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Induction and Multi-Sensitive End-Product Repression in Two Converging Pathways Degrading Aromatic Substances in *Pseudomonas fluorescens*

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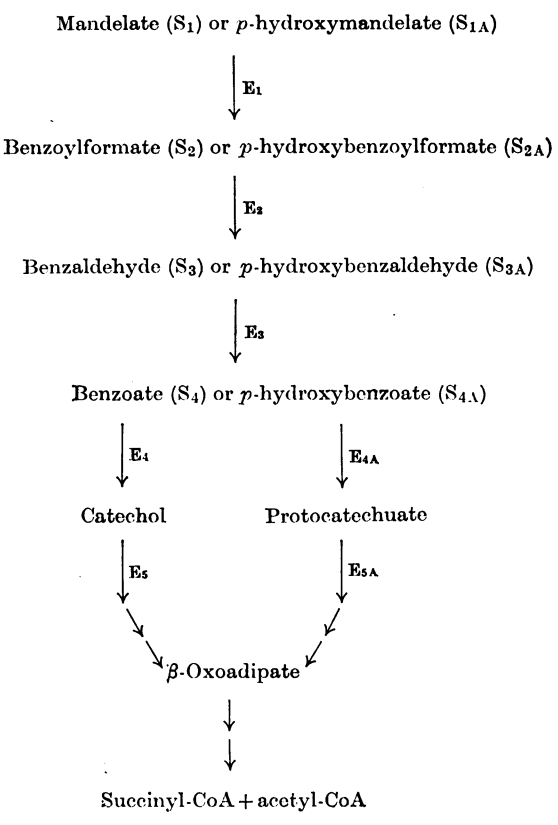
1. Control of enzyme formation has been examined in the pathways degrading mandelate and *p*-hydroxymandelate in *Pseudomonas fluorescens*. 2. The first three enzymes form a group which is common to both pathways and which is co-ordinately induced or repressed. The genes controlling these enzymes are assumed to form a 'regulon'. This group of enzymes is induced by mandelate or *p*-hydroxymandelate and repressed by benzoate and by *p*-hydroxybenzoate (the immediate end products resulting from the action of this group of enzymes). 3. Repression is independently exerted by end products of enzymes controlled by succeeding regulons, i.e. by catechol, by protocatechuate and finally by succinate and acetate. 4. The pattern is repeated further along the pathway, so that benzoate oxidase (controlled by the second regulon) is repressed by its immediate end product, catechol, and again by succinate and acetate. 5. Pyrocatechase, an enzyme controlled by the third regulon, is repressed by succinate and acetate. 6. There is a parallel system of multi-sensitive repression mechanisms controlling production of the enzymes that degrade the hydroxy compounds. Again, the enzymes of each regulon are repressed by the immediate end product of their action and by the end products of each succeeding group of enzymes. 7. Repressor activity appears to be exerted by compounds that are likely to occur as such in the external environment or that occur at points of convergence of the degradative pathways of the cell. 8. The net effect of this control system, involving both induction and end-product repression, appears to be that cells will not form inducible degradative enzymes if the end products are already being supplied from without or are being produced by degradation of some alternative source of carbon and energy.

In the classical example of sequential induction, mandelate is degraded with the formation of the following intermediates (Stanier, 1951):

$$\text{Mandelate} \xrightarrow{E_1} \text{benzoylformate} \xrightarrow{E_2} \text{benzaldehyde} \xrightarrow{E_3} \text{benzoate} \xrightarrow{E_4} \text{catechol} \xrightarrow{E_5} \beta\text{-oxoadipate} \rightarrow\rightarrow \text{succinyl-CoA} + \text{acetyl-CoA}$$

(Scheme 1). The original findings suggested the hypothesis that such inductions proceed in single sequential steps, with the product of each reaction acting as an inducer for the next enzyme in the sequence (Stanier, 1947). It has now been shown that enzyme induction in a number of degradative pathways (Stanier, Hegeman & Ornston, 1963; Palleroni & Stanier, 1964) is the result of the co-ordinate induction of blocks of enzymes that are connected by sequential steps. Thus in the mandelate pathway E_1, E_2 and E_3 have been shown to be induced simultaneously by the primary substrate, S_1, of the pathway; E_4 is induced by benzoate, and E_5 and the immediately subsequent enzymes are induced by catechol (Stanier *et al.* 1963). Because E_1, E_2 and E_3 are related in function and regulated together by inducers (Stanier *et al.* 1965) and by repressors (Mandelstam, 1964; Mandelstam & Jacoby, 1965) they may be under the control of a single 'operon', i.e. a group of genes that are transcribed and regulated as a unit. Strictly speaking, the term 'operon' should be reserved for a system where the genes are known to be closely linked genetically, as in the histidine operon. Since there is no evidence that the genes determining the structure of E_1–E_3 are linked, the term 'regulon', coined by Maas & McFall (1964), is used in the present paper to describe the group of genes controlling these enzymes. This term was intended to describe a system (e.g. the arginine synthetic



Scheme 1. Pathways of degradation of mandelate and *p*-hydroxymandelate in *Pseudomonas fluorescens*.

pathway) in which the enzymes are regulated as a group but where the genes are not necessarily closely linked.

In a parallel pathway, *p*-hydroxymandelate is degraded through the following sequence of intermediates (Gunter, 1953):

$$p\text{-Hydroxymandelate} \xrightarrow{E_1} p\text{-hydroxybenzoylformate} \xrightarrow{E_2} p\text{-hydroxybenzaldehyde} \xrightarrow{E_3} p\text{-hydroxybenzoate} \xrightarrow{E_4} \text{protocatechuate}$$

Protocatechuate, in turn, undergoes a series of reactions (Evans, 1963) to form β-oxoadipate, at which point the *p*-hydroxymandelate pathway converges with that of mandelate (Scheme 1). It has been suggested that enzymes E_1–E_3 of the mandelate and *p*-hydroxymandelate pathways are common (Gunter, 1953; Stanier, Gunsalus & Gunsalus, 1953). Further evidence of this is presented below.

Mandelstam & Jacoby (1965) investigated the repression of the enzymes E_1–E_3, controlled by the first regulon, by the end product of their action (benzoate) and by the end products of subsequent sequences in the system (catechol and succinate). All three substances repressed the formation of the enzymes of the first regulon. The possibility that these repression effects were due to 'sequential repression' or to the formation of a single common end-product repressor, e.g. succinate or some closely related metabolite, was ruled out. The term 'multi-sensitive' was introduced to describe this regulatory mechanism in which the mandelate regulon was shown to be activated independently by at least three metabolites of the pathway.

The present paper describes induction and repression of the mandelate pathway by intermediates of the *p*-hydroxymandelate pathway. In addition, the enzymes controlled by the second and third regulons of the mandelate and *p*-hydroxymandelate pathways have been examined and shown to be subject both to induction and to multi-sensitive repression exerted by the immediate end products resulting from their action and by the end products of succeeding regulons.

METHODS

Organism. Pseudomonas fluorescens A3.12 (obtained from Dr R. Y. Stanier) or mutants derived from this strain were used.

Isolation of mutants. Mutants with specific enzymic defects in the mandelate or *p*-hydroxymandelate sequences were obtained after treatment of wild-type cultures of *P. fluorescens* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as mutagen (Mandelstam & Jacoby, 1965). Mutants retained for use had no measurable enzymic activity, and failed to show growth on the substrate in question added as sole carbon source for at least 24hr.

Induction in growing cultures. Induction was carried out with cultures growing exponentially (0·06–0·1mg./ml.) in the glucose–mineral salts medium described by Mandelstam & Jacoby (1965). Inducers were added at a concentration of 250μg./ml. unless stated otherwise. The cell density was normally allowed to double in the presence of the inducer. Under these conditions, intermediates of the mandelate and *p*-hydroxymandelate pathways, with the exception of *p*-hydroxymandelate, proved to be excellent inducers. *p*-Hydroxymandelate required an 18–24hr. induction period before appreciable amounts of enzyme could be detected, and for this reason cells grown from a small inoculum on *p*-hydroxymandelate as the sole carbon source were used in some experiments. Samples for assay were treated as described below.

Preparation of cells for assay. At the end of the growth period samples were removed and chloramphenicol was added to a final concentration of 0·1mg./ml. The suspensions were centrifuged, the cells washed with phosphate buffer (0·1M-KH_2PO_4, adjusted with NaOH to pH7), and resuspended in the same buffer to a concentration of 1·0mg. dry wt./ml. for determination of oxygen uptake by whole cells.

Cell-free extracts were prepared after ultrasonically disrupting 8·5ml. of a suspension of cells (1·0mg./ml. in 0·9% NaCl) for 5min. in an oscillator, as described by Mandelstam & Jacoby (1965).

Toluene-treated cells for estimation of benzoylformate decarboxylase were prepared as described by Mandelstam & Jacoby (1965).

Oxygen uptake by whole cells. Portions (2·5ml.) containing 1·0mg. of whole cells suspended in buffer (0·1M-KH_2PO_4, pH 7) were assayed by conventional manometric techniques with substrates (0·5ml.) in the following concentrations: mandelate, *p*-hydroxymandelate, protocatechuate and *p*-hydroxybenzaldehyde, 2·0mg./ml.; catechol and benzaldehyde, 1·0mg./ml. The total volume/flask was 3·0ml. Values were corrected for endogenous respiration, and are expressed as mμmoles of O_2/min.

E_1 (mandelate dehydrogenase), E_2 (benzoylformate decarboxylase), E_4 (benzoate oxidase) and E_5 (pyrocatechase). Activities of these enzymes were determined by the methods described, or referred to, by Mandelstam & Jacoby (1965).

E_3 [benzaldehyde dehydrogenase (NAD- and NADP-linked) (benzaldehyde–NAD oxidoreductase, EC 1.2.1.6, and benzaldehyde–NADP oxidoreductase, EC 1.2.1.7)]. These enzymes were assayed spectrophotometrically by following the reduction of NAD and NADP by ultrasonically disrupted cell extracts in the presence of benzaldehyde. In these studies it was found that the NAD-linked and the NADP-linked dehydrogenases were present in equivalent amounts, and that assay of extracts with the two nucleotides together resulted in a doubling of the individual values. For this reason the enzymes were assayed together. The procedure adopted was to add the following to a 1·0cm. cell in the order given: 1·4ml. of phosphate buffer, pH 8·5 (0·1M); 0·3ml. of NaCN (1·0mg./ml.); 0·5ml. of cell-free extract (containing about 0·5mg. dry wt. of bacteria); 0·3ml. of NAD (1·0mg./ml.); 0·3ml. of NADP (1·0mg./ml.). The reaction was started by adding 0·2ml. of benzaldehyde (1·0mg./ml.) and allowed to proceed at room temperature (about 18°). The change in extinction at 340mμ was measured at 1min. intervals for a period of 5min. against a blank containing water instead of benzaldehyde.

p-Hydroxybenzaldehyde dehydrogenase. Owing to the high absorption of *p*-hydroxybenzaldehyde at 340mμ, no satisfactory spectrophotometric assay was possible. In a number of instances extracts of cells induced with *p*-hydroxybenzaldehyde were measured with benzaldehyde as the substrate, but usually activity was determined by whole-cell oxidation.

E_{4A} (p-hydroxybenzoate oxidase). Assay of this enzyme was based on the loss of *p*-hydroxybenzoate from a buffered suspension of whole cells in the presence of chloramphenicol. The colorimetric method used was a modification of the Millon reaction for blood tyrosyl compounds (Folin & Marenzi, 1929; Bernhart & Schneider, 1943). The procedure was as follows. To a flask (125ml.) were added: whole cells (2mg. dry wt. in a volume of 17ml. of 0·1M-phosphate buffer, pH 7); 2ml. of chloramphenicol (1mg./ml.). After equilibration in a water bath at 30° for 5min., 1ml. of 0·4% sodium *p*-hydroxybenzoate was added. Then 1ml. samples were removed immediately, and after being shaken for 20, 40 and 60min. they were added directly to tubes containing 2·5ml. of 0·8N-H_2SO_4 and 1ml. of $HgSO_4$ (11·3%, w/v) in 4·5N-H_2SO_4. The tubes were then heated in boiling water for 8min., cooled rapidly and centrifuged at 5000rev./min. for 5min. in an MSE centrifuge. To 3·5ml. of the supernatant 0·05ml. of fresh aqueous $NaNO_2$ (2·5%, w/v) was added and the tubes were incubated for 2hr. at 35°. The extinction was read at 500mμ, and the amount of *p*-hydroxybenzoate remaining undestroyed was calculated.

E_{5A} (protocatechuate oxygenase, EC 1.99.2.2). Measurement of this enzyme was based on the disappearance of protocatechuate from a suspension of whole cells. Protocatechuate was determined colorimetrically by a modification of the method of Child, Simpson & Westlake (1963). The general procedure was as described for *p*-hydroxybenzoate except that 1ml. of 0·4% sodium protocatechuate was substituted to give a final concentration of 200μg./ml. At 0, 30 and 60min., 4ml. samples were removed and added directly to tubes containing carbonate (3·75%, w/v). After 20min. incubation at room temperature, the tubes were centrifuged to remove cell debris and the extinction of the supernatant solution was measured at 540mμ. The uptake of protocatechuate was then calculated.

Enzyme units. All units are expressed as mμmoles of substrate destroyed/min.; specific activities are units/mg. dry wt. of bacteria.

Reagents. Benzoylformic acid was synthesized by the method of Oakwood & Weisgerber (1955). *p*-Hydroxymandelic acid was synthesized by the method of Ladenburg, Folkers & Major (1936). Benzaldehyde (AnalaR grade) was deacidified and redistilled according to the method of Vogel (1954).

RESULTS

Comparison of enzymes E_1, E_2 and E_3 of the mandelate and p-hydroxymandelate pathways. In view of earlier studies suggesting that E_1–E_3 are common to both the mandelate and *p*-hydroxymandelate pathways (Gunter, 1953; Stanier *et al.* 1953) and later evidence (see the introduction) showing that E_1–E_3 are induced co-ordinately, these enzymes were reinvestigated with both mandelate and *p*-hydroxymandelate as substrates.

Cultures grown in a minimal medium with mandelate, *p*-hydroxymandelate or benzoylformate as the sole carbon source were assayed for mandelate dehydrogenase (E_1). The enzyme oxidizing *p*-hydroxymandelate was assayed in the same manner as E_1 but with *p*-hydroxymandelate as the substrate (see the Methods section).

Table 1 illustrates the activities of E_1–E_3 of fully induced cells growing exponentially at the expense of mandelate and benzoylformate. As reported by Stanier *et al.* (1963), S_1 and S_2 are equally good inducers. Extracts of such cells also oxidized *p*-hydroxymandelate. Similarly, cells grown on *p*-hydroxymandelate possessed activities of E_1 and E_2 similar to those of mandelate- and benzoylformate-grown cells, and exhibited the same activity of the *p*-hydroxymandelate oxidizing enzyme. An anomalously low value for E_3 was consistently found in cultures induced with *p*-hydroxymandelate. The reason for this is not known. This cross-induction supported, but of

Table 1. *Specific activities of the mandelate group of enzymes in cultures fully adapted to mandelate, benzoylformate and* p-*hydroxymandelate*

Cultures of *P. fluorescens* A3.12 (wild type) were grown to a density of about 0·1 mg./ml. in minimal medium containing as sole carbon source mandelate or benzoylformate or *p*-hydroxymandelate (final concn. 250 μg./ml.).

	Mandelate-grown	*p*-Hydroxymandelate-grown	Benzoylformate-grown
E_1 (mandelate dehydrogenase)	94	56	88
p-Hydroxymandelate dehydrogenase*	30	27	28
E_2 (benzoylformate decarboxylase)	715	560	708
E_3 (benzaldehyde dehydrogenase)	67	9	60

* Determined in the same way as E_1, but with *p*-hydroxymandelate as substrate.

Table 2. *Repression of the mandelate group of enzymes by* p-*hydroxybenzoate, protocatechuate, succinate and acetate*

Cultures of *P. fluorescens* A3.12 (wild type) growing exponentially in glucose medium were induced with mandelate, with and without the addition of *p*-hydroxybenzoate or protocatechuate (each at 250 μg./ml.) or succinate (500 μg./ml.) or acetate (500 μg./ml.).

	E_1 (mandelate dehydrogenase)		E_2 (benzoylformate decarboxylase)		E_3 (benzaldehyde dehydrogenase)	
Induction medium	Specific activity	Repression (%)	Specific activity	Repression (%)	Specific activity	Repression (%)
Mandelate	107	0	315	0	21	0
Mandelate + *p*-hydroxybenzoate	12	89	9	97	3	86
Mandelate + protocatechuate	96	11	235	25	17	19
Mandelate + succinate	35	67	111	65	12	43
Mandelate + acetate	55	49	111	65	15	29

course did not prove, the suggestion that the same enzymes catalysed the degradation of the two series of compounds. Further evidence was sought and was provided by a mutant (*md*$^-$) lacking E_1. This organism, isolated for inability to grow on mandelate, also failed to grow on *p*-hydroxymandelate. At the same time, benzoylformate, which induced both mandelate- and *p*-hydroxymandelate-oxidizing enzyme in the wild type (Table 1), failed to induce either in the *md*$^-$ mutant.

Since *p*-hydroxymandelate was a poor inducer, and since the evidence was consistent with the assumption that E_1–E_3 were common to both pathways, mandelate was usually used as inducer.

Repression of enzymes controlled by the first regulon [E_1 (mandelate dehydrogenase), E_2 (benzoylformate decarboxylase) and E_3 (benzaldehyde dehydrogenase)] by intermediates of the p-*hydroxymandelate pathway.* A series of experiments was carried out in which, with mandelate as the inducer, intermediates of the *p*-hydroxymandelate pathway were tested in turn as repressors. The experiments were all done with cultures growing in glucose as the carbon source with inducers and repressors added as indicated. At the concentrations used none of these substances inhibited growth.

p-Hydroxybenzoate was found to repress enzymes E_1, E_2 and E_3 by about 90% (Table 2). At the same time, it was found that enzymes subsequent to *p*-hydroxybenzoate (*p*-hydroxybenzoate oxidase and protocatechuate oxygenase) were fully induced. This repression by *p*-hydroxybenzoate was similar to that produced by benzoate (see Mandelstam & Jacoby, 1965).

Protocatechuate had a low and somewhat variable effect on the enzymes controlled by the first regulon (11–25% repression). This was in contrast with the 50–80% repression observed previously for catechol, the corresponding intermediate of the mandelate pathway (Mandelstam & Jacoby, 1965).

Succinate and acetate, as final products of the pathway, were tested and shown to repress E_1, E_2 and E_3 by about 30–70% (Table 2). Repression by these compounds was again variable and less than that found with *p*-hydroxybenzoate. Repression

by acetate and succinate not only varied from day to day but with the duration of the experiments. When the time-course was followed, the enzymes 'escaped' from repression at any time between 40 and 80 min. after addition of the repressor. The actual degree of repression found at the end of 1 hr. (the usual duration of an experiment) was thus dependent on the time at which 'escape' had begun.

In cultures that had been pre-adapted by continued growth in the presence of succinate or acetate, there was no repression at all, i.e. full activities of the enzymes were obtained on induction. In such pre-adapted cells it is likely that the repressors are removed much more efficiently, presumably by expansion of the tricarboxylic cycle, and that an intracellular concentration of the substrate sufficiently high to cause repression cannot be achieved.

Experiments were then done to determine whether *p*-hydroxybenzoate and protocatechuate were themselves repressors or whether they acted only after conversion into some common repressor (e.g. succinate or acetate). Mutants (p-*hydroxybenz*$^-$ and *protocat*$^-$), unable to utilize the substrates in question, were isolated and the repression effects measured. *p*-Hydroxybenzoate repressed the enzymes of the first regulon almost completely, although it was not itself being metabolized, and the degree of repression was comparable with that found in a mutant unable to oxidize benzoate (Table 3). Similar results were obtained with protocatechuate in a *protocat*$^-$ mutant. Here again repression was about the same as that observed with protocatechuate in the wild type (see Table 2). These data add to the evidence (see also Mandelstam & Jacoby, 1965) that intermediates of these pathways act as repressors as such, and not as a result of their being metabolized to a common end-product repressor.

Repression of enzymes of the first regulon by benzaldehyde and p-*hydroxybenzaldehyde.* For completeness, the effect of benzaldehyde and *p*-hydroxybenzaldehyde on induction of the enzymes of the first regulon by mandelate was determined. Rather unexpectedly, both aldehydes were found to be very strong repressors of the first regulon (Table 4). The repression by benzaldehyde and *p*-hydroxybenzaldehyde is unusual in that it presents a case where a substrate gives virtually complete repression of its own enzyme. The possibility that the aldehydes exert their effect only

Table 3. *Repression of enzymes of the mandelate group of enzymes by benzoate and by* p-*hydroxybenzoate in mutants unable to utilize these compounds*

P. fluorescens cells growing exponentially in glucose–minimal medium were induced with mandelate alone or with benzoate for the *benz*$^-$ mutant and with *p*-hydroxybenzoate for the p-*hydroxybenz*$^-$ mutant.

	Specific activity (units/mg.)					
	benz$^-$ mutant			p-*hydroxybenz*$^-$ mutant		
Induction medium ...	Mandelate	Mandelate + benzoate	Repression (%)	Mandelate	Mandelate + *p*-hydroxybenzoate	Repression (%)
E_1 (mandelate dehydrogenase)	36	2	95	19	0	100
E_2 (benzoylformate decarboxylase)	670	26	96	293	17	94
E_3 (benzaldehyde dehydrogenase)	40	4	90	18	1	94

Table 4. *Repression of enzymes in the mandelate group by benzaldehyde and* p-*hydroxybenzaldehyde*

Exponentially growing cultures of *P. fluorescens* A3.12 (wild type) were induced with mandelate with and without the addition of benzaldehyde or *p*-hydroxybenzaldehyde.

	Specific activity (units/mg.)				
Induction medium ...	Mandelate	Mandelate + benzaldehyde	Repression (%)	Mandelate + *p*-hydroxybenzaldehyde	Repression (%)
E_1 (mandelate dehydrogenase)	82	0	100	0	100
E_2 (benzoylformate decarboxylase)	450	0	100	0	100
E_3 (benzaldehyde dehydrogenase)	35	6	83	6	83

after they have been oxidized to the corresponding aromatic acid is unlikely for kinetic reasons, but has not been formally excluded.

Existence of an alternative oxidation system for benzaldehyde and p-*hydroxybenzaldehyde.* Since the cells are known to be able to grow with *p*-hydroxybenzaldehyde as sole carbon source, the fact that this compound acted as a potent repressor of a group of enzymes including its own appeared to create an anomalous situation. This was resolved when it was found that the cells had another inducible enzyme which could oxidize benzaldehyde or *p*-hydroxybenzaldehyde and was not controlled by the same regulon. Three types of evidence supported this conclusion.

First, although E_3, as measured in cell-free extracts, was repressed by benzaldehyde or *p*-hydroxybenzaldehyde, the whole cells, when tested, oxidized the aldehydes at rates equivalent to those of mandelate-induced cells (Table 5). Also, treatment with the aldehyde alone induced whole-cell activity, but little or no E_3. These facts suggested that the cells had an additional aldehyde dehydrogenase which was induced by benzaldehyde and subsequently destroyed during preparation of the cell-free extract.

Finally, the assumption that there was a benzaldehyde dehydrogenase separate from E_3 was strengthened by the finding that benzoate and *p*-hydroxybenzoate, which are repressors for E_3 (cf. Table 3), failed to repress the whole-cell oxidative activity induced by benzaldehyde or *p*-hydroxybenzaldehyde. Benzaldehyde oxidation in mandelate-induced cells was repressed about 95% by *p*-hydroxybenzoate (cf. also Table 3), but, in contrast, this compound had virtually no repressor effect in the benzaldehyde-induced system. Similarly, benzoate also failed to repress activity in cells induced with benzaldehyde or with *p*-hydroxybenzaldehyde.

Confirmation of the presence of an aldehyde-oxidizing enzyme separate from E_3 was obtained by isolation of a mutant (*benzald*$^-$) unable to grow on either benzaldehyde or *p*-hydroxybenzaldehyde. The mutant retained the ability to grow on mandelate (or *p*-hydroxymandelate), and E_3 was readily demonstrable in such cultures. However, in the mutant whole-cell oxidative capacity could not be induced by the aldehydes as in the wild type.

Repression of enzymes controlled by the second and third regulons of the mandelate pathway. As stated in the introduction, E_4 (benzoate oxidase) is a separately controlled system, whereas E_5 (pyrocatechase) is the first enzyme of a third group. These were now examined in turn for repression effects.

Cultures growing in glucose medium were induced with benzoate alone or with catechol (125 μg./ml.) or succinate (500 μg./ml.) as repressors. Catechol produced a weak and rather variable repression (usually about 20–30%). Succinate was a much more potent repressor, causing inhibition of synthesis of benzoate oxidase to the extent of 60–95%. Since benzoate-oxidase activity is largely destroyed during the preparation of cell-free extracts (see Mandelstam & Jacoby, 1965), the activity had to be measured with whole cells. The observed values thus represent oxygen uptake not only for the oxidase step but for the whole of the pathway. To ensure that the observed repression was, in fact, due to repression of E_4 and not to an effect on an enzyme lower in the pathway, oxygen uptake was always measured with catechol as the substrate. The catechol oxidation values were substantially the same in the control cultures and in those treated with catechol or succinate. Thus the pathway from E_5 onwards had been fully induced and the observed effects on benzoate oxidation could be properly ascribed to repression of E_4.

Acetate (500 μg./ml.) was also tested and produced about the same degree of repression as succinate. β-Oxoadipate was not tested because it fails to enter the cells (see Mandelstam & Jacoby, 1965).

The effect of succinate and acetate on the formation of E_5 (pyrocatechase) was then measured. Succinate was a very variable repressor, inhibiting synthesis by 30–90%. Acetate, however, was a consistently stronger repressor for this enzyme (Table 6).

Repression of enzymes controlled by the second and third regulons of the p-*hydroxymandelate pathway.* Data for an analogous experiment on E_{4A} (*p*-hydroxybenzoate oxidase) are given in Table 7. Protocatechuate, the immediate product of enzyme action, was a rather weak repressor, whereas

Table 5. *Comparison of whole-cell oxidation of benzaldehyde with the activity of E_3 (benzaldehyde dehydrogenase) in wild-type cultures induced with mandelate, with mandelate and benzaldehyde, or with benzaldehyde alone*

P. fluorescens cultures growing exponentially in glucose medium were exposed to the substrates for one generation. Benzaldehyde oxidation was then measured in washed whole cells and in cell-free extracts.

Induction medium	Benzaldehyde oxidation (whole cells) (mμmoles of O_2/min./mg.)	E_3 (benzaldehyde dehydrogenase) (units/mg.)
Mandelate	560	70
Mandelate + benzaldehyde	547	7
Benzaldehyde	555	7

Table 6. *Repression of pyrocatechase by succinate and acetate*

P. fluorescens cells growing exponentially in glucose–minimal medium were induced with catechol (125 μg./ml.) alone, or with succinate or acetate (both at 1mg./ml.). After one generation the pyrocatechase was estimated in cell-free extract. Values for two experiments are shown.

	Pyrocatechase	
Induction medium	Specific activity	Repression (%)
Catechol	110, 85	0
Catechol + succinate	14, 56	87, 34
Catechol + acetate	7, 18	94, 79

Table 7. *Repression of* p-*hydroxybenzoate oxidase by protocatechuate, succinate and acetate*

Wild-type *P. fluorescens* (A3.12) growing exponentially in glucose–minimal medium was induced with *p*-hydroxybenzoate (250 μg./ml.) with and without the addition of protocatechuate (250 μg./ml.), succinate (500 μg./ml.) or acetate (500 μg./ml.).

	E_{4A} (*p*-hydroxybenzoate oxidase)	
Induction medium	Specific activity	Repression (%)
p-Hydroxybenzoate	68	0
p-Hydroxybenzoate + protocatechuate	50	27
p-Hydroxybenzoate + succinate	3	96
p-Hydroxybenzoate + acetate	5	93

succinate and acetate produced a much more marked effect.

Finally, the effects of acetate and succinate on the formation of E_{5A} (protocatechuate oxidase) were measured. Both compounds produced moderate repression, reducing the specific activity in a typical experiment from 54 units/mg. to 27 and 33 units/mg. respectively.

DISCUSSION

It has been tacitly assumed that inhibition of synthesis is due to interference with the enzyme-synthesizing mechanism. Theoretically, however, a repressor could exert its effect by preventing the induction or the functioning of a permease needed for uptake of the inducer. G. D. Hegeman & L. D. Ornston (personal communication) looked for, and were unable to find, any evidence for the existence of such a permease. Mandelate appears to enter the cells freely, and to reach the same concentration inside the cells as in the external medium. The interaction between the inducer and the various repressors thus appears to occur within the cell and to affect the enzyme-forming mechanism itself. An analogous case is the repressor action of succinate on the inducible amidase of *Pseudomonas aeruginosa*, which has been examined and shown not to occur at the permease level (Clarke & Brammar, 1964).

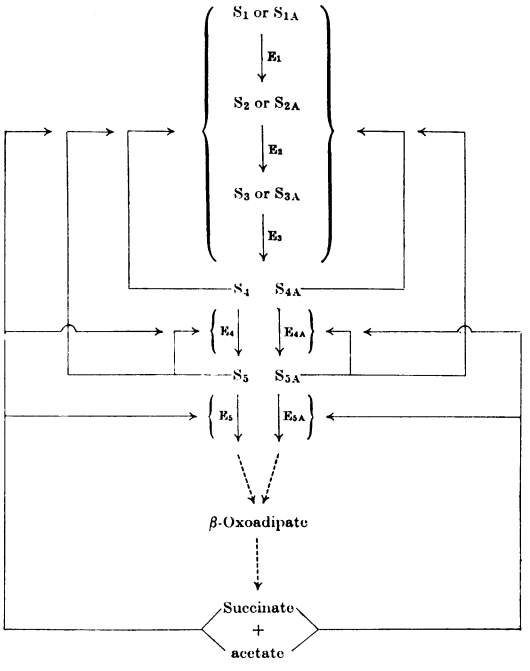
It is convenient to consider the control of one regulon at a time. The enzymes of the first regulon were previously shown to be repressed by benzoate, the end product of action of these enzymes, and by the more distal end products catechol and succinate (or acetate) (Mandelstam & Jacoby, 1965). Since the repression mechanism could be activated independently by any one of these repressors, it was called a 'multi-sensitive repression mechanism'. The present experiments show that the corresponding compounds, S_{4A} and S_{5A} (see Scheme 2), of the *p*-hydroxymandelate pathway also act as repressors of this regulon.

In addition, benzaldehyde and *p*-hydroxybenzaldehyde repress the first group of enzymes including E_3, which is required for their metabolism. We believe that this is the first reported instance of an enzyme being virtually completely repressed by its own substrate. One would expect such a property to be a disadvantage to the organism. In this case it is not, because an alternative inducible enzyme is available which oxidizes aromatic aldehydes. The existence of such an enzyme, separate from E_3, is supported by the following observations: (i) it is induced by benzaldehyde or *p*-hydroxybenzaldehyde, whereas E_3 is repressed; (ii) it is not repressed by benzoate or *p*-hydroxybenzoate, whereas E_3 is repressed; (iii) mutants can be obtained in which neither benzaldehyde nor its *p*-hydroxy derivative can induce an oxidative activity. These mutants have at the same time lost the ability to grow on *p*-hydroxybenzaldehyde. They nevertheless have a normal amount of E_3 when induced by mandelate.

In the second regulon in each pathway, multi-sensitive repression systems are again found. Thus benzoate oxidase is repressed by the immediate end product catechol and by the distal end products succinate and acetate. The corresponding enzyme in the parallel pathway is similarly repressed by protocatechuate and, again, by succinate and acetate (Scheme 2).

Finally, the enzymes controlled by the third regulon in each pathway are repressed by the distal products succinate and acetate.

There is a selective advantage to the cell in possessing the capacity to 'switch on' simultaneously the synthesis of a whole group of enzymes in a pathway. Stanier *et al.* (1963) have shown that there is an appreciable lag (about 40 min.) between the appearance of enzymes controlled by the first regulon of the mandelate pathway and those



Scheme 2. Multi-sensitive end-product repression in two convergent degradative pathways. S_1 is mandelate and the ensuing intermediates are S_2 etc. (see Scheme 1). The corresponding enzymes are E_1 etc. The hydroxy series is S_{1A} etc. The first three compounds in both pathways are degraded by the same enzymes (E_1, E_2 and E_3), which are induced or repressed as a group assumed to be controlled by a set of genes (regulon). Repression is produced by S_4, the product formed by the action of the first group of enzymes. There is also repression by end products of more distal groups (i.e. by S_5 and also by acetate or succinate). The enzyme controlled by the second regulon, E_4, is repressed by S_5, its end product, and again by acetate and succinate. Finally, the enzymes of the third regulon, represented by E_5, are repressed by acetate and succinate. (β-Oxoadipate, the immediate end product, does not enter the cells and could not be tested.) There is a corresponding symmetrical set of repression mechanisms for the parallel pathway that degrades the hydroxy derivatives.

controlled by the third regulon. This lag would presumably be even longer if the enzymes had to be induced one at a time. The number of sequential steps between mandelate and β-oxoadipate is reduced to three by the existence of the regulons, instead of being eight, or possibly more. The consequent reduction in lag time when the cells become adapted to growth on mandelate is, at a guess, about $2\frac{1}{2}$ hr., i.e. about three generation times. Thus, in the course of such an adaptation, a cell possessing the regulon type of mechanism would be subject to an eightfold enrichment relative to a cell that did not possess the mechanism.

Against this selective advantage must be set the disadvantage in terms of protein economy that would be manifest if the cells formed enzymes to produce a substrate which was already available from some other source, either endogenous or exogenous. The control mechanism we have described prevents this from happening since, whenever the end product of a group of enzymes is present in excess, it represses the formation of all the preceding enzymes in the degradative pathway. It is significant that new regulons begin at points where degradative pathways converge or where the compounds concerned are likely to occur in the external environment (see Stanier *et al.* 1963). Thus benzoate is found as such under natural conditions; catechol is the point at which the tryptophan and mandelate pathways converge; *p*-hydroxybenzoic acid is an intermediate in the degradation of *p*-cresol (Dagley & Patel, 1957), and thus represents another junction of two pathways. From our findings we would predict that β-oxoadipate, again a point of convergence, would act as a repressor if it entered the cells.

It appears that this rather complex system of controls has been evolved to strike a balance between two selective pressures. The first is the pressure to adapt to the utilization of carbon substrates in the minimum time: the second is to preserve a maximum economy in protein synthesis. These pathways in *Pseudomonas* thus exhibit the same dual-control mechanism by induction and by end-product repression that has been described for inducible degradative enzymes in *Escherichia coli* (McFall & Mandelstam, 1963*a*,*b*). In both types of organism the end result is the same: an enzyme is not synthesized unless its substrate is present, and even then it is not synthesized if end products required for cell growth are already present in excess.

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