Methods section.

		Enzyme activity (munits/mg of protein)									
Strain ...		NF 0410		NA 36		NF 2405		NF 1706			
Inducer ...		L-Mandelate (5mM)	Phenylglyoxylate (5mM)	L-Mandelate (5mM)	Phenylglyoxylate (5mM)	L-Mandelate (5mM)	Phenylglyoxylate (5mM)	L-Mandelate (5mM)	Phenylglyoxylate (5mM)	Thiophenoxyacetate (1mM)
Enzyme										
L-Mandelate dehydrogenase		0	0	165	195	4.4	5.4	138	158	97
Phenylglyoxylate carboxy-lyase		9.0	224	2.5	3.6	2.6	1.7	8.5	9.0	3.5
Benzaldehyde dehydrogenase I		2.0	28	40	56	4.4	4.3	46	49	28
Benzyl alcohol dehydrogenase		7.4	23	22	24	384	303	190	394	6.0
Benzaldehyde dehydrogenase II		8.0	15	21	17	185	239	150	295	<1
Catechol 1,2-oxygenase		1.4	177	15	57	81	65	42	47	1.8

but the enzymes he measured were well separated in the metabolic sequence. Nevertheless the first three enzymes of the pathway in bacterium N.C.I.B. 8250 are formed before the others; a feature not observed for all inducible catabolic pathways (e.g. Hosokawa, 1970). The rapid appearance of phenylglyoxylate carboxy-lyase is difficult to reconcile with the generally accepted models of enzyme induction. It is remarkable that benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II are formed at about the same time whether induced by L-mandelate or phenylglyoxylate or by benzyl alcohol or benzaldehyde. In the former case the induction soon slows down whereas with benzyl alcohol or benzaldehyde the differential rate remains constant for long periods. This presumably means that because of the rate limitation of benzaldehyde dehydrogenase I (see Table 1) L-mandelate and phenylglyoxylate lead to the rapid accumulation of benzaldehyde or benzyl alcohol, which serve as inducers. We have confirmed that there is a transitory accumulation of benzaldehyde and benzyl alcohol when bacterium N.C.I.B. 8250 is grown in the presence of L-mandelate or phenylglyoxylate (C. A. Fewson, A. M. Cook & J. D. Beggs, unpublished work). Any fundamental linkage between the mandelate enzymes and the benzyl alcohol enzymes is ruled out by the specific gratuitous induction of the mandelate enzymes by thiophenoxyacetate (Table 1) and the specific gratuitous induction of the benzyl alcohol enzymes by pyridylcarbinols and other compounds (Livingstone *et al.*, 1972). Also, in mutant strain NF 1706 (Table 4) where there was hyperinduction of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase II in the presence of L-mandelate or phenylglyoxylate, there was no induction by thiophenoxyacetate. The hyperinduction of the benzyl alcohol enzymes in mutant strains NF 2405 and NF 1706 is difficult to understand as both possess very low activities of phenylglyoxylate carboxy-lyase and would not be expected to form either benzaldehyde or benzyl alcohol at appreciable rates.

Benzoate oxidase and catechol 1,2-oxygenase appear very close together and, if anything, in the inverse order of their metabolic roles. They are, however, clearly regulated by separate genetic elements as catechol 1,2-oxygenase, but not benzoate oxidase, is induced by growth of bacterium N.C.I.B. 8250 on catechol (Kennedy & Fewson, 1968*b*).

Although the first three enzymes of mandelate oxidation are co-ordinately regulated in bacterium N.C.I.B. 8250 there is no evidence as to whether their structural genes are contiguous.

Specificity of induction

The primary inducer of the mandelate enzymes appears to be phenylglyoxylate. The best evidence

for this is the failure of L-mandelate to induce the other two enzymes in mutant strain NF 0410 which lacks L-mandelate dehydrogenase (Table 4). This is another example of a catabolic pathway that is not induced by the initial substrate (see Ornston, 1971). Presumably L-mandelate serves as inducer after being converted into phenylglyoxylate by the basal amount of L-mandelate dehydrogenase (Table 1). It is therefore a little surprising that the kinetics of induction of the mandelate enzymes are so similar with either L-mandelate or phenylglyoxylate as inducer, but the 'K_{ind}' for phenylglyoxylate could be very low. Several derivatives of mandelate, or presumably of phenylglyoxylate, which are substituted in the aromatic ring can also serve as inducers. This, together with the relatively non-specific nature of the enzymes involved (Kennedy & Fewson, 1968*b*) allows a wide range of compounds substituted in the aromatic ring to serve as growth substrates for this organism (Fewson, 1967*b*; Kennedy & Fewson, 1968*b*). A similar, but probably more restricted, situation occurs in *P. putida* (Stanier *et al.*, 1953; Stevenson & Mandelstam, 1965). Gunsalus *et al.* (1965) have pointed out how relative non-specificity may serve for economy of protein biosynthesis and of genetic material.

Our search for gratuitous inducers of this regulon was based on the discovery of Hegeman (1966*a*) that phenoxyacetate, and to a lesser extent thiophenoxyacetate, induced the mandelate enzymes in *P. putida*. We found that thiophenoxyacetate was quite a good inducer in bacterium N.C.I.B. 8250, as are phenoxyacetate and a few substituted phenoxyacetates. Nevertheless, the overall specificity is fairly strict and it is interesting, in view of other differences, that the same type of compound should serve as gratuitous inducer in both *P. putida* and bacterium N.C.I.B. 8250. We have not yet been successful in finding a non-inducing substrate. A compound of this type would be useful as it might simplify the search for constitutive mutant strains.

Comparative biochemistry of mandelate oxidation

The results in the present paper show that in bacterium N.C.I.B. 8250 L-mandelate is oxidized to benzoate by three co-ordinately controlled enzymes induced by phenylglyoxylate. In *P. putida* both D- and L-mandelate are oxidized to benzoate by a co-ordinately controlled group of five enzymes which are relatively non-specifically induced by L- and D-mandelate or phenylglyoxylate (Hegeman, 1966*a*,*b*,*c*). In *P. aeruginosa*, conversely, L-mandelate dehydrogenase forms an independent regulon induced by L-mandelate, and phenylglyoxylate carboxy-lyase and benzaldehyde dehydrogenase constitute a second group induced by phenylglyoxylate (Rosenberg, 1971). In view of these differences in regulation it seems possible that the enzymes for mandelate oxidation have had a separate evolutionary origin in these three bacteria (see Cánovas *et al.*, 1967) but much more work will have to be done on the corresponding enzymes of different organisms before any firm conclusions can be drawn.

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