Induction and Multi-Sensitive End-Product Repression in the Enzymic Pathway Degrading Mandelate in *Pseudomonas fluorescens*

By J. MANDELSTAM AND G. A. JACOBY*

National Institute for Medical Research, Mill Hill, London, N.W. 7

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1. The first five enzymes involved in the degradation of mandelate in *Pseudomonas fluorescens* have been examined. 2. Induction is not significantly affected by glucose. 3. The first three enzymes form a group inducible by mandelate and repressible by benzoate, catechol and succinate. 4. The possibility that benzoate and catechol act as repressors only after they have been degraded to succinate is unlikely since mutants blocked at suitable points in the pathway have the same repression pattern as the wild type. 5. It is concluded that synthesis of the enzymes is subject to a multi-sensitive repression mechanism that can be independently activated by benzoate or catechol or succinate. 6. In each case the repression can be largely overcome by increasing the concentration of the inducer. 7. The enzymes of the first group are thus controlled by a dual system in which induction by the first substrate is opposed by repression exerted by the end product of the first group and by the products of succeeding groups.

A previous study (McFall & Mandelstam, 1963*a,b*) on the behaviour of three enzymes in *Escherichia coli* suggested that the production of an inducible catabolic enzyme is controlled not only by its inducer but also by an end-product repression that is specific. Thus β -galactosidase is induced by lactose, or other β -D-galactosides, and the induction is prevented by galactose or some close metabolic derivative. Similarly L-tryptophanase and D-serine deaminase are each induced by their substrates and both are repressed by pyruvate, an end product of enzyme action in both instances. Other systems have been described in which enzyme synthesis is controlled by induction and by end-product repression, e.g. nitrate reductase in *Chlorella vulgaris* (Morris & Syrett, 1963), amidase in *Pseudomonas aeruginosa* (Brammar & Clarke, 1963) and glucosamine 6-phosphate deaminase in *Bacillus subtilis* (Clarke & Pasternak, 1962).

The present paper describes the interaction of induction and end-product repression in a longer and more complex pathway, the series of enzymes that degrades mandelic acid in *Pseudomonas fluorescens*. This pathway is as follows:

* Present address: Massachusetts General Hospital, Boston, Mass., U.S.A.

This system was extensively investigated by Stanier and his colleagues (see Stanier, 1951) and is the classic example of 'sequential induction', since the addition of any intermediate caused the successively induced formation of all the ensuing enzymes in the pathway. Stanier, Hegeman & Ornston (1964) have since provided evidence that enzymes E_1 , E_2 , E_{2a} and E_{2b} form a single group apparently controlled by an operon, induced simultaneously and equally well by mandelate or benzoylformate. The benzoic acid produced by the action of this group of enzymes then induces E_4 , which appears to be separately controlled operon. The catechol formed by E_4 then induces pyrocatechase (E_5) and the other enzymes leading to β -oxoadipate. These enzymes form a third group. According to this later work the pathway is thus still sequentially induced, but the number of steps is less than was originally thought. The criteria of an operon in this system are physiological (Stanier *et al.* 1964) only, and there is as yet no genetic evidence that the genes determining the structures of these enzymes are closely linked.

The present work, which has been published in a preliminary form (Mandelstam, 1964*a,b,c*), deals with the first five enzymes and points to the conclusion that the enzymes of the first operon are subject not only to induction but to repression by the end product resulting from the action of that group of enzymes and independently by the end products of each succeeding group.

METHODS

Organism. *Pseudomonas fluorescens* A3.12 (obtained from Dr R. Y. Stanier) was used. It was grown with shaking at 30° in a basal mineral salts medium containing (per l.): KH_2PO_4 , 13.6 g.; NH_4Cl , 1.0 g.; $(\text{NH}_4)_2\text{SO}_4$, 1.0 g.; MgSO_4 , 20 mg.; $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 1.56 mg. The pH was adjusted to pH 7 with NaOH. Unless otherwise stated, the carbon source was glucose (5 mg./ml.). The bacteria were washed with a solution containing KH_2PO_4 (13.6 g./l.) adjusted with NaOH to pH 7.

Isolation of mutants. Mutants defective in one of the enzymes in the sequence were obtained as follows. Cells from an exponential culture were suspended in 0.2 M-acetate buffer, pH 5.0, at a density of 3 mg. dry wt./ml., and to 4 ml. of this was added 1.0 ml. of a solution of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (4 mg./ml.). After incubation at 35° for 30 min. the suspension was centrifuged, the cells were washed once with phosphate buffer (10 ml.) and suspended in 250 ml. of broth medium (hydrolysed casein plus yeast extract). The culture was divided into five equal portions and left to grow overnight at room temperature. The cells were subcultured twice in basal medium with sodium succinate (5 mg./ml.) as carbon source. Inocula (1000–2000 cells/plate) were plated on basal medium containing mandelic acid (250 μg ./ml.) solidified with agar (1.5 g./100 ml.). Wild-type bacteria produced colonies of very uniform size after incubation for 24 hr. at 35°. Plates were then left at room temperature (18–20°) for a further 3–6 days. Mutants, blocked in the pathway and therefore unable to utilize mandelic acid, then developed as pin-point colonies readily distinguished from the wild type under a low-power microscope. These mutants were picked and tested for ability to utilize the intermediates of the pathway as growth substrates. When the position of the block had thus been determined, it was quantitatively checked, by inducing with mandelate a culture growing in glucose medium (see below) and measuring the enzyme in question. Those mutants that were 'leaky' (i.e. that had low activity after induction by growth in the presence of mandelate) were discarded. The mutants retained for use had no measurable enzymic activity and failed to show growth on the substrate in question for at least 24 hr.

Induction in growing cultures. Except in the preliminary experiments (see below) induction was carried out with cells in exponential-growth phase (0.1 mg./ml.) in glucose medium with inducer at 250 μg ./ml., except when stated otherwise. Samples for assay were treated as described below.

Oxygen uptake by whole cells. Samples (10 ml.) were centrifuged, washed and resuspended in phosphate buffer (6 ml.). Portions (2.5 ml.) were assayed by conventional manometric methods with the substrates at the following concentrations: mandelic acid, 2.5 mg./ml.; benzoic acid, 2.5 mg./ml.; catechol, 300 μg ./ml. Values were corrected for endogenous respiration, and are expressed in units of $\mu\text{moles of O}_2/\text{min}$.

***E*₁: mandelate dehydrogenase.** Samples (100 ml.) were centrifuged, washed once with buffer, resuspended in 8.5 ml. of 0.9% NaCl solution and disrupted by ultrasonic vibration in an oscillator (25 kc./sec.; 250 w) for 3 min. Unbroken cells were removed by centrifuging (2500 g for 10 min.) and the enzyme was determined as described by Stanier (1955).

***E*₂: benzoylformate decarboxylase (benzoylformate carboxylase, EC 4.1.1.7).** In some experiments the enzyme was determined with a portion (1.0 ml.) of the cell-free extract obtained as above, after ultrasonic disruption (see Stanier, 1955). However, similar values were more simply obtained with toluene-treated samples prepared as follows. Portions (10 ml.) were centrifuged and the cells washed and resuspended in 6 ml. of 0.1 M-phosphate buffer, pH 6.0, containing thiamine pyrophosphate (100 μg ./ml.). Two drops of toluene were added, and the tubes stoppered, shaken vigorously and left for 30 min. Toluene was driven off by blowing air through the suspension for about 2 min., and 2.5 ml. portions were taken for manometric determination of the CO_2 produced anaerobically after the addition of benzoylformate (final concn. 1 mg./ml.). Values were corrected for CO_2 retention.

***E*₃: benzaldehyde dehydrogenase (*NAD*⁺-linked) (benzaldehyde-*NAD* oxidoreductase, EC 1.2.1.6).** When attempts were made to measure this enzyme in ultrasonically disrupted preparations by following the reduction of *NAD*⁺ (Stanier, 1955), values were erratic and frequently no activity was detectable even with extracts of fully adapted cells. The reason was found to be the presence, in cells grown under our conditions, of a potent *NADH* oxidase. The interfering activity was abolished in the presence of NaCN (final concn. 100 μg ./ml.). The procedure finally adopted was as follows. The following additions were made, in the order given, to a spectrophotometer cell: 1.2 ml. of 1.0 M-phosphate buffer, pH 8.5; 0.3 ml. of NaCN solution (1 mg./ml.); 1.0 ml. of cell-free extract (equivalent to about 2 mg. dry wt. of bacteria); 0.3 ml. of *NAD*⁺ solution (1 mg./ml.). The reaction was started by adding 0.2 ml. of aqueous benzaldehyde (1.0 mg./ml.). The change in extinction at 340 $\text{m}\mu$ was measured at 15 or 30 sec. intervals against a blank containing water instead of benzaldehyde.

***E*_{3b}: benzaldehyde dehydrogenase (*NADP*-linked).** This enzyme was not investigated.

***E*₄: benzoate oxidase.** No method was found of breaking the cells without losing most or all of the activity. This is in keeping with the findings of other workers.

***E*₅: pyrocatechase.** The enzyme was estimated manometrically (Hayaishi, Katagiri & Rothberg, 1957) with cell-free extracts prepared as above by ultrasonic disruption.

Enzyme units. All units are expressed as $\text{m}\mu\text{moles}$ 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); *NAD*⁺ (90% pure) was obtained from British Drug Houses Ltd., Poole, Dorset.

RESULTS

Effect of glucose on induction of mandelate pathway in non-growing cells. Before carrying out induction experiments in growing cultures it was necessary to find a source of carbon that would not act as a repressor. Succinate, which is a useful non-repressing carbon source for β -galactosidase in *E. coli*, was found to be a strong repressor in *Pseudomonas* (see below).

Glucose was then tested for repressor activity on the mandelate-pathway enzymes in non-growing cells. In other systems the non-growing state

causes maximum accumulation of carbon-source repressors (see Mandelstam, 1961), and it was therefore expected that any repressor action of glucose would be most strongly expressed under these conditions.

Cells in exponential growth in glucose-minimal medium were harvested, washed, suspended in phosphate buffer at 0.5 mg. dry wt./ml. and induced with mandelic acid (5 mg./ml.) in the absence and in the presence of glucose (5 mg./ml.). Samples (10 ml.) were taken at intervals and the ability of the whole cells to oxidize mandelic acid was measured. Under these conditions, not only did glucose fail to repress, but it actually caused a fivefold stimulation in the rate of formation of mandelate-pathway enzymes as measured by oxidative capacity (Fig. 1).

Fig. 1. Effect of glucose on adaptation to mandelate in non-growing cells. Cells were suspended in phosphate buffer at 0.5 mg. dry wt./ml. with mandelate alone (1 mg./ml.) (●) or with glucose (5 mg./ml.) (○) in addition. The suspensions were shaken at 30° and samples removed. The ability of washed whole cells to oxidize mandelate was measured.

In growing cultures there was again no strong repressor action of glucose. Cultures grown for 3–5 hr. in the presence of mandelate (2 mg./ml.) together with glucose (5 mg./ml.) had 80–100% of the enzymic activity of cultures grown with mandelate alone. Under comparable conditions the inducible systems that degrade amino acids in the same strain of bacteria show a much more marked glucose effect (Jacoby, 1964).

Since glucose was relatively non-repressive for this enzyme sequence, it was subsequently used as a carbon source, and the remaining experiments described below were done with growing cultures.

End-product repressions in the mandelate pathway. A series of experiments was done in which, with mandelate as inducer, subsequent metabolites were tested in turn as repressors. The experiments were all done with cultures growing with glucose as carbon source and with inducers and repressors added as indicated. At the concentrations used none of these substances inhibited growth, but succinate (see below) caused some stimulation. The results can be most easily appraised by considering first the effects these substances have on the subsequent ability of whole cells to oxidize mandelate or benzoate. It was shown (see below) that oxidation by whole cells is, in fact, a reliable reflexion of what is happening to the individual enzymes.

Benzoylformate had no significant repressor activity. Benzaldehyde was not initially tested, but subsequent work showed that it did repress mandelate dehydrogenase (I. L. Stevenson & J. Mandelstam, unpublished work). Benzoate in the presence of mandelate repressed mandelate-oxidative capacity by about 80–90% but induced benzoate-oxidative capacity as well as mandelate did, and occasionally better. Catechol repressed both activities. A typical result is shown in Table 1.

The findings suggested that benzoate was repressing some or all of the preceding enzymes (i.e. E_1 – E_3) but as originally suggested (see Stanier, 1951), inducing E_4 and subsequent enzymes,

Table 1. *Effect of benzoate and catechol on induction of the capacity of whole cells to oxidize mandelate and benzoate*

A culture growing in glucose medium was treated with mandelate alone or with mandelate in the presence of benzoate or catechol. Inducer and repressor concentrations were 250 μ g./ml. After one generation samples were taken and the oxidative capacity of washed whole cells was measured with either mandelate or benzoate as substrate.

Induction medium	Mandelate oxidation		Benzoate oxidation	
	Units/mg.	Repression (%)	Units/mg.	Repression (%)
Mandelate	270	—	142	—
Mandelate + benzoate	25	91	106	25
Mandelate + catechol	10	96	6	96

Table 2. *Repression of enzymes in the mandelate pathway by benzoate*

Bacteria were grown in glucose medium with mandelate (250 $\mu\text{g./ml.}$) or with mandelate + benzoate (250 $\mu\text{g./ml.}$). The culture density increased in both cases from 0.09 to 0.18 mg./ml. Enzyme activities were determined in ultrasonically disrupted cells (see the Methods section). Values are specific activities expressed as units/mg. dry wt. of bacteria before disruption.

Enzyme	Sp. activity (units/mg. dry wt.)		Repression (%)
	Mandelate	Mandelate + Benzoate	
E ₁ (mandelate dehydrogenase)	135	10	92
E ₂ (benzoylformate decarboxylase)	685	41	94
E _{3a} (benzaldehyde dehydrogenase)	38	9.4	75
E ₄ * (benzoate oxidation)	127	130	0
E ₅ (pyrocatechase)	193	198	0

* E₄ activity is lost during ultrasonic disruption: values refer to O₂ uptake by whole cells (m μ moles/min./mg. dry wt.).

Table 3. *Repression of enzymes in the mandelate pathway by catechol*

The experiment was done as described in Table 2 except that benzoate was replaced by catechol (250 $\mu\text{g./ml.}$).

Enzyme	Sp. activity (units/mg. dry wt.)		Repression (%)
	Mandelate	Mandelate + catechol	
E ₁ (mandelate dehydrogenase)	108	25	77
E ₂ (benzoylformate decarboxylase)	420	143	66
E ₃ (benzaldehyde dehydrogenase)	34	16	53
E ₄ * (benzoate oxidation)	120	7	94
E ₅ (pyrocatechase)	123	135	(-10)†

* O₂ uptake by whole cells (see footnote to Table 2).

† Negative value indicates stimulation.

whereas catechol was repressing all the preceding enzymes including E₄.

The effects of benzoate on the formation of individual enzymes were confirmed by disrupting the cells after induction and measuring the enzymic activities of the cell-free preparation. The first three enzymes, i.e. those preceding benzoate in the pathway, were all repressed by about 75–95% (Table 2). E₅ (pyrocatechase), on the other hand, was fully induced, and E₄, though the measurements had to be made with whole cells, also appeared to be fully induced.

The effect of catechol on the production of the same enzymes is shown in Table 3. E₁, E₂ and E₃ were repressed 50–80%, whereas E₅ was fully induced. The fact that E₅ was present in high amounts indicates that the low values for benzoate oxidation by cells grown in the presence of catechol (see also Table 1) were due to repression of E₄.

The results thus far may be summarized as follows. Benzoate represses E₁, E₂ and E₃, and induces first E₄, and then, as would be expected on the basis of sequential induction, E₅ and the subsequent enzymes: catechol represses E₁–E₄, but

induces E₅ and the subsequent enzymes (again as suggested by Stanier, 1951).

An attempt was made to determine whether β -oxoadipate, the end product of the group of enzymes beginning at E₅, acted as a repressor, but this substance was apparently unable to enter the cells. Organisms adapted to growth on mandelate, and therefore presumably adapted to β -oxoadipate, failed to utilize it as a carbon source for growth, and showed no significant oxidation when tested manometrically.

Succinate, as a final product of the pathway, was also tested. This usually produced a somewhat weaker repression (40–60%) of E₁, E₂ and E₃, but had more effect on the synthesis of E₅ (about 85% repression). A typical result is shown in Table 4. Oxidation of benzoate by whole cells was also repressed but, since E₅ activity was also low, it cannot be categorically stated that E₄ was repressed. Repressor effects produced by succinate were more variable than those produced by benzoate or catechol, and repression of E₁–E₃ was occasionally much greater than the result shown in Table 4.

Acetate, when tested as a repressor, had much the

Table 4. *Repression of enzymes in the mandelate pathway by succinate*

The experiment was done as described in Table 2 except that benzoate was replaced by succinate (500 $\mu\text{g./ml.}$).

Enzyme	Sp. activity (units/mg. dry wt.)		Repression (%)
	Induction medium.....	Mandelate + succinate	
E ₁ (mandelate dehydrogenase)			
E ₂ (benzoylformate decarboxylase)			
E ₃ (benzaldehyde dehydrogenase)			
E ₄ * (benzoate oxidation)			
E ₅ (pyrocatechase)			

* O₂ uptake by whole cells (see footnote to Table 2).

same effect as succinate, i.e. it appeared to repress all the activities E₁–E₅. Other substances were not investigated, but presumably any intermediate in the tricarboxylic acid cycle or anything contributing material to the cycle would tend to produce effects resembling those of succinate.

Specificity of repression. Since the repressors, at the concentrations used, did not affect growth, it was apparent that they were not general inhibitors of protein synthesis, but a more detailed examination of their specificity was clearly desirable. Their effect was accordingly examined on the formation of the induced enzyme sequence oxidizing *p*-hydroxybenzoate. This pathway converges with the mandelate pathway at β -oxoadipate. A culture in glucose medium was allowed to double in the presence of *p*-hydroxybenzoate (250 $\mu\text{g./ml.}$) and the substance being tested as a repressor (also at 250 $\mu\text{g./ml.}$, but at 500 $\mu\text{g./ml.}$ for succinate). Samples were taken and the oxygen uptake was measured with *p*-hydroxybenzoate as substrate.

There were obvious differences in the repression patterns of the two pathways. Benzoate, a very potent repressor for the mandelate-pathway enzymes, had no effect on adaptation to *p*-hydroxybenzoate, and catechol acted only as a weak repressor in the latter system (giving less than 50% repression). Acetate, which inhibited mandelate adaptation by about 90%, decreased adaptation to *p*-hydroxybenzoate by only about 30%. Succinate, however, affected both systems more or less equally.

It seems reasonable to conclude that the repressor effects, at any rate those produced by benzoate and catechol, were specific and were not the result of an effect on enzyme induction in general.

Possible modes of repressor action. Considering for the moment the repression of enzymes of the first operon by benzoate, catechol and succinate, the following explanations seem possible:

(a) Multi-sensitive repression system. The repression mechanism is sensitive to, and can be

independently activated by, each of the repressor substances.

(b) Repression by one end product. There is only one repressor (e.g. succinate itself or some compound closely related to it metabolically), and benzoate and catechol are repressors because they are both converted into it. This possibility was ruled out by the use of suitable mutants. Thus a strain *benz*[–] (unable to form benzoate-oxidizing enzyme) was isolated and induced with mandelic acid in the presence and absence of benzoate. In this organism benzoate repressed the development of mandelate-oxidative capacity, and catechol and succinate were also repressors (Fig. 2), and the results did not differ significantly from those found with the wild type. The effect of benzoate on the formation of benzoylformate decarboxylase was checked and the results were qualitatively similar to those shown in Fig. 2.

Although the *benz*[–] mutant had no measurable benzoate-oxidase activity, the possibility had to be considered that it was 'leaky' and that an amount of enzyme too small to be measured was nevertheless providing a trickle of succinate that was the cause of the repression. One would then expect that, if benzoate caused repression only after degradation to succinate, the addition of benzoate to a culture containing an excess of succinate should produce no further effect. An experiment was accordingly carried out in which succinate at 1 mg./ml. (i.e. twice the concentration used previously) and benzoate (250 $\mu\text{g./ml.}$) were tested separately and together. In the presence of both substances the repression was greater than that with either alone (Fig. 3).

The experiments with the *benz*[–] mutant suggest that benzoate and succinate act separately as repressors and that their effects are additive.

A number of mutants were also obtained that were blocked between catechol and β -oxoadipate. These mutants displayed the same effects as the *benz*[–] and wild type, i.e. catechol remained a

Fig. 2. Effect of benzoate, catechol and succinate on adaptation to mandelate in a *benz*⁻ mutant. A mutant blocked at *E*₄ (benzoate oxidation) was used. Cells growing exponentially in glucose-minimal medium were induced with mandelate (250 μ g./ml.) alone ($\circ$) or with one of the following present as well: benzoate (250 μ g./ml.) ($\bullet$), catechol (250 μ g./ml.) ($\blacktriangle$), or succinate (500 μ g./ml.) ($\blacksquare$). Samples were taken at 20 min. intervals for 1 hr. and the ability of washed whole cells to oxidize mandelate was measured.

potent repressor though it could not be converted into succinate. Benzoate and succinate, as expected, were still repressors in this strain.

(c) 'Sequential repression.' On this hypothesis each group of enzymes in the pathway is repressed only by the immediate end product of action of the enzymes of that group. Thus if we assume, as suggested by Stanier *et al.* (1964), that *E*₁-*E*₃ comprise a co-ordinated group, then benzoate is its repressor. The second operon, determining *E*₄, is repressed by catechol. The third operon, determining *E*₅, and the next few enzymes are repressed by β -oxoadipate, and so on until succinate represses the last enzymes of the induced pathway. If, then, cells are induced by adding mandelate, some induction will occur and material will begin to flow down the pathway. If succinate has also been introduced into the medium it will begin by repressing the last enzymes of the sequence, thus leading to accumulation of β -oxoadipate. This will,

Fig. 3. Additive repression produced by benzoate and succinate in a *benz*⁻ mutant. For procedure see the legend to Fig. 2. Additions were as follows: $\circ$, mandelate (250 μ g./ml.) alone; $\bullet$, mandelate + benzoate (250 μ g./ml.); $\blacktriangle$, mandelate + succinate (1 mg./ml.); $\blacksquare$, mandelate + benzoate + succinate.

Fig. 4. Repression of benzoylformate decarboxylase (*E*₂) in an *md*⁻ mutant. The mutant was blocked at *E*₁ (mandelate dehydrogenase). Cells growing exponentially in glucose-minimal medium were treated with mandelate, which acts as an inducer for *E*₂ and *E*₃ (see Stanier *et al.* 1964). Samples were taken at 20 min. intervals for 1 hr. and the decarboxylase was measured in toluene-treated preparations (see the Methods section). Additions were as follows: $\circ$, mandelate (250 μ g./ml.); $\bullet$, mandelate + benzoate (250 μ g./ml.); $\blacktriangle$, mandelate + catechol (250 μ g./ml.); $\blacksquare$, mandelate + succinate (500 μ g./ml.).

in turn, repress the enzymes from *E*₅ onwards so that catechol will accumulate etc. etc., until finally the enzymes of the first operon are repressed.

Fig. 5. Effect of inducer concentration on repression produced by succinate. Wild-type cells growing in glucose-minimal medium were treated with succinate (500 $\mu\text{g./ml.}$) as repressor and with various amounts of inducer, as follows: ●, mandelate (250 $\mu\text{g./ml.}$); ▲, mandelate (500 $\mu\text{g./ml.}$); ■, mandelate (1000 $\mu\text{g./ml.}$); ○, control culture containing no succinate and induced with mandelate at 250 $\mu\text{g./ml.}$

The possibility was ruled out by the use of a mutant *md⁻* (lacking mandelate hydrogenase). It was treated with mandelate, and the observation of Stanier *et al.* (1964) was confirmed that this caused induced formation of benzoylformate decarboxylase. In this mutant material does not pass down the pathway because of the block at E_1 ; nevertheless, benzoate and the other two repressors were still active (Fig. 4) and the results again resembled those obtained with the wild type.

The elimination of possibilities (b) and (c) leaves (a) as the most likely explanation.

Reversibility of repression. With β -galactosidase in *E. coli* the repression produced by metabolic products could not be reversed by inducer (Mandelstam, 1962).

The reversibility of end-product repression in the mandelate pathway was examined as follows.

Fig. 6. Effect of inducer concentration on repression produced by benzoate. Details are as in Fig. 5, with benzoate (250 $\mu\text{g./ml.}$) in place of succinate.

Several flasks of culture were set up with the usual glucose medium and a fixed concentration (500 $\mu\text{g./ml.}$) of succinate as repressor. Inducer (mandelate) was added at concentrations varying from 250 to 1000 or, occasionally, 2000 $\mu\text{g./ml.}$ (Preliminary experiments had shown that the lowest concentration of inducer was sufficient to give a maximum rate of induction when no succinate was present.) The repression resulting from the presence of succinate was readily reversed by increasing the concentration of inducer (Fig. 5).

The repression induced by benzoate (250 $\mu\text{g./ml.}$) or by catechol (125 $\mu\text{g./ml.}$) was also reversed by increasing the amount of inducer. An experiment with benzoate is illustrated in Fig. 6.

In case the measurements of oxidation by whole cells might be misleading, some of the experiments were checked by using mandelate as inducer and measuring the benzoylformate decarboxylase in toluene-treated preparations. The results were similar to those obtained with whole cells.

DISCUSSION

Reference has already been made (see the introduction) to the fact that, in *E. coli*, the production of β -galactosidase, tryptophanase and D-serine deaminase appears in each case to be jointly determined by induction and by end-product repression. In all three a single metabolic step degrades the inducing substrate to one of the common intermediary metabolites of the cell: galactose in the first case and pyruvate in the other two. One of the reasons for choosing the mandelate

pathway is that a sequence of about eight enzymes is involved. This is roughly the number of enzymes concerned in biosynthetic sequences producing arginine or histidine, which are all controlled by end-product repression. Though the mandelate pathway also has this property, its regulation presents a number of features that distinguish it from other pathways, both catabolic and biosynthetic.

An obvious difference between the β -galactosidase of *E. coli* and the mandelate-pathway enzymes is that, in the former, the effects of metabolic repression could not be reversed by inducer (Mandelstam, 1962). This suggested that the metabolic repressor was independent of the repressor postulated by Jacob & Monod (1961) as the cause of inducibility. Supporting evidence was the occurrence of metabolic repression in mutant types (o^+ or i^-) that are constitutive for β -galactosidase (Brown, 1961; Mandelstam, 1962). These mutants have been postulated to be either unable to make functional repressor (i^-) or to be largely insensitive to its action (o^+), so that the enzyme is produced constitutively.

These facts led to the conclusion (Mandelstam, 1962) that the formation of β -galactosidase is controlled by two distinct types of repressor, one of which is controlled by the i gene whereas the other is formed during the metabolism of carbon compounds. The second repressor was tentatively identified as galactose or some metabolically related compound (McFall & Mandelstam, 1963b). The case for the separate existence of two repressors for the β -galactosidase system of *E. coli* is supported by the observations of Loomis & Magasanik (1964).

In the mandelate-pathway enzymes the repression of the first operon, whether produced by benzoate, catechol or succinate, is readily reversed by the inducer. A strict demonstration of competitive interaction is not possible since both the inducer and the repressors are being metabolized. However, the mere fact that there is interaction between inducer and repressor raises the possibility that they may be acting at the same site. Gorini (1963) has described an analogous reaction in the system of enzymes synthesizing arginine from glutamate in *E. coli*. The whole sequence is repressed by the end product, arginine, and the repression is reversed, more or less competitively, by glutamate, the first substrate. This interaction between the first and last substrates is formally analogous to that found in the mandelate pathway. Both instances suggest that these control mechanisms may differ substantially from those governing the formation of the lactose system of *E. coli*.

A distinctive property of the mandelate system is the repression of enzymes of the first operon by at least three separate substances: (a) benzoate, (b)

catechol (or one of the immediately succeeding metabolites) and (c) succinate (or some related metabolite). The possibility of a 'sequential repression' (see the Methods section) seems to be ruled out. Another possibility, that benzoate and catechol repress only after being degraded to succinate, is equally unlikely in view of the fact that mutants blocked at E_4 and E_5 exhibit the same repression pattern as the wild-type parent. The remaining explanation is that formation of enzymes of the first operon is governed by a multi-sensitive repression mechanism that can be activated independently by at least three metabolites in the pathway. We have introduced the term 'multi-sensitive' repression to describe this mechanism and to distinguish it from the 'multivalent' repression system found in coliform bacteria (see Umbarger, 1964). (In the latter a number of enzymes supplying intermediates used in the synthesis of the branched amino acids are only repressed when all the end products are present together in excess.)

The experiments described in the present paper in no way indicate the site of repressor action, and the possibilities are as follows:

(a) Effect on transport of inducer into the cell, i.e. the repressors inhibit the functioning of a specific permease. This would imply the existence of a permease molecule with specific sites for the 'substrate' (mandelate) and for each of the repressors, that is an 'allosteric' molecule (see Monod, Changeux & Jacob, 1963) with at least four specific combining sites.

(b) An effect on the protein-synthesizing mechanism. There are a number of possible sites where a repressor might act: at the gene level, as suggested by Jacob & Monod (1961), or at the ribosome level, as suggested by Hauge, MacQuillan, Cline & Halvorson (1961). In either case, if we assume that the inducer and the repressors act on the same molecule, it is necessary to postulate that this molecule has at least five recognition sites: one for the inducer, one for each of three repressors and one for the relevant portion of DNA or messenger RNA where it is supposed to act. The alternative is to postulate a rather non-specific site with affinity for repressors of widely differing chemical characteristics.

(c) Finally, the possibility must be considered that the inducer and the repressors do not all react with the same molecule.

The experimental resolution of this question will probably not be possible until a reliable cell-free system for forming the induced enzymes becomes available.

The advantage to the cell, in terms of protein economy, of having control by induction and by end-product repression has been discussed by

Scheme 1. Representation of induction and repression in the mandelate pathway. Mandelate (S_1) is degraded by a series of enzymes E_1 etc. to the tricarboxylic cycle. E_1 – E_3 are determined by an operon and are inducible by S_1 ; E_4 is independently inducible by benzoate; E_5 and the ensuing enzymes before β -oxadipate are determined by a third operon inducible by catechol (see Stanier *et al.* 1964). The enzymes of the first operon are repressed by S_4 (the end product to which they give rise), by S_5 (the end product of the second group) and also by succinate (β -oxadipate may also be a repressor, but it fails to enter cells and thus cannot be tested). The repressors act additively, and their effect can be counteracted by increasing the concentration of S_1 . S_1 , mandelate; S_2 , benzoylformate; S_3 , benzaldehyde; S_4 , benzoate; S_5 , catechol.

McFall & Mandelstam (1963a). The mandelate pathway exhibits the same general property but in a much more elaborate form (Scheme 1). This complexity may have been evolved to deal with the intricate system of converging pathways by which the pseudomonads can degrade a large variety of aromatic carbon compounds to components of the tricarboxylic acid cycle.

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