REGULATION OF THE ENZYMES CONCERNED IN THE DEGRADATION OF AROMATIC COMPOUNDS IN PSEUDOMONAS PUTIDA

A thesis submitted in part fulfilment of the requirements for the degree of D. Phil. in the University of Oxford.

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ABSTRACT

ABSTRACT

Pseudomonas putida metabolises aromatic carboxylic acids by convergent pathways. The regulons for the synthesis of the enzymes involved are controlled by induction and repression by intermediates of the pathways. The results of previous studies in this system suggest that the organism should exhibit a selective enzymic response to mixed aromatic substrates. It has been the aim of this study to predict the enzymic response to mixtures of mandelate, benzoate and parahydroxybenzoate and then to check these predictions experimentally.

The affinities of these three inducer substrates for their regulons have been determined by measuring the initial rates of enzyme synthesis over a range of inducer concentrations in cultures growing on glucose. Glucose itself was shown to have very little catabolite repressor effect in this system. From the results, an induction constant, defined as the inducer concentration for half-maximum rate of induction, was estimated for each substrate. Those for the benzoate and mandelate regulons were similar (c. 0.5 mM) and much higher than that for parahydroxybenzoate hydroxylase (< 0.01 mM).

By measuring the repressor effect of each aromatic substrate on the induction by one of the other substrates, repression constants (K_{rep}) were also obtained that are a quantitative measure of the affinity of the repressor for that regulon. Mandelate had no repressor activity and the benzoate regulon was not repressed by parahydroxybenzoate. The mandelate regulon was severely repressed by p-OH benzoate (K_{rep} = 0.02 mM) while the effect of benzoate on the parahydroxybenzoate regulon was much less pronounced (K_{rep} =1.0 mM). Benzoate had a transient, severe effect on the mandelate regulon and, at high concentrations, a moderate permanent form of repression was observed.

The enzymic reponse of the organism following a changeover from growth on a single aromatic carbon source to growth in the presence of an additional aromatic carbon source was predicted by comparing the induction and repression constants for the substrates concerned. Batch cultures were used to provide an indication of the steady state enzymic reponse, which was obtained using a nitrogen-limited chemostat operated at high growth rates. In general, the predictions were experimentally validated.

Benzoate oxidase was always fully induced in mixtures containing benzoate, while the mandelate enzymes were almost completely repressed in the presence of parahydroxybenzoate. The mandelate regulon was only partially induced in mixtures containing mandelate and benzoate as was the parahydroxybenzoate regulon in cells grown on parahydroxybenzoate and benzoate. The extent to which the enzymes for the less preferred carbon source were induced was affected by the relative concentrations of the substrates.

The results indicate that comparison of induction and repression constants can be used to predict the enzymic behaviour of this organism towards mixed aromatic substrates and it is suggested that this may form the basis of a general method for predicting enzymic adaptation to mixed substrates.

Relative substrate utilisation can only be inferred from the induction and repression constants if complete repression of an enzyme system is predicted. Further data are necessary whenever the predictions indicate that more than one enzyme system will be induced. Thus, in this study, it was predicted, and experimentally verified in the chemostat, that mandelate would not be used by cells growing in

the presence of mandelate + p-OH benzoate. When induction of two enzyme systems was predicted, the relative utilisation of the substrates was qualitatively related to the enzymic content of the cells.

In contrast to other systems where the reaction to mixed substrates seems to be directed towards the selection of the substrate capable of supporting the fastest growth rate, Pseudomonas putida selects, not the best growth substrate (mandelate), but the substrate requiring the synthesis of fewer enzymes for its metabolism (benzoate). Factors in the environment acting as possible selective agents are discussed.

In the course of this study, it was also found that the synthesis of the mandelate enzymes was delayed when low external inducer concentrations were used. At high concentrations, enzyme synthesis rapidly followed the addition of inducer. A pre-induction or maintenance effect was also observed in which cells induced at saturating concentrations of mandelate were able to maintain a maximum rate of induction when transferred to sub-saturating inducer concentrations. Induced cells were able to accumulate

mandelate from low external concentrations. These results are discussed in terms of an inducible mandelate transport system.

The severe transient repression of the mandelate regulon by benzoate was greatly reduced by pre-induction with mandelate. Benzoate inhibited the accumulation of mandelate by the cells and the action of benzoate on the mandelate regulon is discussed in relation to the possible induction mechanisms of the mandelate regulon.

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CHAPTER ONE

INTRODUCTION

INTRODUCTION

Enzymic adaptation

Microorganisms are unique among living organisms in being able to use a wide variety of simple organic and inorganic compounds to satisfy their nutritional requirements. This is particularly obvious in the case of carbon sources and nowhere is this metabolic diversity more pronounced than in the aerobic pseudomonads (Stanier, Palleroni and Doudoroff, 1966). Some strains isolated by den Dooren de Jong (1926) were able to grow well on over ninety simple carbon sources.

These carbon sources are metabolised by specific enzyme sequences which are usually inducible (Karström, 1938) and which produce as end-products a limited number of small molecule metabolites that can enter the central metabolic routes of the cell. Thereby, energy and carbon skeletons are provided for biosyntheses.

Mechanism of induction

Most of our knowledge of control in inducible systems stems from detailed biochemical and genetic studies in the enteric bacteria (see reviews by Maas and McFall, 1964;

Epstein and Beckwith, 1968; Martin, 1969). The genes containing the information for the inducible enzymes of a biochemical pathway are often clustered together, at least in coliform bacteria, in genetic units of function, the operons (Jacob, Perrin, Sanchez and Monod, 1960; Ames and Martin, 1964). Within each operon, the genes are contiguous and are regulated as a unit by control elements located at one end of the operon (Maas and McFall, 1964; Epstein and Beckwith, 1968; Martin, 1969).

From their results with pleiotropic constitutive mutants and partial diploids of the lactose (lac) operon of Escherichia coli, Jacob and Monod (1961) formulated their well-known, and by now widely accepted, model for the control of inducible systems. They proposed that the genes of an operon are normally not transcribed but are under the negative control of a regulator gene. This synthesises constitutively a repressor molecule that combines reversibly with the operator region of the operon and prevents the transcription of polycistronic messenger-ribonucleic acid (m-RNA) for the structural genes of that operon. Inducers can combine with the repressor, prevent its association with the operator, release repressors already attached to the operator and

permit mRNA synthesis.

Considerable support for and further refinement of this model have come from *in vivo* and *in vitro* work on the *lac* operon in *E. coli* (Attardi, Naono, Rouvière, Jacob and Gros, 1963; Nakada and Magasanik, 1964; Gilbert and Müller-Hill, 1966; Reznikoff, Miller, Scaife and Beckwith, 1969; Zubay and Chambers, 1969; Riggs, Newby and Bourgeois, 1970; De Crombrugghe, Chen, Gottesman, Pastan, Varmus, Emmer and Perlman, 1971). Repressible systems (which behave in essentially the same way as inducible systems but are usually concerned with biosynthetic enzymes) and other inducible systems in this and other organisms seem to be controlled in a similar fashion, e.g. the galactose operon (Epstein and Beckwith, 1968), the biosynthetic operons for histidine (Ames and Hartman, 1963; Roth, Silbert, Fink, Voll, Antón, Hartman and Ames, 1966) and tryptophan (Imamoto, Morikawa and Sato, 1965; Hiraga, 1969; Morse and Yanofsky, 1969b; Morse, Mosteller and Yanofsky, 1969).

However it should be pointed out that in some systems, e.g. the arabinose operon of *E. coli*, a *positive* rather than negative control over transcription operates

(Englesberg, Irr, Power and Lee, 1965; Sheppard and Englesberg, 1967; Englesberg, Sheppard, Squires and Meronk, 1969), while translation of mRNA may also be controlled in some systems (Roth et al., 1966; Berberich, Kovach and Goldberger, 1967; Kovach, Phang, Ference and Goldberger, 1969; Lavallé and De Hauwer, 1970).

Enzyme induction by mixed substrates

So far the enzymic response to a single substrate has been considered. Other substrates in the medium may modify the enzymic response to an inducing substrate. This was first observed when yeasts and bacteria were adapted to mixed carbohydrates (for review, see Gale, 1943). Glucose repressed the enzymes for alternative carbon sources but, although the effect became known as the 'glucose effect', it was not unique to glucose and many other readily metabolisable substrates can repress the enzymes for alternative substrates (Neidhardt and Magasanik, 1957; Mandelstam 1961, 1962). Succinate, a poor repressor in E. coli (Mandelstam, 1962; Okinaka and Dobrogosz, 1967) is a powerful repressor and a good carbon source in Pseudomonas spp. It represses amidase and histidase in P. aeruginosa (Brammer, Clarke and Skinner, 1967; Lessie and Neidhardt, 1967b), the enzymes for aromatic acid catabolism in P. putida (Mandelstam and Jacoby, 1965; Stevenson and Mandelstam ,1965) and those for

autotrophic utilisation of formate in P. oxalaticus (Blackmore and Quayle, 1968).

In some organisms, even the enzymes for glucose can be repressed; citrate is the repressor in P. aeruginosa (Hamilton and Dawes, 1960, 1961) and succinate in Arthrobacter crystallopoietes (Krulwich and Ensign, 1969). Enzymes for substrates other than carbon sources can also be repressed by alternative compounds, e.g. inorganic phosphate represses organophosphorus uptake and metabolism in Bacillus cereus and E. coli (Torriani, 1960; Rosenberg and LaNauze, 1967). Glucose and NH_4^+-salts repress histidase by which Klebsiella aerogenes uses histidine as a nitrogen source (Neidhardt and Magasanik, 1957).

Magasanik (1961) summarised the data on all these effects and proposed the term 'catabolite repression' to describe them all.

Mechanism of catabolite repression

If 'repression' is loosely defined as 'inhibition of enzyme synthesis', then catabolite repression could be direct or indirect. Direct repression will be defined as an effect of the catabolite repressor on the protein

synthesising machinery as such. It might cause, for example, a reduced rate of transcription or translation. Indirect repression, on the other hand, will be defined as interference with the processes whereby the inducer gains access to the cell or is converted to an active form within the cell. A particular catabolite could act in both ways.

Direct repression

Much of the work on catabolite repression has been done in the lac system of E. coli which is repressed by a number of carbon sources (see review Paigen and Williams, 1970). In this system, there is a permease for the uptake of β-galactoside inducers which is coordinately induced with the other lac enzymes (Rickenberg, Cohen, Buttin and Monod, 1956; Cohen and Monod, 1957) and which can also be a point at which catabolite effectors could act indirectly (see later). However, Herzenberg (1959) and Cohn and Horibata (1959) showed that catabolite repression occurred in permease-less strains, while Mandelstam (1962) and Loomis and Magasanik (1964) showed that the effect could also be produced in constitutive strains (both i^- and o^c) that synthesise the lac enzymes in the absence of inducer.

In the lac system, the catabolite effector seems to act at the level of transcription of the lac genes and does not alter the stability or translation of the lac-mRNA (Nakada and Magasanik, 1964; Képès, 1963). No specific catabolite repression locus has been defined but catabolite repression is dependent on an intact promoter (Pastan and Perlman, 1968; Silverstone, Magasanik, Reznikoff, Miller and Beckwith, 1969). This is thought to be the site of attachment of the RNA polymerase (Ippen, Miller, Scaife and Beckwith, 1968), an oligomeric protein that may use different subunits to distinguish between those operons that are subject to catabolite repression and those that are not (Burgess, Travers, Dunn and Bautz, 1969; Travers, 1971).

Magasanik (1961) suggested that substrates giving rise to catabolite repression do so because their metabolism gives rise to metabolites that are the twue catabolite effectors. Efforts to identify the catabolite effectors in various systems met with little success (see Paigen and Williams, 1970) but recently adenosine-3',5'-monophosphate (cyclic AMP) has been implicated in induction and catabolite repression (for review, see Pastan and Perlman, 1971). Glucose causes a reduction in the internal concentration

of cyclic AMP in E. coli (Makman and Sutherland, 1965) and cyclic AMP reverses catabolite repression in a wide range of systems (Ullmann and Monod, 1968; De Crombrugghe, Perlman, Varmus and Pastan, 1969; Perlman, De Crombrugghe and Pastan, 1969). Both in vivo and in vitro, cyclic AMP increases the rate of β-galactosidase synthesis in the presence of inducers by increasing the synthesis of lac mRNA (Varmus, Perlman and Pastan, 1970; De Crombrugghe, Varmus, Perlman and Pastan, 1970; De Crombrugghe et al., 1971).

Thus catabolite repression may not be the result of an increase in the concentration of an effector but rather a decrease in the concentration of cyclic AMP.

Indirect repression

1. Inducer entry. In principle, indirect repression could operate at the site of inducer entry, the uptake of many substances being mediated by specific transport systems (Cohen and Monod, 1957; Kepes and Cohen, 1962; Lin, 1970). Some can concentrate the transported molecule against a concentration gradient (Koch, 1964; Winkler and Wilson, 1966). These concentrating permeases are coupled to the energy-yielding processes of the cell and may be inducible, like the β-galactoside permease of E. coli (Rickenberg et al.,1956;

Cohen and Monod, 1957), or constitutive like the amide permease of P. aeruginosa (Brammar, McFarlane and Clarke, 1966). Facilitated diffusion systems do not concentrate the transported molecule but may be specific and passively promote the passage of substrates across the membrane (Lin, 1970). They too may be inducible, e.g. that for glycerol in E. coli (Sanno, Wilson and Lin, 1968) or constitutive, e.g. that for thiamine in the same organism (Kawasaki, Miyata, Esaki and Nose, 1969; Kawasaki, Miyata and Nose, 1969). The genes for inducible transport systems may or may not be linked to those specifying the metabolic enzymes that they serve (Englesberg et al., 1965; Jacob and Monod, 1961).

Catabolite repressors can, under certain circumstances, inhibit the function of specific transport systems responsible for inducer entry. For instance, Adhya and Echols (1966) showed that glucose inhibits the galactose permease in E. coli. In the same organism, it can also inhibit permeases for tryptophan (Boezi and De Moss, 1961) and galactosides (Cohn and Horibata, 1959; Koch, 1964; Winkler and Wilson, 1967).

Besides this, the repressor may act by preventing the synthesis of inducible permeases. It will then act both directly and indirectly.

Though it can be demonstrated under certain experimental conditions in many inducible systems, the role indirect repression at inducer entry plays under 'natural' conditions is not always easy to ascertain. However, Adhya and Echols (1966) showed that glucose had little repressor effect on the galactose operon in constitutive strains of E. coli or when the inducer (galactose) was generated internally from lactose. Glucose nonetheless severely inhibits galactose entry and they concluded that normally repression is indirect. On the other hand, inhibition of inducer entry is thought to be of minor importance in the repression of tryptophanase in E. coli (Boezi and De Moss, 1961) and histidase in K. aerogenes (Neidhardt and Magasanik, 1956). In the lac system, both direct and indirect repression undoubtedly operate but the precise contribution of each in different circumstances is unclear (see Paigen and Williams, 1970).

2. Formation of active inducer. The external and internal inducers are not always identical; the external inducer is often converted to an active form by the first metabolic enzyme. Thus urocanate induces histidase in K. aerogenes (Schlesinger, Scotto and Magasanik, 1965) and P. aeruginosa

(Lessie and Neidhardt, 1967 b), α-glycerophosphate induces the glycerol system in E. coli (Koch, Hayashi and Lin, 1964), while in the lac operon a transgalactosylation product of lactose is the active natural inducer (Burstein, Cohn, Kepes and Monod, 1965).

Catabolite repression could, in principle, be mediated by inhibition of the conversion of the external inducer to the active molecular species, but there appear to be no clear-cut examples of this form of indirect repression.

Factors determining the extent to which the enzymes for the less preferred substrate are repressed.

1. Relationship between catabolite repressor activity and the property of being a good growth substrate. In general, those substrates capable of supporting rapid growth (good growth substrates) are also good repressors of the enzymes for alternative carbon sources (Neidhardt and Magasanik, 1956, 1957). E. coli can be grown at different growth rates on a number of carbon sources. In a constitutive mutant (i^-), Mandelstam (1962) found that the rate of synthesis of β-galactosidase was inversely related to the growth rate, as did Okinaka and Dobrogosz (1967) working with an inducible (i^+) strain and the galactoside inducer, isopropyl thio-β-D-galactoside (IPTG).

Blackmore and Quayle (1968) also showed that good growth substrates were potent catabolite repressors in Pseudomonas oxalaticus. This organism metabolises many carbon compounds heterotrophically but assimilates the carbon of formate autotrophically (Blackmore, Quayle and Walker, 1968) via the key inducible enzyme, ribulose diphosphate carboxylase. When grown on formate and compounds that are much better growth substrates than it, e.g. succinate, ribulose diphosphate carboxylase is completely repressed. It is fully induced, however, when poor substrates such as glycerol are used with formate. Oxalate can be regarded as a better growth substrate than formate in terms of NADH and ATP yield per mole metabolised (Blackmore et al., 1968), but supports a growth rate 30% less than formate. In mixtures of formate and oxalate, formate is metabolised in preference and oxalyl-CoA reductase is repressed (Blackmore and Quayle, 1968).

These examples show that the faster the growth rate, the greater is the extent of catabolite repression of those inducible enzymes involved in the metabolism of alternative, poorer carbon sources. Catabolite repression, therefore,

seems to be the mechanism whereby the cell selects from a mixture of substrates the carbon source that can support the fastest growth rate, and it would appear that a fast growth rate is associated with a high intracellular concentration of catabolite repressors (or a low cyclic AMP concentration if its deficiency is the cause of repression).

Catabolite repression can be made more severe than in batch cultures by growing cells in a nitrogen-limited chemostat at a low growth rate. Thus, Mandelstam (1962) showed that the synthesis of β-galactosidase was severely repressed when a constitutive strain of E. coli was grown in such a manner. In contrast, when carbon was the growth-limiting nutrient at low growth rates, catabolite repression was lifted and the rates of synthesis of β-galactosidase in the presence of glucose or glycerol were greater than in the corresponding batch cultures.

Silver and Mateles (1969) also studied the effect of growth rate on β-galactosidase synthesis in carbon-limited chemostats but used an inducible strain of E. coli. Grown on glucose and lactose, the cells synthesised β-galactosidase at progressively greater rates as the growth rate was decreased. A similar effect was observed in the amidase

system of P. aeruginosa by Clarke and her colleagues. This enzyme is induced by amides (Brammar and Clarke, 1964) and repressed by succinate (Brammar et al., 1967). In chemostats limited on acetamide + succinate, amidase was repressed at high growth rates and became derepressed as the growth rate was decreased (Clarke, Houldsworth and Lilly, 1968).

Other workers have studied substrate utilisation by cells growing under carbon limitation and have found that the less preferred substrate is not used at high dilution rates, but is progressively more used as the growth rate is decreased (Baidya, Webb and Lilly, 1967; Harte and Webb, 1967; Mateles, Chian and Silver, 1967). At low dilution rates, both substrates may be completely consumed. However, in these examples, enzyme activities were not measured and substrate utilisation has been interpreted as indicating the extent to which each enzyme system was induced. This assumption may not always be valid (see later).

2. Effect of relative inducer and repressor concentrations on catabolite repression. In the lactose operon, glucose exerts its catabolite repressor effect independently of the lac regulator gene (Brown, 1961; Mandelstam, 1962; Loomis and Magasanik, 1964), suggesting that the inducer,

the catabolite repressor and the aporepressor do not interact. In other systems, however, inducers and repressors do interact; for instance, in the aromatic acid catabolic system of P. putida repression can be reversed by increasing the concentration of inducer (Mandelstam and Jacoby, 1965). In these systems, the extent to which the enzymes for different substrates are induced in mixed substrate media will, thus, depend on the relative inducer and repressor concentrations.

In the amidase system of P.aeruginosa repression by succinate is reversed by the amide inducers. The specific activity of the amidase showed a peak at moderate dilution rates in cultures grown on limiting amounts of acetamide and succinate (Clarke et al., 1968; Clarke and Lilly, 1969). At lower and higher dilution rates, the enzyme was repressed. By comparing the behaviour of the wild-type with that of several regulatory mutants, Clarke and her colleagues concluded that the variation was due to competition between the inducer and repressor at the induction site. Thus, as the growth rate was increased, the effective inducer concentration at the induction site increased, but as the growth rate increased still further, catabolite repressor molecules

also increased until a point was reached at which catabolite repression eventually became dominant to induction.

In the same organism, Dawes and co-workers studied the enzymic reponse to mixtures of citrate and glucose. The enzymes for glucose metabolism are inducible (Hamilton and Dawes, 1960, 1961). By limiting the nitrogen, cells could be grown in a chemostat at a constant growth rate on different steady state concentrations of these two carbon sources. The extent to which the glucose enzymes were induced could be increased either by raising the glucose concentration or by lowering that of citrate (Hamlin, Ng and Dawes, 1967).

Diauxic growth

The extent to which the enzymes for the less preferred of two substrates are induced in a two-substrate medium is thus determined by a number of factors. In the extreme, the enzymes for the less preferred substrate could be completely repressed by the other substrate, and so severe might this repression be that growth would be diauxic, i.e. take place in two stages.

Monod (1942, 1947) showed that, when this occurs, growth in the first stage takes place at the expense of one substrate. When this has been completely consumed, growth stops until sufficient inducible enzyme has been formed to utilise the second substrate. During the diauxic lag, protein turnover provides amino acids for enzyme synthesis (Mandelstam, 1958).

Diauxic growth has been observed in innumerable organisms on a wide range of substrate mixtures. Paigen and Williams (1970) have cited many of the examples involving carbohydrates. In these cases glucose is very often the primary carbohydrate used, but it can sometimes be the secondary carbon source (Hamilton and Dawes, 1959). Diauxic growth can also occur on pairs of substrates other than carbon sources, e.g. nitrogen sources (Neidhardt and Magasanik, 1957; Bourgeois, Wiame and Lelouchier-Dagnelie, 1960) and phosphorus sources (Torriani, 1960; Rosenberg and La Nauze, 1967).

The requirements for diauxic growth have been extensively investigated especially in E. coli grown on glucose and lactose. Severe repression of the enzymes for lactose is not sufficient in this case; active inducer must also be limiting. Thus in lac-constitutive (but catabolite

sensitive) strains, diauxic growth is not observed (Silver and Mateles, 1969). High lactose concentrations or inducers that enter the cell but do not have to be converted to the active inducer also eliminate diauxie (Moses and Prevost, 1966; Silver and Mateles, 1969). In other systems, it has been shown that providing an alternative entry route for the secondary substrate or adding cyclic AMP to overcome repression also eliminate diauxic growth (Adhya and Echolls, 1966; Ullmann and Monod, 1968).

Prediction of the enzymic response to mixed substrates

These examples of enzyme adaptation to mixed substrates show that two opposing factors are involved, those of induction and catabolite repression. If the effects of induction and repression can be quantitatively assessed, it should be possible to predict the enzymic response to substrate mixtures.

Although it is not essential for the argument, it will be assumed that the Jacob-Monod model for enzyme induction is generally valid. The inducer can then be assumed to interact with another molecule (the aporepressor) and this relieves the repression of the inducible enzymes. If we assume also that this interaction has the form of an adsorption isotherm, an induction constant (K_{ind}) can be defined that is a measure of the affinity of the inducer

for the aporepressor. The K_{ind} will be the inducer concentration that supports half the maximum induction rate.

The catabolite repressor may act in a number of ways (see above), but again it must be presumed to act at some site and this reaction will also have a half-saturation value defined by a repression constant (K_{rep}).

It should be possible to determine K_{ind} and K_{rep} from the effects of inducers and repressors on the rate of enzyme synthesis, and indeed they have been in the amidase system of P. aeruginosa (Brammar, 1965; Clarke, 1970). Once determined, the induction and repression constants could be used to predict the enzymic behaviour of an organism towards mixed substrates.

Substrate utilisation in mixed substrates

Although it should be possible from induction and repression constants to predict the enzymic response of an organism to mixed substrates, the rates at which those substrates will be used cannot, in general, be predicted without more information. Obviously a substrate will not be used if its enzymes are largely or completely repressed. However, when both enzyme systems are present, the relative

rates of substrate utilisation would depend not only on the amounts of their respective enzymes, but also on the K_m and turnover number.

The activity of key enzymes can also be altered by metabolites other than the substrate(s) or product(s). Feedback inhibition is common in biosynthetic pathways where the end product often inhibits the first enzyme of the pathway (Stadtman, 1963; Cohen, 1965; Umbarger, 1969). Catabolic enzymes can also be inhibited by the products of the catabolic pathways, e.g. succinate inhibits urocanase in P. putida and the urocanate that accumulates inhibits histidase (Hug, Roth and Hunter, 1968). Other catabolic enzymes that can be inhibited include the glycerol kinase of E. coli (Zwaig and Lin, 1966) and glucose-6-phosphate dehydrogenase in P. aeruginosa (Lessie and Neidhardt, 1967a).

Though there may be inhibitory effects on substrate utilisation, in many cases the substrate consumption in mixed substrates can often be accounted for by the enzyme activities. (Hamlin et al., (1967) reported that there was good correlation between the rate of glucose utilisation and the specific activity of glucose-6-phosphate dehydrogenase in cultures of P. aeruginosa growing on glucose + citrate.

In cultures of P. oxalaticus growing on mixtures of formate and oxalate, the consumption of each substrate was proportional to the specific activity of their enzyme system (Blackmore and Quayle, 1968).

Aromatic carboxylic acid metabolism in Pseudomonas putida

Pseudomonas putida can grow well at the expense of several aromatic carbon sources. The pathways by which these carbon sources are metabolized involve several enzymes that are subject to control by induction (Stanier et al.,1965; Hegeman, 1966; Ornston, 1966) and end-product repression (Mandelstam and Jacoby, 1965; Stevenson and Mandelstam, 1965). These pathways have a number of features that make them suitable for predicting the enzymic response of the organism to mixed (aromatic) substrates.

The biochemistry and enzymology of the pathways are illustrated in Figures 1.1 and 1.2. Stanier and his colleagues used the techniques of simultaneous adaptation and isolation of intermediates together with many blocked mutants to establish the stages of metabolism of these aromatic compounds. Mandelate is metabolised by a group of five enzymes (the mandelate group) to benzoate.(Gunsalus, Gunsalus and Stanier, 1953; Gunsalus, Stanier and Gunsalus, 1953; Stanier, Gunsalus and Gunsalus, 1953). The same enzymes

FIGURE 1.1

Convergent pathways for the metabolism of aromatic carboxylic acids by Pseudomonas putida : Biochemistry.

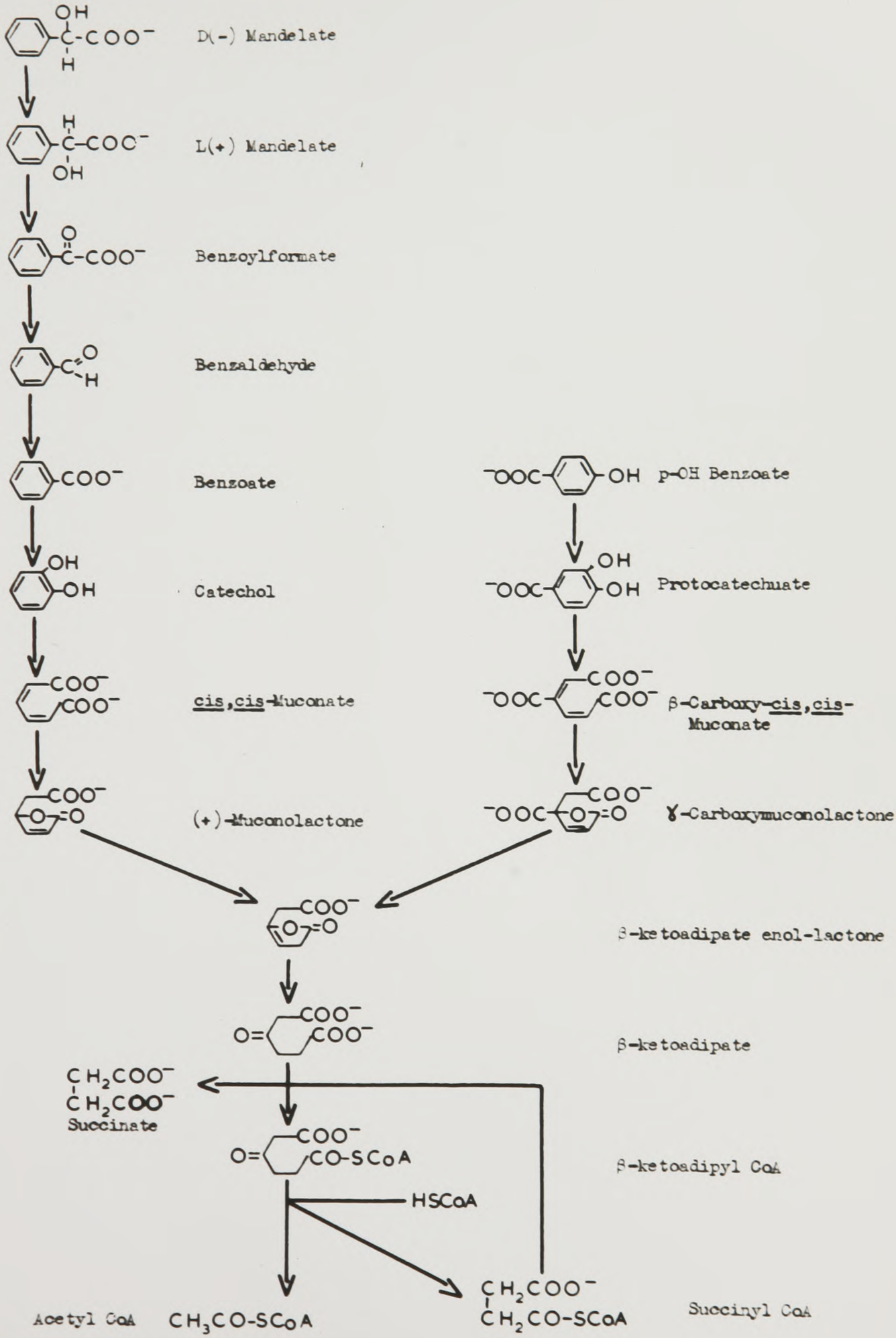

D(-) Mandelate
L(+) Mandelate
Benzoylformate
Benzaldehyde
Benzoate
Catechol
cis,cis-Muconate
(+)-Muconolactone
p-OH Benzoate
Protocatechuate
β-Carboxy-cis,cis-Muconate
γ-Carboxymuconolactone
β-ketoadipate enol-lactone
β-ketoadipate
Succinate
β-ketoadipyl CoA
HSCoA
Acetyl CoA
CH3CO-SCoA
Succinyl CoA

FIGURE 1.2

Convergent pathways for the metabolism of aromatic carboxylic acids by Pseudomonas putida : Enzymology.

The enzymes catalysing the reactions of the pathways in the diagram on the facing page are :

1. Mandelate racemase
2. L(+)-mandelate dehydrogenase
3. Benzoylformate decarboxylase
4. Benzaldehyde dehydrogenases (NAD^+ and $NADP^+$-linked)
5. Benzoate oxidase

5A. p-OH Benzoate hydroxylase

6. Catechol-1,2-oxygenase

6A. Protocatechuate-3,4-oxygenase

7. cis,cis-Muconate lactonising enzyme

7A. β-Carboxy-cis,cis-muconate lactonising enzyme

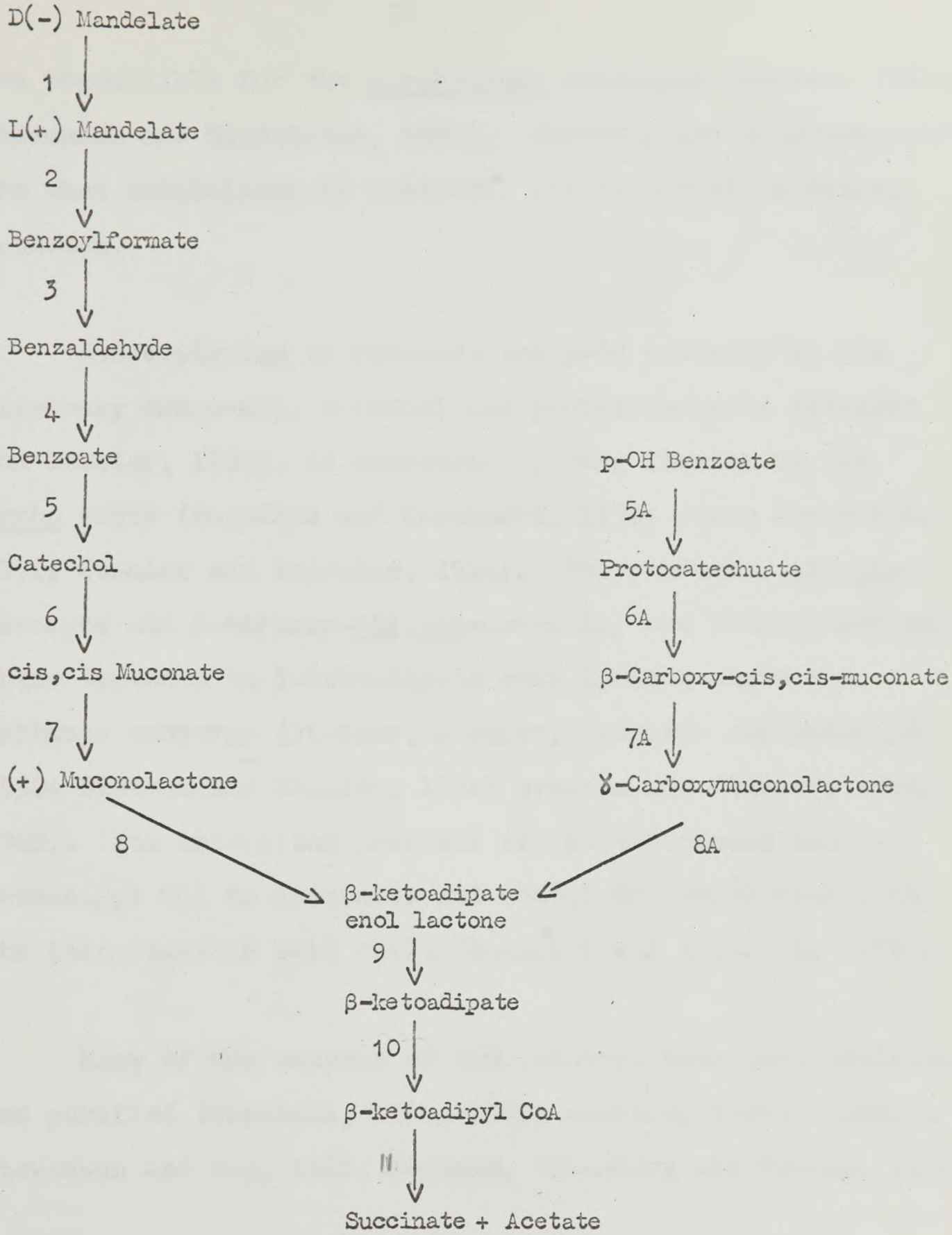
8. Muconolactone isomerase

8A. ϒ-Carboxymuconolactone decarboxylase

9. β-Ketoadipate enol lactone hydrolase

10. β-Ketoadipate succinyl-CoA transferase

11. β-Ketoadipyl CoA thiolase


D(-) Mandelate
1
L(+) Mandelate
2
Benzoylformate
3
Benzaldehyde
4
Benzoate
5
Catechol
6
cis,cis Muconate
7
(+) Muconolactone
8
p-OH Benzoate
5A
Protocatechuate
6A
β-Carboxy-cis,cis-muconate
7A
ɣ-Carboxymuconolactone
8A
β-ketoadipate
enol lactone
9
β-ketoadipate
10
β-ketoadipyl CoA
11
Succinate + Acetate

are responsible for the parahydroxy analogues (Gunter, 1953; Stevenson and Mandelstam, 1965). Benzoate and p-OH benzoate are then metabolised by distinct, yet chemically similar, pathways.

Hydroxylation of benzoate and p-OH benzoate to the dihydroxy compounds, catechol and protocatechuate (Sleeper and Stanier, 1950), is succeeded by ring fission by the ortho route (Hayaishi and Hashimoto, 1950; Evans and Smith, 1951; Stanier and Ingraham, 1954). The products, cis,cis-muconate and β-carboxy-cis,cis-muconate, are metabolised via their lactones to β-ketoadipate enol lactone where the pathways converge (Stanier, Sleeper, Tsuchida and McDonald, 1950; Sistrom and Stanier, 1954; Ornston and Stanier, 1964, 1966). Its metabolism proceeds via β-ketoadipate and β-ketoadipyl CoA to succinate and acetyl CoA which feed into the tricarboxylic acid cycle (Katagiri and Hayaishi, 1957).

Many of the enzymes of the pathways have been isolated and purified (Hayaishi, 1966, 1969; Ornston, 1966; Stachow, Stevenson and Day, 1967; Hegeman, Rosenberg and Kenyon, 1970)

Induction. Cells not previously grown on aromatic carbon sources are characterised by very low basal levels of all

FIGURE 1-3.

Convergent pathways for the metabolism of aromatic carboxylic acids by Pseudomonas putida : Induction of the enzymes.

In the diagram on the facing page, coordinately induced enzymes are indicated by the brackets.

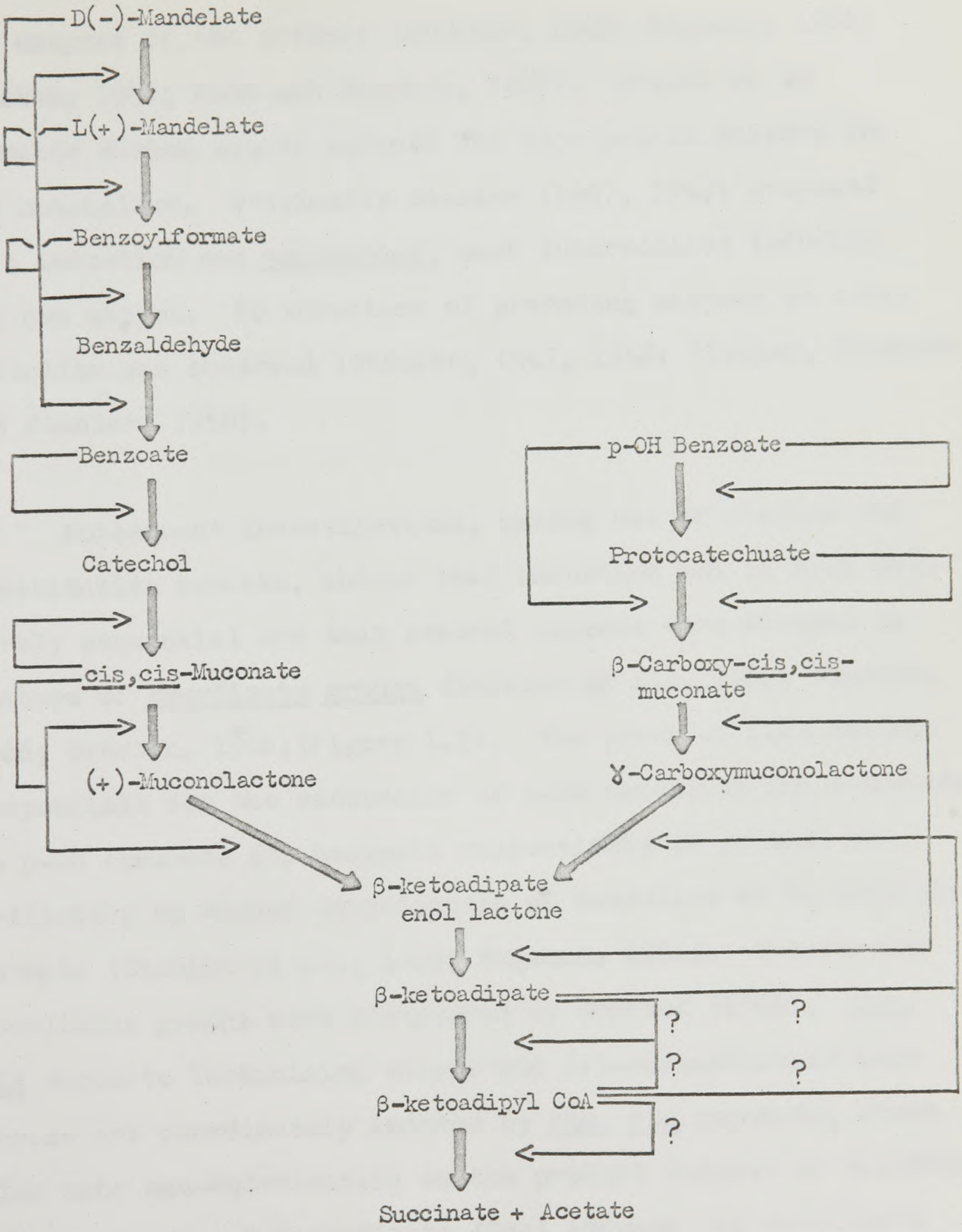
Data are from : Hegeman (1966).

Hosokawa (1970).

Kemp and Hegeman (1968).

Ornston (1966).

Stanier, et al. (1965).


D(-)-Mandelate
L(+)-Mandelate
Benzoylformate
Benzaldehyde
Benzoate
Catechol
cis,cis-Muconate
(+)-Muconolactone
p-OH Benzoate
Protocatechuate
β-Carboxy-cis,cis-muconate
γ-Carboxymuconolactone
β-ketoadipate enol lactone
β-ketoadipate
β-ketoadipyl CoA
Succinate + Acetate
?
?
?
?
?

the enzymes of the pathway (Stanier, 1948; Hegeman, 1966; Ornston, 1966; Kemp and Hegeman, 1968). Growth on an aromatic carbon source induces the appropriate enzymes for its metabolism. Originally Stanier (1947, 1948) proposed that induction was sequential, each intermediate inducing its own enzyme. No induction of preceding enzymes or cross induction was observed (Stanier, 1947, 1948; Sleeper, Tsuchida and Stanier, 1950).

Subsequent investigations, making use of blocked and constitutive mutants, showed that induction was in fact only partly sequential and that several enzymes were induced as members of coordinate groups (Stanier et al., 1965; Hegeman, 1966; Ornston, 1966)(Figure 1.3). The group of five enzymes responsible for the conversion of p-OH mandelate and mandelate to p-OH benzoate and benzoate respectively is induced co-ordinately by either enantiomorph of mandelate or by benzoyl-formate (Stanier et al., 1965; Hegeman, 1966). Two further coordinate groups were discovered by Ornston (1966). Cis, cis muconate lactonising enzyme and (+)-muconolactone isomerase are coordinately induced by cis, cis muconate, which also acts non-coordinately as the product inducer of catechol-1,2-oxygenase. β-ketoadipate (CoA) induces the coordinate group comprising β-carboxy-cis, cis-muconate lactonising enzyme, γ-carboxy-muconolactone decarboxylase and β-keto-adipate enol lactone hydrolase. Since benzoate-grown cells

produce β-ketoadipate (CoA), its function as a coordinate inducer leads to the redundant synthesis of two of the p-OH benzoate pathway enzymes in these cells.

Sequential induction steps occur at benzoate oxidase and p-OH benzoate hydroxylase where each is induced by its substrate (Hegeman, 1966; Hosokawa, 1970). Protocatechuate-3,4-oxygenase is induced by its substrate and also by p-OH benzoate, though non-coordinately with p-OH benzoate hydroxylase (Hosokawa, 1970). β-Ketoadipate or possibly its CoA derivative acts as the inducer of the remaining enzymes of the β-keto-adipate pathway (Kemp and Hegeman, 1968).

Genetic analysis in Pseudomonas spp. is not as advanced as in the enteric bacteria, but transducing phages have been used for preliminary mapping in P. putida and the related P. aeruginosa (Holloway, 1969; Rosenberg and Hegeman, 1969). The genes for the mandelate enzymes of P. putida form a gene cluster (Chakrabarty, Gunsalus and Gunsalus, 1968; Chakrabarty and Gunsalus, 1969) that is co-transduced with a presumptive o^c mutation (Hegeman, 1966) which confers pleiotropic constitutivity for the mandelate group. Wheelis (1970) has shown that the enzymes of the cis, cis muconate group, catechol-1,2-oxygenase and benzoate oxidase are coded for

by a gene cluster that is loosely linked to the mandelate group of genes. The genes for the enzymes of the β-keto-adipate group, protocatechuate-3,4-oxygenase and p-OH benzoate hydroxylase also form a cluster but this is unlinked to either of the previous clusters (Wheelis, 1970).

The existence of coordinacy has prompted Hegeman (1966) and Ornston (1966) to postulate that the genes for those enzyme groups may be organised in operons. However, coordinacy is not a sufficient indicator of an operon. Thus, the enzymes of the tryptophan operon are not always synthesised coordinately (Bauerle and Margolin, 1967; Morse and Yanofsky, 1969 a) and coordinacy has been observed for unlinked genes (Ames and Martin, 1964). Stevenson and Mandelstam (1965) preferred to use the term regulon, coined by Maas and McFall (1964), to describe these control groups. Polarity might be a better indicator of an operon (Kemp and Hegeman, 1968).

Repression. Repression of the enzymes of the aromatic carboxylic acid pathways in P. putida has been observed by Gunter (1951), Ornston (1966) and Sakaki, Kageyama and Egami (1969), but the most comprehensive study was made by Mandelstam and Jacoby (1965) and Stevenson and Mandelstam (1965). They found that the specific end-product of each regulon

represses the induction of that regulon and preceding regulons (Fig. 1.4). Blocked mutants were used to discount the possibilities that the repression was due to metabolism to a common end product, e.g. succinate, or to sequential repression. Mandelstam and Jacoby (1965) referred to this form of repression as 'multi-sensitive' to distinguish it from 'multi-valent' repression observed in the pathways for the biosynthesis of the branched chain amino acids (Freundlich, Burns and Umbarger, 1962).

The mechanism of repression is not known but it is additive and can be reversed at high inducer concentrations (Mandelstam and Jacoby, 1965). Feedback inhibition does not seem to occur in this pathway (Stanier et al., 1965; Hegeman, 1966).

Selective adaptation to mixed aromatic substrates: the aims of this study

The pattern of repression in this system suggests that there ought to be a selective enzymic response to media containing more than one aromatic carbon source. For example, if mandelate and p-OH benzoate are both present, it would be expected that, depending on the inducer concentration, the mandelate enzymes would be at best partly induced and

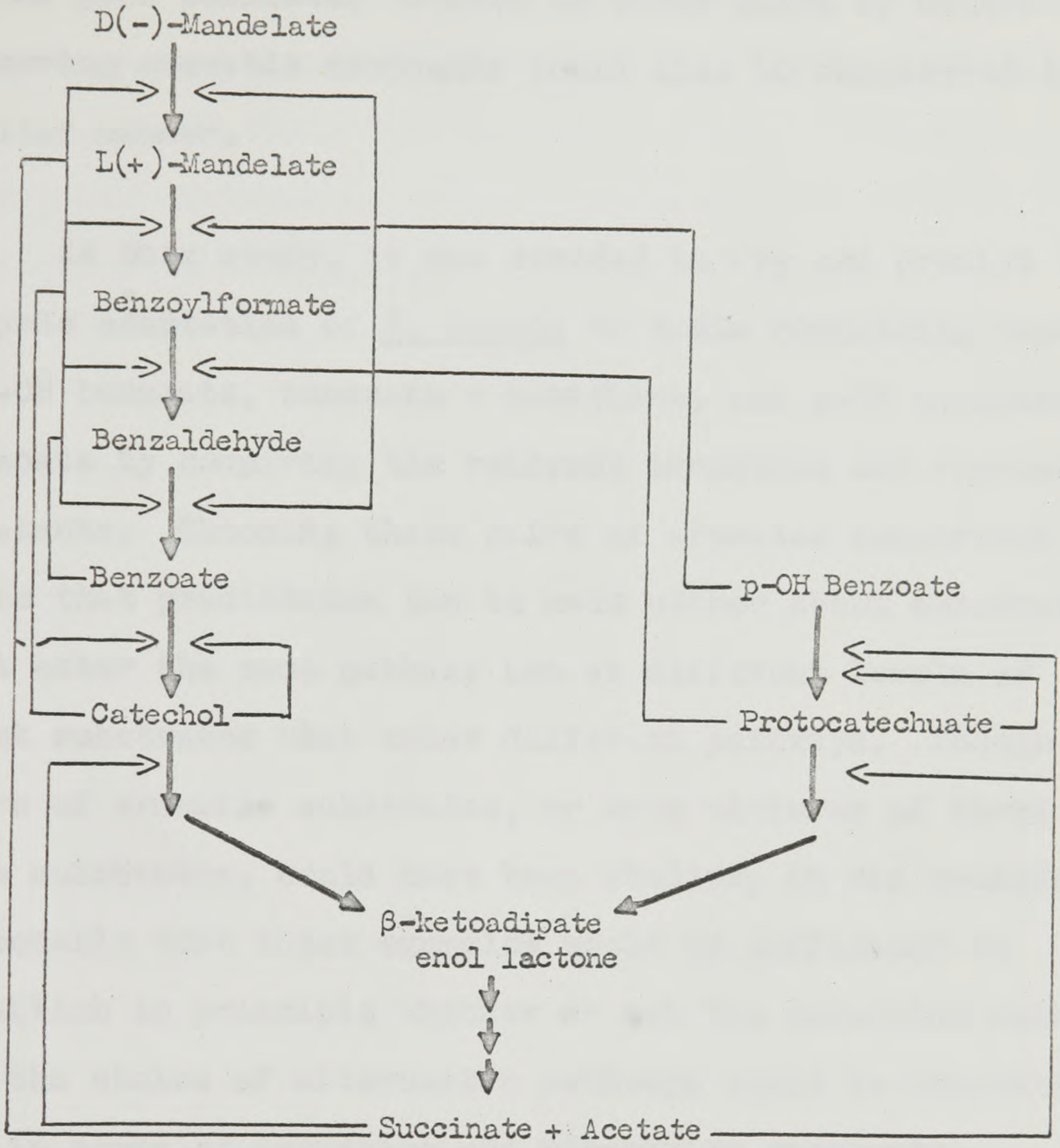
FIGURE 1-4.

Convergent pathways for the metabolism of aromatic carboxylic acids by Pseudomonas putida : Repression of the enzymes.

In the diagram on the facing page, enzymes that are coordinately induced and repressed are indicated by brackets. Succinate and acetate had almost equivalent effects.

Data are from : Mandelstam and Jacoby (1965).

Stevenson and Mandelstam (1965).


D(-)-Mandelate
L(+)-Mandelate
Benzoylformate
Benzaldehyde
Benzoate
Catechol
p-OH Benzoate
Protocatechuate
β-ketoadipate
enol lactone
Succinate + Acetate

that there might, therefore, be a preferential utilisation of the p-OH benzoate. Growth on other pairs of naturally occurring aromatic compounds could also be considered in a similar manner.

In this study, it was decided to try and predict the enzymic adaptation of P. putida to media containing mandelate + p-OH benzoate, benzoate + mandelate, and p-OH benzoate + benzoate by comparing the relevant induction and repression constants. Choosing these pairs of aromatic substrates means that predictions can be made either about substrates that enter the same pathway but at different levels or about substrates that enter different pathways. Though more pairs of aromatic substrates, or even mixtures of three or more substrates, could have been studied, it was considered reasonable that these examples would be sufficient to establish in principle whether or not the induction behaviour and the choice of alternative pathways could be accounted for in terms of induction and repression constants.

The primary intention was to examine the response to mixed substrates from the standpoint of the protein economy of the cell, i.e. the way in which protein synthesis is channelled into each of two pathways when the cells are

presented with a choice. Accordingly, no specific attempts were made to predict the substrate utilisation that would result. As discussed above, more kinetic data would be necessary to do this, but, in fact, it will be seen that the enzymic response is often clear enough to indicate the relative rates of substrate utilisation.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

ORGANISM AND MUTANTS

Pseudomonas putida biotype A strain 90 (Stanier et al., 1966), A.T.C.C. 12633, was used throughout this study. This organism, originally known as P. fluorescens (Stanier, 1947), had previously been classified as P. putida A3.12 (Ornston & Stanier, 1966).

Mutants defective in one or more of the enzymes of the aromatic sequence were obtained from Professor J. Mandelstam. They were all derived from the parent strain after treatment with the mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (Mandelstam & Jacoby, 1965). They were checked for their ability to grow on the intermediates of the aromatic pathways and the position of the block determined. The enzymes concerned displayed negligible activity and no growth was observed for at least 24 hr on minimal agar plates or in liquid medium supplemented with the relevant aromatic carbon source. The mutants used in this study are listed in Table 2.1.

The benz$^-$ mutants used in this study are unable to grow on benzoate and show negligible rates of oxidation with benzoate as substrate. They are still able to de-

TABLE 2.1

Mutants of *Pseudomonas putida* used in this study.

Mutant	Designation	Enzyme(s) blocked
Y 65	*md*$^-$	L(+)-mandelate dehydrogenase
D 57	*md*$^-$	L(+)-mandelate dehydrogenase
D 5701*, D 5703*, Y 6503*	*md*$^-$, *benz*$^-$	L(+)-mandelate dehydrogenase; Benzoate oxidase
C 167	*benz*$^-$	Benzoate oxidase§
A 2	p-OH *benz*$^-$	p-OH Benzoate hydroxylase
Cat 2	*cat*$^-$	Catechol-1,2-oxygenase

* derived from the single mutant Y 65 or D 57.

§ also carries a streptomycin-resistance marker.

carboxylate benzoate (see Chapter 4; Table 4.4) and so the nature of the defect in their benzoate metabolism is unknown.

MEDIA

1. Minimal salts medium

The basal mineral salts medium of Mandelstam & Jacoby (1965) was used in all experiments except where otherwise stated. Its composition (per litre) was:

KH_2PO_4	13.6 g.
NH_4Cl	1.0 g.
$(NH_4)_2SO_4$	1.0 g.
$MgSO_4.7H_2O$	40 mg.
$Fe(NH_4)_2(SO_4)_2.6H_2O$	15.6 mg.

The pH was adjusted to 7.0 with NaOH and the solution autoclaved at 15 lb.sq.in.$^{-1}$ for 15 min. For solid media, 1.5% (w/v) Oxoid N° 3 agar was added before autoclaving. The carbon source was sterilised separately.

2. Nutrient media

Nutrient broth (Oxoid), containing beef extract, yeast extract, peptone and NaCl, was made up as a 1.3% (w/v)

solution and sterilised in capped tubes at 15 lb.sq.in.$^{-1}$. Nutrient agar medium contained in addition 1.5% (w/v) agar and was sterilised in screw-capped bottles. It was allowed to solidify as slopes.

3. Modified minimal salts medium for continuous cultivation.

For use in the chemostat, the nitrogen in the minimal salts medium was made growth-limiting. The $(NH_4)_2SO_4$ was reduced to 0.2 or 0.25 g.l.$^{-1}$. Sodium chloride (1.0 g.l.$^{-1}$) replaced the NH_4Cl and the sulphate was made up with Na_2SO_4 (0.8 g.l.$^{-1}$). The other ingredients were as above except that silicone emulsion (0.1 ml. l.$^{-1}$) was added to control foaming during growth. The modified medium was sterilised at 17 lb. sq.in.$^{-1}$ for 30 min.

In the early part of this study, chemostats were run with 0.2 g.l.$^{-1}$ $(NH_4)_2$ SO_4 which resulted in a steady state culture density of 300-350 µg dry wt. ml.$^{-1}$ at the dilution rates normally employed. This proved to be insufficient for some purposes and later experiments were performed using 0.25 g.l.$^{-1}$ $(NH_4)_2$ SO_4. This raised the culture density to 350 - 400 µg. dry wt. ml.$^{-1}$.

MAINTENANCE AND GROWTH OF ORGANISM

Maintenance of organism

The parent and mutant strains were maintained on nutrient agar slopes, subcultured monthly. After 12 hr. growth at 30°, the slopes were kept at room temperature in the dark. Every 2-3 months, lyophilised samples were resuscitated in nutrient broth and fresh slopes prepared. Periodically, the cells were checked for their microscopic appearance and aromatic growth requirements on plates or in liquid medium.

Growth of organism

For the routine growth of the organism, conical flasks of varying size were used, filled to no more than one-fifth of their capacity. The flasks were shaken on a reciprocating platform at 30° in a hot room or a water bath. This temperature was used in all experiments.

When exponentially growing cultures were required, cells were inoculated from the storage slopes into nutrient broth tubes and shaken at 30° for 6-8 hours. These cultures were then used to inoculate flasks of minimal medium containing an appropriate carbon source. After overnight growth, the cells were centrifuged and resuspended at an appropriate density in more of the same medium. Two generations of

growth were usually sufficient to establish exponential growth and the culture could then be induced.

MATERIALS

1. Chemicals

British Drug Houses (Poole, Dorset) was the supplier of all the chemicals used except where indicated below. Wherever possible "Analar" grade reagents were used. In general, the aromatic carbon sources were only available as the 'Laboratory Grade' but, with the exception of catechol, further purification proved to be unnecessary. Catechol was recrystallised from benzene. DL-mandelic acid was used throughout this study.

The Sigma Chemical Company (St. Louis, Missouri) supplied Antifoam B emulsion (10% silicone) and thiamine pyrophosphate (cocarboxylase). Benzoic acid (Na^+ salt) was made by May and Baker Ltd. (Dagenham, Essex). Chloramphenicol was purchased from Parke Davis, Ltd. (Greenford, Middx). Butyl-PBD [2-(4-tert-butylphenyl)-5-(4-biphenyl)-1-oxa-3,4-diazole] was a product of Ciba (ARL) Ltd., Duxford, Cambs. Blue dextran 2000 was obtained from Pharmacia, Ltd., Sweden. Benzoylformate (Na^+ salt) was a gift from Professor J. Mandelstam, and had been synthesised by the method of Oakwood and Weisgerber(1955). Oxoid products were from

2. Radiochemicals

Sodium cyanide (^{14}C) had a specific activity of 52.8 mCi,mmole^{-1}. ^{131}I-serum albumin (human) was supplied as a sterile solution containing 20 mg. albumin and 1.0 mCi.ml^{-1} in 0.7% NaCl. Benzoic acid (^{14}C-carboxyl) had a specific activity of 29.5 mCi.mmole^{-1}. All were supplied by the Radiochemical Centre, Amersham, Bucks.

3. Carbon sources.

Carbon sources were made up separately at the concentrations indicated below, sterilised and added aseptically to the minimal salts medium at the final desired concentration. Except for glucose and asparagine which were always used at 0.5 and 0.4% (w/v) respectively, the final concentration is indicated in the Text.

With the exception of glucose, which was made up at 50% w/v, carbon sources were made up as 0.5M solutions. D-glucose, sodium succinate, sodium benzoate and L-asparagine. H_2O were then sterilised at 10 lb.sq.in.$^{-1}$ for 10 min.

Other aromatic carbon sources were dissolved with the aid of NaOH, the pH adjusted to about 6.0 and the solution sterilised by filtration through a sterile membrane filter

(Oxoid 4 cm. dia.). Solutions were kept at 4°C in the dark to minimise photo-oxidation.

4. Preparation of mandelic acid (^{14}C-carboxyl)

This was prepared essentially by Hegeman's (1966) modification of the method of Fieser (1957). Benzaldehyde (1.0 ml) was added with mixing to sodium metabisulphite (1.1 g) in water (3 ml). On cooling, the bisulphite addition compound crystallised. It was washed with cold water and ether and then recrystallised from warm water.

Sodium cyanide (12 mg., containing cyanide-^{14}C, 500 μCi) was added to a solution of bisulphite addition compound (100 mg in 1.0 ml water) and the solution mixed thoroughly for 30 minutes at room temperature. The mandelonitrile was extracted with three lots of ether (0.5 ml) and the ether layer was then washed with water (1.0 ml) and saturated sodium chloride (1.0 ml).

The ether layer was acidified with HCl (1.0 ml, 50% w/v) and the ether evaporated in an air stream. The acid solution was refluxed on a water bath for 2 hr. with continuous mixing. On cooling, the mandelic acid was extracted with

ether and applied as a narrow band to preparative chromatography paper (Whatman N.° 3). The chromatogram was developed by descending chromatography in ethanol, acetic acid, water (8:1:1 v/v) for 12 hrs. The radioactive acid was located (R_F = 0.8) by autoradiography (Kodak Blue Brand X-ray film, 6 hours) and eluted with potassium phosphate buffer (0.1M, pH 6). The solution was kept frozen at -20°C. The specific activity was about 2.0 $\mu Ci.\mu mole^{-1}$ and the yield about 70% (calculated on the basis of the cyanide used.)

Purity of the radioactive mandelate. A small amount (0.1 µCi) of the product was compared with authentic mandelic acid by descending paper chromatography (Whatman N.° 1) in four different solvent systems:

A. Ethanol, acetic acid, water (8:1:1 v/v)
B. Benzene, acetic acid, water (125:72:3)
C. Isopropanol, ammonia, water (20:1:2)
D. n-Butanol, pyridine, water (14:3:3)

Radioactive mandelate was detected by radioautography (3 days) and the unlabelled marker by spraying the dried papers with bromophenol green (0.04% in 95% ethanol with a few drops of 0.88 s.g. ammonia)(Dawson, Elliott, Elliott and Jones, 1969). In all cases, the labelled acid moved as a single spot with the same R_F as the authentic acid.

Metabolism of radioactive mandelate by induced and uninduced cells. The metabolism of the labelled acid was also checked. Wild-type cells were grown in glucose-minimal medium, induced with mandelate (10 mM) for three generations, then washed with phosphate buffer (0.1M, pH 7) and finally resuspended (at 500 $\mu g.ml.^{-1}$) in the same buffer, containing chloramphenicol (50 $\mu g.ml.^{-1}$). Portions of the washed cells (2.5 ml) were incubated with shaking at 30° C in closed manometer vessels with mandelate (6.0 μmole) containing the labelled compound (0.02 μCi). The $^{14}CO_2$ evolved was trapped in soda and estimated as in the assay of benzoate oxidase (Below). The mandelate-induced cells were compared with uninduced cells (glucose-grown) which were used at a density of 2500 $\mu g.ml.^{-1}$ The results (Table 2.2) show that more than 99% of the radioactive mandelate was decarboxylated by induced cells and only about 1% by uninduced cells.

ESTIMATIONS

1. Enzyme Assays

Culture samples (5-200 ml) were removed into centrifuge tubes containing chloramphenicol (50 $\mu g.ml.^{-1}$ final concentration), and centrifuged at 3,000 x g for 20 min. Subsequent treatment of the cell pellet depended on the enzyme to be assayed. Except where the enzyme assay involved whole

TABLE 2.2

Metabolism of mandelate-^{14}COOH by wild-type cells.

Wild-type cells were induced for 3-4 generations in glucose-minimal medium containing mandelate (10 mM), washed and resuspended (500 μg. dry wt. ml.$^{-1}$) in phosphate buffer (0.1 M, pH 7) containing chloramphenicol (50 μg. ml.$^{-1}$). Portions (2.5 ml) of this cell suspension were incubated in sealed manometer vessels with mandelate-^{14}COOH (6 μmoles, 0.02 μCi.) for 1 hr. at 30°. The $^{14}CO_2$ evolved was trapped in NaOH, estimated as in the assay for benzoate oxidase (Methods) and compared with that produced by uninduced cells (at 2.5 mg. dry wt. ml.$^{-1}$). The background was 30.7 c.p.m.

	Total mandelate added (c.p.m.)	$^{14}CO_2$ evolved (c.p.m.)
Induced cells	23,500	23,250
	23,240	23,160
Uninduced cells	23,450	264.4
	22,970	273.1

cell oxidation, the samples were kept at 0°C throughout. Exposure to low temperatures was detrimental to whole cells, but they could be kept at room temperature in chloramphenicol for a few hours without appreciable loss of activity.

All enzyme activities are expressed in the same units, namely: 1 unit is equivalent to 1 nmole substrate destroyed or 1 nmole of O_2 consumed per minute.

L(+)-mandelate dehydrogenase: The assay procedure of Hegeman (1966) was followed with minor modifications. The method involves the spectrophotometric measurement of mandelate oxidation in sonicated cell preparations. The oxidation is coupled to phenol-indo-2:6-dichlorophenol (DCPIP).

The cell pellet (above) was washed in phosphate buffer (0.1 M, pH 7) and resuspended at 40-50 mg.ml.$^{-1}$ in more of the same buffer. The cells were disrupted by ultrasonic vibration in an oscillator for 2 minutes at 0°C (Mullard type E 7590B, 25 kcycle.sec.$^{-1}$, 600 watts).

Quite satisfactory assays could be performed on this broken cell suspension but for the assay of mandelate (see later) the sonicated extracts were further purified.

L(+)-mandelate dehydrogenase is a particle-bound preparation and so the particles were separated by high speed centrifugation (100,000 x g, 60 min), after the cell debris and unbroken cells had first been removed at low speed (5,000 x g., 10 min.)

The particle-pellet was washed by dispersing it in buffer (5 ml) in a homogeniser with a close-fitting Teflon pestle and the particles were again separated by centrifugation. The washed particle preparation was once more resuspended by homogenisation in aqueous ethane diol (40% v/v diol) and stored at -20° C until required for assay.

Spectrophotometric assays were carried out at 600 nm in glass cuvettes (3 ml, 1 cm. light path) at 30°C against a water blank in a Cary 14 recording spectrophotometer. Each cuvette contained phosphate buffer (0.1 M, pH 7, 1.0 ml), KCN (0.01 M, 0.3 ml), DCPIP (0.29 mg ml^{-1} 0.6 ml), DL-mandelate(0.15 M, 0.1 ml), particle preparation and water (to 3.0 ml). The reaction was started with the mandelate after the blank rate due to the particles had been noted. Under these conditions, the molar extinction coefficient (ϵ) of DCPIP is 20.6 x $10^3.cm^2$

Benzoylformate decarboxylase : (E.C. N.° 4.1.1.7, benzoylformate carboxy-lyase). This enzyme was assayed by measuring manometrically the rate of carbon dioxide production from benzoylformate using toluene-treated cell samples in the presence of thiamine pyrophosphate (Mandelstam & Jacoby, 1965). The carbon dioxide production was corrected for retention by the buffer (pH 6).

Benzoate oxidase: Because this enzyme is wholly or partly destroyed by the usual procedures used for breaking cells (Sleeper, Tsuchida and Stanier, 1950; Mandelstam and Jacoby, 1965), most workers have used whole cell preparations to assay this enzyme (Mandelstam and Jacoby, 1965; Kemp and Hegeman, 1968; Kennedy and Fewson, 1968). In this study, the manometric technique of Mandelstam and Jacoby (1965) was used to measure the oxygen uptake associated with the metabolism of benzoate by washed cell suspensions.

The cell pellet was washed in phosphate buffer (pH 7, 0.1M, 10 ml) and resuspended in fresh buffer (6.0 ml) containing chloramphenicol (50 $\mu g.ml.^{-1}$). Portions (2.5 ml) of this suspension were used in the assay.

This method was used routinely whenever the samples contained reasonable amounts of enzyme. It was found to

be too insensitive for the assay of samples with low specific activities (see Chapter 3). A more sensitive assay was therefore developed for the determinations of small amounts of enzyme. It is based on the release of $^{14}CO_2$ from benzoic acid ($^{14}COOH$).

Suspensions of washed cells (2.5 ml portions), prepared as above, were incubated at 30°C with shaking in the main compartment of a sealed, double side arm manometer vessel. After 10 minutes for equilibration, labelled benzoate (0.5 ml, 6.0 µmole, 0.02 µCi) was tipped into the main vessel from one side arm. The $^{14}CO_2$ evolved was trapped in the centre well which contained NaOH (0.2 ml, 2N). The reaction was terminated after 0, 20, 40 or 60' by tipping in H_2SO_4 (0.5 ml, 7N) from the other side arm. A further 30 minutes at 30°C was allowed for complete absorption of the $^{14}CO_2$.

The centre well was then rinsed out with 0.8 ml H_2O and the radioactivity determined in 0.4 ml samples (see below). Samples of the original benzoate solution were also counted. Controls were incubated to measure the activity of uninduced cells (about 0.01 - 0.02 nmole mg.dry wt.$^{-1}$).

p-OH Benzoate hydroxylase: p-OH Benzoate hydroxylase from P. putida has been purified and a cell-free assay system developed (Hosokawa & Stanier, 1966). This assay depends on the spectrophotometric measurement of the substrate-dependent oxidation of NADPH in extracts of sonicated cells and requires the complete removal of particle-bound NADPH oxidase from the soluble enzyme (Hosokawa & Stanier, 1966; Cánovas & Stanier, 1967). This would have been especially important in the assay of low specific activity samples such as those which were involved in the determination of the p-OH benzoate hydroxylase induction constant (Chapter 3). In view of the rather time-consuming preparative procedure, it was decided to assay the enzyme by observing the rate of disappearance of p-OH benzoate from buffered cell suspensions (Stevenson & Mandelstam, 1965).

Cell pellets were washed in 10 ml of 0.1M potassium phosphate buffer (pH 7) and resuspended in 9.5 ml of buffer containing chloramphenicol (50 $\mu g.ml^{-1}$ final concentration) in a 50 ml conical flask which was then shaken at 30°. After ten minutes, p-OH benzoate (0.4%, 0.5 ml) was added and duplicate samples (1.0 ml) were removed immediately and after a further 20, 40 or 60 min. incubation. The p-OH benzoate remaining in the samples was determined as below.

2. SUBSTRATE ASSAYS

Mandelate: An assay for this substrate was devised based on the reduction of an electron acceptor. Washed particles containing L(+)-mandelate dehydrogenase were prepared as above and used with potassium ferricyanide as acceptor. DCPIP, used in the assay of this enzyme, was unsuitable for the measurement of the substrate since its greater absorption would have necessitated using it at submaximal concentrations.

Culture samples (1.0 ml) were filtered free of cells (Oxoid membrane filter, 2 cm. diameter) and the filtrate assayed at 30° in cuvettes (1 ml capacity, 1 cm. path) containing $K_3Fe(CN)_6$ (10mM, 0.1 ml), KCN (10mM, 0.1 ml), enzyme, filtrate and phosphate buffer (0.1M, pH 7) to 1.0 ml. The mandelate sample was added last and sufficient enzyme was used to completely oxidise the mandelate within 10-15 min.

Using a water blank, the optical density decrease at 420 nm was followed to completion in a Cary 14 recording spectrophotometer. The assay was standardised with known amounts of DL-mandelate. By this method, only the L(+) isomer is measured.

This spectrophotometric method proved quite satisfactory for samples from cultures grown on mandelate or on mandelate and p-OH benzoate. However, those grown in the presence of benzoate contained a compound the identity of which is unknown (it was not benzoate itself) but which reduced ferricyanide in the presence of the particle preparation. Under these circumstances, mandelate could not be assayed by this method and ^{14}C-mandelate was used instead.

Mandelate (^{14}C-COOH) was added to the growth medium at 0.001 - 0.002 $\mu Ci.ml.^{-1}$. Since the metabolism of mandelate involves the release of the 1-carbon (as CO_2), the radioactivity remaining in the medium is a measure of the amount of mandelate in the sample. Samples (1.0 ml) were acidified with concentrated H_2SO_4 (to pH 1.0 or less) and left for 30 minutes at room temperature to expel $^{14}CO_2$. After neutralisation with NaOH (10N), samples (0.4 ml) were counted in a scintillation counter (see below). It proved unnecessary to remove the cells from culture samples as no significant assimilation of label occurred.

Benzoate: In the past this substrate has been assayed spectrophotometrically after acidification of the incubation

mixtures and ether or chloroform extraction (Fewson et al., 1970; Jamalludin, Subba Rao and Vaidyanathan, 1970). For the purposes of this study it was found to be more convenient to estimate benzoate using labelled benzoate ($^{14}COOH$), since metabolism of this acid also releases the carboxyl carbon as CO_2. The method followed closely that for mandelate.

p-OH Benzoate: The colorimetric assay described by Stevenson and Mandelstam (1965) was used. This is based on a modification of the Millon reaction for blood tyrosyl groups (Folin and Marenzi, 1929; Bernhart and Schneider, 1943). The sample, diluted if necessary to 1.0 ml, was mixed with 1.0 ml $HgSO_4$ (11.3% w/v in 4.5N H_2SO_4) and 2.5 ml H_2SO_4 (0.8N) and heated at 100°C for 8 minutes in a hard glass centrifuge tube. After rapid cooling, the tube was centrifuged at 3,000 x g for 10 minutes and the supernatant decanted. Freshly prepared sodium nitrite (0.05 ml, 2.5% w/v) was added to the supernatant and, after incubation at 35°C for 2 hr., the extinction at 500 nm was read against a water blank (Unicam S.P.500). A standard curve was constructed using p-OH benzoate in the range 0-250 µg.

3. CELL DENSITY DETERMINATION

The turbidity of cultures was estimated at 600 nm in glass cuvettes (1 cm, 3 ml) against a water blank (Unicam SP600 spectrophotometer). The extinction was related to bacterial dry weight using a calibration curve.

4. MEASUREMENT OF RADIOACTIVITY

All counting was carried out in a liquid scintillation counter (Beckman L200 or Wallac Decem-NTL^{314}). The scintillation fluid contained Butyl-PBD (0.7% w/v) and naphthalene (8.0% w/v) in toluene:2-methoxyethanol (3:2 v/v). This will accept up to 4% (v/v) of aqueous sample. It has a high alkali tolerance, but acidic samples had to be neutralised before counting.

Aqueous samples (0.4 ml) were mixed with scintillant (10 ml) in glass vials and counted. In some cases (see Text) samples were dried in the vials and, when cool, the scintillant was added and the samples counted. At least 1000 counts were registered for each sample (standard error $<3\%$).

CHEMOSTAT DESIGN AND OPERATION

The arrangement of the chemostat and ancillary equipment followed that of Dawes (1969) and is illustrated in Fig. 2.1. A medium reservoir (R) of 5l capacity was fitted with air filters (F) and set up as a Mariotte flask to maintain a constant head of medium above the pump (P). The pump, of the peristaltic type, supplied medium through silicone rubber tubing to the growth vessel (V) at a rate that could be set at any value up to 2.0 ml.min.$^{-1}$. The design of the growth vessel (volume 500 ml) is shown in greater detail in Fig. 2.2. Medium was admitted through a fine bore capillary tube (CT) set high above the surface of the culture to minimise back-contamination of the reservoir. The culture volume (100 ml) was maintained constant by a syphon tube (S) and filtered, humidified air was admitted to the culture via a sintered glass disc (I) in the base of the vessel. The air flow rate was measured by a rotameter-type flow meter (FM) and set at about 1 litre min.$^{-1}$ which was sufficient for culture aeration and instantaneous mixing of the incoming medium. The air left the vessel by an outlet port (OP) fitted with a bubble-cap (BC) to prevent contamination. The culture was maintained at 30° by partial immersion of the growth vessel and air humidifiers (H) in a water bath.

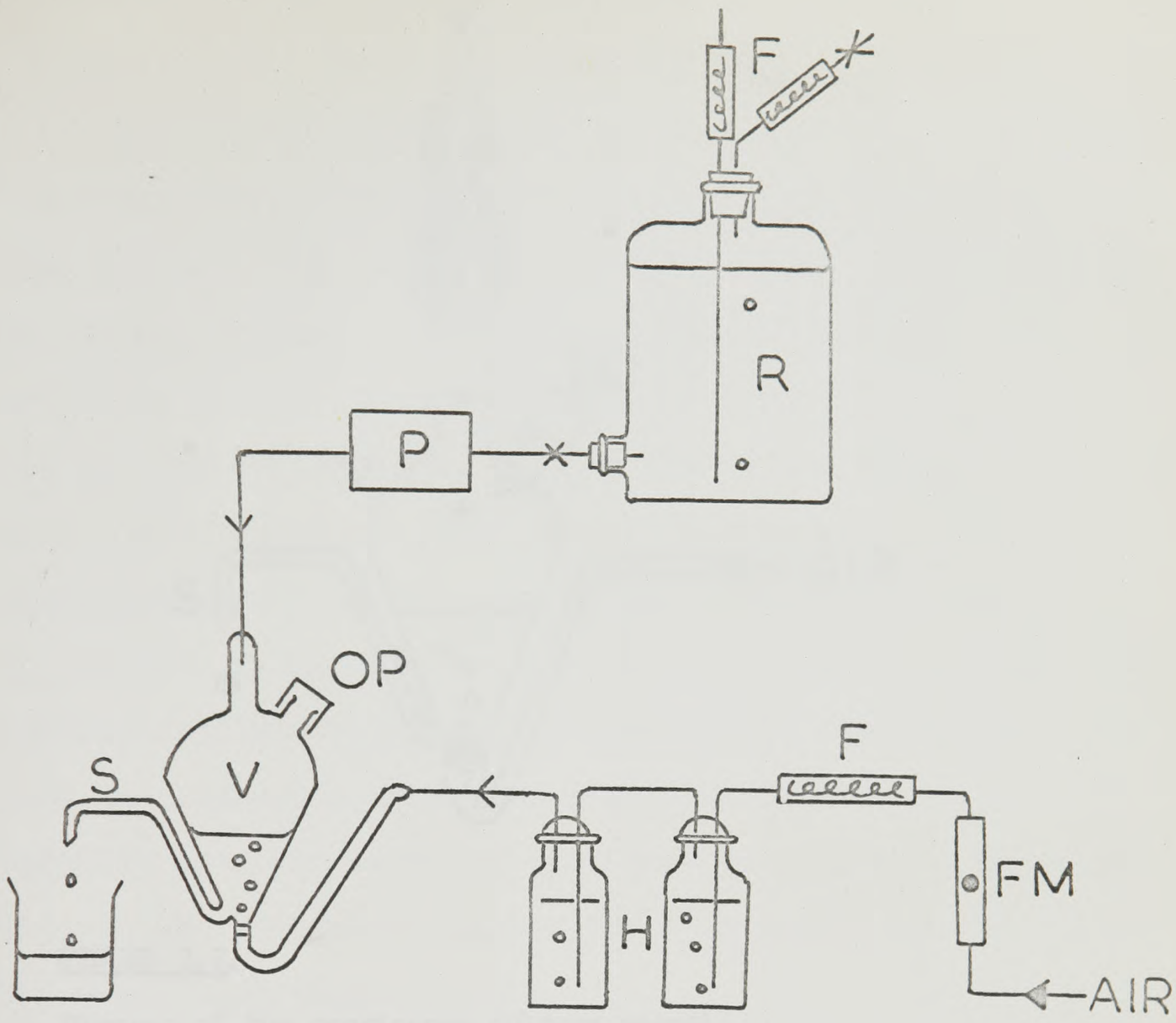


FIGURE 2.1.

Schematic diagram of the arrangement of the chemostat and ancillary equipment.

F = air filter (packed with cotton wool).

FM = air flow meter (rotameter type).

H = air humidifiers

OP = air outlet port.

P = peristaltic pump.

R = medium reservoir (5l.).

S = syphon.

V = [illegible] ml.).

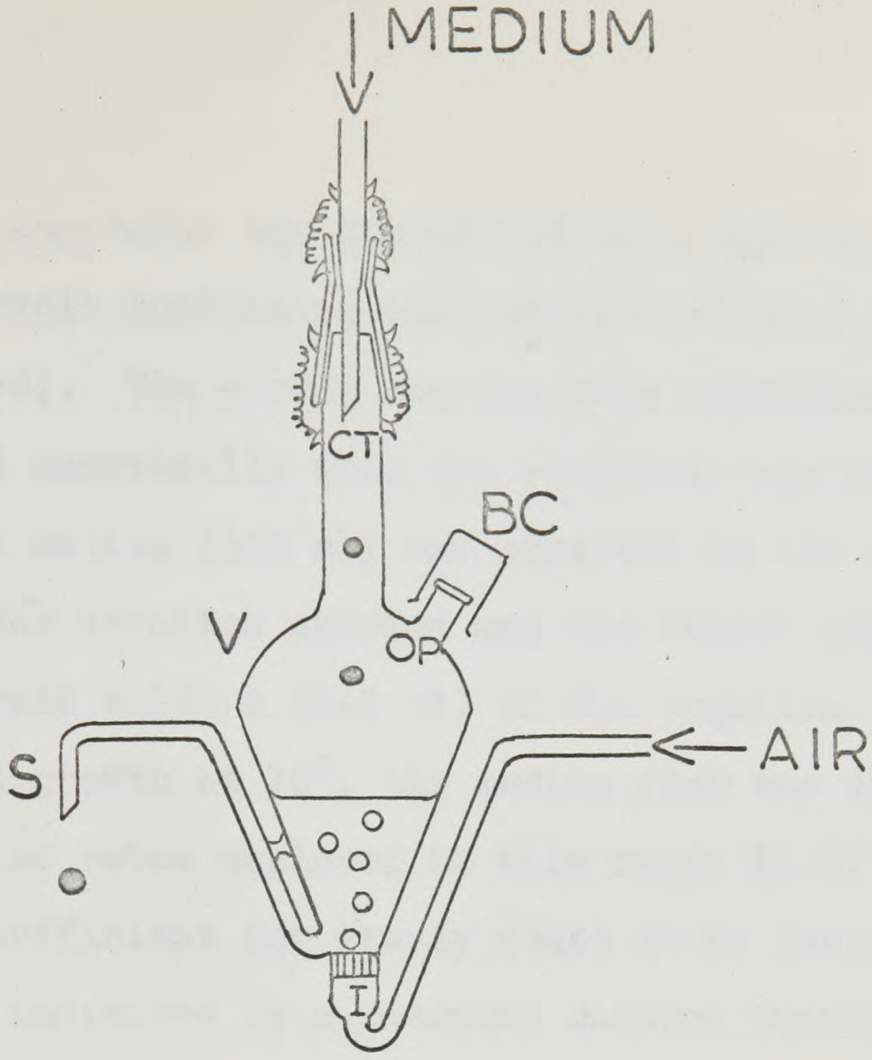


FIGURE 2.2.

Diagram of the continuous culture vessel.

BC = bubble cap.

CT = capillary tube (medium feed).

I = air inlet (sintered glass disc).

OP = air outlet port (also used for sampling the culture).

S = syphon.

V = culture vessel (500 ml.).

The apparatus was sterilised as a unit in an autoclave, the reservoir containing the modified minimal salts medium (see above). The carbon sources were sterilised separately and added aseptically when the reservoir was cool. The completed medium (100 ml) was admitted to the growth vessel, the aeration started and the vessel inoculated with a broth culture (1-2 ml) of the organism. After overnight growth at 30°, the medium flow was started. At the flow rates employed in this study 12-15 hr. was usually sufficient for steady state to be established. This was indicated by a constant culture density and a constant specific activity of inducible enzymes. Normally, however, the chemostat was run for a further 9-12 hr. before the experiment was started.

The medium flow rate was determined by measuring the time taken to collect a known volume of culture from the syphon tube. Nitrogen limitation was checked by the absence of an orange colour or precipitate after treating the effluent medium with Nessler's reagent. It was also checked at the end of the experiment by observing the rapid increase in cell density after the addition of $(NH_4)_2SO_4$ directly to the growth vessel.

In experiments where the enzymic adaptation to mixed aromatic substrates was being investigated, the inflow could be switched from one reservoir to another by a three-way tap. Throughout a run, the flow rate and culture density were periodically monitored. The culture volume was measured at the end of the experiment. The pH of the culture was never more than 0.1 different from that of the inflow medium. For the assay of enzymes and substrates in continuous cultures, samples were sometimes taken directly from the growth vessel via the outlet port but samples obtained by collecting the culture from the outflow syphon were just as satisfactory at the fast flow rates employed. To facilitate the comparison of data from continuous and batch cultures, the flow rates of the chemostats were adjusted to give growth rates as close as possible to the maximum rates observed on the substrates under investigation (Table 2.3).

TABLE 2.3

Growth rates of Pseudomonas putida on various carbon sources.

Wild-type cells were grown from a small inoculum in minimal medium containing the relevant carbon source as the sole source of carbon. The growth was measured by observing the optical density of the culture at 600 nm.

Substrate	Concentration	Growth rate (min.)
Nutrient broth	1.3% w/v	39
Succinate	10 mM	42
Glucose	0.5% w/v	70-80
Mandelate	10 mM	45-50
Benzoate	10 mM	48-55
p-OH Benzoate	10 mM	60-70

CHAPTER THREE

INDUCTION AND REPRESSION IN THE AROMATIC PATHWAYS OF PSEUDOMONAS PUTIDA

INDUCTION AND REPRESSION IN THE AROMATIC PATHWAYS OF PSEUDOMONAS PUTIDA

SECTION ONE : INTRODUCTION

This chapter describes experiments designed to provide data for the determination of induction constants for mandelate, benzoate and p-OH benzoate in Pseudomonas putida. The study of repression in the aromatic pathways of this organism, begun by Mandelstam & Jacoby (1965) and Stevenson & Mandelstam (1965), was extended and repression constants determined.

However, it was first necessary to find a carbon source that would support growth of the organism but that would not itself act as a catabolite repressor of the aromatic acid pathways. By using such a carbon source, the kinetics of induction are easier to study than they would be in uninduced cells added directly to a medium containing an aromatic compound as the only carbon source.

Glucose was shown to be a weak catabolite repressor in this system by Mandelstam & Jacoby (1965), who examined the induction of the mandelate group of enzymes in nitrogen-

starved, non-growing cells. These are the conditions that intensify catabolite repression of the lac system of E. coli (Mandelstam, 1962), so that any repressor effects of glucose in the aromatic system of P. putida should have been manifest in these conditions.

Glucose-grown cells were incubated in nitrogen-free medium containing mandelate in the presence and absence of glucose. Their ability to oxidise mandelate was measured at intervals and it was found that glucose failed to act as a catabolite repressor. It also had no repressor activity in growing cells.

In the present study additional experiments have been carried out to confirm that glucose is not a significant catabolite repressor in the aromatic system of P. putida.

1. Effect of glucose on the induction of the aromatic system in continuous culture.

Another way of looking for an effect of glucose on the induction of the aromatic enzymes is to compare the steady state specific activities of the aromatic enzymes in cultures grown in nitrogen-limited chemostats on an aromatic

inducer with and without glucose. Any repressor effect of glucose should reveal itself as a decrease in the specific activity.

Wild-type cells were therefore grown in such a chemostat (see Methods) supplied with an aromatic inducer (10 mM) and glucose (0.5% w/v). The dilution rate (0.45 vol.hr.$^{-1}$) was set so that the cells were growing at almost the maximum growth rate observed with glucose as sole carbon source (Table 2.3). The steady state specific activity of the appropriate enzyme was determined, the glucose was then removed from the inflowing medium and the enzyme level re-determined, when the new steady state had been established. The results of a number of such experiments are set out in Table 3.1.

Glucose, under these conditions, exerted very little repressor effect on the induction of benzoylformate decarboxylase by mandelate or of benzoate oxidase by benzoate. It appeared, however, to have a more significant effect on the induction of p-OH benzoate hydroxylase by p-OH benzoate.

TABLE 3.1

Effect of glucose on the induction of the enzymes of the aromatic pathway in continuous culture.

Wild-type cells were grown in a nitrogen-limited chemostat on glucose (0.5 % w/v) and an aromatic inducer (10 mM). The specific activity of the inducible enzyme was measured at steady state. The glucose was then removed and the specific activity redetermined when the new steady state had been reached. The dilution rate was set at 0.45 vol. hr.$^{-1}$ and the cell density was constant in the range 350-400 µg. dry wt. ml.$^{-1}$

ENZYME	SPECIFIC ACTIVITY (units mg. dry wt. ml.$^{-1}$)		REPRESSION %
	+ glucose	- glucose	
Benzoylformate decarboxylase	965	1040	7.5
Benzoate oxidase	355	400	11
p-OH Benzoate hydroxylase	49.5	82.5	40

2. Induction of the aromatic pathways in L-asparagine medium.

Hegeman (1966) has shown that L-asparagine is a non-repressing carbon source for the induction of the mandelate enzymes in P. putida and that the specific activity of benzoylformate decarboxylase induced in the presence of this carbon source was the same as that in a culture grown on mandelate alone.

The finding that glucose is a relatively non-repressing carbon source for the enzymes of the aromatic system in P. putida would be strengthened if it could be shown that glucose supported rates of induction and steady state enzyme levels similar to or even higher than those of L-asparagine.

Therefore the rates of induction of benzoylformate decarboxylase by mandelate, of benzoate oxidase by benzoate, and of p-OH benzoate hydroxylase by p-OH benzoate were compared in wild-type cells growing in minimal medium supplemented with glucose or asparagine. Each inducer was added at a final concentration of 10.0 mM to exponentially growing cells (100 µg.dry wt.ml.$^{-1}$) and samples were taken periodically over the next 1-2 generations for the estimation

of the induced enzyme (Methods). The differential rates of enzyme synthesis (units mg.dry wt.$^{-1}$) were calculated and compared for each carbon source.

Within the limits of accuracy associated with the determination of rates of induction in different cultures (see Discussion), the data presented in Table 3.2 suggest that the repressor effects of glucose and L-asparagine are not significantly different.

Since asparagine serves as both a carbon and a nitrogen source, a nitrogen-limited chemostat could not be used to determine the specific activities of the aromatic enzymes at steady state in asparagine medium. Hence in order to compare the steady state specific activities of these enzymes in the presence of glucose or asparagine, wild-type cells were grown in batch culture from a low density (<5 µg.dry wt.ml.$^{-1}$) through at least six generations in minimal medium containing an aromatic inducer (10 mM) and either glucose or asparagine. The flasks were then sampled and the specific activity of the induced enzyme determined. This length of time should have been sufficient for the cells to have reached a specific activity approaching the final steady state level.

TABLE 3.2

Comparison of the rates of induction and steady state specific activities of enzymes of the aromatic pathways in cells grown on glucose and on L-asparagine.

Wild-type cells (at a density of 100 μg. dry wt. $ml.^{-1}$), growing exponentially in minimal medium containing glucose (0.5%) or L-asparagine (0.4%), were induced with an aromatic inducer (10 mM). The rate of synthesis of the inducible enzyme was determined over the next 2 generations.

In another experiment, the specific activities of the inducible enzymes were determined in cultures grown from a small inoculum (< 5 μg. dry wt. $ml.^{-1}$) to a high density (500 μg. $ml.^{-1}$) in minimal medium containing glucose or asparagine and an aromatic inducer.

INDUCER	RATE OF INDUCTION (units mg. dry $wt.^{-1}$)		STEADY STATE ACTIVITY (units mg. dry $wt.^{-1}$)	
	Asparagine	Glucose	Asparagine	Glucose
Mandelate	960	1130	1035	1270
	1010	1270	1120	1130
Benzoate	575	530	590	600
	497	395	605	620
p-OH Benzoate	100	82	87.5	82
	84	67	92.5	97

The results show that the rates of induction were similar for all three inducers in the two growth media (Table 3.2). It thus seems reasonable to hope that induction constants measured in glucose medium would be very similar to those in the presence of only the aromatic inducer and that predictions made on this basis would not be grossly affected by catabolite repression.

SECTION TWO: MEASUREMENT OF INDUCTION CONSTANTS

1. K_{ind} for the mandelate group of enzymes

Without implying that the interaction of aporepressor and inducer is exactly analogous to the interaction of an enzyme with its substrate, a method used in the measurement of the Michaelis constant of an enzyme-substrate system was used where possible to determine the induction constants of the aromatic system (Lineweaver and Burk, 1934). This requires a series of submaximum, linear, initial rates of enzyme induction over a reasonable inducer concentration range. It is of course essential that the inducer concentration be maintained constant over the period of the measurement and this can be best achieved by using gratuitous inducers, i.e., those that are not metabolised (Monod and Cohn, 1952).

The coordinacy of the synthesis of the mandelate enzymes (Stanier et al., 1965; Hegeman, 1966) can be exploited to permit DL-mandelate to function as a gratuitous inducer. Mutants blocked at L(+)-mandelate dehydrogenase (md⁻) are unable to metabolise L(+)-mandelate, though the other four enzymes of the group are induced. Of these, benzoylformate decarboxylase was selected as an easily measurable 'indicator enzyme' for the mandelate system.

Portions of a culture of mutant Y65 (md⁻), growing in glucose-minimal medium, were induced with DL-mandelate in the concentration range 0.25-2.0 mM. At suitable intervals after the addition of the inducer, samples were taken for the assay of benzoylformate decarboxylase. Growth was followed throughout. The results of a typical experiment are presented in Figure 3.1. The differential plot gave linear rates of induction that were dependent on the inducer concentration (below 1.0 mM), but which could not be analysed in the manner suggested by Lineweaver and Burk (1934). This method involves the use of a double reciprocal plot of induction rate and inducer concentration. In these experiments the plot was non-linear with a positive rather

FIGURE 3-1.

Induction of benzoylformate decarboxylase by mandelate : effect of inducer concentration.

Cells of mutant Y65 (md$^-$) growing exponentially in glucose-minimal medium were induced with DL-mandelate and samples of the cultures were taken at intervals for the assay of benzoylformate decarboxylase (Methods). The inducer was added at the points indicated by the arrows and the inducer concentrations were :

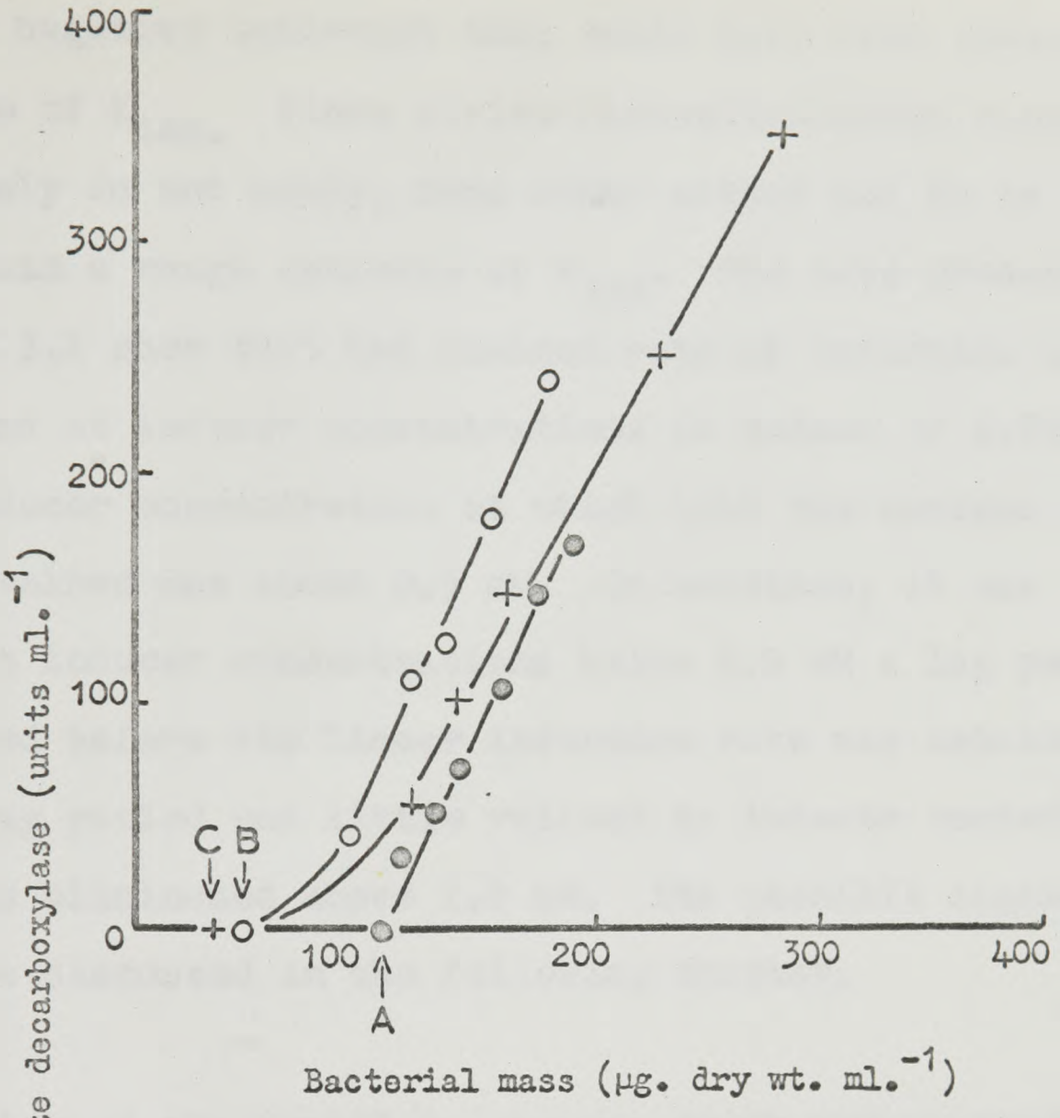
Flask A = 2.0 mM (●).

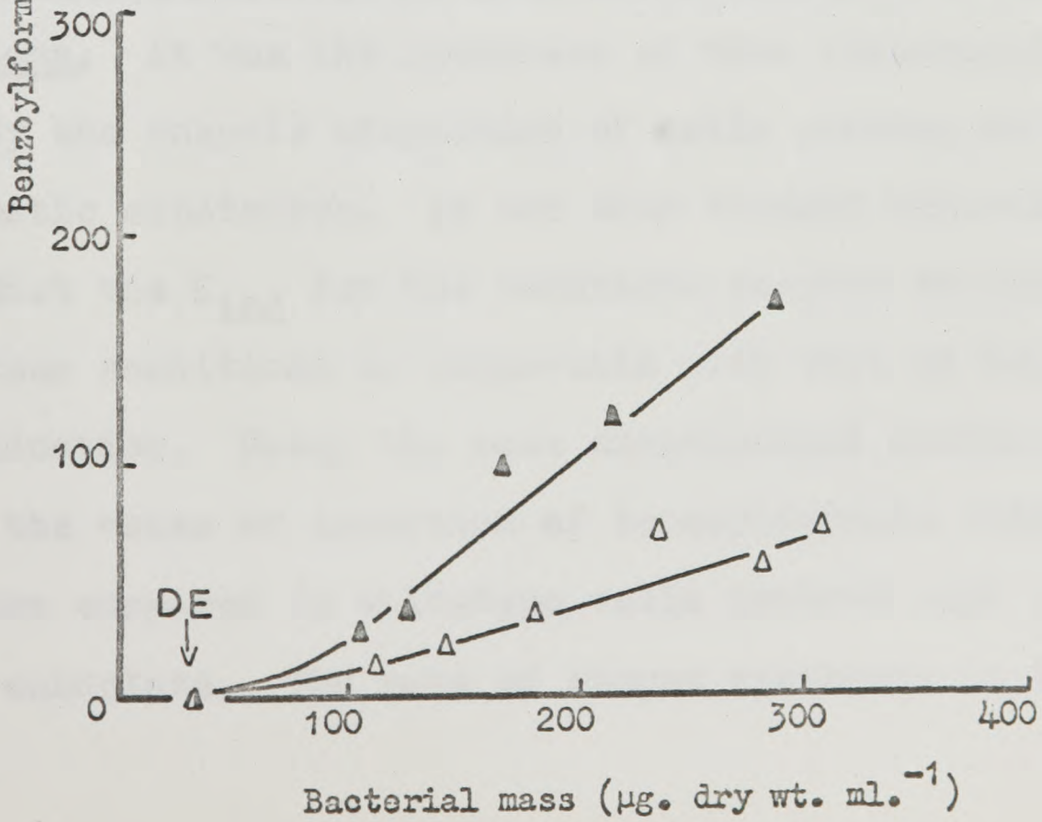
Flask B = 1.0 mM (○).

Flask C = 0.75 mM (+).

Flask D = 0.50 mM (▲).

Flask E = 0.25 mM (△).


400
300
200
100
0
C B
A
100
200
300
400
Benzoylformate decarboxylase (units ml.$^{-1}$)
Bacterial mass (µg. dry wt. ml.$^{-1}$)


300
200
100
0
DE
100
200
300
400
Bacterial mass (µg. dry wt. ml.$^{-1}$)

than a negative intercept that could have been taken as a measure of K_{ind}. Since strict Michaelis-Menten kinetics obviously do not apply, some other method had to be used to obtain a rough estimate of K_{ind}. The data presented in Figure 3.1 show that the maximum rate of induction was obtained at inducer concentrations in excess of 0.75 mM. The inducer concentration at which half the maximum rate was obtained was about 0.5 mM. In addition, it was found that at inducer concentrations below 2.0 mM a lag period appeared before the linear induction rate was established. This lag period was little related to inducer concentration, but was eliminated above 2.0 mM. Its possible significance will be discussed in the following chapter.

Induction of the mandelate enzymes under non-gratuitous conditions. It was the intention of this investigation to study the enzymic adaptation of cells growing on mixtures of aromatic substrates. It was thus thought desirable to check that the K_{ind} for the mandelate enzymes obtained under gratuitous conditions is comparable with that of non-gratuitous induction. Using the same experimental method as above, the rates of induction of benzoylformate decarboxylase were compared in wild-type cells induced with 5 mM and 0.5 mM mandelate. The rate of enzyme synthesis at the

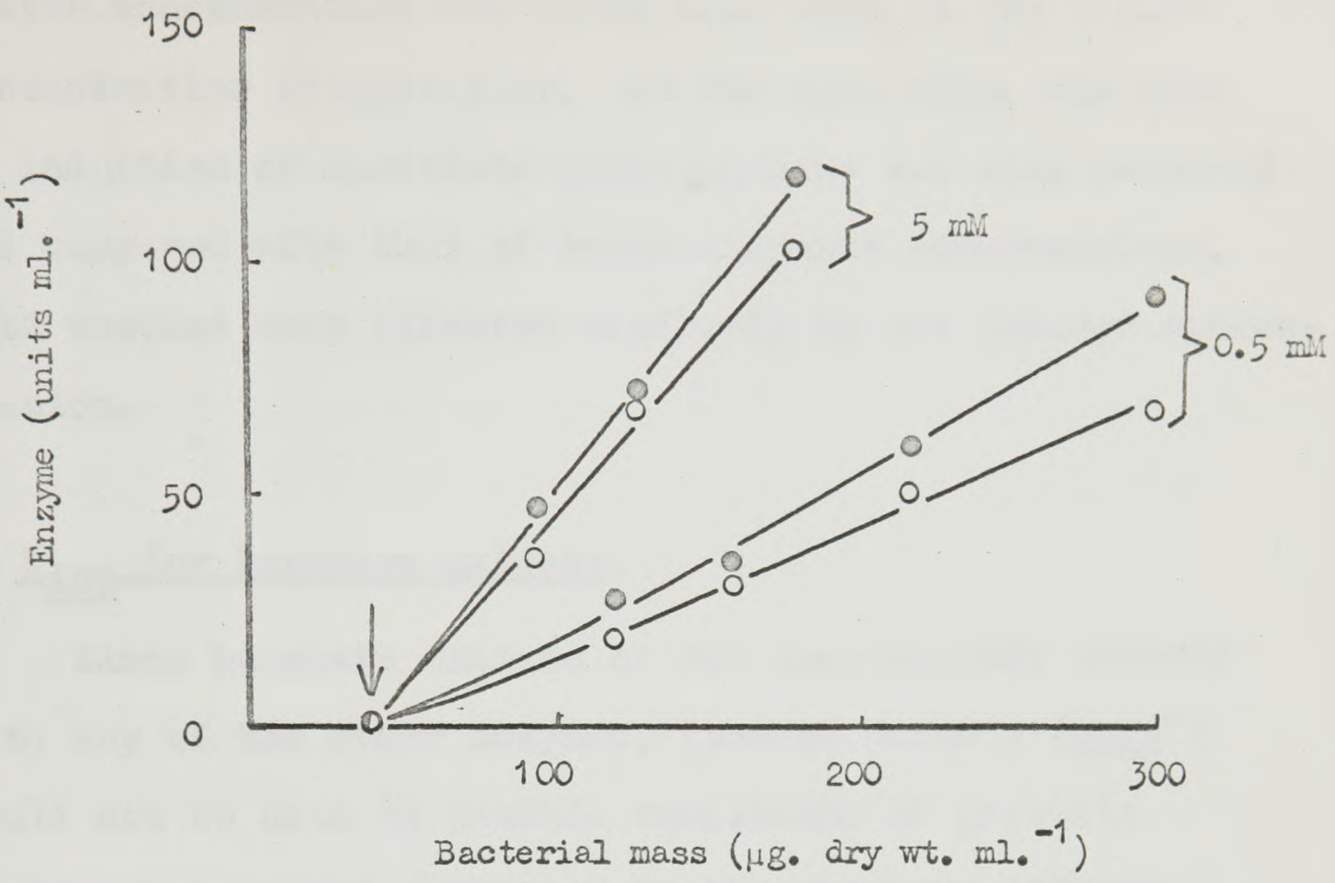


FIGURE 3.2

Induction of benzoylformate decarboxylase and mandelate dehydrogenase by mandelate in wild-type cells.

Wild-type cells, growing exponentially in glucose-minimal medium, were induced with 5.0 or 0.5 mM mandelate at the point indicated by the arrow. Samples were taken at intervals and assayed for benzoylformate decarboxylase (●) and mandelate dehydrogenase (○) as in Methods.

latter concentration was about half that at the higher concentration (Figure 3.2). At the same time, the rate of induction of mandelate dehydrogenase was also measured and compared with that of benzoylformate decarboxylase. Both enzymes were affected similarly by the inducer concentration.

2. K_{ind} for benzoate oxidase.

Since benzoate oxidase is not coordinately induced with any of the other enzymes, blocked mutants (*benz*$^-$) could not be used to provide conditions of gratuity analogous to those described in the previous section. Wild-type cells had to be used. Furthermore, non-metabolisable inducer analogues have not been described for this enzyme and their use could, in any case, be criticised on the grounds that their affinities for the induction system may be greatly different from that of the natural inducer, benzoate.

Since benzoate is a very efficient inducer, low concentrations had to be used to achieve submaximum rates of induction suitable for the calculation of K_{ind}. These low concentrations could not be kept constant for long periods,

so it was necessary to determine the initial rates of induction before significant metabolism of the inducer had occurred. Samples taken during this initial period had very low specific activities, and these were difficult to assay by the whole cell oxidation method. This difficulty was overcome by using the assay method based on the decarboxylation of benzoate-^{14}COOH, which is not troubled by the endogenous respiration of the cells.

The experimental procedure used was similar to that described above. Wild-type cells, growing exponentially in glucose-minimal medium, were induced at different benzoate concentrations and samples were taken for the assay of benzoate oxidase by the decarboxylation method. There was a lag period (20-40 min.) between the addition of the inducer and the start of enzyme synthesis. The initial rates of enzyme synthesis were linear over the inducer concentration range 0.125 - 0.5 mM (Figure 3.3) enabling the K_{ind} to be calculated by the method of Lineweaver and Burk (1934), (Figure 3.4). Its value was 0.5 mM. Maximum rates of induction, measured by the whole cell oxidation method, were obtained with benzoate concentrations in the range 0.5-1.0 mM.

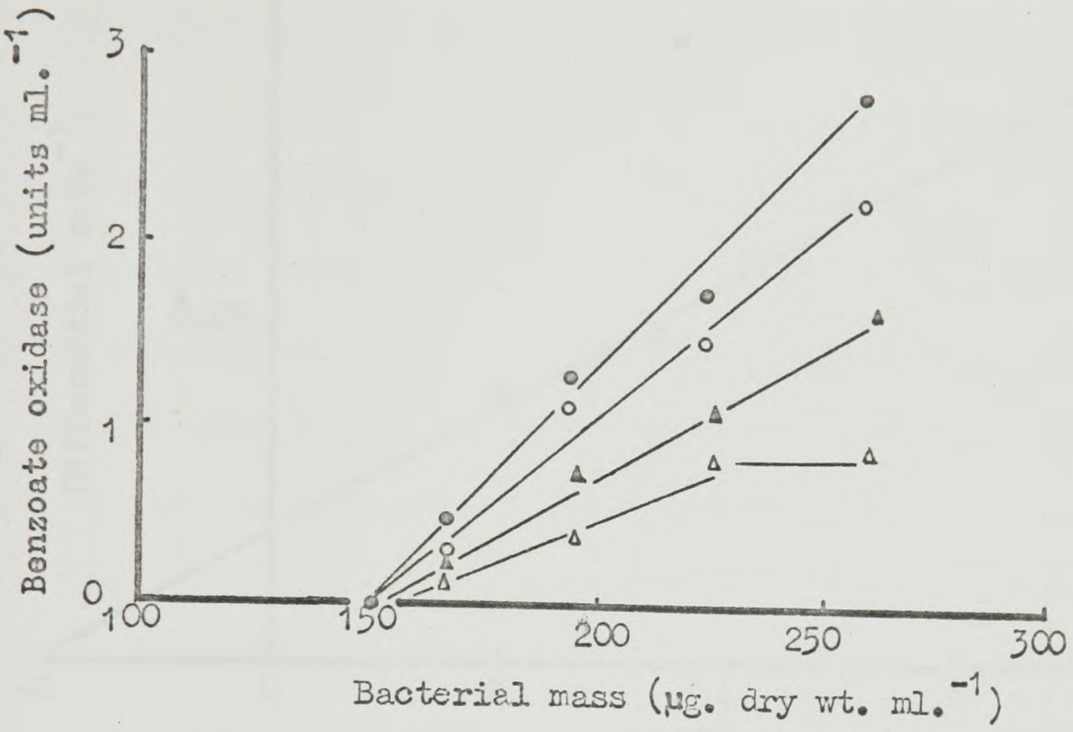


FIGURE 3.3

Induction of benzoate oxidase by benzoate : effect of inducer concentration.

Wild-type cells, growing exponentially in glucose-minimal medium, were induced with benzoate at 0.5 (●), 0.3 (○), 0.2 (▲) and 0.125 mM (△). Samples were removed at intervals and assayed for benzoate oxidase (decarboxylation of $C_6H_5{}^{14}COOH$). The inducer was added when the culture density was 100 µg. dry wt. ml.$^{-1}$

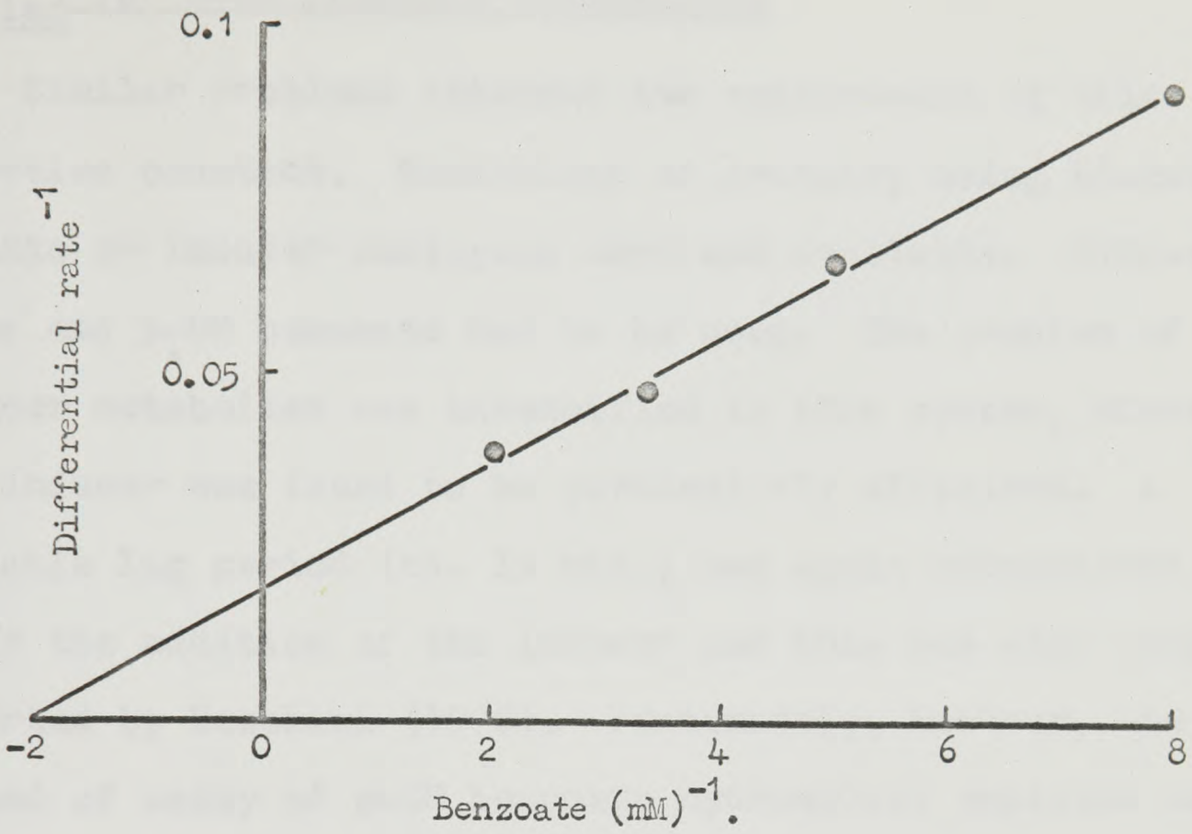


FIGURE 3.4

Induction constant for benzoate oxidase.

Differential rates of synthesis of benzoate oxidase (units mg. dry wt.$^{-1}$) were calculated from the data of Fig. 3.3 and plotted by the method of Lineweaver and Burk (1934). The value of the induction constant (K_{ind}) was 0.5 mM.

3. K_{ind} for p-OH benzoate hydroxylase

Similar problems attended the measurement of this induction constant. Conditions of gratuity using blocked mutants or inducer analogues were not available. Wild-type cells and p-OH benzoate had to be used. The problem of inducer metabolism was intensified in this system, where the inducer was found to be particularly efficient. A variable lag period (ca. 15 min.) was again encountered after the addition of the inducer and this has also been reported by Hosokawa (1970). Fortunately, however, the method of assay of p-OH benzoate hydroxylase employed was sufficiently sensitive thatsamples with very low specific activities could be assayed with little difficulty.

The experimental procedure used for determining the other two induction constants was again followed. Exponentially growing wild-type cells in glucose-minimal medium were induced over a range of p-OH benzoate concentrations, and the rate of synthesis of the hydroxylase determined. The extremely low inducer concentrations necessary to observe submaximum induction rates and the problems associated with inducer metabolism and initial rate determinations prevented accurate data being obtained below 0.01 mM. At 0.02 mM, induction appeared to be still at a maximum (Figure 3.5). While a value for K_{ind} could not be obtained from the data,

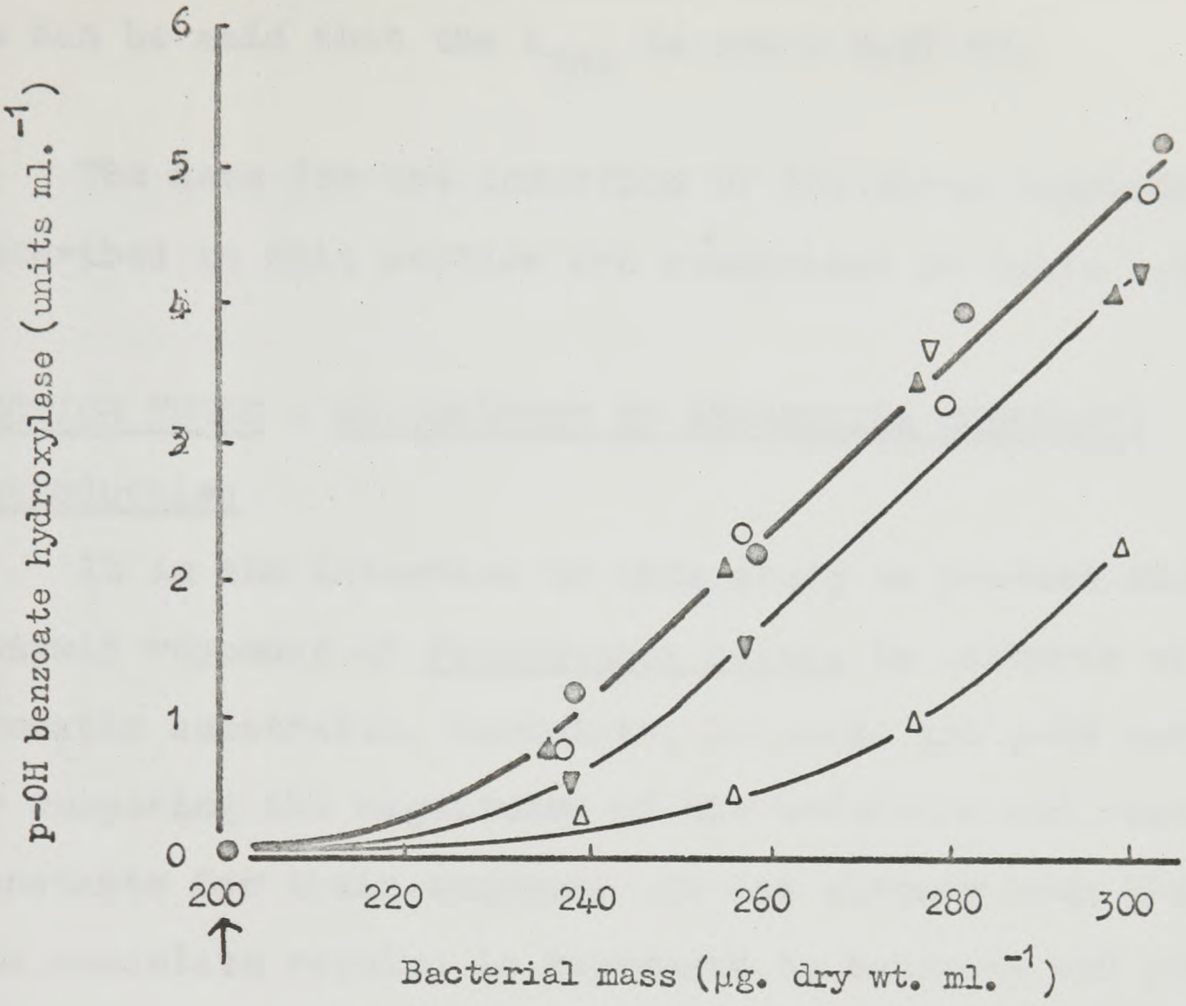


FIGURE 3.5

Induction of p-OH benzoate oxidase : effect of p-OH benzoate concentration.

Wild-type cells, growing exponentially in glucose-minimal medium, were induced with p-OH benzoate at 0.2 (●), 0.1 (○), 0.05 (▲), 0.02 (▽) and 0.01 mM (△). The inducer was added at the point indicated by the arrow and samples were then taken for the assay of p-OH benzoate hydroxylase (Methods).

it can be said that the K_{ind} is below 0.01 mM.

The data for the induction of the three regulons described in this section are summarised in Table 3.3.

SECTION THREE : MEASUREMENT OF REPRESSION CONSTANTS

Introduction

It is the intention of this study to predict the enzymic response of Pseudomonas putida to mixtures of the aromatic substrates, mandelate, benzoate and p-OH benzoate, by comparing the magnitudes of the induction and repression constants for their enzymes. It has already been shown that the mandelate regulon is repressed by benzoate and p-OH benzoate (Mandelstam and Jacoby, 1965; Stevenson and Mandelstam, 1965). This section describes experiments designed to measure constants for the repressor activities that each of these substrates may have on the induction of the regulons described above.

The mechanisms of these repressor effects, like those of the inducers, are unknown and, for the purposes of determining the K_{rep}, do not concern us at present. However, an observation made by Stevenson and Mandelstam (1965) may be of importance in this study. They found that 'escape' could

TABLE 3.3

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

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

Inducer	Concentration for maximum induction	K_{ind}
Mandelate	1.0-2.0 mM	$\bar{c}$. 0.5 mM
Benzoate	0.5-1.0 mM	$\bar{c}$. 0.5 mM
p-OH Benzoate	$\bar{c}$. 0.02 mM	$<$0.01 mM

occur from the repression normally exerted by succinate on the mandelate regulon and that there was little repression of this regulon by succinate in succinate-adapted cells. When this type of transient repression occurs, it means that the steady state enzymic response of the organism to substrate mixtures may differ from that observed in short-term experiments. For this reason, the actions of the repressors were examined not only with a view to determining the K_{rep} values but, wherever possible, to check that 'escape' is not involved.

Since intermediates of the aromatic pathways that are produced by the metabolism of mandelate, benzoate and p-OH benzoate can also act as repressors of the regulons induced by these substrates (Fig. 1.4), it was also considered necessary to investigate the repression caused by some of these intermediates in case they are important in determining enzymic adaptation to the main substrates. Succinate and acetate were found to be similar in the type and degree of repression that they caused (Mandelstam and Jacoby, 1965; Stevenson and Mandelstam, 1965), so only succinate was investigated in this study. Catechol was also used, but not protocatechuate since the original work of Stevenson and Mandelstam (1965) showed its repressor effects to be relatively mild.

In all the cases of repression studied, a comparison was initially made of the kinetics of induction in the presence and absence of the repressor. As far as possible, conditions of gratuity were employed for both inducer and repressor by using the appropriately blocked mutant. Under these conditions not only is the repressor concentration maintained but its metabolism to an intermediate with a different repressor effect is prevented.

Cells growing exponentially in glucose-minimal medium were induced with a concentration of inducer sufficient for the maximum induction rate (see Table 3.3). At the same time, repressor at a suitable concentration was added to part of the culture and samples were taken for the assay of the inducible enzyme. If the rate or time-course of the induction was altered in the presence of the repressor, the investigation was extended. If no repressor action was observed, the experiment was repeated using a higher ratio of repressor to inducer, but in no case were repressors used at concentrations that measurably affected the growth rate of the organism.

REPRESSION CAUSED BY MANDELATE, BENZOATE AND p-OH BENZOATE

1. Repression of benzoylformate decarboxylase by p-OH benzoate

Conditions of gratuity for both mandelate and p-OH benzoate could have been obtained by using a double mutant (p-OH benz$^-$, md$^-$) but this was not available. The single mutant A2 (p-OH benz$^-$) had therefore to be used. Since the repression observed was severe, the low p-OH benzoate concentrations necessary for the measurement of K_{rep} were safeguarded by the mutation. At the same time, the high mandelate concentrations used were not significantly decreased during the course of the experiments.

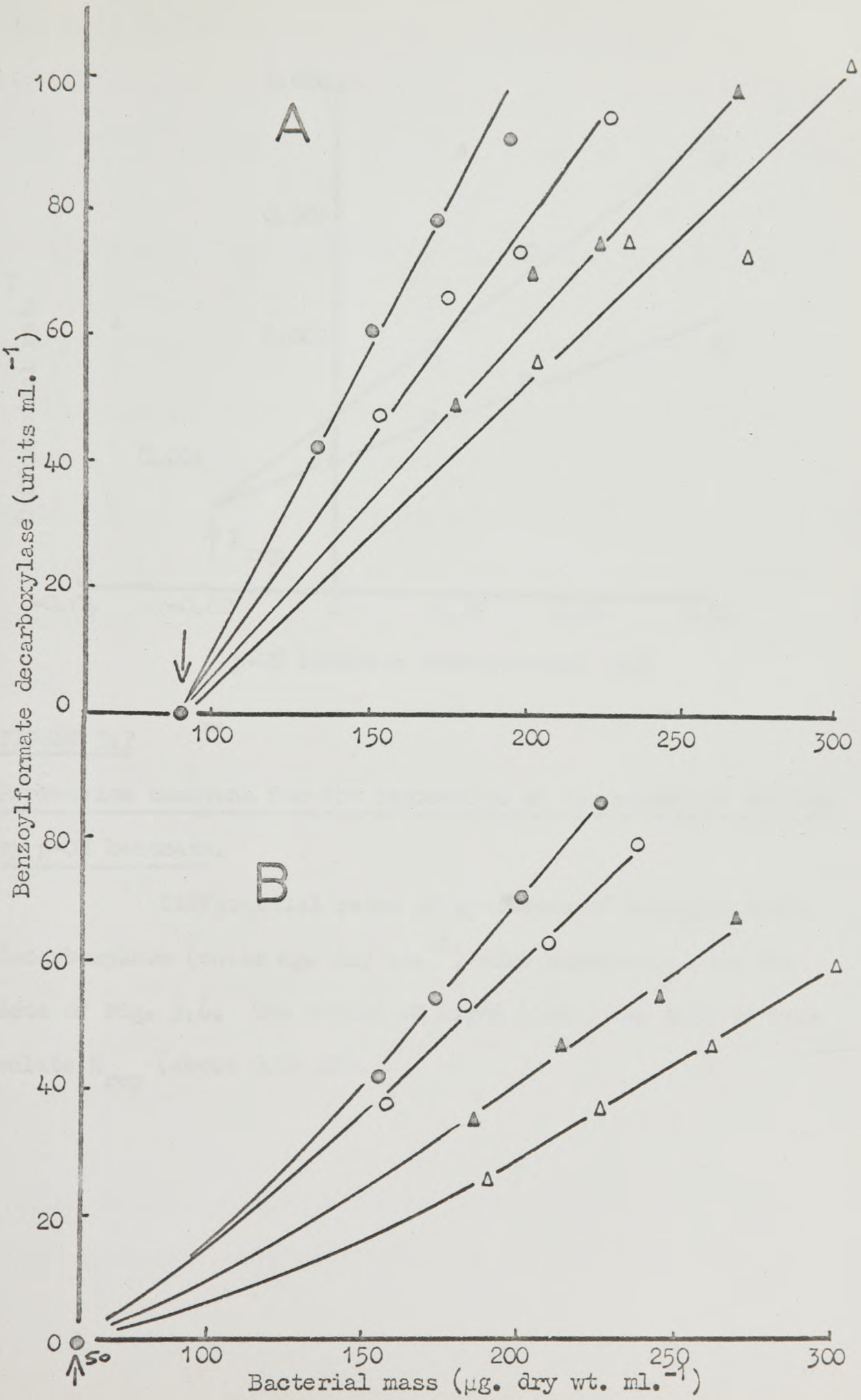
The results of a typical experiment are shown in Figure 3.6. The presence of quite low concentrations of p-OH benzoate (0.015-0.060 mM) was sufficient to cause a marked decrease in the rate of synthesis of benzoylformate decarboxylase. When the inducer was used at two concentrations, (1.5 and 5.0 mM), a Dixon analysis of the results was possible (Dixon, 1953). This gave a value of 0.02 mM for the repression constant, (Figure 3.7). The results also show that the repression by p-OH benzoate was reversed at increased inducer concentrations.

No delay in the onset of enzyme synthesis was caused by p-OH benzoate. [A delay is observed with 1.5 mM mandelate

FIGURE 3.6

Effect of p-OH benzoate on the induction of the mandelate regulon.

Cells of mutant A 2 (p-OH benz$^-$), growing exponentially in glucose-minimal medium, were induced ($\downarrow$) with 5.0 mM (A) or 1.5 mM (B) mandelate. p-OH Benzoate was added at the same time at 0 (●), 0.015 (○), 0.03 (△) or 0.06 mM (△). Samples were taken at intervals thereafter and assayed for benzoylformate decarboxylase.


A
B
100
80
60
40
20
0
100
150
200
250
300
Benzoylformate decarboxylase (units ml.$^{-1}$)
Bacterial mass (µg. dry wt. ml.$^{-1}$)
50

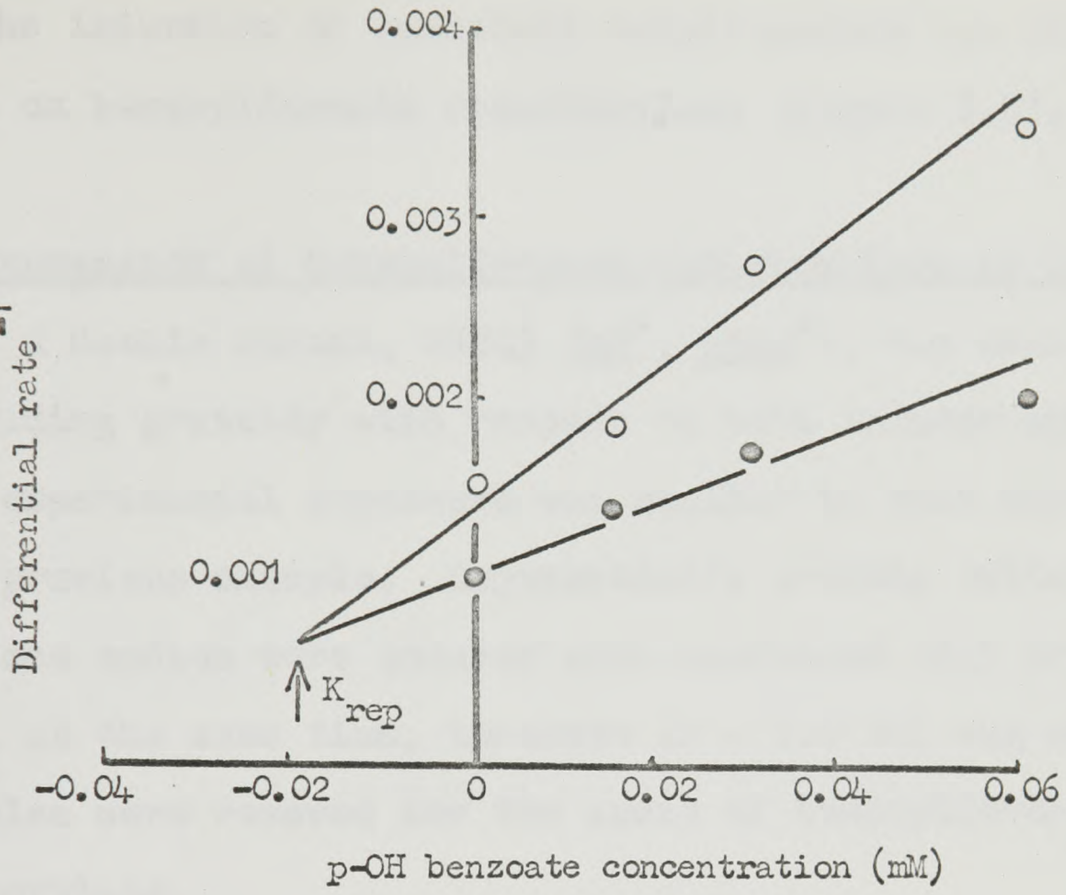


FIGURE 3.7

Repression constant for the repression of the mandelate regulon by p-OH benzoate.

Differential rates of synthesis of benzoylformate decarboxylase (units mg. dry wt.$^{-1}$) were calculated from the data of Fig. 3.6. The method of Dixon (1953) was used to calculate K_{rep} (about 0.02 mM).

in any case]. As expected, the effect of p-OH benzoate on the induction of mandelate dehydrogenase was similar to that on benzoylformate decarboxylase (Figure 3.8).

2. Repression of benzoylformate decarboxylase by benzoate.

A double mutant, D5703 (md⁻, benz⁻), was available, providing gratuity with respect to both inducer and repressor. The experimental procedure was similar to that adopted in the previous example. Exponentially growing cultures in glucose medium were induced with mandelate (2.5 or 5.0 mM) and, at the same time, benzoate (0 - 1.0 mM) was added. Samples were removed for the assay of benzoylformate decarboxylase.

The results showed that benzoate prevented the induction of the mandelate enzymes for a considerable time (up to 2 generations), after which induction started at a rate indistinguishable from that of the control (Figure 3.9). [In this experiment, the different cultures were started at different cell densities so that 'escape' occurred at about the same stage in exponential growth].

This effect of benzoate on the induction by mandelate will be examined in greater detail in the next chapter.

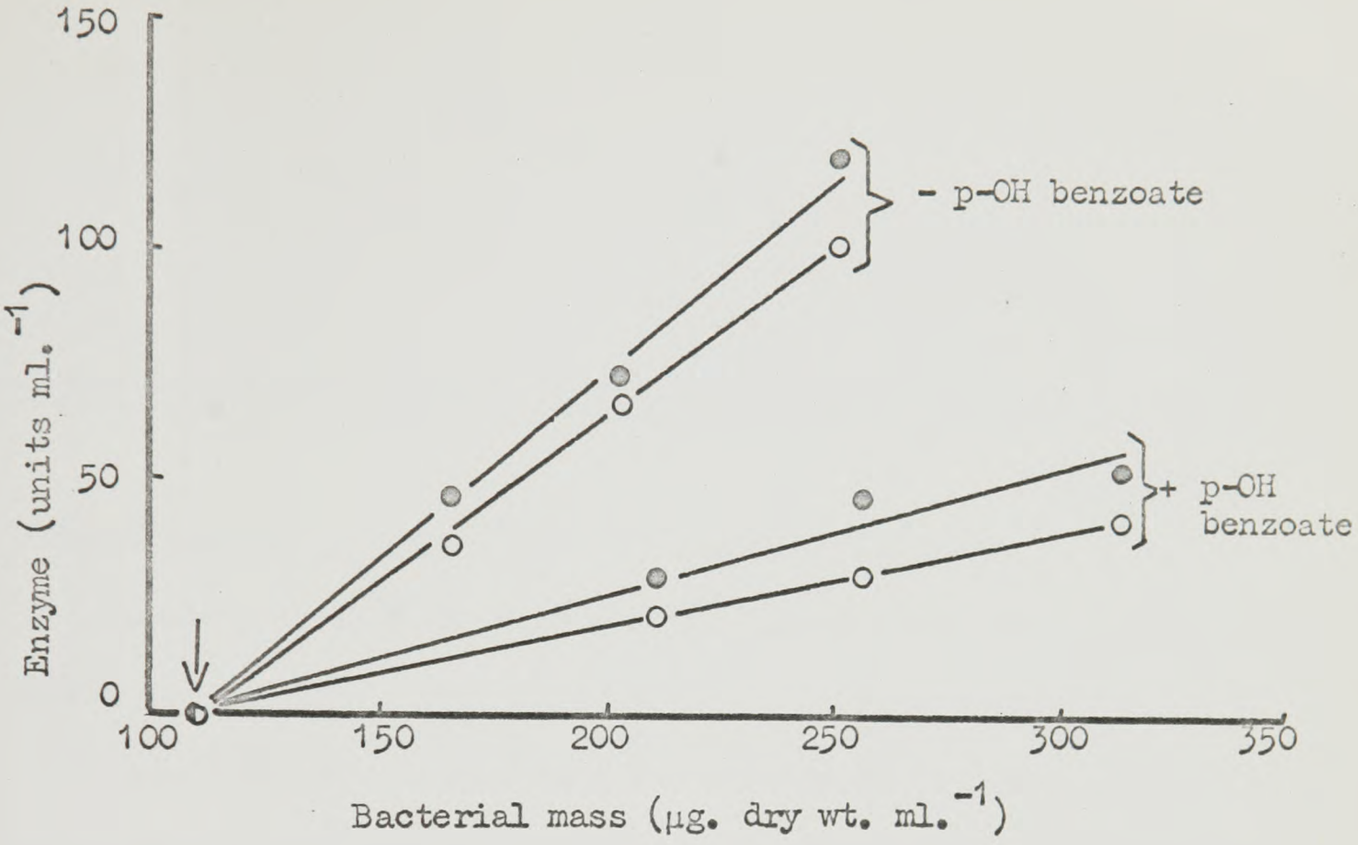


FIGURE 3.8

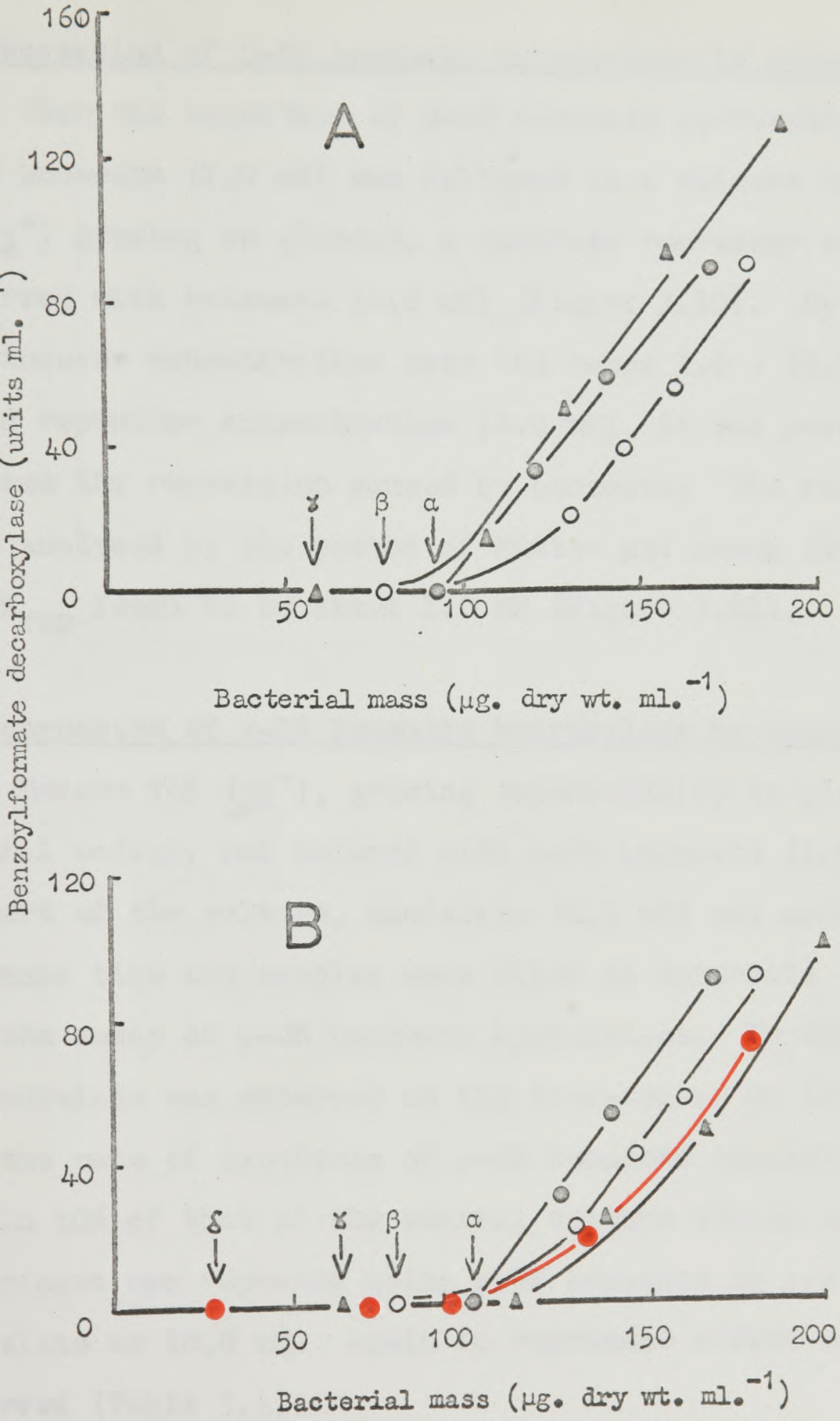
Effect of p-OH benzoate on the induction of benzoylformate decarboxylase and mandelate dehydrogenase.

Mutant A 2 (p-OH benz$^-$) was grown on glucose and induced (↓) with mandelate (5 mM). One portion of the culture also received p-OH benzoate (0.2 mM). Samples were taken and assayed for benzoylformate decarboxylase (●) and mandelate dehydrogenase (O).

FIGURE 3.9

Effect of benzoate on the induction of benzoylformate decarboxylase by mandelate.

Cells of mutant D 5701 (md$^-$, benz$^-$), growing exponentially in glucose-minimal medium, were induced with 5.0 mM (A) or 2.5 mM mandelate (B). At the same time, benzoate was added at a final concentration of α = 0 (◎), β = 0.05 (○), γ = 0.2 (△) and δ = 1.0 mM (●). The inducer and repressor were added at the time indicated by the arrows and samples were taken at intervals and assayed for benzoylformate decarboxylase.


A
B
160
120
80
40
0
50
100
150
200
γ
β
α
δ
Benzoylformate decarboxylase (units ml.$^{-1}$)
Bacterial mass (µg. dry wt. ml.$^{-1}$)
Bacterial mass (µg. dry wt. ml.$^{-1}$)

3. Repression of p-OH benzoate hydroxylase by benzoate

When the induction of p-OH benzoate hydroxylase by p-OH benzoate (1.0 mM) was followed in a culture of C167 (*benz*$^-$) growing on glucose, a moderate repressor effect was observed with benzoate (2.0 mM) (Figure 3.10). By increasing the inducer concentration over the range 1.0 - 10.0 mM at a fixed repressor concentration (2.0 mM), it was possible to reverse the repression caused by benzoate. The results were analysed by the method of Hunter and Downs (1945) and the K_{rep} found to be about 1.0 mM (Figure 3.11).

4. Repression of p-OH benzoate hydroxylase by mandelate

Mutant Y65 (*md*$^-$), growing exponentially in glucose-minimal medium, was induced with p-OH benzoate (1.0 mM). To part of the culture, mandelate (2.0 mM) was added at the same time and samples were taken at intervals for the assay of p-OH benzoate hydroxylase. No effect of mandelate was observed on the time-course of induction and the rate of synthesis of p-OH benzoate oxidase was within 10% of that of the control culture (Table 3.4). The experiment was repeated using p-OH benzoate at 1.0 mM and mandelate at 10.0 mM. Again no repressor effect was observed (Table 3.4).

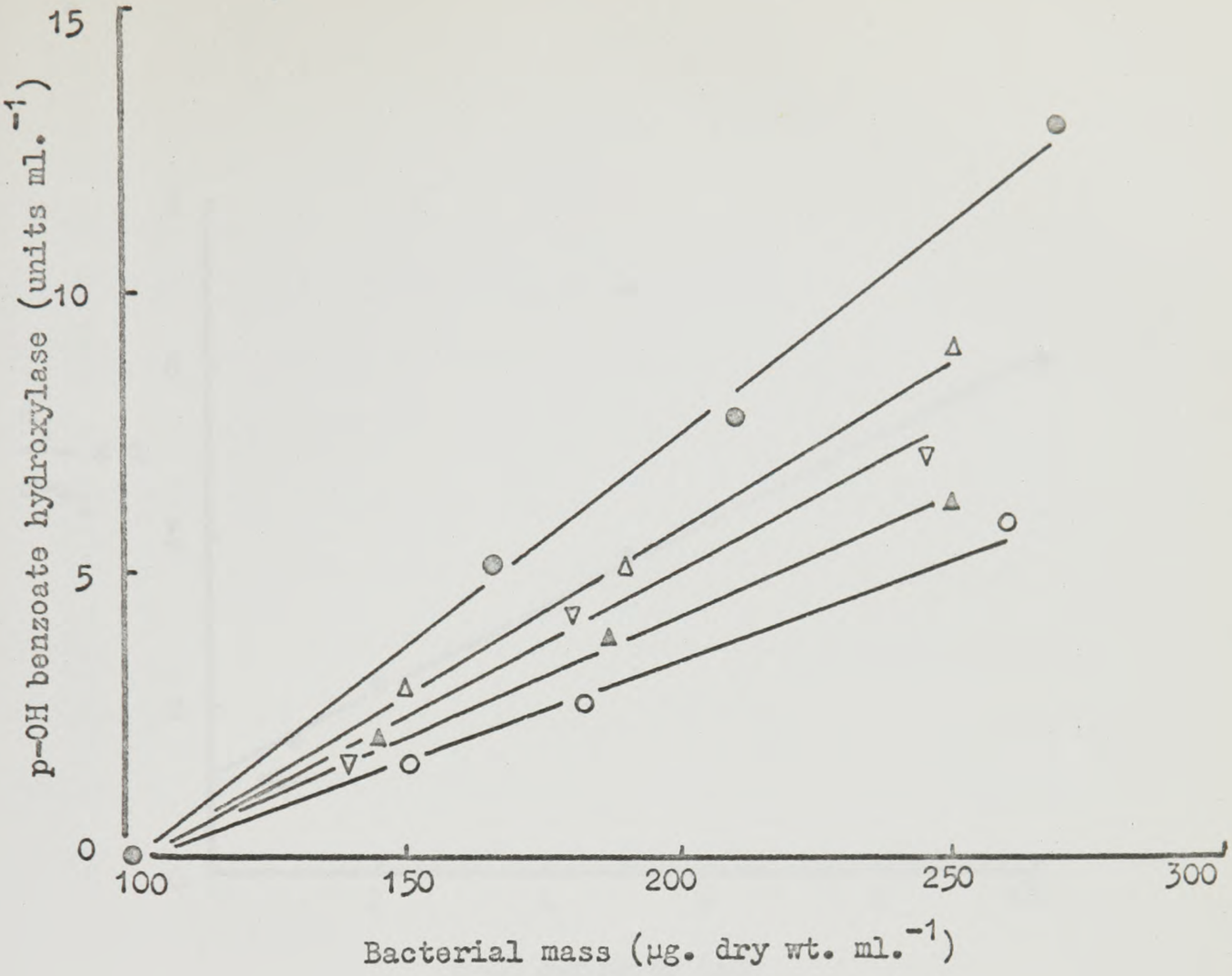


FIGURE 3.10

Effect of benzoate on the induction of p-OH benzoate hydroxylase.

Cells of mutant C 167 (benz$^-$), growing exponentially in glucose-minimal medium, were induced with 1.0 (O), 2.0 (Δ), 5.0 (∇) and 10.0 mM (Δ) p-OH benzoate in the presence of 2.0 mM benzoate. A control flask received only p-OH benzoate (1.0 mM) (●). Inducer and repressor were both added when the cell density was 100 µg. dry wt. ml.$^{-1}$ Samples were taken at intervals thereafter and assayed for p-OH benzoate hydroxylase.

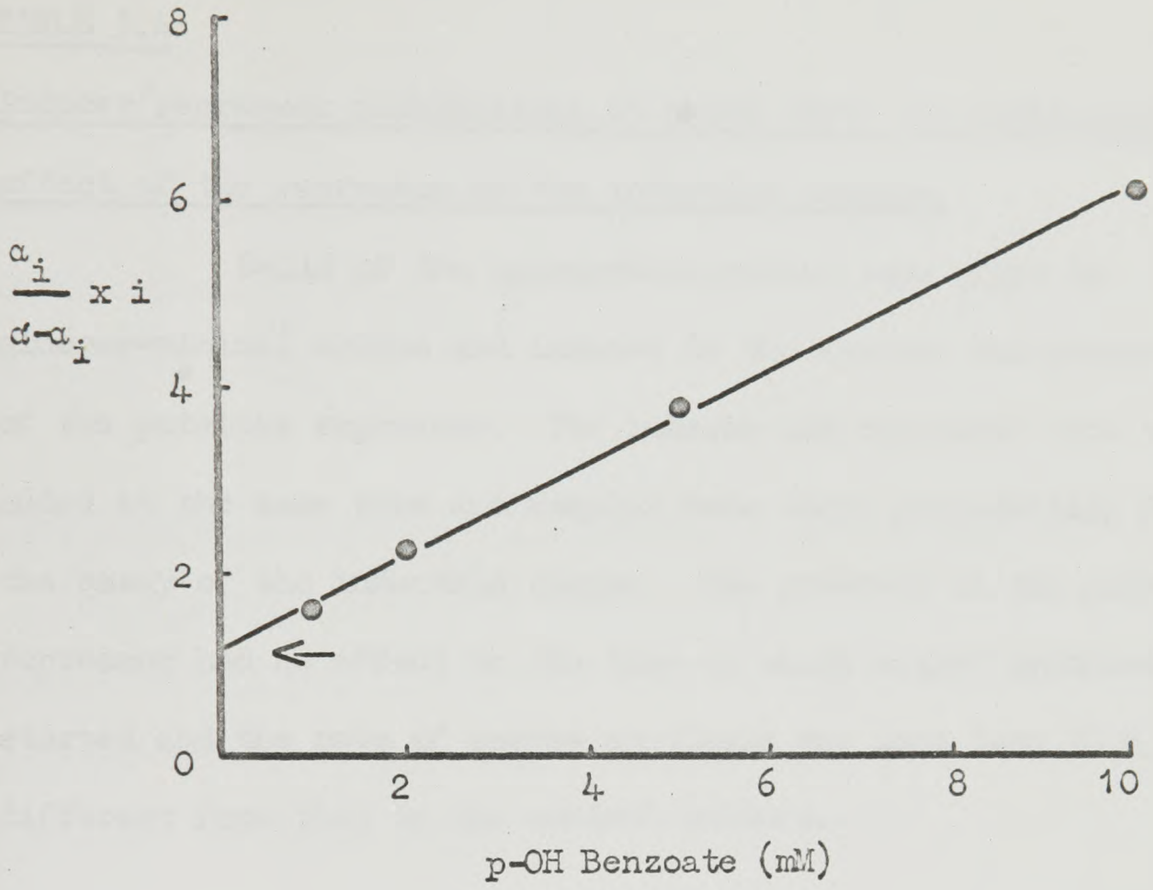


FIGURE 3.11

Repression constant for repression of p-OH benzoate hydroxylase by benzoate.

Differential rates of synthesis of p-OH benzoate hydroxylase (units mg. dry wt.$^{-1}$) in the presence (a_i) and absence (a) of benzoate were calculated from the data of Fig. 3.10. The method of calculation of K_{rep} (↓) was that of Hunter and Downs (1945) and its value was about 1.0 mM.

Concentration of repressor = i.

TABLE 3.4

Inducer/repressor combinations in which there was negligible effect of the repressor on the induction process.

Cells of the appropriate mutant were grown in glucose-minimal medium and induced in the absence and presence of the putative repressor. The inducer and repressor were both added at the same time and samples were taken periodically for the assay of the inducible enzyme. The presence of the putative repressor had no effect on the time at which enzyme synthesis started and the rate of enzyme synthesis was less than 10 % different from that in the control culture.

Inducer (mM)	Repressor (mM)	Mutant
p-OH Benzoate 1.0	Mandelate 2.0	Y 65 (md$^-$)
1.0	10.0	
Benzoate 1.0	Mandelate 2.0	Y 65 (md$^-$)
0.5	5.0	
Benzoate 1.0	p-OH Benzoate 2.0	A 2 (p-OH benz$^-$)
1.0	10.0	

5. Repression of benzoate oxidase by mandelate

The above experiment was repeated using benzoate (1.0 mM) as inducer with mandelate at 2.0 mM. No repressor effect of mandelate was observed with these concentrations or when benzoate at 0.5 mM and mandelate at 5.0 mM were used (Table 3.4).

6. Repression of benzoate oxidase by p-OH benzoate

The experimental procedure described in the two previous cases was again adopted to investigate the repressor activity of p-OH benzoate on benzoate oxidase in the mutant A2 (p-OH benz$^-$). With benzoate at 1.0 mM and p-OH benzoate at 2.0 mM, no repression was observed nor did increasing the p-OH benzoate concentration to 10.0 mM reveal any effect (Table 3.4).

REPRESSOR EFFECTS OF CATECHOL

7. Repression of benzoylformate decarboxylase by catechol.

The double mutant, blocked at mandelate dehydrogenase and catechol-1,2-oxygenase (md$^-$, cat$^-$), was unavailable, and the single mutant Cat 2 (cat$^-$) had to be used. Exponentially growing cells in glucose minimal medium received catechol (2.0 mM) and mandelate (2.0, 4.0 or 10.0 mM) and the rates of synthesis of benzoylformate decarboxylase was determined. The rates were compared with that of a control culture induced with mandelate (2.0 mM) alone (Figure 3.12).

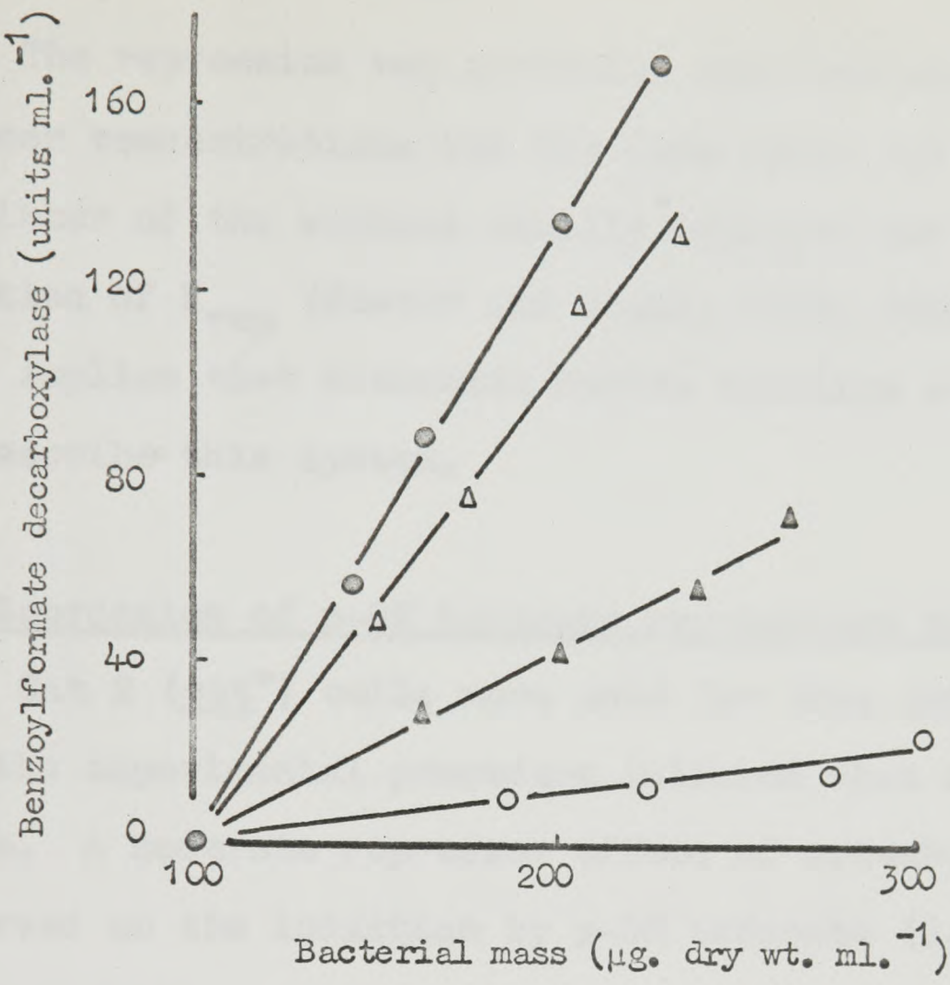


FIGURE 3.12

Effect of catechol on the induction of the mandelate regulon.

Cat 2 (cat$^-$) cells, growing exponentially in glucose-minimal medium, were induced with mandelate at 2.0 (O), 4.0 (Δ) and 10.0 mM (Δ). Catechol (2.0 mM) was added at the same time. Another culture was induced with mandelate (2.0 mM) alone (●). Samples were taken at intervals and assayed for benzoylformate decarboxylase. The induction was started when the culture density was 100 µg. dry wt. ml.$^{-1}$

The repression was partially reversed at increased inducer concentrations but the data could not be analysed by either of the methods usually employed for the determination of K_{rep} (Hunter and Downs, 1945; Dixon, 1953). This implies that Michaelis Menten kinetics are inadequate to describe this system.

8. Repression of p-OH benzoate hydroxylase by catechol

Cat 2 (cat$^-$) cells were used for this investigation and the experimental procedure followed that described above. A moderate repressor effect of catechol (2 mM) was observed on the induction by p-OH benzoate (2.5 mM) (Figure 3.13).

9. Repression of benzoate oxidase by catechol

A slight lag was observed in the synthesis of benzoate oxidase when mutant Cat2 (cat$^-$) was induced in glucose medium, with benzoate (1 mM) and catechol (0.5 mM). The subsequent induction rate was similar to that of the control which did not receive catechol (Figure 3.14).

It is clear from the above that the repressor effects of catechol on the mandelate, benzoate and p-OH benzoate regulons are much less pronounced than, for instance, the

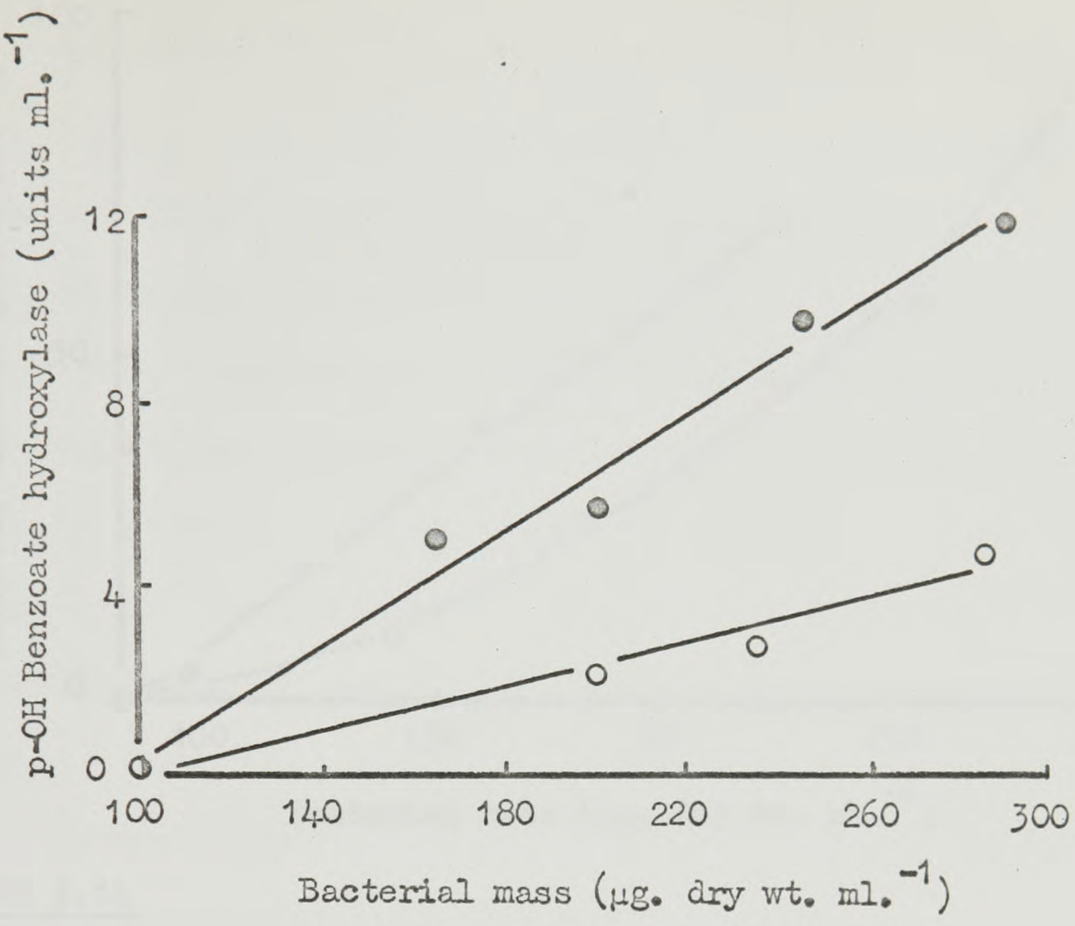


FIGURE 3.13

Effect of catechol on the induction of p-OH benzoate hydroxylase.

Cat 2 (cat$^-$) cells, growing exponentially in glucose-minimal medium, were induced with 2.5 mM p-OH benzoate alone (●) or together with 2.0 mM catechol (O). Samples were taken and assayed for p-OH benzoate hydroxylase. The inducer was added when the culture density was 100 µg. dry wt. ml.$^{-1}$

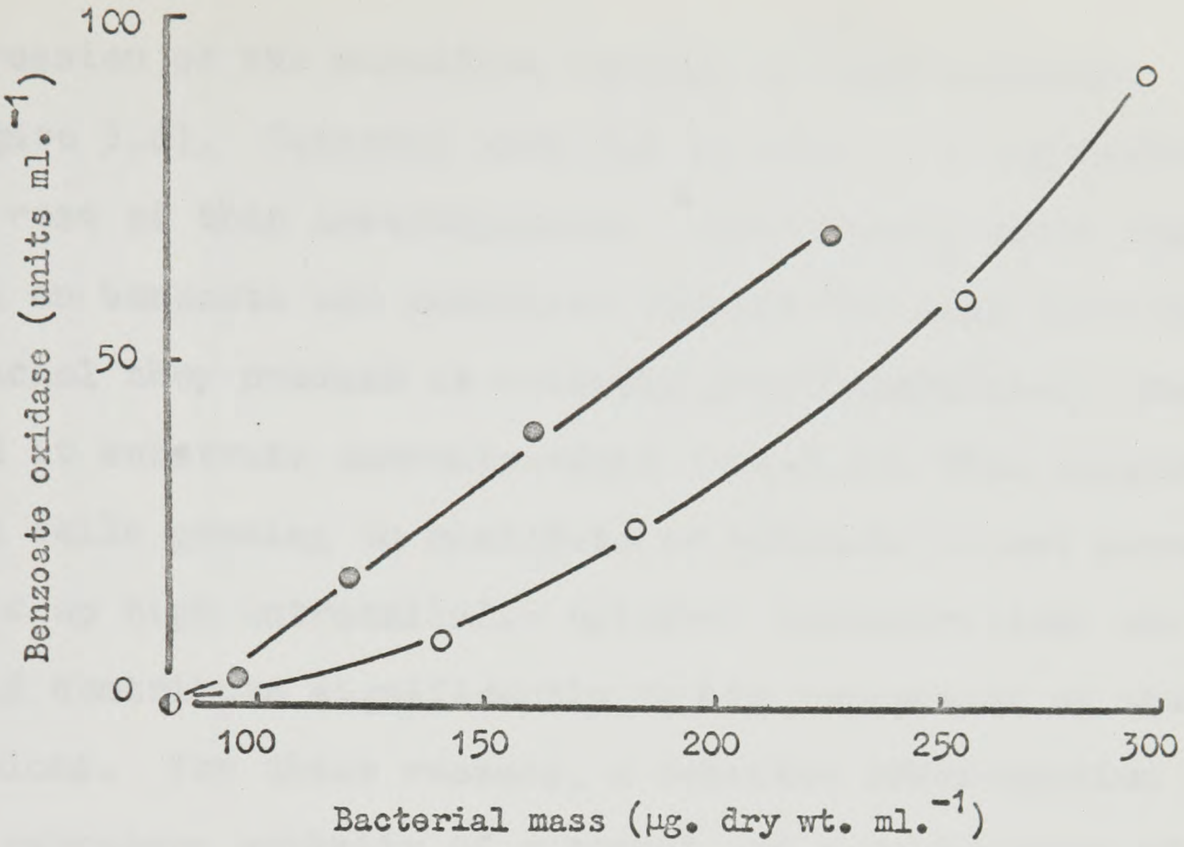


FIGURE 3.14

Effect of catechol on the induction of benzoate oxidase.

Cells of mutant Cat 2 (cat$^-$), growing exponentially in glucose-minimal medium, were induced with 1.0 mM benzoate in the presence (O) and absence (●) of 0.5 mM catechol. Inducer and repressor were added when the culture density was 80 µg. dry wt. ml.$^{-1}$ Samples were taken at intervals thereafter and assayed for benzoate oxidase.

repression of the mandelate regulon by p-OH benzoate (Figure 3.6). Catechol will not be used as a substrate in the rest of this investigation. Furthermore, cells grow well on benzoate and mandelate despite the fact that the catechol they produce is markedly growth-inhibitory when used at substrate concentrations (<2-3 mM). This suggests that cells growing on mandelate or benzoate do not normally build up high intracellular catechol concentrations that could contribute significantly to the repression of their regulons. For these reasons, a detailed investigation of the repressor activity of catechol and a measurement of its repression constants were not undertaken.

REPRESSOR EFFECTS OF SUCCINATE

10. Repression of benzoylformate decarboxylase by succinate

In Y65 (*md*$^-$) cells growing in glucose-minimal medium, there was a slight effect of succinate (7.5 mM) on the induction by mandelate (2.5 mM). A short lag period eventually gave rise to a period in which the rate of synthesis of benzoylformate decarboxylase approached that in a control culture induced with mandelate alone (Figure 3.15).

11. Repression of benzoate oxidase by succinate

When wild-type cells, growing exponentially in glucose-

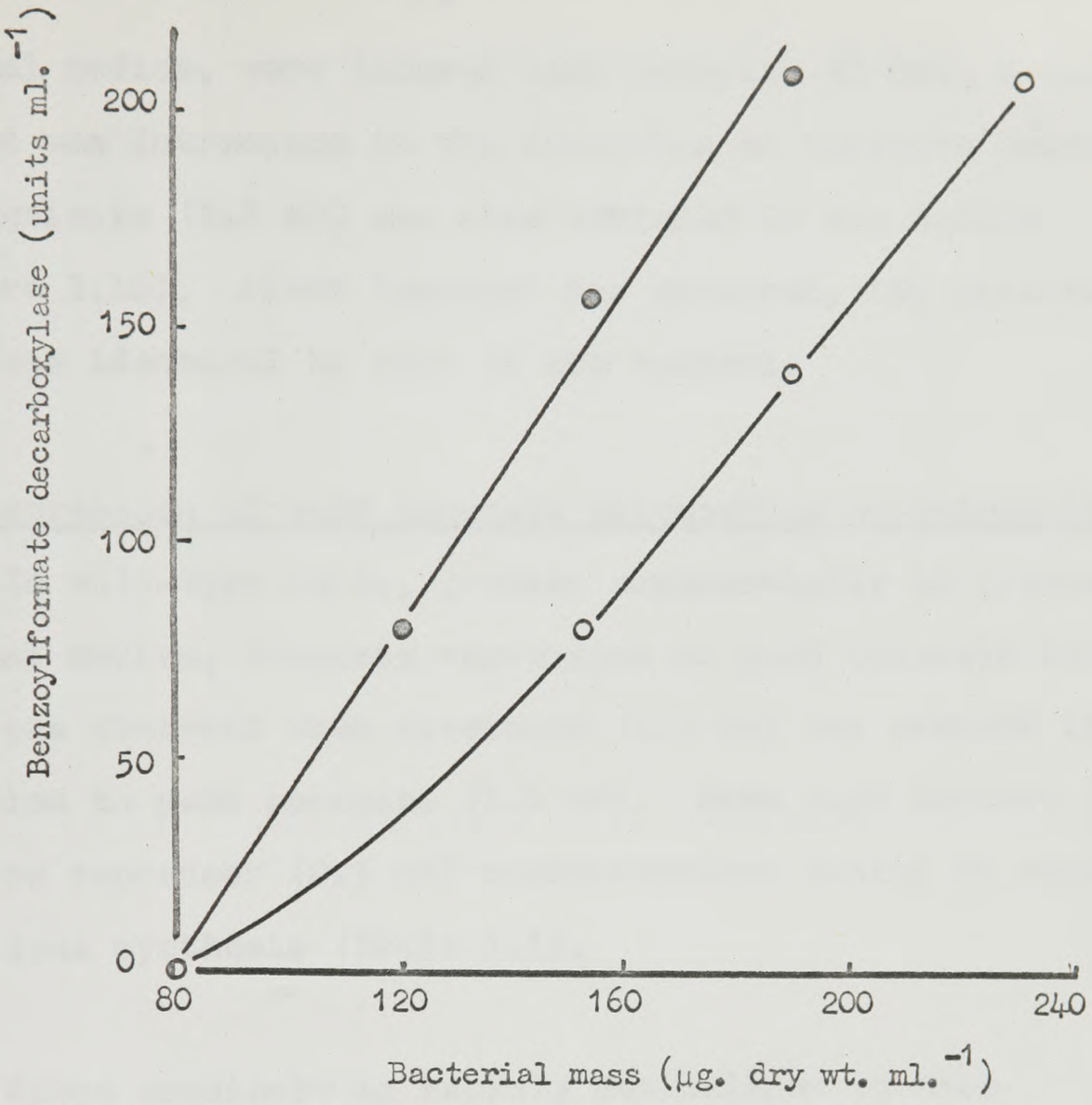


FIGURE 3.15.

Effect of succinate on the induction of benzoylformate decarboxylase by mandelate.

Mutant Y 65 (md$^-$), growing exponentially in glucose-minimal medium, were induced at 80 µg. dry wt. ml.$^{-1}$ with 2.5 mM mandelate alone (●) or together with 7.5 mM succinate (○). Samples were taken at intervals for the assay of benzoylformate decarboxylase (Methods).

minimal medium, were induced with benzoate (1 mM), a lag period was introduced in the induction of benzoate oxidase if succinate (2.0 mM) was also included in the medium (Figure 3.16). After 'escape' had occurred, the induction rate was identical to that of the control.

12. Repression of p-OH benzoate hydroxylase by succinate

In wild-type cells, growing exponentially in glucose-minimal medium, complete repression of p-OH benzoate hydroxylase was observed when succinate (7.5 mM) was present in addition to p-OH benzoate (1.0 mM). Even high inducer (2.5mM) and low repressor (0.5 mM) concentrations failed to result in enzyme synthesis (Table 3.5).

Since succinate is rapidly metabolised by this organism (Table 2.3), it is difficult to extend this investigation for longer periods or to lower repressor concentrations. Although succinate will not be used as a substrate in the rest of this investigation, it should be borne in mind, however, that the intracellular concentration of this repressor, produced by metabolism of the aromatic substrates, is very likely to be high enough to cause considerable repression of p-OH benzoate hydroxylase. It may thus be a factor to consider in explaining the enzymic adaptation to mixtures containing p-OH benzoate.

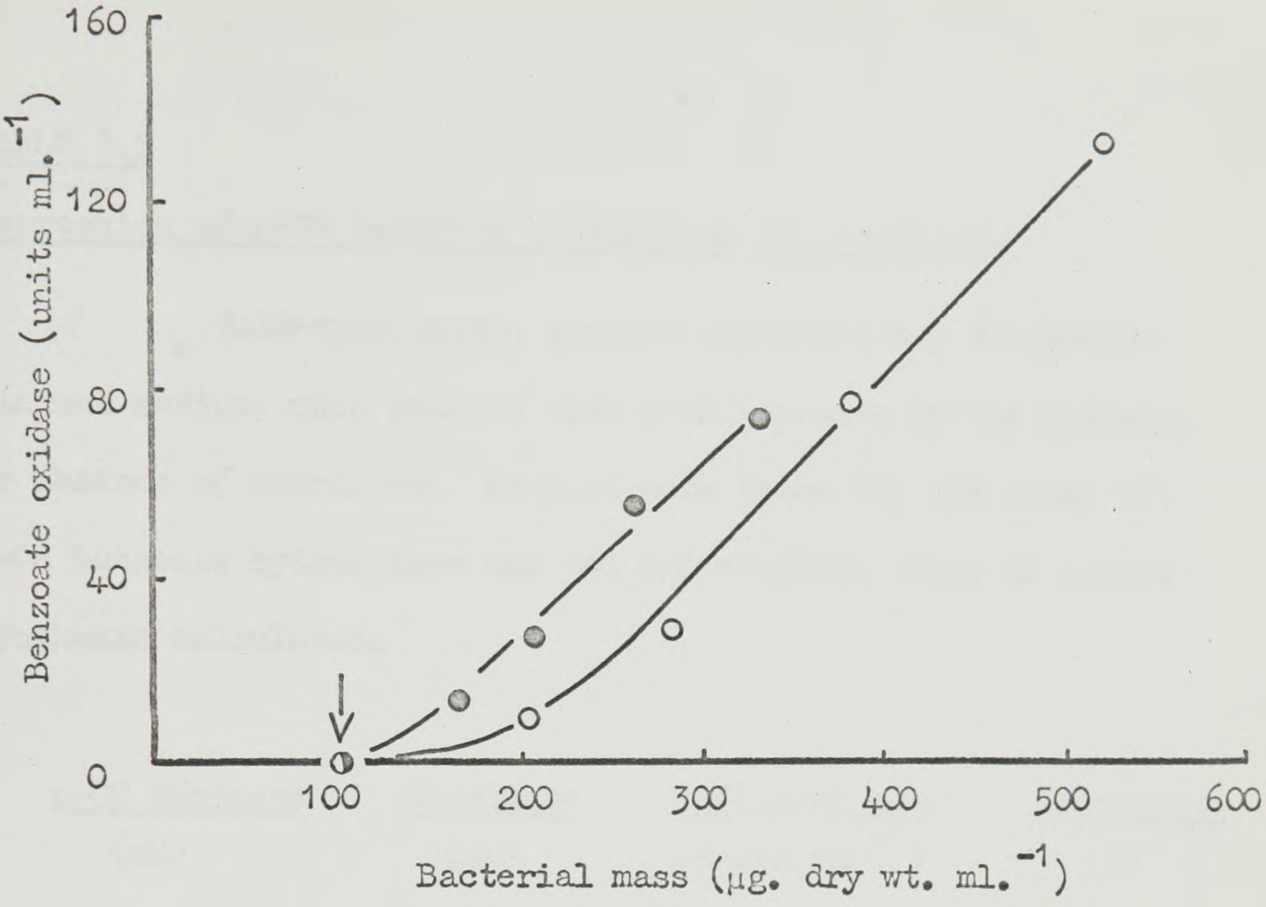


FIGURE 3.16

Effect of succinate on the induction of benzoate oxidase.

Wild-type cells, growing exponentially in glucose-minimal medium, were induced (⇓) with 1.0 mM benzoate alone (●) or with 2.0 mM succinate (O). Samples were taken and assayed for benzoate oxidase.

TABLE 3.5

Repression of p-OH benzoate hydroxylase by succinate.

Wild-type cells, growing exponentially in glucose minimal medium, were induced with p-OH benzoate in the presence or absence of succinate. Samples were taken for the assay of p-OH benzoate hydroxylase and the differential rates of enzyme synthesis calculated.

p-OH Benzoate (mM)	Succinate (mM)	Induction rate (units $mg.^{-1}$)	Repression %
1.0	0	66.0	--
1.0	7.5	0	100
2.5	0	66.5	--
2.5	0.5	0	100

In contrast, the repressor effects of succinate on the other two regulons were small enough to be ignored in the prediction of enzymic adaptation to mixtures of mandelate and benzoate.

SECTION FOUR: DISCUSSION AND SUMMARY

This chapter has been concerned with the investigation of induction and repression in the aromatic acid pathway of P. putida (Figure 1.1). The complexity of this induction and 'multi-sensitive' repression has already been described (Introduction) and is summarised in Figures 1.3 and 1.4. From this, it seems reasonable to assume that, in a medium containing mixed aromatic substrates, the enzymic response of the organism will be selective. It was the aim of this study to predict this response to mixed substrates (mandelate, benzoate and p-OH benzoate). To do this, it was necessary to assess the quantitative importance of induction and repression in the three regulons concerned.

Induction was first examined to obtain induction constants (K_{ind}). The data for induction are set out in Table 3.3. Some problems were encountered especially when investigating induction by p-OH benzoate. Here the affinity

of the inducer was so high that a precise determination of K_{ind} was not possible. The absence of gratuitous induction prevented more than an upper limit being set on the induction constant.

The induction constants were somewhat variable, since the method employed in their calculation relies on the accurate measurement of induction rates. Under apparently identical conditions of induction, these rates were found to vary from day to day by as much as $\pm 50\%$. Similar variations have been observed before in this and other inducible systems (Mandelstam, 1962; Hegeman, 1966; Yudkin, 1969). Mandelstam (1962) has suggested that factors such as culture age and degree of aeration are important in determining the intracellular levels of those metabolites that are thought to be the effectors of catabolite repression (Magasanik, 1961; Mandelstam, 1961; McFall & Mandelstam, 1963).

In predicting the enzymic response of the organism to media containing two substrates, the induction constants will be compared and repressor effects taken into account. Gross differences in the K_{ind} are what is of importance,

so that it does not matter that a precise value for the K_{ind} of p-OH benzoate hydroxylase could not be obtained or that the constants for benzoate oxidase and the mandelate regulon are somewhat variable.

Since the induction constants for benzoate oxidase and the mandelate enzymes are very similar (about 0.5 mM) both inducer and repressor concentrations will be of importance in determining enzymic adaptation to mixtures of mandelate and benzoate. On the other hand, the induction constant of p-OH benzoate hydroxylase is so low ($<$0.01 mM) and so different from the other induction constants that p-OH benzoate hydroxylase should be induced at all reasonable concentrations. The degree of induction in mixed substrate experiments will depend more on the nature and concentration of the repressor than on the concentration of p-OH benzoate.

An independent measurement of the K_{ind} for the mandelate regulon was obtained by using a chemostat (see Chapter 6). The K_{ind} obtained was 0.7 mM (Fig. 6.5) and this value agrees fairly well with that obtained from Figure 3.1 (0.5 mM). The data of Stanier and co-workers (Stanier et al., 1965) also allow a K_{ind} to be calculated

(0.2 mM) which is somewhat less than that obtained in this study.

The kinetics of induction of the mandelate regulon are interesting in that high inducer concentrations ($\geqslant$2.0 mM) elicit almost immediate synthesis of the enzymes at a high rate. Lower concentrations involve a lag period before a constant differential rate of synthesis is established. Such kinetics are similar to those observed in E. coli when gratuitous inducers are used to induce β-galactosidase in permease$^+$ strains (Monod, 1956; Herzenberg, 1959; Cohn and Horibata, 1959). Since low concentrations of mandelate do not initially appear to be effective and may, therefore, modify the response of the organism to mixed aromatic substrates, the induction of the mandelate regulon was studied in more detail (see next Chapter).

The pattern of repression in the aromatic pathways of P. putida was worked out by Mandelstam and Jacoby (1965) and Stevenson and Mandelstam (1965). It has been confirmed and extended in the present study. Additional cross-pathway repression of p-OH benzoate hydroxylase by benzoate and by catechol has been shown to occur. The data on repression are summarised in Table 3.6 and Figure 3.17.

TABLE 3.6

Summary of the repressor effects in the aromatic pathway of Pseudomonas putida.

Wild-type or mutant cells (Text), growing exponentially on glucose, were induced with mandelate, benzoate or p-OH benzoate at concentrations sufficient for maximum induction in the absence of repressors. The effects of various repressors on the time-course and rate of induction were noted. The repressors were added at the same time as the inducers.

In the following table, an effect on the rate of induction is designated 'R' and a transient effect or 'escape' (see Text) as 'E'. The effects were severe (++) or moderate (+) or absent (-).

REPRESSOR	INDUCER: Mandelate	Benzoate	p-OH Benzoate
Mandelate		E - R -	E - R -
Benzoate	E ++ R -		E - R +
p-OH Benzoate	E - R ++	E - R -	
Catechol	E - R +	E + R -	E - R +
Succinate	E + R -	E + R -	E/R ++*

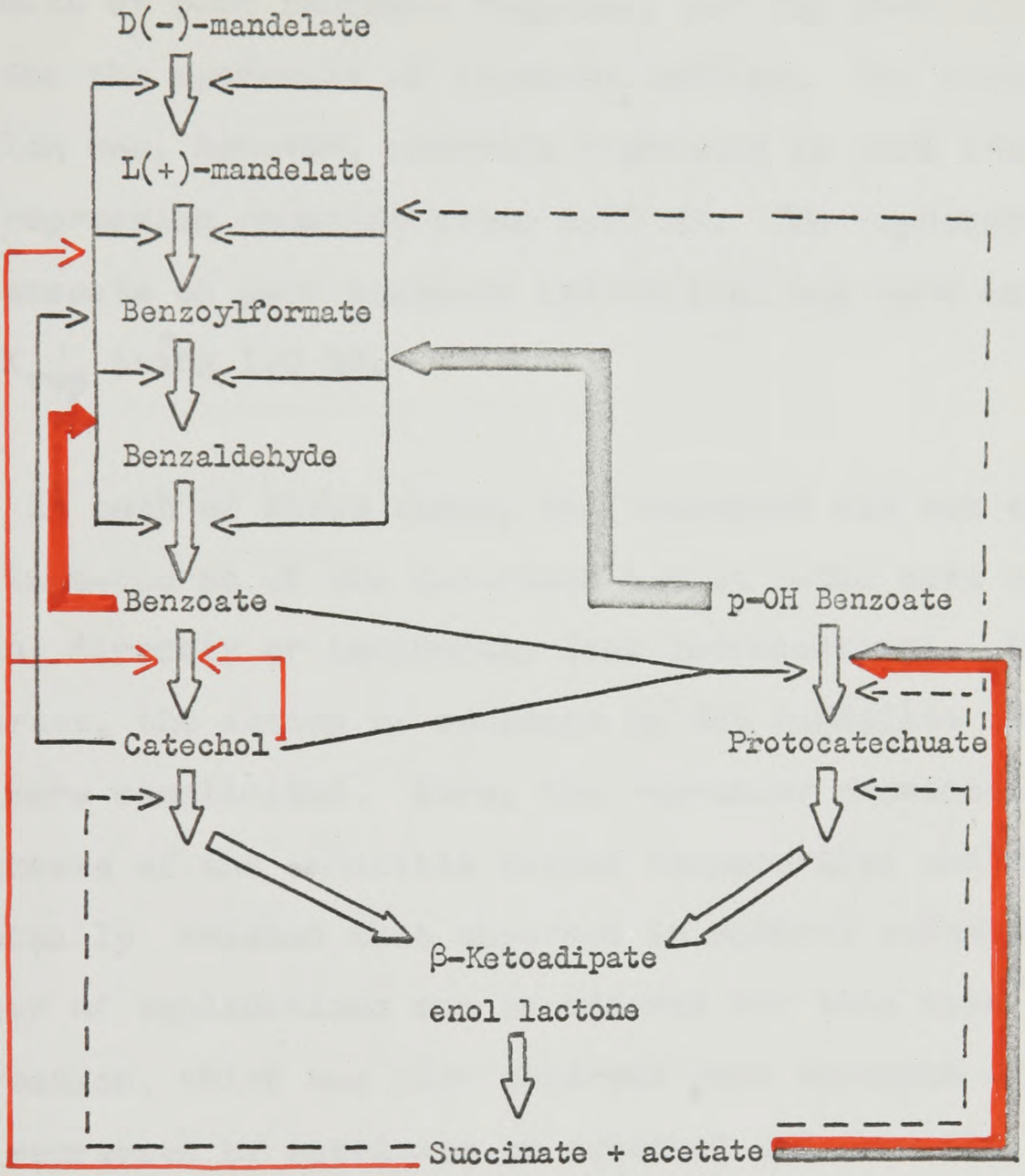
* escape/rate effects were not differentiated.

FIGURE 3.17

Repression in the aromatic pathways of *Pseudomonas putida*.

The diagram on the facing page summarises repressor effects found in the pathways of aromatic carboxylic acid catabolism in *Pseudomonas putida*.

Black lines indicate that the effect is on the rate of induction. Red lines indicate transient repression. Red/black indicates undefined effect. Broken lines are repressor effects described by Stevenson and Mandelstam (1965) but not investigated in the present study. The width of the line is a measure of the severity of the effect (see Text).


D(-)-mandelate
L(+)-mandelate
Benzoylformate
Benzaldehyde
Benzoate
p-OH Benzoate
Catechol
Protocatechuate
β-Ketoadipate
enol lactone
Succinate + acetate

Mandelate appeared to have no repressor effects on the benzoate or p-OH benzoate regulons, nor did p-OH benzoate repress the synthesis of benzoate oxidase. The mandelate regulon was, however, severely repressed by p-OH benzoate, the repression constant being 0.02 mM. The repressor action of benzoate on p-OH benzoate hydroxylase was more modest, the K_{rep} being 1.0 mM.

In both of these cases, the repressor did not affect the time-course of the induction and it could have been acting directly or indirectly (see Introduction). In contrast, the action of benzoate on the mandelate regulon was more complicated. Here, the repressor prevented the synthesis of the inducible enzyme temporarily; the rate eventually reached that observed in control cultures. A number of explanations can be offered for this type of repression, which was also observed when benzoate oxidase was repressed by succinate or catechol and when the mandelate regulon was repressed by succinate.

1. The repressor may inhibit the entry of the inducer into the cells. If this occurred it is unlikely to be complete unless excessive repressor concentrations are used. Inducer would eventually build up inside the cell, albeit slowly, and, under gratuitous conditions, would

finally attain a concentration similar to that in the control. The kinetics of induction should in this case consist of an initial period, in which the induction rate accelerates towards that of the control, followed by a constant differential rate of enzyme synthesis indistinguishable from that in a control culture. The higher the repressor concentration, the longer will the inducer take to accumulate inside the cells and the later will the 'escape' take place.

If induction is not gratuitous, the inducer may not reach a high enough internal concentration for full induction (this will depend on the external concentration and the K_{ind}). Increased repressor concentrations should nonetheless decrease the steady state induction rate, and a lag period should still be observed, during which equilibrium is established between the rate of inducer entry, the rate of enzyme synthesis and the rate of inducer metabolism.

2. The repression may be like the transient repression found in the lactose system of E. coli. When glucose is added to cells growing on a non-repressing carbon source such as glycerol, the induced synthesis of β-galactosidase is almost completely stopped

for up to one generation. Thereafter, the repressed rate of synthesis normally associated with glucose-grown cells is observed (Moses and Prevost, 1966; Tyler, Loomis and Magasanik, 1967).

The detailed mechanism of this example of repression is unknown but, since the effect is observed in constitutive, cryptic and pre-induced cells, the action of the repressor cannot be simply the result of an effect on inducer entry (Moses and Prevost, 1966; Tyler et al., 1967). Furthermore, glucose does not chase thiomethyl-β-D-galactoside out of loaded cells (Tyler et al., 1967).

Pastan and Perlman (1968) have implicated the lac promoter in transient as well as catabolite repression, and cyclic AMP overcomes both types of repression (Pastan and Perlman, 1968; Perlman and Pastan, 1968; Perlman et al., 1969). Transient repression may thus be a period of intense catabolite repression, which follows the addition of glucose and results from a sudden alteration in the catabolite effector pools. Eventually growth is established on glucose and these pools are adjusted to the new steady state. In support of this view, it has been observed that cells pre-adapted to growth on glucose do not experience

transient repression (Moses & Prevost, 1966). Furthermore, Makman and Sutherland (1965) showed that immediately after its addition to the medium, glucose caused a massive loss of cyclic AMP from E. coli cells. In the steady state, glucose suppressed the formation of the nucleotide in proportion to its repressor activity.

In the present study, the repressors were often not acting gratuitously and the 'escape' could certainly have been due to transient alterations in the internal concentration of the catabolite effector(s), but this explanation seems inadequate to explain 'escape' in situations where the repressor was acting gratuitously, e.g., the effect of benzoate on the mandelate regulon. Whatever their cause, these 'escape' effects will be important in determining the enzymic response to mixed substrates in the transition period before the steady state is reached. Consequently a more detailed investigation of the effect of benzoate on the mandelate regulon was thought desirable and is reported in the next chapter. This chapter also describes further experiments on the induction of the mandelate regulon by mandelate, which may not be as simple a process as was first thought.

CHAPTER FOUR

INDUCTION OF THE MANDELATE REGULON AND ITS REPRESSION BY BENZOATE

INDUCTION OF THE MANDELATE REGULON AND ITS REPRESSION BY BENZOATE

SECTION ONE : INTRODUCTION

1. Effect of mandelate concentration on induction of the mandelate regulon

In the previous chapter, the rate of induction of the mandelate enzymes was measured at different mandelate concentrations in md$^-$ cells growing on glucose (Figure 3.1). At mandelate concentrations below 2.0 mM, there was a manifest delay in the onset of induction. The duration of this lag period (calculated by extrapolation of the linear portion of the differential plot to the abscissa) did not vary significantly over the mandelate concentration range 0.25 - 1.0 mM. It was eliminated at higher concentrations (Table 4.1). The kinetics of induction at low mandelate concentrations (below 2.0 mM) were not confined to conditions of gratuity (Figure 3.2).

The delay in the onset of induction at low concentrations of mandelate could be due to:

(i) Conversion of mandelate to an active inducer. Such a conversion might be rate-limiting at low external mandelate concentrations.

TABLE 4.1

Duration of the lag period between the addition of the inducer and the synthesis of benzoylformate decarboxylase : effect of inducer concentration.

Using the data of Figure 3.1, the time that elapsed between the addition of mandelate and the establishment of a constant rate of synthesis of benzoylformate decarboxylase was calculated by extrapolation (see Text).

Mandelate (mM)	Lag (generations)
2	0
1	0.95
0.75	1.35
0.5	1.25
0.25	1.25

(ii) Slow diffusion of mandelate into the cells. The entry of mandelate into the cell might be rate-limiting at low external concentrations. The rate of diffusion should be proportional to the concentration gradient across the cell membrane. Accordingly, a high external mandelate concentration should rapidly produce an intracellular concentration high enough to support the maximum rate of induction of the mandelate enzymes. At lower mandelate concentrations, one would expect a lag followed, eventually, by a constant rate of induction once the internal mandelate concentration had reached steady-state.

This was, in fact, what was observed (Figure 3.1).

2. Effect of benzoate on induction by mandelate.

Also in the previous chapter, it was shown that benzoate repressed the induction of the mandelate regulon, although induction eventually did start and the rate soon approached that of an unrepressed culture (Figure 3.9)

Since the mechanism of induction by mandelate is unknown, the effect on it of benzoate must also be uncertain. Clearly the effect of benzoate is short-lived. At first, benzoate could be inhibiting the entry of mandelate or its conversion to an active inducer, but in time sufficient

mandelate (or active inducer) might build up inside the cell, albeit slowly, to maintain a maximum rate of induction. Alternatively, an uptake (or conversion) system might become induced in sufficiently large amounts to reverse the inhibitory effects of benzoate.

The remainder of this chapter describes experiments to investigate further the mechanism of induction by mandelate and the effect on it of benzoate, but first it was necessary to make certain that the submaximum rate of induction at low mandelate concentrations was maintained for a significant length of time.

SECTION TWO : INDUCTION BY MANDELATE

Accordingly, mutant Y65 (*md*$^-$), growing exponentially in glucose-minimal medium, was induced with 0.25 mM mandelate at a low cell density (20 µg.dry wt.ml.$^{-1}$). When the culture density reached 330 µg.ml.$^{-1}$, the cells were quickly centrifuged and resuspended at a lower density (50 µg.ml.$^{-1}$) in fresh medium. This procedure had no noticeable effect on growth and prolonged the exponential phase so that the synthesis of benzoylformate decarboxylase, once it started, could be measured over 3-4 generations.

The results (Figure 4.1) show that, in these cells, the enzyme was synthesised at a constant rate (which was 25% of that in a culture induced with 5 mM mandelate). No acceleration towards the maximum rate was observed.

1. Conversion of mandelate to an active inducer

As far as is known, mandelate is metabolised only via benzoylformate in this organism. This is also an inducer of the mandelate regulon, but its effectiveness compared with mandelate is not known. The mutant used in the experiment described at the beginning of this chapter lacks the enzyme, mandelate dehydrogenase, to effect this conversion. This was confirmed by measuring the rate of decarboxylation of labelled mandelate by this mutant (Y65) and another like it (D57) after they had been induced with mandelate.

Both mutants were grown in glucose-minimal medium supplemented with mandelate (10 mM). After three generations of growth in this medium the cells were harvested and washed in phosphate buffer (0.1 M, pH 7). The washed cells were resuspended (2.5 mg. dry wt.ml.$^{-1}$) in the same buffer, containing chloramphenicol (50 μg.ml.$^{-1}$). Portions (2.5 ml)

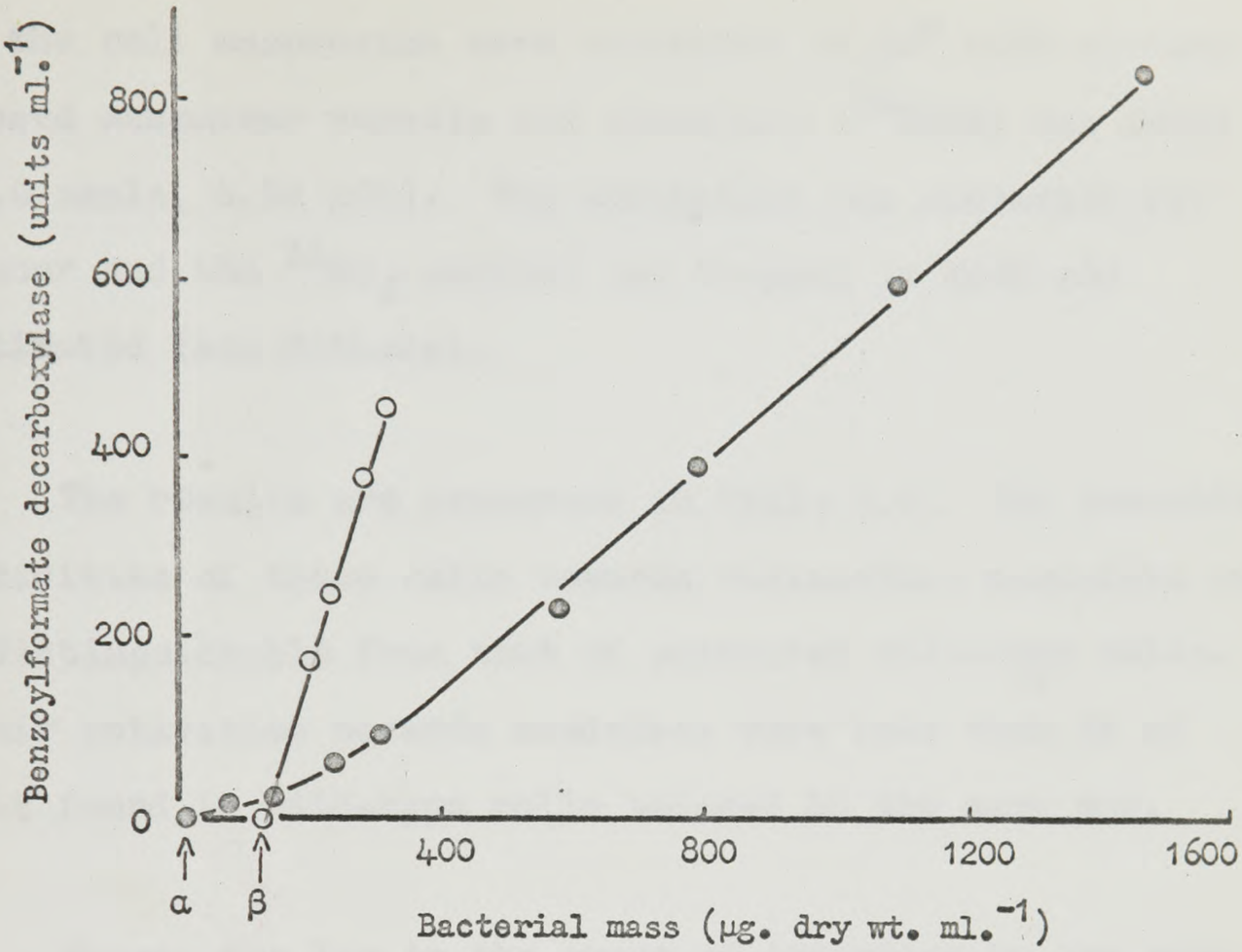


FIGURE 4.1

Induction of benzoylformate decarboxylase by 0.25 mM mandelate.

Y 65 (md$^-$) cells, growing exponentially in glucose-minimal medium, were induced at α with 0.25 mM mandelate. Samples were taken at intervals thereafter and assayed for benzoylformate decarboxylase (●). When the cell density reached 330 μg. dry wt. ml.$^{-1}$, the cells were centrifuged and resuspended in fresh medium (at 50 μg. ml.$^{-1}$). The enzyme synthesis was followed as before and the results are presented as though growth had continued without intermission.

A control culture was induced at β with 5.0 mM mandelate

(○)

of the cell suspension were incubated at 30° with shaking in closed manometer vessels and mandelate ($^{14}COOH$) was added (6.0 µmole, 0.02 µCi). The incubation was continued for 1 hour and the $^{14}CO_2$ evolved was trapped in NaOH and estimated (see Methods).

The results are presented in Table 4.2. The metabolic activities of these cells towards radioactive mandelate were indistinguishable from that of uninduced wild-type cells. Their activities towards mandelate were less than 2% of that found in wild-type cells induced in the same way.

Hence, the lag in the onset of induction by low concentrations of mandelate could not have been due to conversion of the mandelate in significant amounts to benzoylformate or to any other compound that involved decarboxylation. Nevertheless, it is not possible to rule out conversion to a compound which retains the 1-carbon of mandelate or to an unknown compound which does not retain this carbon atom but which is produced in very small amounts. It was thought more profitable to postpone investigation of these possibilities and to turn to the alternative explanation for the lag, namely that the inducer does not enter the cells readily at low external concentrations.

TABLE 4.2

Metabolism of mandelate-^{14}COOH by md$^-$ cells.

Cells of mutants Y 65 and D 57 (md$^-$) were induced for 3 generations with mandelate (10 mM) in glucose-minimal medium, washed and resuspended (2.5 mg. dry wt. ml.$^{-1}$) in phosphate buffer (0.1 M, pH 7) containing chloramphenicol (50 µg. ml.$^{-1}$). Portions (2.5 ml) of these cell suspensions were incubated with shaking in sealed manometer vessels containing mandelate-^{14}COOH (6 µmoles, 0.02 µCi.) for 1 hr. at 30°. The $^{14}CO_2$ evolved was trapped in soda and estimated as in the assay for benzoate oxidase (Methods). The activity of the mutants is compared with that of the wild-type (taken from Table 2.2). The background was 30.7 cpm.

Cells	Total mandelate added (cpm)	$^{14}CO_2$ evolved (cpm)
Y 65	23,600	223.2
D 57	23,200	450.8
Wild-type (induced)	23,500	23,250
	23,240	23,160
Wild-type (uninduced)	23,450	264.4
	22,970	273.1

2. Effect on induction at low mandelate concentration of loading the cells with mandelate

If the lag period at low mandelate concentrations is due to the slow diffusion of the inducer into the cells, cells that are already 'loaded' with mandelate should show no lag in the induction of the mandelate regulon at low mandelate concentrations. Cells may be 'loaded' with mandelate by incubating them with inducer in the presence of chloramphenicol to prevent induction from beginning. To remove the antibiotic, the cells are then washed with medium containing mandelate so as to maintain the internal mandelate pool. Re-incubation in fresh medium containing mandelate at the same low concentration should result in an immediate induction of the mandelate system.

Since in previous experiments a lag of about 70 minutes (1 generation) was observed during induction with 0.25 mM mandelate (Figure 4.2, curve A), the cells of a culture (100 ml) were 'loaded' with mandelate by incubating them for 70 minutes in glucose medium containing 0.25 mM mandelate and chloramphenicol (50 $\mu g.ml.^{-1}$). They were then centrifuged, washed twice with warm medium containing 0.25 mM mandelate (50 ml) and resuspended to their original density (50 $\mu g.$dry wt.$ml.^{-1}$) in fresh, warm glucose medium

FIGURE 4.2

Induction of benzoylformate decarboxylase by 0.25 mM mandelate in cells 'loaded' with mandelate.

Cells of mutant D 57 (md^-) were treated as follows :

A. (●) at α 0.25 mM mandelate was added.

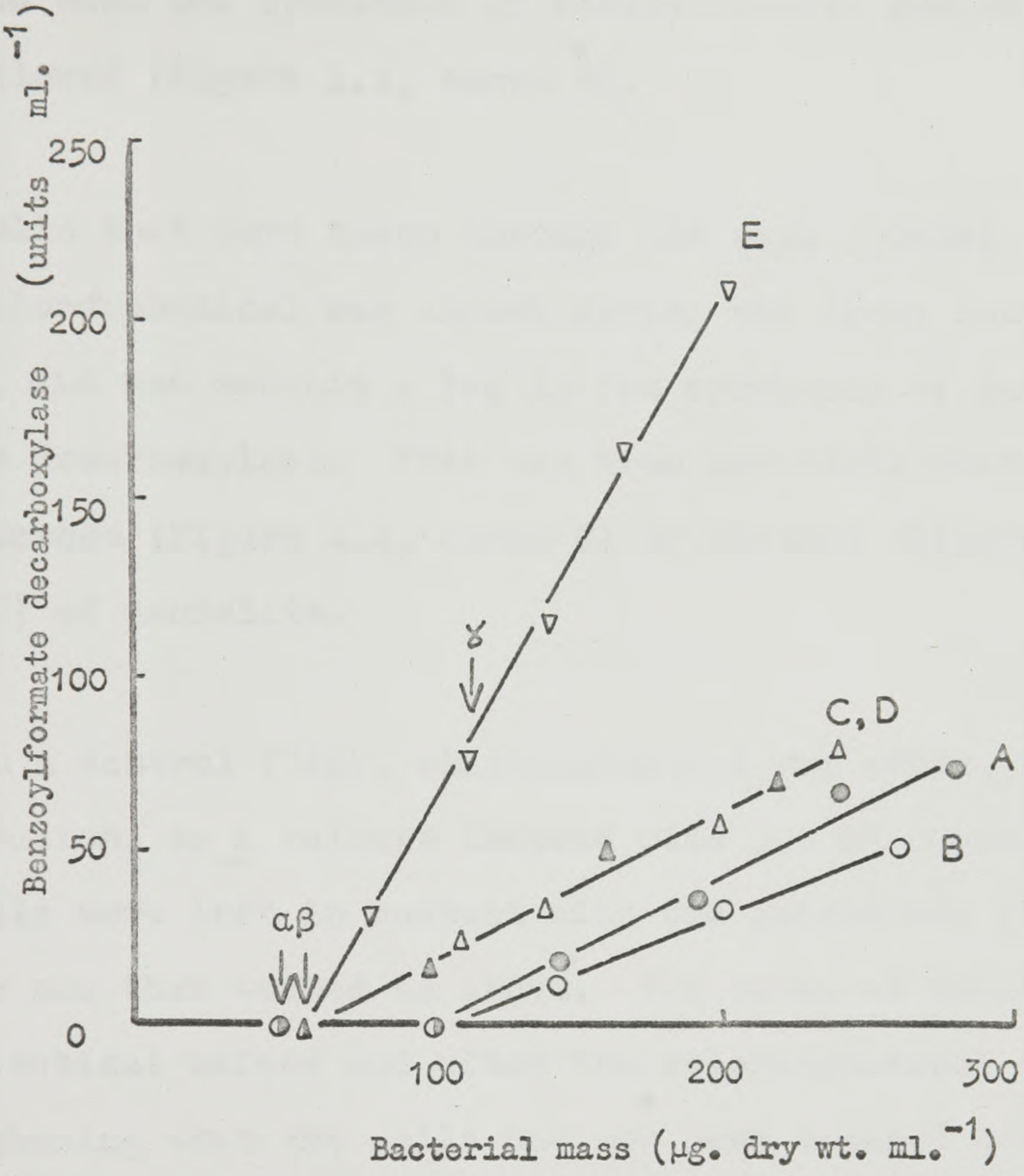
B. (○) The cells were pre-incubated for 70 min. in glucose medium containing 0.25 mM mandelate and chloramphenicol (50 μg. $ml.^{-1}$). The antibiotic was removed by washing the cells with fresh, warm medium containing mandelate (0.25 mM). The washed cells were resuspended in fresh glucose medium containing 0.25 mM mandelate (at α).

C. (Δ). The procedure of B was followed except that chloramphenicol was omitted. The washed cells were resuspended with 0.25 mM mandelate at β.

D. (▲). As for C except that the washing was done in the absence of mandelate.

E. (▽). At β cells were induced in glucose medium with 5.0 mM mandelate and the synthesis of benzoylformate decarboxylase allowed to proceed. At γ chloramphenicol (50 μg. $ml.^{-1}$) was added and the culture incubated with shaking for 70 min. The antibiotic was then removed in the usual way and the cells resuspended in fresh medium containing 5.0 mM mandelate.

In all cases, samples were taken during the final incubation period and assayed for benzoylformate decarboxylase.


Benzoylformate decarboxylase (units ml.$^{-1}$)
250
200
150
100
50
0
100
200
300
Bacterial mass (μg. dry wt. ml.$^{-1}$)
E
C, D
A
B
αβ
γ

also containing 0.25 mM mandelate. A lag was still observed when the synthesis of benzoylformate decarboxylase was followed (Figure 4.2, curve B).

Cells that were taken through the same process, except that chloramphenicol was absent during the first incubation period, did not exhibit a lag in the synthesis of benzoylformate decarboxylase. This was true for cells washed in the presence (Figure 4.2, curve C) or absence (Figure 4.2, curve D) of mandelate.

In a control flask, chloramphenicol was added, after 1 generation, to a culture induced with 5.0 mM mandelate. The cells were left in contact with the antibiotic for 70 minutes and then washed as above. The rates of induction were identical before and after the chloramphenicol treatment, showing that the cells had not been damaged and that the antibiotic had been effectively removed by the washing process (Figure 4.2, curve E).

These results of the 'loading' experiments do not support the hypothesis that slow diffusion is responsible for the lag in the induction of benzoylformate decarboxylase at low mandelate concentrations. What they do suggest

is that a protein is synthesised during the lag period and that this may be responsible for the uptake of mandelate or its conversion to an active inducer.

This being so, it is reasonable to propose that cells, already fully or partially induced at high mandelate concentrations, should be able to synthesise the mandelate enzymes without a lag when transferred to a low concentration of mandelate.

3. Effect of pre-induction at high mandelate concentrations on subsequent induction at low mandelate concentrations

It was known that mandelate at high concentrations (2-10 mM) rapidly induces the mandelate enzymes without a lag. A concentration in this range (5 mM) was, therefore, used to induce cells of mutant D57 (md$^-$), growing in glucose-minimal medium. Benzoylformate decarboxylase was followed for about 1 generation and then the cells were centrifuged, washed twice in mandelate-free medium and resuspended in fresh glucose-minimal medium containing 0, 5.0 or 0.25 mM mandelate. Growth and benzoylformate decarboxylase were followed for another two generations. The results were compared with those of a control culture receiving 0.25 mM mandelate throughout.

It can be seen from Figure 4.3 that:

(a) without pre-induction, there was the expected lag of about 1 generation with 0.25 mM mandelate.

(b) with pre-induction, cells resuspended in 5.0 mM mandelate recommenced synthesis of benzoylformate decarboxylase with only a very slight lag and at a rate three times that of the culture (a) above.

(c) with pre-induction, cells resuspended in 0.25 mM mandelate exhibited no lag in resuming their synthesis of benzoylformate decarboxylase. Rather surprisingly, the rate of induction was the same as at the higher concentration.

(d) the washing procedure effectively removed mandelate from the cells, since pre-induced cells resuspended in mandelate-free medium failed to synthesise any more enzyme. There could not have been any significant mandelate left in the cells after washing that could account for the pre-induction effect.

4. Maintenance of the induced state at low concentrations of mandelate.

The pre-induction effect was also examined in another type of experiment, illustrated in Figure 4.4. D57 (*md*$^-$) cells were grown in glucose-minimal medium with mandelate

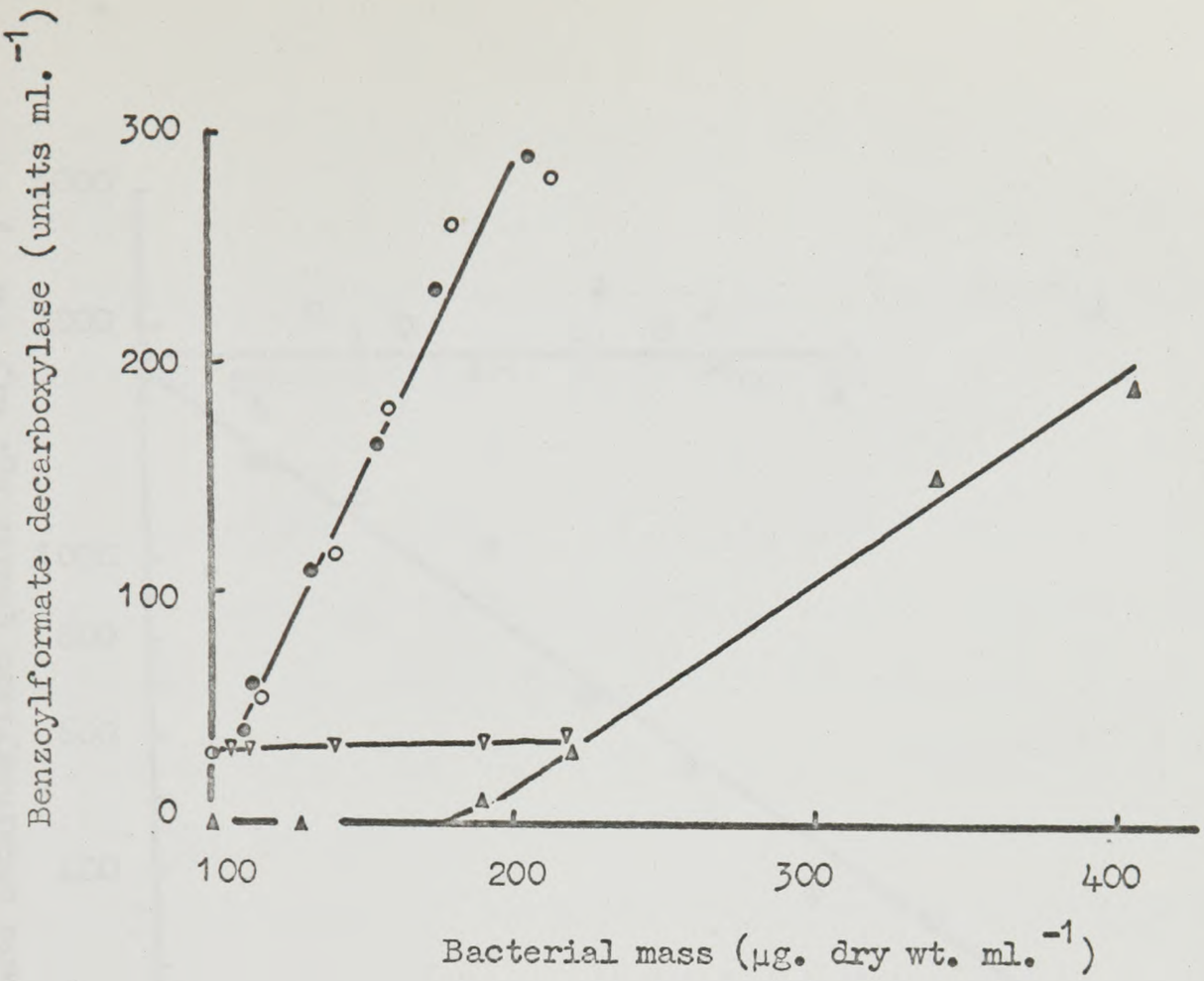


FIGURE 4.3

Effect of pre-induction of benzoylformate decarboxylase with 5.0 mM mandelate on the subsequent induction at 0.25 mM mandelate.

Mutant D 57 (md$^-$), growing exponentially in glucose-minimal medium, was induced for one generation with 5.0 mM mandelate. The cells were then washed in mandelate-free medium and resuspended in fresh glucose-minimal medium containing 0 (▽), 0.25 (○) or 5.0 mM (●) mandelate. The rates of synthesis of benzoylformate decarboxylase were determined and compared with that in uninduced cells induced with 0.25 mM mandelate (▲).

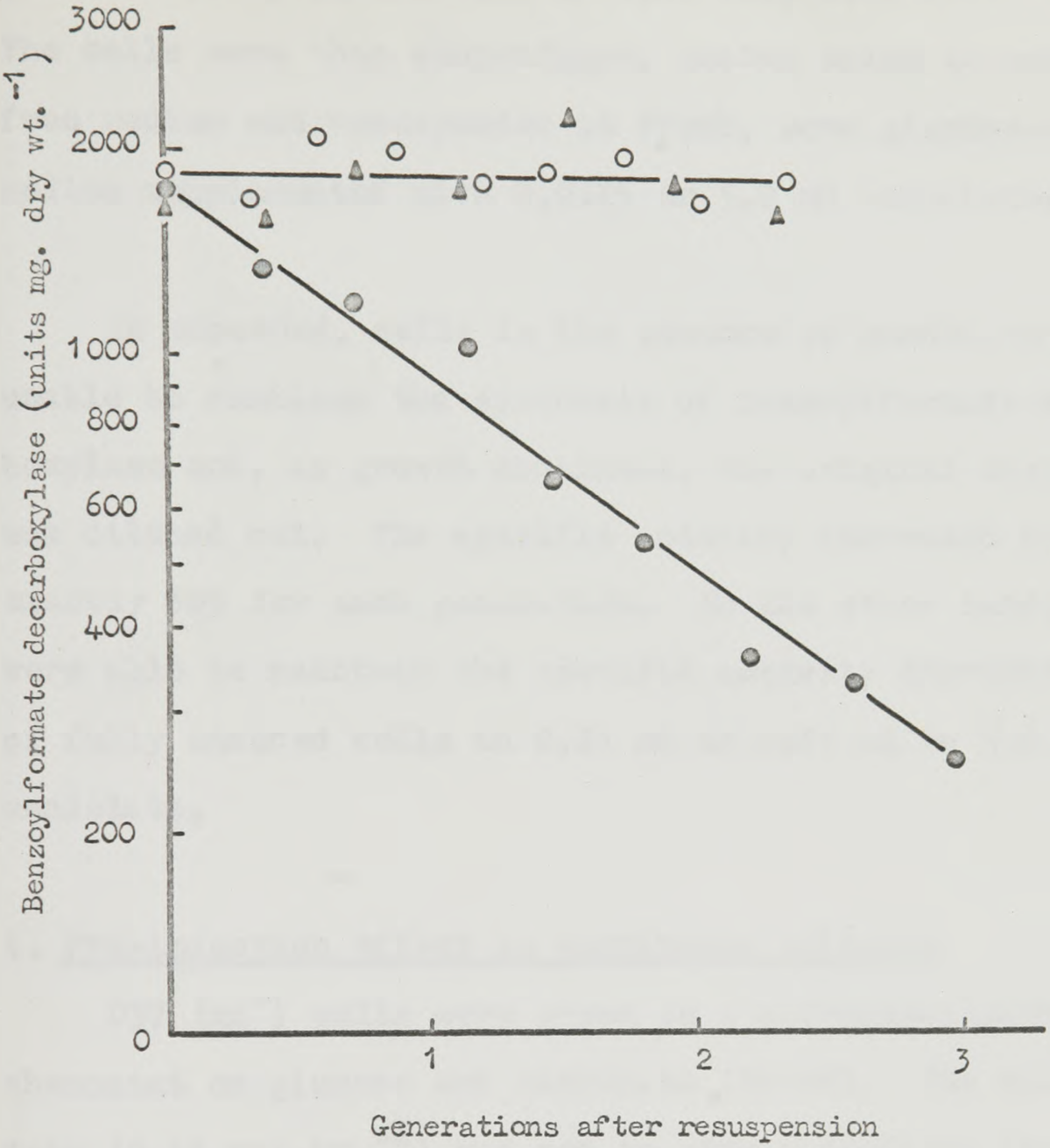


FIGURE 4.4

Maintenance of benzoylformate decarboxylase induction in induced cells with 0.25 mM mandelate.

Mutant D 57 (md$^-$) was grown for five generations in glucose-minimal medium with 5.0 mM mandelate, washed in mandelate-free medium and resuspended in fresh glucose-minimal medium with 0 (●), 0.25 (▲) or 5.0 mM (○) mandelate. Samples were taken for the assay of benzoylformate decarboxylase over the next three

(5.0 mM) for 5 generations so that they were fully induced. The cells were then centrifuged, washed twice in mandelate-free medium and resuspended in fresh, warm glucose-minimal medium supplemented with 0,0225 or 5.0 mM mandelate.

As expected, cells in the absence of mandelate were unable to continue the synthesis of benzoylformate decarboxylase and, as growth continued, the original enzyme was diluted out. The specific activity decreased by almost exactly 50% for each generation. On the other hand, cells were able to maintain the specific activity characteristic of fully induced cells in 0.25 mM as well as in 5.0 mM mandelate.

5. Pre-induction effect in continuous cultures

D57 (*md*$^-$) cells were grown in a nitrogen-limited chemostat on glucose and mandelate (10 mM). The dilution rate (0.45 vol.hr.$^{-1}$) was set to give a doubling time of 90 min., close to the maximum for glucose (Table 2.3). The specific activity of benzoylformate decarboxylase was measured at steady state and then the inducer concentration was reduced in a step-wise manner, the enzyme being re-determined at each steady state. The results are shown in Figure 4.5 (curve B) and may be compared with the changes

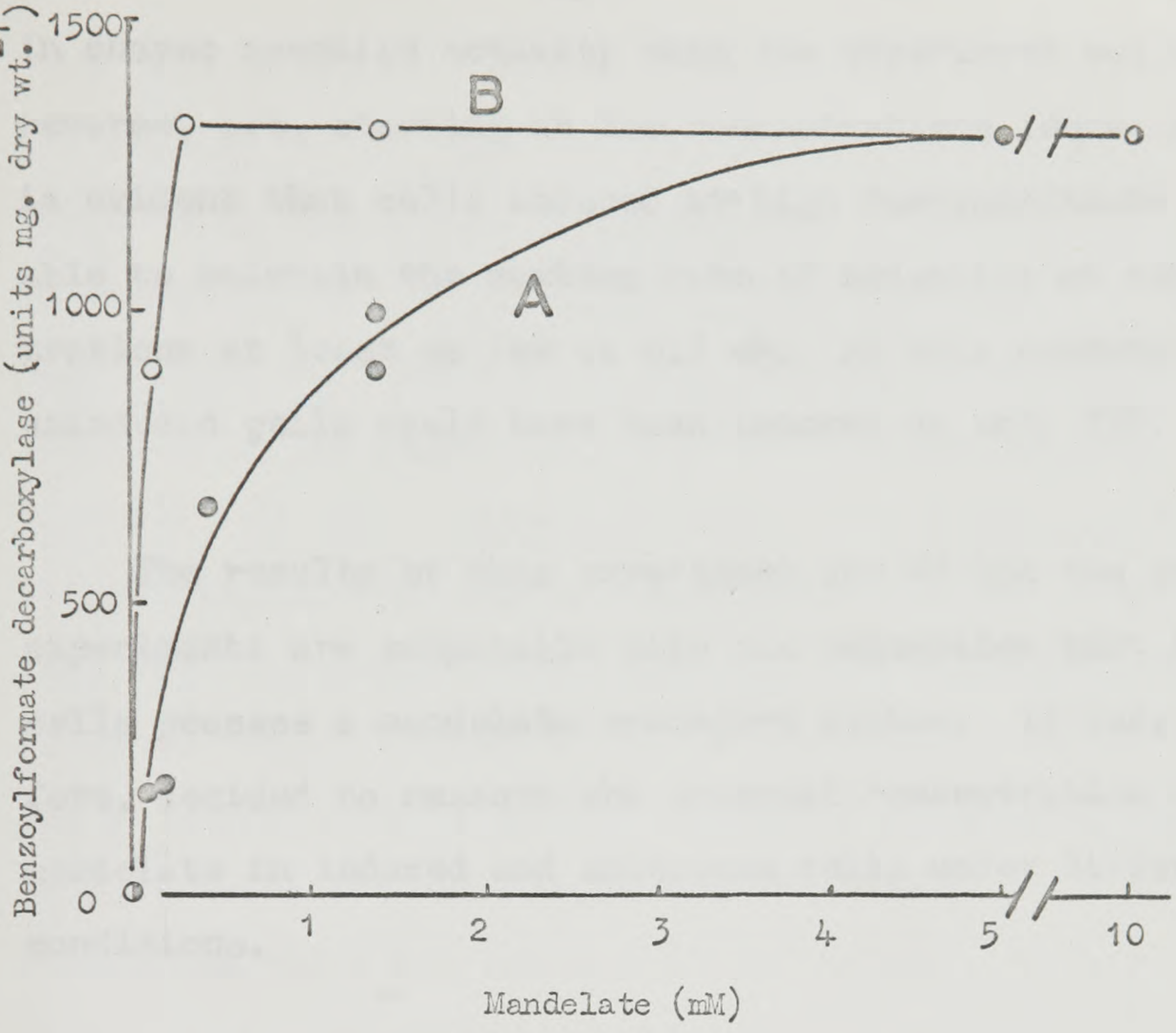


FIGURE 4.5

Induction of benzoylformate decarboxylase by mandelate in continuous culture.

Mutant D 57 (md$^-$) was grown to steady state in a nitrogen-limited chemostat on glucose (0.5 % w/v). Increasing concentrations of mandelate were added to the inflowing medium and the specific activity of benzoylformate decarboxylase determined at each steady state (●). After the culture had reached steady state with 10 mM mandelate, the experiment was repeated using decreasing concentrations of inducer (○). The dilution rate was 0.45 vol. hr.$^{-1}$ and the culture density 350 µg. dry wt.ml.$^{-1}$

in enzyme specific activity when the experiment was done in reverse, i.e. starting at low concentrations (curve A). It is evident that cells induced at high concentrations are able to maintain the maximum rate of induction at concentrations at least as low as 0.3 mM. At this concentration uninduced cells would have been induced to only 35%.

The results of this experiment and of the two previous experiments are compatible with the assumption that induced cells possess a mandelate transport system. It was, therefore, decided to measure the internal concentration of mandelate in induced and uninduced cells under different conditions.

6. Uptake of radioactive mandelate by md⁻ cells

The uptake of inducers into cells possessing active, concentrating permeases has been examined by a number of workers using essentially similar methods. These usually involve incubation of the cells in medium containing an energy source and radioactively labelled inducer. Samples are then removed and the cells collected on a membrane filter, washed quickly and counted to estimate the radioactive inducer accumulated by the cells (Képès, 1960; Winkler and Wilson, 1966).

This procedure was also used in this study to examine the ability of D57 (md$^-$) cells, preinduced by growth in the presence of mandelate, to accumulate radioactive mandelate. No evidence could be obtained by this method for any significant accumulation of the inducer. This agrees with the findings of Hegeman (1966) and it would appear that accumulation, if it occurred at all, was quite modest. Alternatively, the accumulated mandelate might have been lost from the cells during washing. Problems were encountered in trying to extend the sensitivity of this method and it was decided to resort to the older centrifugation method to separate the cells from the medium. This is capable of detecting lower internal concentrations and does not involve washing the cells. It has been widely used before (Gale, 1947; Roberts, Cowie, Abelson, Bolton and Britten, 1955; Cohen and Rickenberg, 1956; Boezi and De Moss, 1960). The method used in this study was a modification of the one used by Roberts et al. (1955).

Cells of mutant D57 (md$^-$) were grown in glucose-minimal medium with and without mandelate (10 mM) through at least three generations. At a density of 400-500 µg.dry wt.ml.$^{-1}$ they were harvested, washed twice in mandelate-free medium and resuspended at 100 times their original density in

fresh glucose-minimal medium containing chloramphenicol (50 $\mu g.ml.^{-1}$). Portions (10 ml) of this cell suspension were incubated with shaking at 30° with mandelate (^{14}C) at different external concentrations. The radioactive label was present at 1-2 x 10^5 $c.p.m.ml.^{-1}$. Samples (2.5 ml) were removed at different times and centrifuged at 20,000 x g for 5 minutes. The supernatant was carefully decanted, the walls of the centrifuge tube were wiped, the tube was carefully rinsed with 10 ml of medium without disturbing the pellet and, finally, the tube inverted to drain for 1-2 min. The cell pellet was then dispersed in trichloracetic acid (TCA) (10%, 1.0 ml) at 0° to release the intracellular mandelic acid. The cells were then recentrifuged in the cold, the supernatant removed and the pellet dried to constant weight in a vacuum oven at 70°. The dry weight of the pellet was then determined.

The amounts of radioactivity in the original supernatant from the incubation and from TCA-extracted cells were determined for each sample by scintillation counting. To ensure that the counting efficiencies in all the samples were comparable, an equal volume of 10 % TCA was added to the supernatant from the original incubation. Samples (0.5 - 1.0 ml) were prepared for counting by drying them in vials under vacuum at 70° for 1 hour. A disc of glass

fibre (Whatman, GFA) was placed in each vial to prevent splashing of the contents during the drying process. These conditions effectively dried the samples and removed trichloracetic acid without destroying the mandelate. Scintillation fluid (5 ml) was added when the vials were cool.

The radioactive mandelate that is present in the cell pellet, however, is not all contained within the cells; a certain amount of extracellular fluid is trapped in the interstices of the cells. Hence , determinations of the total water content of the pellets and the fraction that is intracellular had to be made.

Determination of the total water content of cell pellets. The water content of a cell pellet was calculated from its dry weight. Cell samples were centrifuged as above and the wet weight measured. After drying to constant weight, the dry weight was determined. This accounted for 20% of the wet weight and is similar to the figures obtained for E. coli by Roberts et al. (1955).

Determination of the intracellular and extracellular spaces of cell pellets. Compounds that are excluded from the interior of the cell can be used to estimate the extracellular and intracellular phases. Roberts et al. (1955)

have used serum albumin while Black and Gerhardt (1961) and Gerhardt and Black (1961) have used dextrans.

were
Cells/equilibrated in the presence of ^{131}I-serum albumin (human, M.W. 6.1×10^4, 0.3 $\mu Ci.ml.^{-1}$) or dextran (M.W. 2×10^6, 25 $mg.ml.^{-1}$) labelled with a blue dye (absorption maximum = 610 nm). The cells were separated by centrifugation and the supernatant decanted. The walls of the tube and the pellet surface were rinsed and the tube drained (as above). The pellet was dispersed in minimal medium (1.0 ml) and the cells recentrifuged. The second supernatant was removed and its radioactivity or optical density determined and compared with that of the supernatant from the original incubation. The cell pellets were dried to constant weight and their dry weights determined. The data were then sufficient to calculate the proportions of intracellular and extracellular water in the wet cell pellet (see Table 4.3).

The results obtained by the dextran and albumin methods were similar and showed that the extracellular fluid accounted for approximately 16% of the total water.

TABLE 4.3 Determination of the extracellular medium in cell pellets.

Cells were suspended in minimal medium (80 mg. in 4 ml) containing ^{131}I - serum albumin with carrier (1.5 μCi. 2 mg.). The cells were removed by centrifugation, the supernatant decanted and the pellet dispersed in unlabelled medium (1 ml). The cells were again centrifuged and the liquid removed (see Text). The ^{131}I in this and the original supernatant was estimated and the weighed pellet dried and reweighed. In a similar manner, the experiment was repeated with Dextran 2000 labelled with a blue dye (abs. max. 610 nm) and the optical densities of the original supernatant and the medium from the pellet resuspension measured.

Water content of pellet	Extracellular medium	Pellet resuspension	Medium in pellet	%
^{131}I experiment.				
0.3130 ml	556,736 cpm ml^{-1}	36,750 cpm	0.066 ml	17.0
0.3205	541,922	41,200	0.076	23.5
0.2910	430,180	23,200	0.054	18.5
Dextran experiment.				
0.3055 ml	4.60 (610 nm)	0.210 (610 nm)	0.060 ml	15.6
0.3125	4.60	0.202	0.058	14.7
0.2820	4.55	0.202	0.058	16.5

When the uptake of mandelate by cells was measured by the centrifugation method, it was shown (Figures 4.6 and 4.7) that:-

(i) there was a rapid equilibration in both induced and uninduced cells over the external mandelate concentration range 0.02 - 10.0 mM.

(ii) induced cells were able to accumulate mandelate at lower concentrations (0.02 and 0.25 mM) but, at higher concentrations, no accumulation was observed.

(iii) uninduced cells did not accumulate mandelate at any of the concentrations, but no exclusion of mandelate was noted.

SECTION THREE : REPRESSION OF THE MANDELATE REGULON BY BENZOATE

In the introduction to this chapter, it was suggested that the transient repression exerted by benzoate on the mandelate regulon (Figure 3.9) could have been due to inhibition of mandelate uptake or its conversion to an active inducer. Since the experiment involved the double mutant D570 1 (*mdl*$^-$, *benz*$^-$), metabolism of the benzoate was dismissed as an explanation for the 'escape' phenomenon. However, before proceeding further with an investigation of this example of repression, it was thought desirable to check that the mutant was indeed inactive towards benzoate.

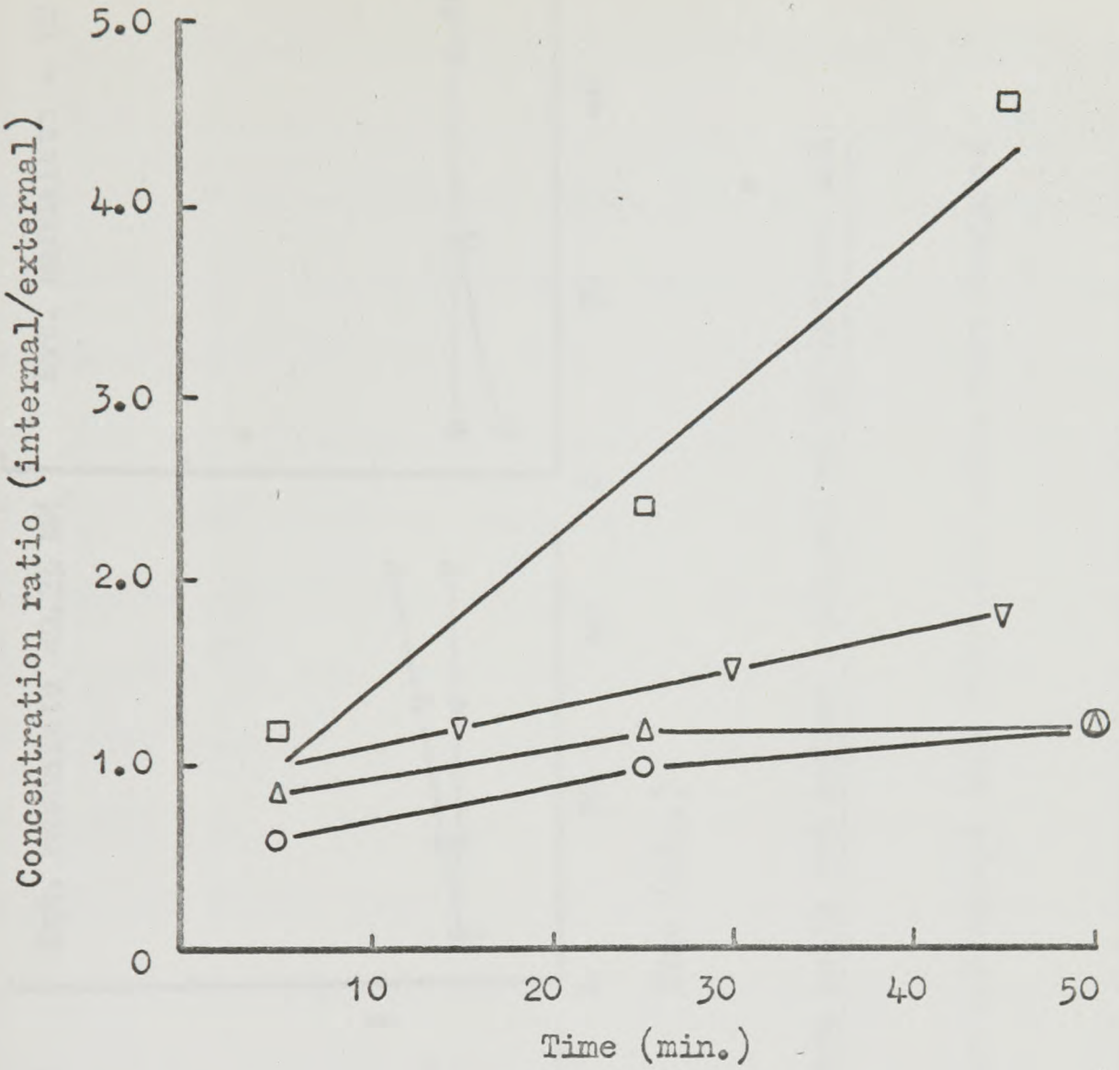


FIGURE 4.6

Distribution of mandelate between cells and medium : effect of external mandelate concentration.

Mutant D 57 (md$^-$) was pre-induced in glucose-minimal medium containing mandelate (10 mM). The cells were washed in mandelate-free medium and resuspended (40-50 mg. dry wt. ml.$^{-1}$) in fresh glucose-medium containing chloramphenicol (50 µg. ml.$^{-1}$). Mandelate-^{14}C (1-2 X 10^5 cpm. ml.$^{-1}$) was added with carrier to the desired external concentration. The ratio of internal to external label was then calculated (see Text) in samples (2.5 ml) removed from the incubation medium at various times. The external concentrations were 0.02 (□) 0.25 (▽) 2.5 (△) & 10 mM (○).

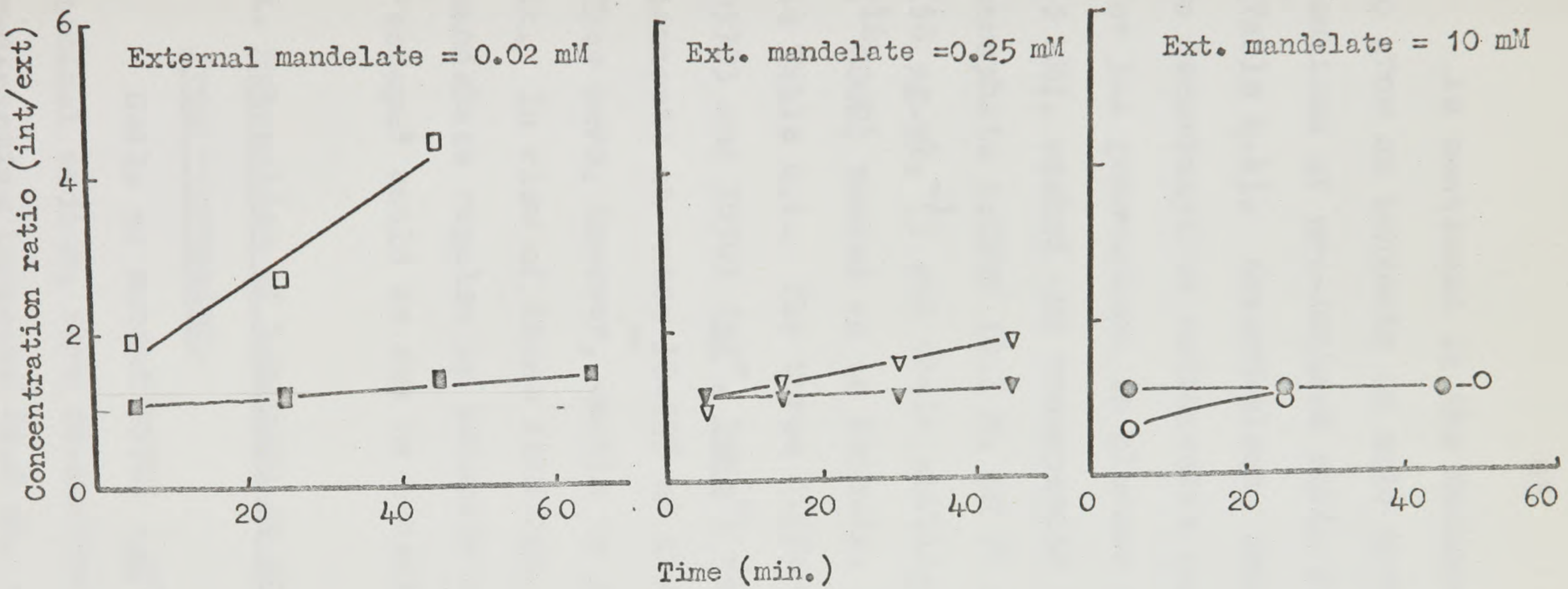


FIGURE 4.7.

Distribution of mandelate between cells and medium : comparison of induced and uninduced cells.

Legend same as for Figure 4.6 except that uninduced cells were compared with induced cells.

The open symbols represent the induced cells and the filled symbols are for the uninduced cells. External mandelate concentrations were 0.02 (□■), 0.25 (△▲) and 10.0 mM (○●).

As mentioned in the Methods Chapter, benz$^-$ mutants failed to grow on benzoate as sole carbon source, and washed suspensions of pre-induced cells failed to oxidise benzoate (Table 4.4). Nevertheless, these cells were found to be able to decarboxylate radioactive benzoate. Cells were grown for 3-4 generations in glucose medium containing benzoate (5 mM), washed and resuspended (500 µg. dry wt. ml.$^{-1}$) in phosphate buffer (0.1 M, pH 7) containing chloramphenicol (50 µg.ml.$^{-1}$) and their ability to decarboxylate benzoate (^{14}COOH) tested as in Methods. The results are presented in Table 4.4. The three different double mutants D5701, D5703 and Y6503 (md$^-$, benz$^-$) were able to decarboxylate benzoate at rates 10-20% of those of induced wild-type cells. They were, however, unable to grow on benzoate or to oxidise it. In view of these findings, the repression of the mandelate regulon by benzoate was re-examined to see whether 'escape' could be due to benzoate metabolism.

1. Metabolism of benzoate as an explanation for the 'escape' from repression.

Cells of mutant D5701 (md$^-$, benz$^-$), growing in glucose-minimal medium, were co-induced with mandelate (2.5 mM) and radioactive benzoate (0.2 mM, containing 0.002 µCi.ml.$^{-1}$). This concentration of benzoate was expected to allow 'escape'

TABLE 4.4.

Activity of benz$^-$ mutants of Pseudomonas putida towards benzoate.

Benz$^-$ cells were grown for 3-4 generations in glucose-minimal medium containing benzoate (5 mM), were washed and resuspended (500 µg. dry wt. ml.$^{-1}$) in phosphate buffer (0.1 M, pH 7) containing chloramphenicol (50 µg. ml.$^{-1}$). Their metabolic activity towards benzoate was measured and compared with wild-type cells similarly treated.

(i) The rate of oxidation of benzoate was measured by manometry.

(ii) The rate of decarboxylation of benzoate-^{14}COOH was determined.

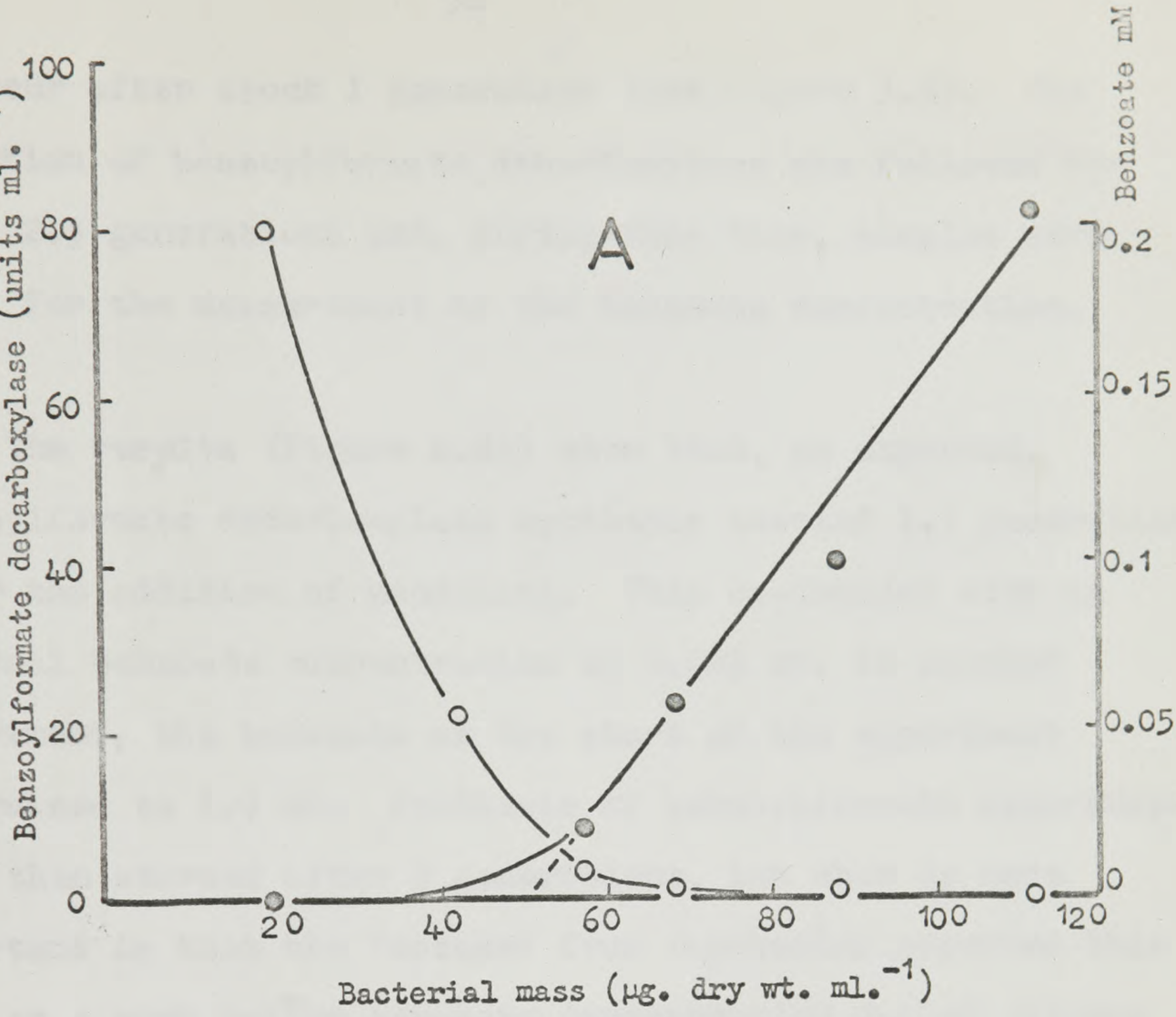
(iii) Growth on benzoate as sole carbon source was checked in liquid medium and on agar plates for 24-36 hr.

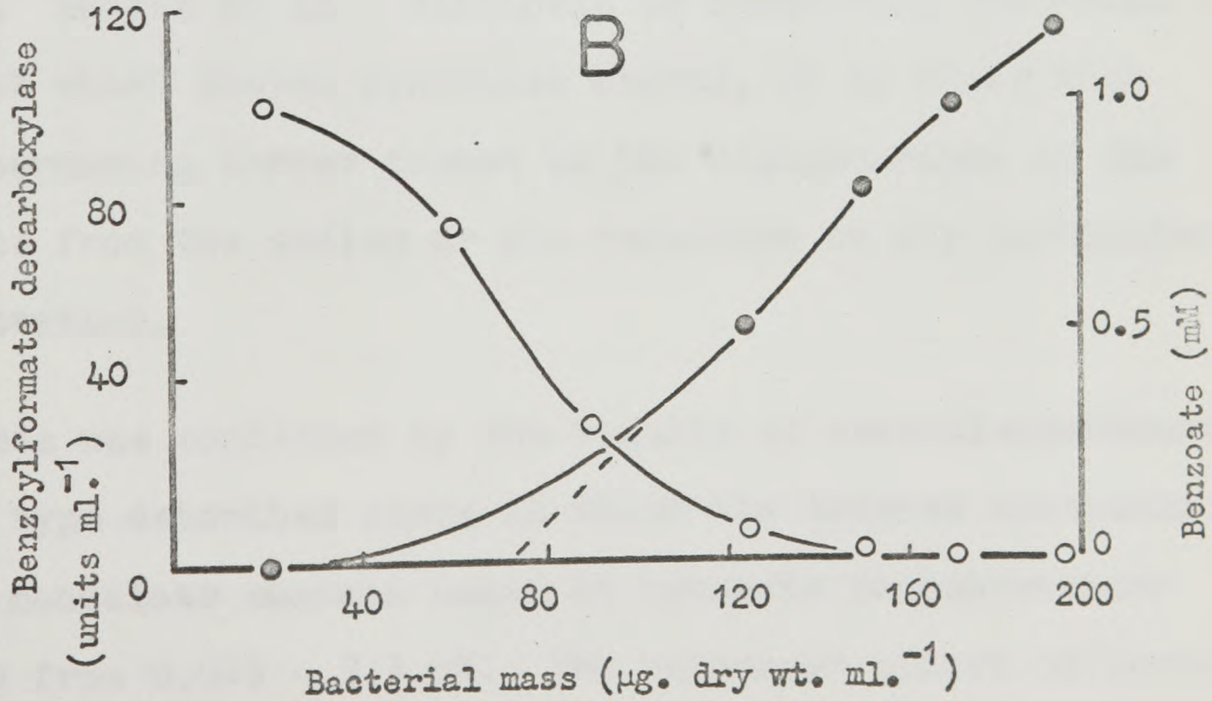
	Growth on benzoate	Benzoate oxidation		Decarboxylation	
		Sp. activity	% of WT	Sp. activity	% of WT
Wild-type	+	590	100	43.0	100
D 5701	-	5.8	1.0	5.8	13
D 5703	-	3.0	0.5	8.3	19
Y 6503	-	2.7	0.5	8.1	19

FIGURE 4.8 A + B

'Escape' of benzoylformate decarboxylase from transient repression by benzoate : effect of benzoate metabolism.

Mutant D 5701 (md$^-$, benz$^-$), growing exponentially in glucose-minimal medium, was induced with mandelate (2.5 mM) when the cell density was 20 µg. dry wt. ml.$^{-1}$ At the same time, benzoate-^{14}C (0.002 µCi. ml.$^{-1}$) was added at 0.2 (A) or 1.0 mM (B). Samples were taken at intervals thereafter and assayed for benzoylformate decarboxylase (●) and benzoate (O).


A
Benzoylformate decarboxylase (units ml.$^{-1}$)
Benzoate mM
100
80
60
40
20
0
0.2
0.15
0.1
0.05
0
20
40
60
80
100
120
Bacterial mass (µg. dry wt. ml.$^{-1}$)


B
Benzoylformate decarboxylase (units ml.$^{-1}$)
Benzoate (mM)
120
80
40
0
1.0
0.5
0
40
80
120
160
200
Bacterial mass (µg. dry wt. ml.$^{-1}$)

to occur after about 1 generation (see Figure 3.9). The induction of benzoylformate decarboxylase was followed for about 2.5 generations and, during this time, samples were taken for the measurement of the benzoate concentration.

The results (Figure 4.8A) show that, as expected, benzoylformate decarboxylase synthesis started 1.3 generations after the addition of mandelate. This co-incided with an external benzoate concentration of 0.025 mM. In another experiment, the benzoate at the start of the experiment was raised to 1.0 mM. Synthesis of benzoylformate decarboxylase then started after 2 generations, but what is more important is that the 'escape' from repression occurred this time at a much higher benzoate concentration 0.5 mM (Figure 4.8 B). Whilst it is difficult to accurately determine the point at which enzyme synthesis starts, it is clear that the determining factor cannot be the disappearance of the benzoate from the medium or its reduction to any particular concentration.

This was confirmed by the results of several experiments of the type described above in which the induced synthesis of the mandelate enzymes began at benzoate concentrations varying from 0.025 - 7.3 mM. The repressor effect of benzoate

was not significantly different in benz$^-$ and benz$^+$ phenotypes (Table 4.5).

A result that is of interest for the purposes of this study is that shown in Figure 4.9. Wild-type cells were induced in glucose medium with 10 mM mandelate and 7.5 mM benzoate. 'Escape' occurred when the benzoate concentration had hardly altered (7.3 mM). However, after the 'escape' there was residual repression; the rate of synthesis of benzoylformate decarboxylase was only 30% of that in the control. These are the sorts of concentrations that were later used in the mixed substrate experiments (see Chapter 6).

2. Effect of preinduction of the mandelate regulon or of the benzoate regulon on the repressor effect of benzoate

Since 'escape' from benzoate repression did not appear to be the result of benzoate metabolism an alternative explanation was sought. One possibility is that when uninduced cells are suddenly exposed to external benzoate, there is a sudden increase in the intracellular pool, and that this results in severe repression of the mandelate enzymes. Later when the cells have adapted to metabolising benzoate, they are able to keep the level of internal benzoate low and the repression is relieved.

TABLE 4.5

Effect of inducer and repressor concentrations on the induction of benzoylformate decarboxylase by mandelate in the presence of benzoate.

Cells of different phenotypes were grown in glucose medium and co-induced with mandelate and benzoate. Benzoylformate decarboxylase was assayed and the benzoate concentration in the medium monitored. The start of enzyme synthesis was determined by extrapolation of the differential plot. The rate of synthesis was compared with that in a control culture, induced in the absence of benzoate.

Phenotype	Mandelate (mM)	Initial benzoate (mM)	Benzoate at start of induction (mM)	Lag gen.	Repression
md⁻ benz⁻	2.5	0.05	ND	0.55	-
		0.2	0.025	1.2	ND
		0.2	ND	0.95	-
		1.0	ND	2.0	-
		1.0	0.4	2.0	ND
		1.0	0.55	1.5	ND
	5.0	0.05	ND	0.55	-
		0.2	ND	0.64	-
Benz⁻	2.5	0.6	0.22	1.6	ND
		1.0	0.6	1.6	ND
md⁻	2.5	1.0	0.6	1.6	ND
Wild-type	2.5	1.0	0.8	1.6	-
		1.0	0.5	1.6	ND
	10.0	7.5	7.3	0.8	+
		7.5	7.3	1.6	+

ND = not determined.

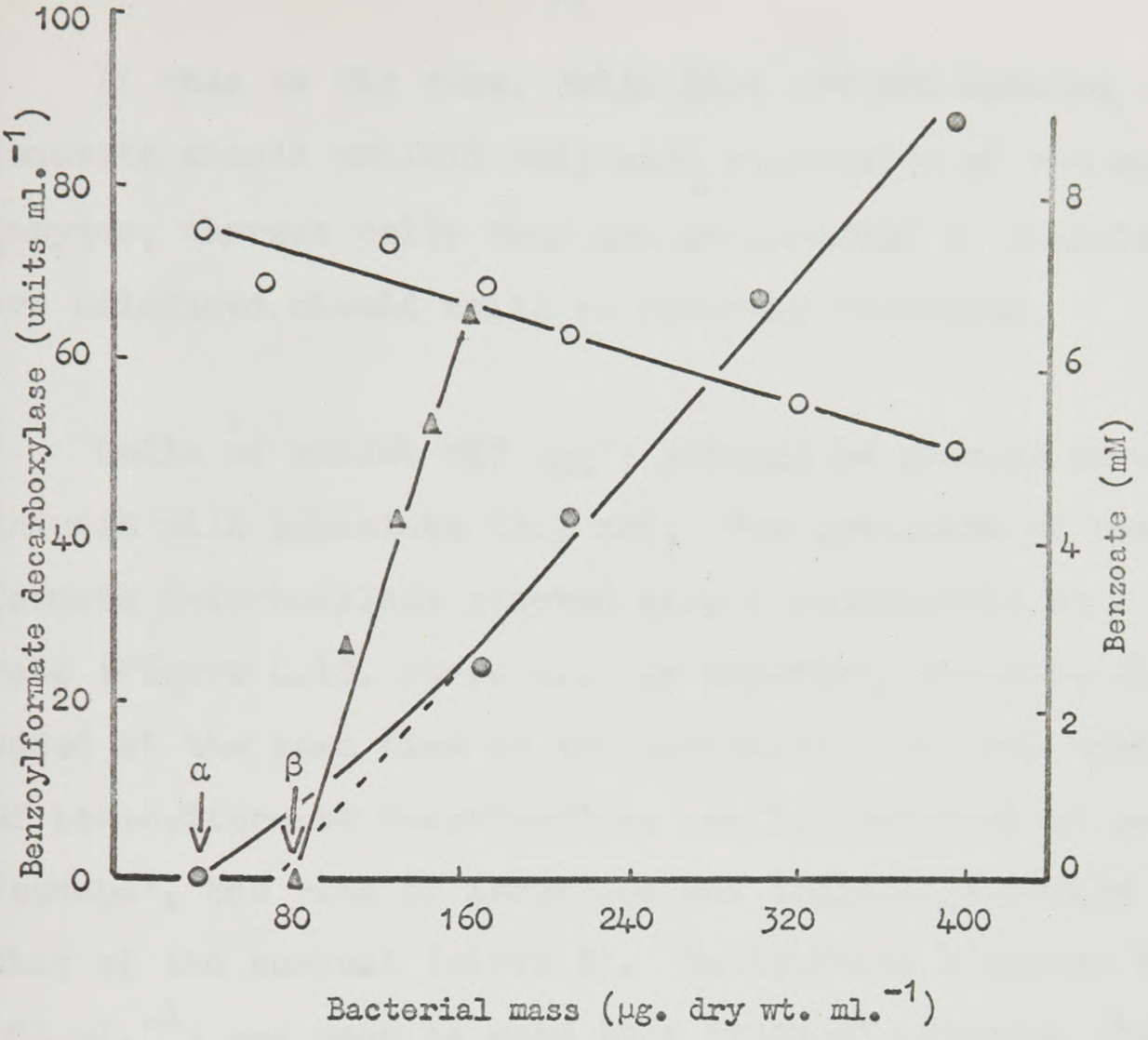


FIGURE 4.9

Repression of the mandelate regulon by benzoate after 'escape' has occurred.

Wild-type cells, growing exponentially in glucose-minimal medium, were induced with mandelate (10 mM) and benzoate-C^{14} 7.5mM (0.002 μCi. ml.$^{-1}$) at α. Samples were taken at intervals thereafter and assayed for benzoylformate decarboxylase (●) and benzoate (O).

In a control flask, benzoylformate decarboxylase was induced with mandelate (10 mM) at β (Δ).

If this is the case, cells that are pre-induced with benzoate should exhibit only mild repression of the mandelate enzymes, whereas cells that are pre-induced by mandelate or are uninduced should still be severely repressed.

Cells of mutant D57 (md$^-$) growing on glucose were induced with mandelate (2.5 mM). The synthesis of benzoylformate decarboxylase started almost immediately at a high rate (Figure 4.10, curve A). As expected, benzoate (1 mM) added at the same time as the mandelate prevented synthesis of benzoylformate decarboxylase for 1.5 generations and, after 'escape', the rate of induction was indistinguishable from that of the control (curve B). Radioactive benzoate (0.002 μCi.ml.$^{-1}$) was used to show that 'escape' occurred when the benzoate concentration had been reduced to 0.25 mM.

In cells that received benzoate about 60 minutes before the mandelate, the lag lasted 1.25 generations (curve C) and enzyme synthesis started when the benzoate concentration had been reduced to 0.2 mM. [The benzoate was originally added at 1.2 mM so that, when the mandelate was added, the benzoate concentration would have been reduced to about 1.0 mM].

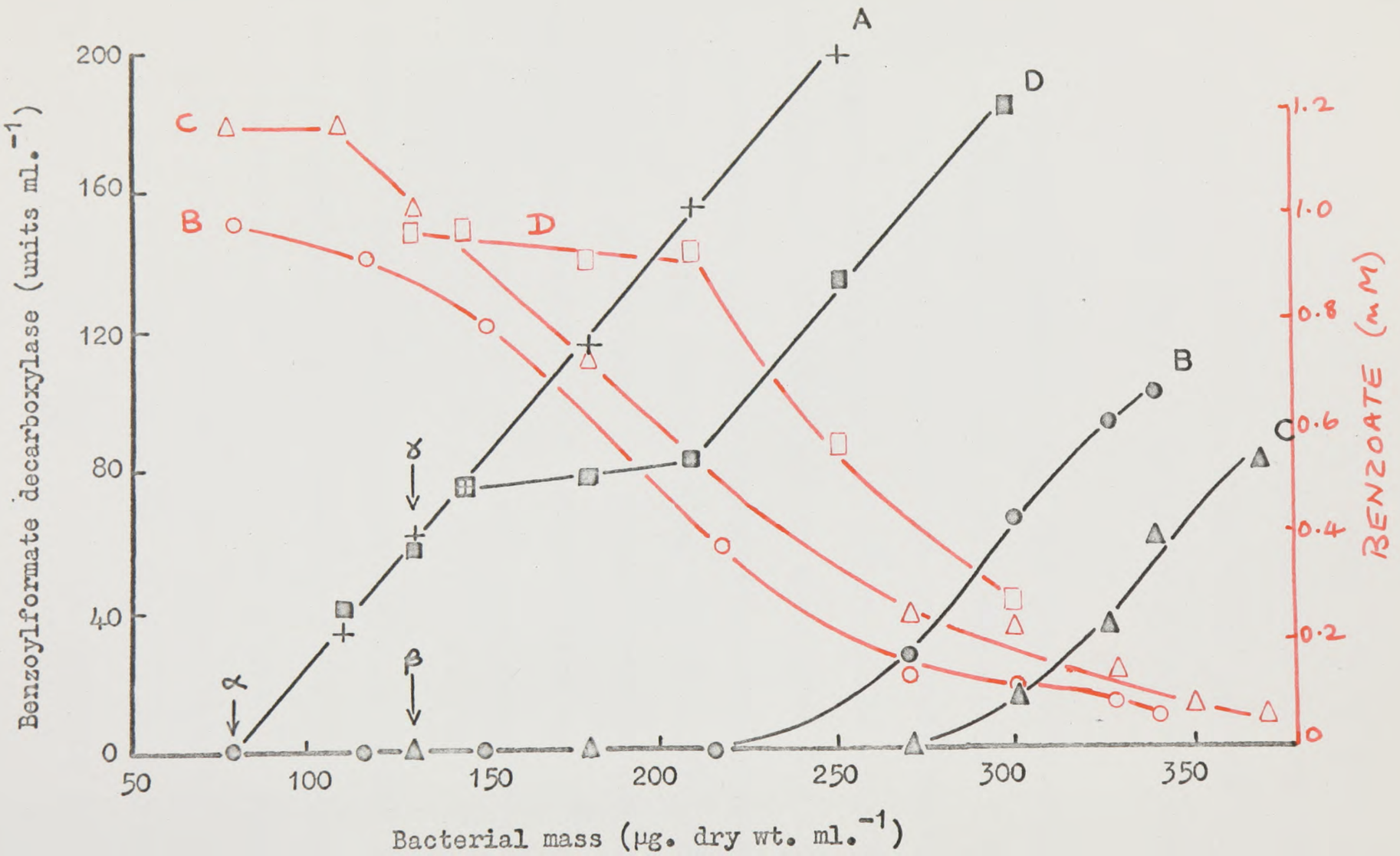

Benzoylformate decarboxylase (units ml.$^{-1}$)
200
160
120
80
40
0
50
100
150
200
250
300
350
Bacterial mass (µg. dry wt. ml.$^{-1}$)
BENZOATE (mM)
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
α
β

FIGURE 4-10.

Effect of pre-induction of the mandelate or benzoate regulons on the transient repression of benzoylformate decarboxylase by benzoate.

Mutant D57 (md$^-$) was grown in glucose-minimal medium and samples of the culture were taken for the assay of benzoylformate decarboxylase (—) and the estimation of the benzoate concentration of the medium (—) after the following additions :

Culture A (crosses). Mandelate (2.5 mM) was added at α.

Culture B (circles). Mandelate (2.5 mM) and benzoate-^{14}COOH (1.0 mM, 0.001 μCi. ml.$^{-1}$) were both added at α.

Culture C (triangles). Benzoate-^{14}COOH (1.2 mM, 0.001 μCi. ml.$^{-1}$) was added at α; mandelate (2.5 mM) was added at β.

Culture D (squares). Mandelate (2.5 mM) was added at α followed by Benzoate-^{14}COOH (1.0 mM, 0.001 μ Ci. ml.$^{-1}$) at γ.

The induction rate was again identical to that in the control. It thus appears that an ability to metabolise benzoate does not prevent the repression of the mandelate enzymes.

In contrast, if mandelate was added 60 min. before the benzoate, the duration of the effect was very much reduced. Shortly after the addition of the benzoate, induction ceased for about 0.5 generations and then recommenced at the same rate as before (curve D). The benzoate concentration at the point of 'escape' was 0.85 mM.

The possession of a partially induced mandelate enzyme system, therefore, mitigates the effects of benzoate on the rate of induction by mandelate much more effectively than does the ability to utilise benzoate. If induced cells possess a mandelate transport system and this is not susceptible to benzoate inhibition, then the difference between mandelate-induced and uninduced cells would be explained. Accordingly, the effect of benzoate on the entry of mandelate into uninduced and induced cells was investigated.

3. Effect of benzoate on the intracellular concentration of mandelate

The centrifugation method described above was used to measure the effect of benzoate on uptake of radioactive mandelate. The relative concentrations of benzoate and mandelate that were used would have produced marked repressor effects in uninduced cells.

Benzoate (1.0 mM) had no effect on the uptake of mandelate (2.5 mM) in induced (md^-) cells; a rapid equilibration of mandelate across the cell membrane was still seen (Figure 4.11A). At these concentrations, benzoate also had no effect on the equilibration of mandelate in uninduced cells (Figure 4.11 B). Only when a low external concentration of mandelate (0.02 mM) was used with induced cells did benzoate (1 mM) have any effect on the uptake of mandelate. The accumulation of mandelate normally observed in this situation was prevented for about 40 minutes. Afterwards, the cells started to accumulate mandelate (Figure 4.11 C).

SECTION FOUR : DISCUSSION

As shown in Chapter 3, submaximum concentrations of mandelate, besides leading to submaximum rates of induction of the mandelate regulon, also introduced a delay in the

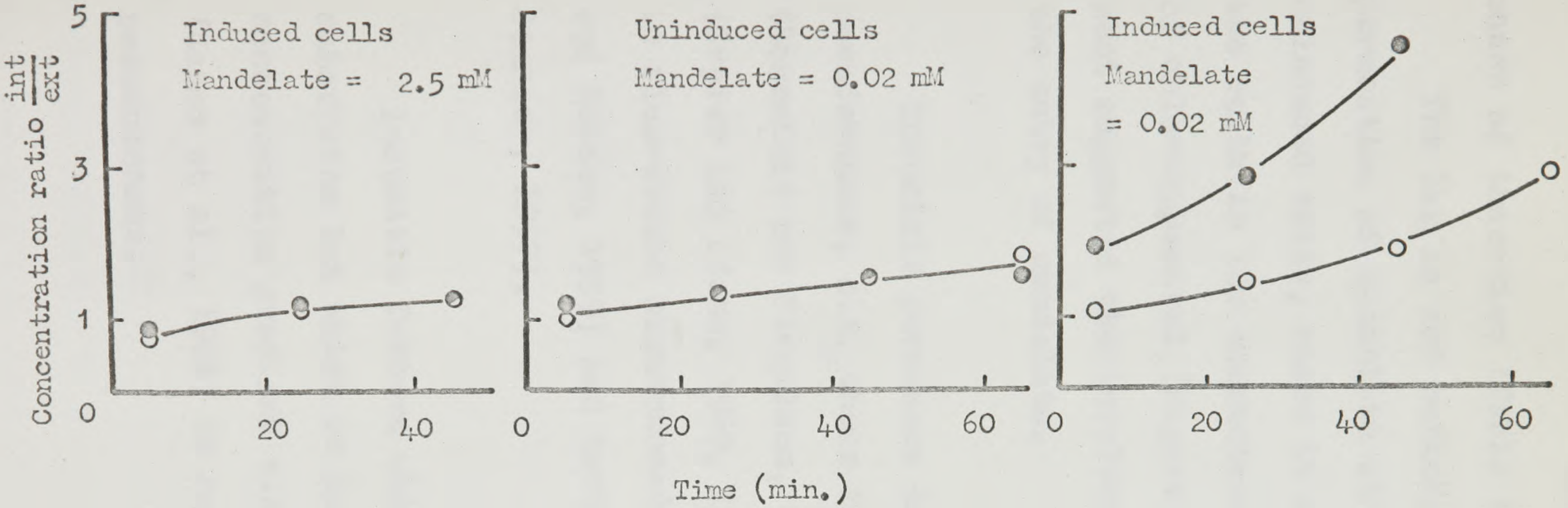


FIGURE 4.11

Distribution of mandelate between cells and medium : effect of external benzoate.

Induced and uninduced cells of mutant D 57 (md$^-$) were incubated in glucose-minimal medium containing chloramphenicol (50 µg. ml.$^{-1}$) with mandelate-^{14}COOH (1-2 X 10^6 cpm. ml.$^{-1}$) at a concentration of 0.02 or 2.5 mM. The ratio of intracellular to extracellular label was calculated as in Text when 1.0 mM benzoate was present (O) or absent (●) from the medium.

onset of induction (Table 4.1).

The lag is not satisfactorily explained by slow permeation of mandelate at low external concentrations into uninduced cells, since it was not eliminated by equilibrating the cells in low concentrations of mandelate in the presence of chloramphenicol (Figure 4.2). The data from this experiment suggested the involvement of an inducible protein in the entry of mandelate.

Inducible permeases have been frequently described in pseudomonads, e.g. those for tryptophan in P. acidovorans (Rosenfeld and Fiegelson, 1969), hydroxyproline in P. putida (Gryder and Adams, 1969, 1970), Krebs cycle intermediates in fluorescent pseudomonads (Kogut and Podoski, 1953; Clarke and Meadow, 1959) and tartrate (Shilo, 1957; Shilo and Stanier, 1957).

Inducible factors which facilitate the passage of substrates but which do not result in accumulation against a concentration gradient e.g. the glycerol factor in E. coli (Sanno et al., 1968) do not seem to have been described in pseudomonads.

Some of the data on the induction of the mandelate system can be interpreted in terms of inducer entry. A pre-induction or maintenance effect was observed under several experimental conditions; cells induced at high inducer concentrations were able to maintain high rates of enzyme synthesis when transferred to lower concentrations that normally resulted in sub-maximal rates of synthesis. Such a maintenance or pre-induction effect has been described for the lactose system of E. coli (Monod, 1956; Novick and Weiner, 1957; Cohn and Horibata, 1959). In this case, it has been explained on the basis of the known action of the inducible β-galactoside permease. This is able to accumulate β-galactoside inducers within the cell even in the presence of quite low external concentrations. A high rate of induction can thus be maintained by pre-induced cells even in low inducer concentrations. Cryptic cells, possessing no permease, are not able to support high rates of β-galactosidase synthesis at low external concentrations of galactoside (Cohen and Monod, 1957; Herzenberg, 1959; Cohn and Horibata, 1959).

Investigation of the mandelate concentrating ability of induced cells showed that, at low external concentrations, a slow accumulation took place. After 45-50 minutes, cells in

0.02 mM mandelate had concentrated the inducer 4-5fold and in 0.25 mM by about two-fold. The internal concentration did not appear to have reached equilibrium and, in time, even higher intracellular concentrations might have been achieved. This degree of internal concentration may well be enough to account for the maintenance effects.

However, in the case of β-galactosidase, the kinetics of induction in uninduced cells differ from those observed in the mandelate system. In the lac system, sub-maximum inducer concentrations, that show maintenance with pre-induced cells, support accelerating, autocatalytic rates of enzyme synthesis (Cohen and Monod, 1957; Monod, 1956; Herzenberg, 1959). Initially uninduced cells have very little galactoside permease and this limits inducer entry. In time, an increasing proportion of the cells become fully induced for the lac operon (including the permease) and thus the rate of β-galactosidase synthesis in the culture accelerates up to the maximum. Once it is fully induced for the lac permease, a cell can maintain itself in this state even in low external inducer concentrations.

In the mandelate system, however, concentrations that nonetheless show maintenance support only submaximum linear

rates of synthesis. To account for this finding it has to be proposed that the rate of synthesis of the mandelate permease is too slow to keep up with the rate of cell division so that the cells divide before they possess enough permease to ensure that each daughter cell also inherits enough permease to maintain itself. This would perhaps not occur once the permease had been fully induced.

The pre-inductive effect could also have been due to the need to synthesise an inducible facilitated diffusion carrier which rapidly equilibrates mandelate in induced but not uninduced cells. However, a direct investigation of the intracellular mandelate concentrations failed to show that mandelate was excluded from uninduced cells; equilibration was rapid in both induced and uninduced cells. The problem of explaining the kinetics of induction of the mandelate regulon in uninduced cells at low inducer concentrations is no easier with this model than with the permease hypothesis.

Conversion of mandelate to an active inducer by an inducible enzyme was another possibility that was considered. Conversion in significant amounts to an unidentified, stable compound that did not retain the 1-carbon of mandelate was

ruled out, but minute amounts of such a compound or a compound retaining the 1-carbon could be involved and function as an active inducer. Once again the kinetics of induction are not easy to explain on this basis.

The action of benzoate on induction by mandelate is quite unlike that of p-OH benzoate. 'Escape' from benzoate repression was not associated with any particular concentration of benzoate nor was repression due to a transient increase in the internal benzoate pool. If this had been so, cells pre-induced by benzoate would have suffered less repression than uninduced cells. No such effect was observed. However, what did seem to reduce the benzoate repression was pre-induction of the mandelate system.

Benzoate might inhibit the entry of mandelate into uninduced cells, but not into induced cells which possess enough mandelate entry factor to overcome the inhibitory effect of benzoate. However a direct investigation of the uptake of mandelate in the presence of benzoate failed to reveal any significant effect of benzoate on uninduced cells especially at the concentrations of benzoate and mandelate that normally repressed the synthesis of the mandelate enzymes.

The only effect of benzoate on mandelate uptake that was found was that the repressor seemed to delay the accumulation of mandelate at low external concentrations by induced cells, but even then, after about 40 minutes, the accumulating ability of the cells seemed to have recovered.

If mandelate is converted to an active inducer, benzoate could be acting as an inhibitor or a repressor of the enzyme concerned. Cells induced by mandelate would suffer a smaller benzoate effect because they would be well endowed with the converting enzyme.

The fact that high benzoate concentrations also result in continued repression after the severe transient repression could be due to the inability of the induced mandelate transport system or converting enzyme to function fully in the presence of such high repressor concentrations. Benzoate might of course also have a direct repressor effect at these concentrations.

Although the mechanisms of mandelate enzyme induction and benzoate repression are not clear, what is important for the purposes of this study is that, because of the pre-inductive effects, the reaction to benzoate of cells already

growing on mandelate should be different from that of un-induced cells. This could affect the transition period following changeover from growth on a single aromatic carbon source to a two-substrate mixture.

CHAPTER FIVE

ENZYMIC ADAPTATION TO MIXED AROMATIC SUBSTRATES : PREDICTIONS

ENZYMIC ADAPTATION TO MIXED AROMATIC SUBSTRATES: PREDICTIONS

Introduction

The purpose of this chapter is to predict the enzymic adaptation of Pseudomonas putida to mixed aromatic substrates using data already described. From the values of the induction and repression constants for the enzyme systems, it should be possible to answer the following questions concerning the steady state enzyme concentrations of cells in media containing more than one aromatic carbon source:-

1. Which enzymes will be preferentially induced?
2. If two pathways are induced, what is the relative extent of their formation and how is it affected by substrate concentration?
3. Is the steady state in a particular mixed substrate medium determined by the previous growth history of the cells, i.e. will the steady state be affected if one of the pathways is already established?

Enzyme adaptation to mixed substrates could be investigated by transferring cells from a medium containing glucose or succinate directly into the medium containing the mixed aromatic substrates and observing the induction of the aromatic enzymes. Under these conditions, the cells

would be initially unable to grow and adaptation to the new medium would be slow. In a growing culture, the induction is more rapid and the kinetics simpler. Hence these predictions are made for a culture growing on one of the aromatic substrates with the second added later.

The substrates chosen for these mixed-substrate investigations were mandelate, benzoate and p-OH benzoate. There are thus three combinations of pairs of substrates to consider, and each steady state can be approached by growth on either substrate of the pair as the first carbon source.

	FIRST SUBSTRATE	MIXTURE
1.	Mandelate	Mandelate + p-OH benzoate
2.	p-OH benzoate	
3.	Benzoate	Benzoate + mandelate
4.	Mandelate	
5.	p-OH Benzoate	p-OH Benzoate + Benzoate
6.	Benzoate	

The enzymic changes occurring in the cultures described by 1-6 above will be predicted by considering the data obtained on induction and repression for the relevant

aromatic carbon sources. However, to predict the behaviour of the organism in media containing more than one aromatic carbon source, it is necessary to assume that the values of K_{ind} and K_{rep} obtained from experiments in which cells were grown on glucose are valid in situations where glucose is absent. There seems to be reasonable evidence to justify this assumption (see Chapter 3).

1. Mandelate
2. p-OH Benzoate

→ Mandelate + p-OH Benzoate

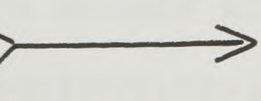
From the data of Table 3.3, p-OH benzoate hydroxylase should be fully induced at 0.02 mM whereas the mandelate enzymes require an inducer concentration 50-100 times higher than this for full induction. Furthermore, while mandelate has no repressor effect on the induction of p-OH benzoate hydroxylase (Table 3.4), p-OH benzoate exercises severe repression on the mandelate enzymes (Table 3.6). The K_{rep} for this repression is about 0.02 mM (Figure 3.7).

Cells growing on p-OH benzoate should fail to synthesise any of the mandelate group of enzymes when mandelate is added at a reasonable concentration, provided of course that the steady state concentration of p-OH benzoate is substantially in excess of the K_{rep} (say 0.1 mM) . Cells

already growing on mandelate should stop synthesising mandelate enzymes immediately a substantial (> 0.1 mM) concentration of p-OH benzoate is added. There should thus be a changeover period in which both enzyme systems would be present. The mandelate enzymes, completely repressed, would be diluted out by growth while the specific activity of p-OH benzoate hydroxylase should increase and eventually reach a value associated with fully induced cells.

Since repression by p-OH benzoate, though severe, is partially reversed by high concentrations of inducer, it should be possible partly to induce the mandelate enzymes at very high ratios of mandelate to p-OH benzoate. Obviously, the mandelate enzymes should be induced in the presence of p-OH benzoate, if the concentration of the latter is lower than the K_{rep}.

3. Benzoate
4. Mandelate



Benzoate + Mandelate

Full induction of the mandelate system should occur at inducer concentrations above 0.75 - 2.0 mM, while benzoate oxidase should be fully induced at slightly lower concentrations, 0.5 - 1.0 mM (Table 3.3).

Mandelate has no repressor effect on benzoate oxidase (Table 3.4), while benzoate has a complex and undetermined repressor effect on the mandelate enzymes (Chapter 4). For the purposes of this study what matters is that benzoate exerts a transient, severe effect on the induction of uninduced cells by mandelate and that mandelate-induced cells are much less affected. The duration of this transient repression seems to be determined by the mandelate and benzoate concentrations used (Table 4.5). At high concentrations of benzoate a further, lasting repression is to be expected after 'escape' has taken place (Figure 4.9).

Thus, when mandelate is added to cells growing on benzoate as sole carbon source, there should be a delay before the mandelate enzymes are synthesised and its duration should depend on the substrate concentrations used. The extent of repression of the mandelate enzymes at steady state should also depend on the steady state substrate concentrations. Benzoate oxidase activity should be maximal at all benzoate concentrations in excess of 1.0 mM.

Cells that are already growing on mandelate should not experience a significant period of intense repression

following the addition of benzoate. The steady state specific activity of mandelate enzymes in this case should again be determined by the substrate concentrations. When a long-term repression is expected, the specific activity of the mandelate enzymes should decrease steadily towards the new steady-state activity. Cells grown on mandelate are, of course, sequentially induced for benzoate oxidase; its activity in such cells will presumably depend on the internal benzoate concentration and this may be sub-optimal. Following the addition of benzoate to these cells, there may thus be an increase in the specific activity of benzoate oxidase.

5. p-OH Benzoate
6. Benzoate → p-OH Benzoate + benzoate

In the absence of repressors, p-OH benzoate hydroxylase should be fully induced by p-OH benzoate concentrations in excess of 0.02 mM (Table 3.3). Benzoate oxidase, on the other hand, should be fully induced by benzoate concentrations 25-50 times higher (Table 3.3). There is little or no repressor effect of p-OH benzoate on induction by benzoate (Table 3.4), while benzoate exerts a moderate repressor effect on p-OH benzoate hydroxylase (Table 3.6). The K_{rep} is about 1.0 mM (Figure 3.11).

When a culture is growing on p-OH benzoate, the addition of benzoate at concentrations substantially in excess of the K_{rep} should cause an immediate marked repression of p-OH benzoate hydroxylase. As a consequence, the specific activity of p-OH benzoate hydroxylase should decline towards its new steady state value. Meanwhile, benzoate at these concentrations should immediately induce benzoate oxidase at maximum rates.

On the other hand, cells already growing on benzoate should maintain their level of benzoate oxidase after p-OH benzoate is added, provided the benzoate concentration remains unaltered. Immediately after the addition of p-OH benzoate, p-OH benzoate hydroxylase should start to be synthesised and its steady state specific activity should be determined by the relative concentrations of benzoate and p-OH benzoate, but should be lower than that obtained by growth on p-OH benzoate alone.

Predictions regarding diauxic growth in media containing aromatic substrates

The conditions that are necessary for establishing diauxic growth have already been discussed; severe repression

of the enzymes of the less preferred substrate is necessary together with a limitation on the rate of entry of the inducer (Introduction).

Diauxic growth should not therefore be observed in cultures grown on p-OH benzoate + benzoate since the repression of p-OH benzoate hydroxylase by benzoate is unlikely to be complete at most substrate concentrations and there is no evidence of any entry effect. The transient and incomplete nature of the repressor effect of benzoate on the induction of the mandelate enzymes makes it unlikely that diauxic growth would be observed on mandelate + benzoate. The repression of the mandelate enzymes by p-OH benzoate is certainly severe and there is possibly an inducible entry system for mandelate. However there is no evidence that p-OH benzoate affects the entry of mandelate. Although diauxie might occur in media containing these two substrates, it could not be confidently predicted.

Substrate utilisation by cultures growing on more than one aromatic substrate

The predictions of this chapter, summarised in Table 5.1, refer only to the enzymic response of cells to more than one aromatic substrate. No predictions have been

TABLE 5-1.

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

The predictions in this Table are based on the data of Chapters 3 and 4. Wild-type cells were considered to be grown on one aromatic substrate and then to receive an additional aromatic carbon source. Both substrates were assumed to be present at concentrations sufficient for full induction in the absence of repressors. The Table summarises the predicted changes in specific activities of the inducible enzymes following the change-over to growth on alternative aromatic carbon sources.

FIRST SUBSTRATE

SECOND SUBSTRATE (added to first)	MANDELATE	BENZOATE	p-OH BENZOATE
MANDELATE		Benzoate Oxidase : Remains fully induced. Mandelate Enzymes : Lag, then rise to partially induced value.	p-OH Benzoate Hydroxylase : Remains fully induced. Mandelate Enzymes : Totally repressed.
BENZOATE	Mandelate Enzymes : Fall to partially induced value. Benzoate Oxidase : May rise to new value.		p-OH Benzoate Hydroxylase : Falls to lower value. Benzoate Oxidase : Rises to fully induced value.
p-OH BENZOATE	Mandelate Enzymes : Totally repressed p-OH Benzoate Hydroxylase : Rises to fully induced value.	Benzoate Oxidase : Remains fully induced. p-OH Benzoate Hydroxylase : Rises to partially induced value.	

made about the relative rates of utilisation of the two substrates. Clearly if the enzymes for one substrate are completely or almost completely repressed, that substrate cannot be metabolised. However, where the induction and repression constants indicate that both enzyme systems should be present, a prediction about the relative rates of utilisation of the two substrates would require additional kinetic data (see Introduction).

Experiments testing the predictions of this chapter are described in the next chapter.

CHAPTER SIX

ENZYMIC ADAPTATION TO MIXED AROMATIC SUBSTRATES: EXPERIMENTAL

ENZYMIC ADAPTATION TO MIXED AROMATIC SUBSTRATES: EXPERIMENTAL

SECTION ONE : INTRODUCTION

Relative merits of batch and continuous culture methods. The enzymic response of the organism to different combinations and proportions of substrates in the medium can be investigated in batch or continuous culture. Batch cultures are convenient in that they are more easily handled, but suffer from certain disadvantages. The worst of these results from the fact that the concentrations of the substrates may vary continuously, particularly when the inducer concentrations are low. Whenever this happens, it will complicate any conclusions one might wish to draw about the response of the organism to competing substrates. Another disadvantage of the batch culture method is concerned with the short time over which meaningful enzyme measurements can be made following a changeover to mixed substrates while still maintaining exponential growth (3-4 generations).

While batch methods can give an indication of the initial enzymic response to two inducers, steady state conditions can be conveniently achieved by means of a chemostat. The theoretical and practical aspects of this

device have been extensively reviewed (Powell, 1965; Málek, 1967; Tempest, 1970). For the purposes of this study, the chemostat confers the ability to investigate enzyme variation under conditions where the substrate concentrations can be held constant at any pre-determined level, and the experiment itself can be run for an indefinite time, enabling steady state to be achieved. As mentioned in the Introduction, the chemostat has been used by many workers to investigate the roles of induction and repression in inducible systems (Mandelstam, 1962; Clarke et al., 1968; Clarke and Lilly, 1969; Jensen and Neidhardt, 1969; Silver and Mateles, 1969).

In this study, batch cultures were used first and they provided a useful indication of the initial enzymic response of cells to mixed substrates. The chemostat was then used to investigate the predictions that were made for the steady state.

SECTION TWO : ENZYMIC ADAPTATION IN BATCH CULTURE

A. METHOD

Wild-type cells were inoculated from a broth culture into minimal medium containing a single aromatic carbon source at 10 mM. Overnight growth in this medium was followed by re-inoculation into more of the same medium.

Within two generations, the growth was usually exponential and the second carbon source, also at 10 mM, was then added. Immediately before this addition, and at about 0.5 generation intervals thereafter, samples of suitable size were removed for the assay of the two enzyme systems. Benzoylformate decarboxylase, benzoate oxidase and p-OH benzoate hydroxylase were the enzymes chosen for assay when mandelate, benzoate and p-OH benzoate were the corresponding substrates. By starting the experiment at a low cell density (40-50 µg.dry wt.ml.$^{-1}$), the enzymic response could be followed for about 3.5 generations when the end of the exponential phase was approaching (500-600 µg.dry wt.ml.$^{-1}$).

B. RESULTS

1. Mandelate ——→ mandelate + p-OH benzoate

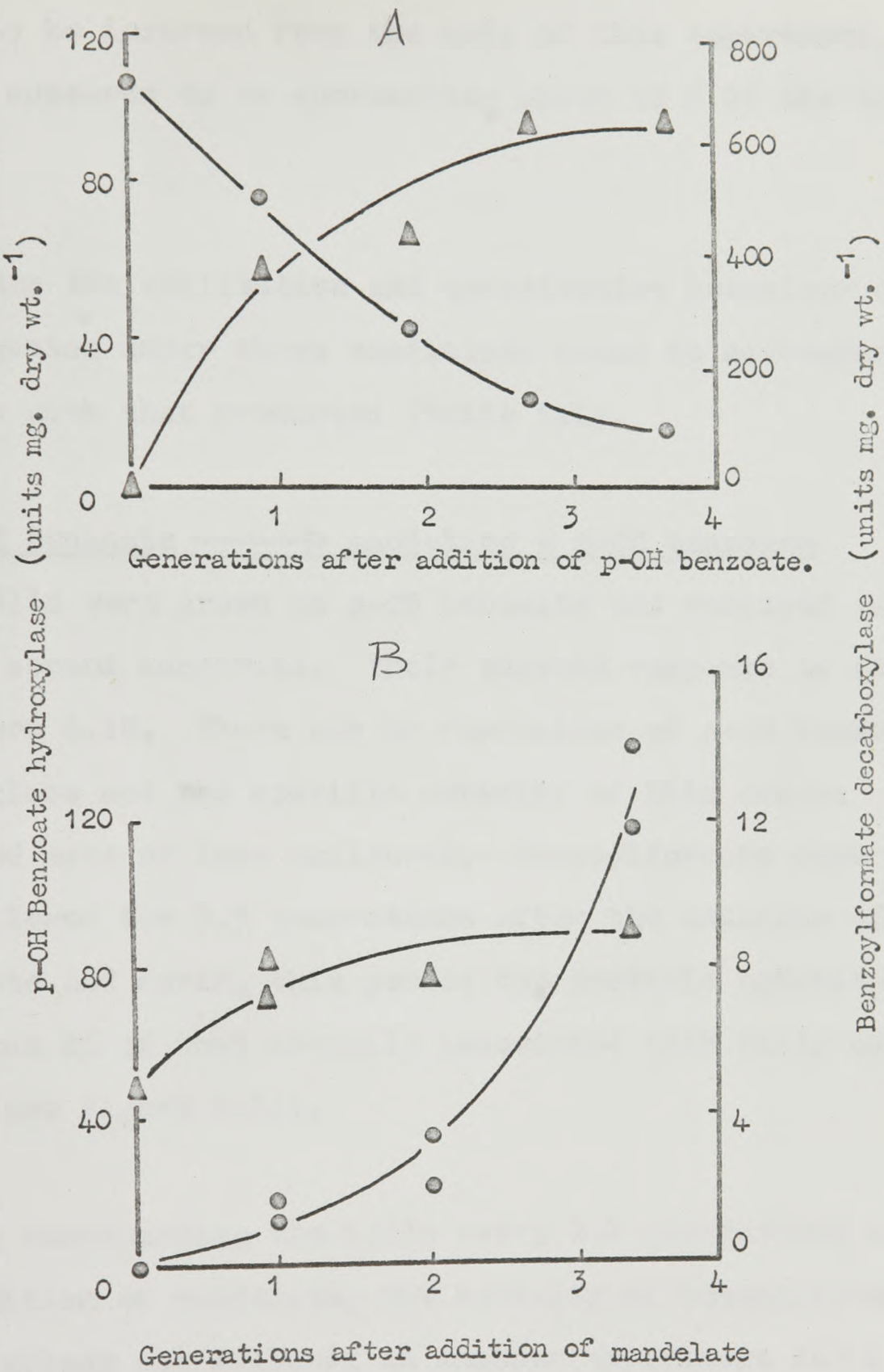
Cells growing on mandelate received p-OH benzoate as the second carbon source. An immediate induction of p-OH benzoate hydroxylase was observed (Figure 6.1A), the specific activity rising towards the value characteristic of full induction. Benzoylformate decarboxylase, on the other hand, was immediately repressed and the enzyme began to be diluted out by growth. However, the decrease in specific activity showed that the repression was not complete.

FIGURE 6.1

Enzymic adaptation to mixed aromatic substrates in batch culture : Mandelate + p-OH benzoate.

A. Wild-type cells, growing exponentially on mandelate (10 mM), received p-OH benzoate (also at 10 mM) at zero-time. Samples were taken at intervals thereafter and assayed for benzoylformate decarboxylase (○) and p-OH benzoate hydroxylase (△). The culture density at zero-time was 45 μg. dry wt. $ml.^{-1}$

B. As for A except that the first substrate was p-OH benzoate and mandelate was added later.


A
120
80
40
0
800
600
400
200
0
1
2
3
4
Generations after addition of p-OH benzoate.
p-OH Benzoate hydroxylase (units mg. dry wt.$^{-1}$)
Benzoylformate decarboxylase (units mg. dry wt.$^{-1}$)
B
16
12
8
4
0
Generations after addition of mandelate

The final, steady state specific activity of this enzyme can only be inferred from the data of this experiment, but it appeared to be approaching about 10 % of the initial value.

Thus the qualitative and quantitative behaviour of the organism under these conditions seems to correspond closely with that predicted (Table 5.1).

2. p-OH Benzoate ——→ mandelate + p-OH benzoate

Cells were grown on p-OH benzoate and received mandelate as the second substrate. Their enzymic response is shown in Figure 6.1B. There was no repression of p-OH benzoate hydroxylase and the specific activity of this enzyme remained more or less unaltered. Benzoylformate decarboxylase was followed for 3.5 generations after the addition of mandelate and during this period the specific activity was less than 2% of that normally associated with fully induced cells (see Figure 6.1A).

By resuspending the cells every 2.5 generations after the addition of mandelate, the activity of benzoylformate decarboxylase was followed in another experiment for 8 generations. The amount of enzyme was $< 1\%$ of that found in a fully induced culture (Table 6.1).

TABLE 6.1

Enzymic adaptation to mixed aromatic substrates in batch culture :

(2) p-OH Benzoate ⟶ Mandelate + p-OH benzoate.

Wild-type cells, growing exponentially in minimal medium with p-OH benzoate (10 mM), received mandelate (10 mM) in addition to the p-OH benzoate at zero-time. Samples were taken at intervals thereafter and assayed for p-OH benzoate hydroxylase and benzoylformate decarboxylase. The density of the culture at zero-time was 45 μg. ml.$^{-1}$. By resuspending the cells in fresh medium at a lower density every 2.5-3.5 generations, exponential growth was prolonged to enable the enzymic adaptation to be followed for 8 generations.

Generations after t = 0	p-OH benzoate hydroxylase (units mg.$^{-1}$)	Benzoylformate decarboxylase (units mg.$^{-1}$)
0	129	0
1.15	132	0.6
2.1	116	0.6
3.25	115	1.0
4.2	98	0.6
5.15	120	0.6
6.15	130	8.2
7.15	105	5.6
8.1	121	4.3

Once again, these results agree with the predictions summarised in Table 5.1, although the repressor effect of p-OH benzoate appears to be somewhat more severe when it is the first growth substrate.

Growth in medium containing p-OH benzoate and mandelate

It was suggested in the previous chapter that diauxic growth might occur in media containing p-OH benzoate and mandelate.

Wild-type cells were grown overnight on p-OH benzoate and transferred at a low cell density (<5 μg.dry wt.ml.$^{-1}$) into a flask containing p-OH benzoate (2.5 mM) and mandelate (5.0 mM). The flask was shaken at 30°C and the culture density measured periodically. Samples were taken for the assay of p-OH benzoate, mandelate, benzoylformate decarboxylase and p-OH benzoate hydroxylase. No diauxic lag was observed and the cells began to synthesise the mandelate enzymes when the p-OH benzoate concentration had been reduced to a very low level (Figure 6.2). The rate of induction of the mandelate enzymes appeared to be rapid enough for the cells to start using mandelate sufficiently rapidly to continue growing exponentially once the p-OH benzoate had been exhausted.

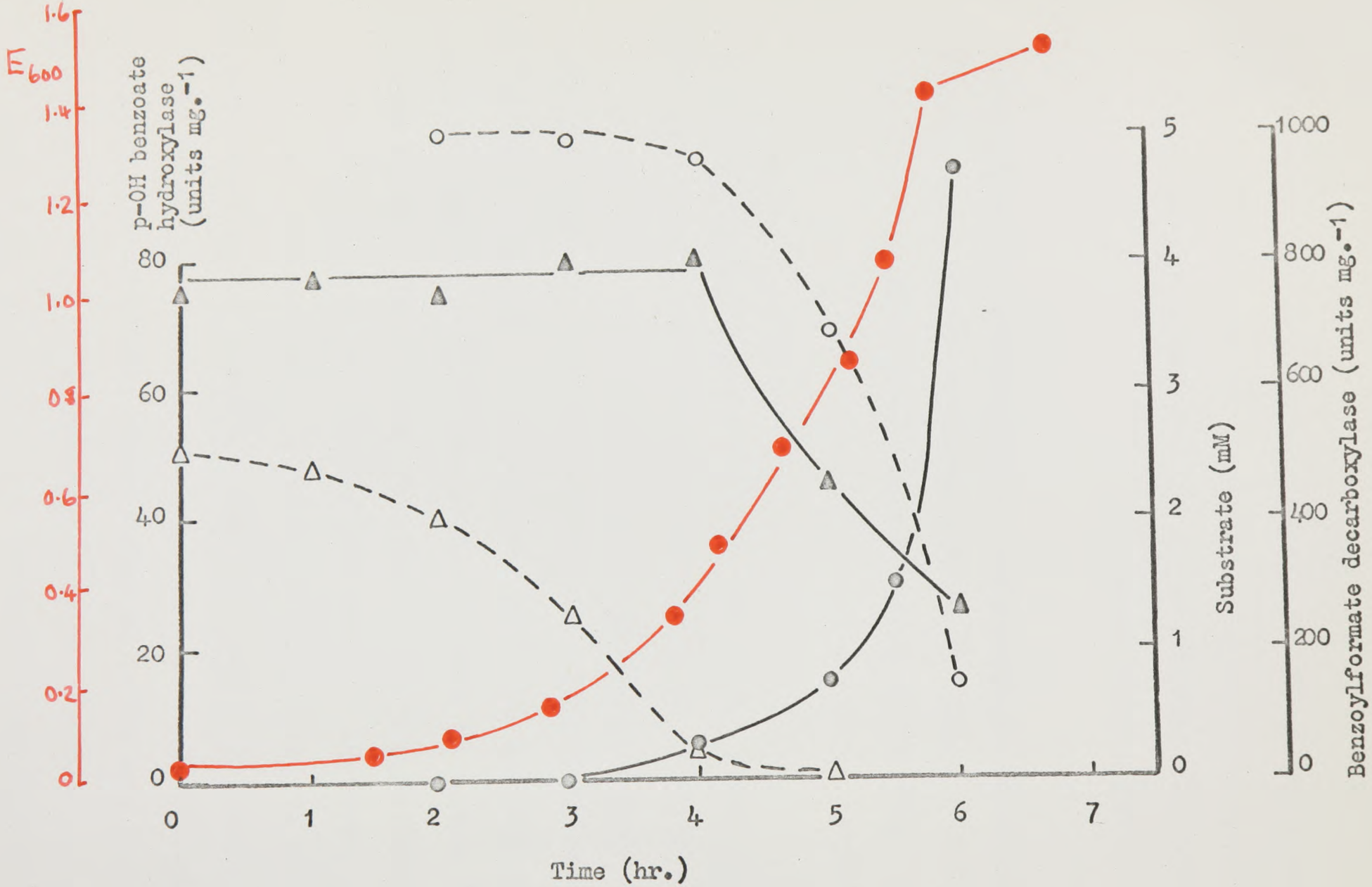

E_{600}
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
p-OH benzoate hydroxylase (units mg.$^{-1}$)
80
60
40
20
0
Substrate (mM)
5
4
3
2
1
0
Benzoylformate decarboxylase (units mg.$^{-1}$)
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
Time (hr.)

FIGURE 6-2.

Growth of Pseudomonas putida in medium containing p-OH benzoate and mandelate.

Wild-type cells, previously adapted to growth on p-OH benzoate, were inoculated into minimal medium containing p-OH benzoate (2.5 mM) and mandelate (5.0 mM). The culture was vigorously aerated by shaking at 30° and the following were measured as in Methods :

1. Optical density (600 nm) ●
2. Concentrations of mandelate (O) and p-OH benzoate (Δ).
3. Activities of benzoylformate decarboxylase (◎) and p-OH benzoate hydroxylase (▲).

3. Benzoate ——→ Benzoate + mandelate

Full induction of benzoate oxidase, observed when wild-type cells were grown on benzoate, was maintained following the addition of mandelate (Figure 6.3A). Synthesis of benzoylformate decarboxylase commenced immediately after the addition of the mandelate, but it is not possible from the data of this experiment to predict the steady state induction level of this enzyme. It appeared to be tending towards 30% of full induction. The predictions (Table 5.1) suggested that benzoate should have exerted a transient severe repression in these uninduced cells, and that there should have been a long lag period instead of immediate induction of benzoylformate decarboxylase.

4. Mandelate ——→ Benzoate + mandelate

On addition of benzoate to cells growing up exponentially on mandelate, benzoate oxidase remained induced at its original level, while the mandelate regulon was partially repressed. The specific activity of benzoylformate decarboxylase decreased towards a value of about 30-40 % of that associated with growth on mandelate alone (Figure 6.3B).

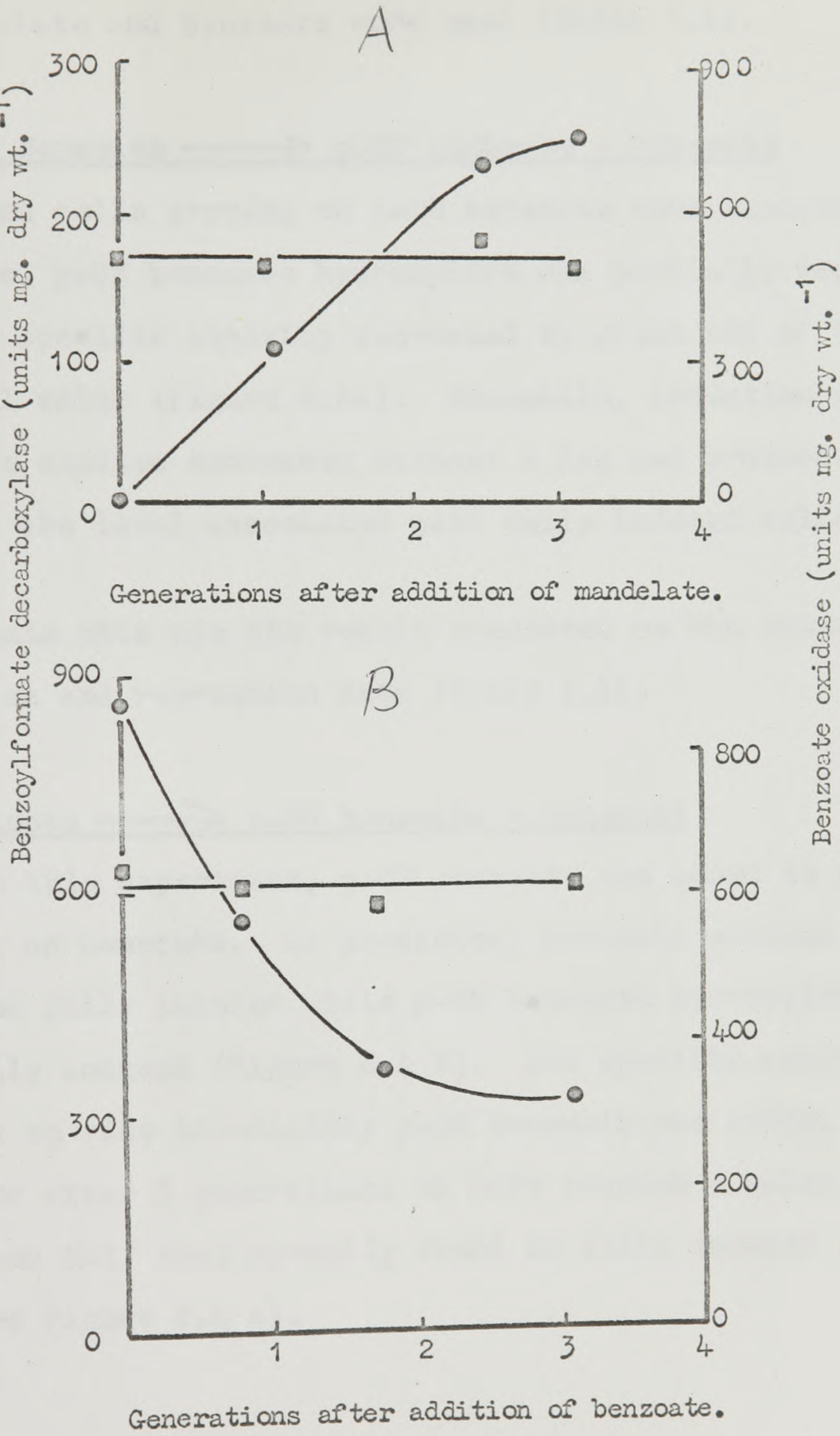
These results agree with the prediction that the mandelate enzymes might be repressed when high concentrations

FIGURE 6.3 A + B

Enzymic adaptation to mixed aromatic substrates in batch culture : Benzoate + mandelate.

A. Wild-type cells, growing exponentially on benzoate (10 mM), received mandelate (also at 10 mM) at zero-time. Samples were taken at intervals thereafter and assayed for benzoate oxidase (□) and benzoylformate decarboxylase (○). The culture density at zero-time was 60 μg. dry wt. $ml.^{-1}$

B. As for A except that mandelate was the first sustrate and benzoate was added as the second substrate when the cell density was 50 μg. $ml.^{-1}$


A
300
200
100
0
900
600
300
0
1
2
3
4
Benzoylformate decarboxylase (units mg. dry wt.$^{-1}$)
Benzoate oxidase (units mg. dry wt.$^{-1}$)
Generations after addition of mandelate.
B
900
600
300
0
800
600
400
200
0
1
2
3
4
Generations after addition of benzoate.

of mandelate and benzoate were used (Table 5.1).

5. p-OH Benzoate ——→ p-OH benzoate + benzoate

When cells growing on p-OH benzoate were exposed to benzoate, p-OH benzoate hydroxylase was partially repressed and its specific activity decreased to about 40% of the original value (Figure 6.4A). Meanwhile, induction of benzoate oxidase commenced without a lag and proceeded towards the level associated with fully induced cells.

Again this was the result predicted on the basis of induction and repression data (Table 5.1).

6. Benzoate ——→ p-OH benzoate + benzoate

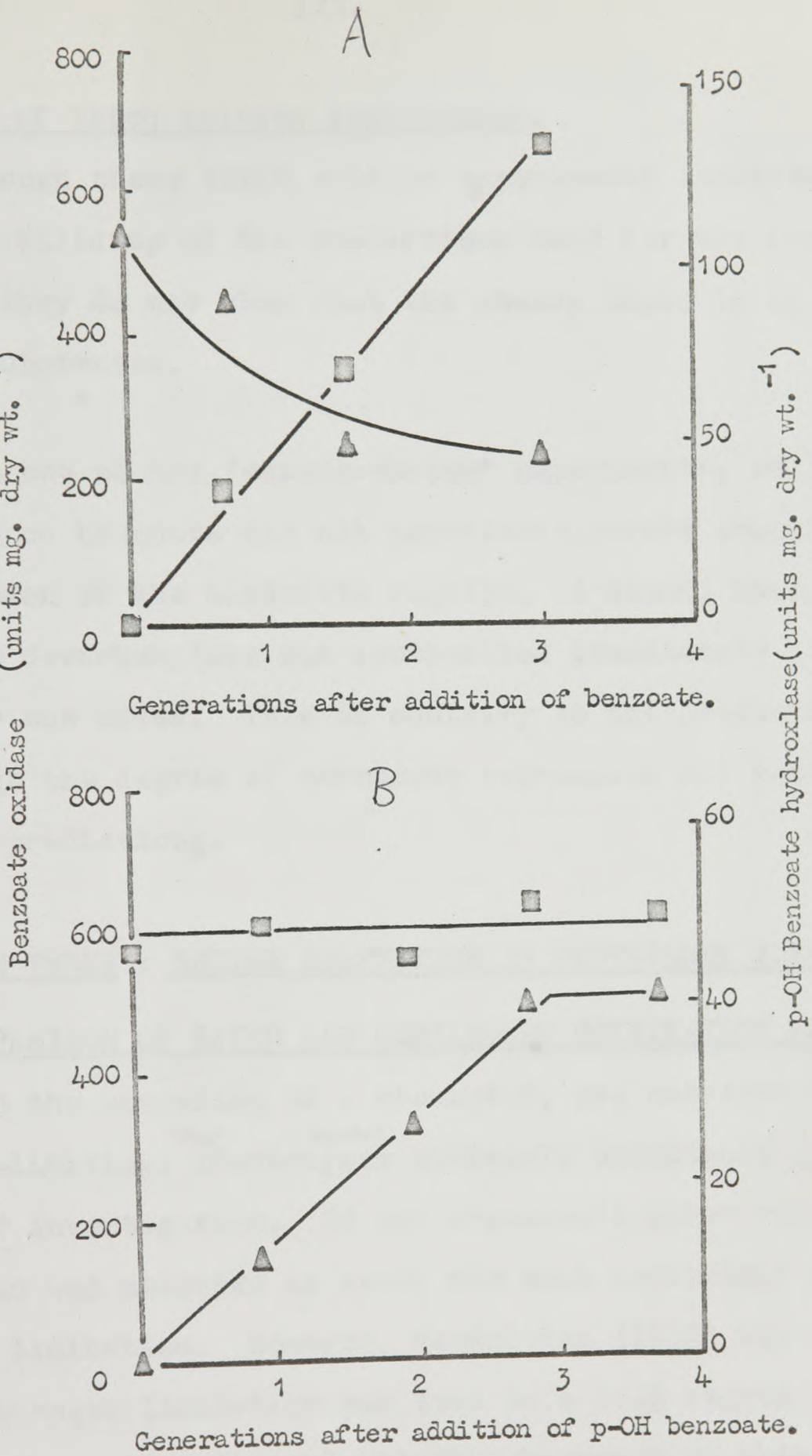
In this experiment, p-OH benzoate was added to cells growing on benzoate. As predicted, benzoate oxidase remained fully induced while p-OH benzoate hydroxylase was partially induced (Figure 6.4 B). Its specific activity started to rise immediately p-OH benzoate was added, and appeared after 3 generations to have reached a value which was about half that normally found in fully induced cells (compare Figure 6.4 A).

FIGURE 6.4 A+B.

Enzymic adaptation to mixed aromatic substrates in batch culture : p-OH Benzoate + benzoate.

A. Wild-type cells, growing exponentially on p-OH benzoate, (10 mM), received benzoate (also at 10 mM) at zero-time. Samples were taken at intervals thereafter and assayed for p-OH benzoate hydroxylase (▲) and benzoate oxidase (■). The culture density at zero-time was 55 µg. dry wt. $ml.^{-1}$

B. As for A except that benzoate was the first substrate and p-OH benzoate the second, and the culture density was 70 µg. $ml.^{-1}$ at the start.


A
800
600
400
200
0
150
100
50
0
1
2
3
4
Benzoate oxidase (units mg. dry wt.$^{-1}$)
p-OH Benzoate hydroxlase(units mg. dry wt.$^{-1}$)
Generations after addition of benzoate.
B
800
600
400
200
0
60
40
20
0
1
2
3
4
Generations after addition of p-OH benzoate.

Summary of batch culture experiments.

Though these batch culture experiments indicate the general validity of the predictions made for the transient state, they do not show what the steady state is in these mixed substrates.

In one of the 'enzyme-choice' experiments, cells growing on benzoate did not experience severe transient repression of the mandelate regulon. Instead, benzoylformate decarboxylase was synthesised immediately its inducer was added. This is contrary to the predictions although the degree of permanent repression may conform to the predictions.

SECTION THREE : ENZYME ADAPTATION IN CONTINUOUS CULTURE

A. COMPARISON OF BATCH AND CONTINUOUS CULTIVATION METHODS

In the operation of a chemostat, one nutrient is made growth-limiting. The carbon source was obviously unsuitable in this type of investigation. Of the organism's other requirements, nitrogen was selected as being the most convenient basis for growth limitation. However, Mandelstam (1962) has shown that nitrogen limitation can lead to a high degree of catabolite repression and, if this happened in this system, interpretation of the results would be complicated. Experi-

ments were, therefore, designed to test the possibility that nitrogen limitation introduces factors invalidating the direct comparison of chemostat and batch culture experiments.

1. Specific activities of aromatic enzymes in fully induced cells: comparison of batch and continuous culture methods

Wild-type cells were grown to steady state in batch and continuous cultures on one of the aromatic carbon sources (10 mM) and the specific activity of the induced enzyme determined. The chemostat was operated at a dilution rate such that the cells were growing at almost the maximum growth rate on the particular carbon source (Table 2.3). Steady state in batch cultures was achieved by growing the cells from a small inoculum (<5 μg.dry wt.ml.$^{-1}$) to a high density ($\bar{c}$. 500 μg.ml.$^{-1}$) and then determining their enzyme content.

The results are presented in Table 6.2 and it is clear that, though there are large variations in specific activities between apparently identical cultures, nitrogen limitation in the chemostat does not significantly repress the induction of these enzymes. Further experiments were carried out to confirm this conclusion.

TABLE 6.2

Specific activities of aromatic enzymes in fully induced cells : comparison of batch and continuous cultures.

Wild-type cells were grown to steady state in nitrogen-limited chemostats on an aromatic carbon source (10 mM). The dilution rates were set so that the doubling time was close to the maximum. The specific activity of the induced enzyme was measured and compared with that of cells grown from a small inoculum (<5 μg. dry wt. $ml.^{-1}$) to a high density (500 μg. $ml.^{-1}$) on the same substrate in batch culture. A number of determinations waa made and the results averaged (the range is in parentheses).

	CHEMOSTAT		BATCH CULTURE	
Substrate	N° of expts.	Sp. Act.	N° of expts.	Sp. Act.
Mandelate	10	993 (835-1100)	3	1093 (880-1270)
Benzoate	5	476 (400-570)	5	585 (500-620)
p-OH Benzoate	10	87.5 (78-115)	6	82 (78-120)

2. Measurement of the induction constant for the mandelate regulon in the chemostat

Assuming that the inducible enzyme is not unstable, its steady state specific activity in cells growing in a chemostat should be a direct measure of its rate of induction in those cells. The data of Figure 4.4 suggest that benzoylformate decarboxylase is stable in growing cells, so that, if its specific activity is measured in a chemostat run to steady state over a range of mandelate concentrations, the K_{ind} could be calculated.

The experimental procedure used has already been described (Chapter 4). Cells of mutant D57 (md$^-$) were grown on glucose and increasing concentrations of mandelate were supplied. At each steady state the specific activity of benzoylformate decarboxylase was determined and the inducer concentration then increased. The variation in specific activity over the mandelate concentration range 0 - 10 mM is shown in Figure 4.5 (curve A). Full induction was observed at 5.0 mM while a Lineweaver and Burk (1934) analysis of the data gave a K_{ind} of 0.7 mM (Figure 6.5). Reference to Table 3.3 shows that in batch culture the maximum rate of induction occurred at 0.75 - 2.0 mM and the K_{ind} was about 0.5 mM. The induction of the mandelate

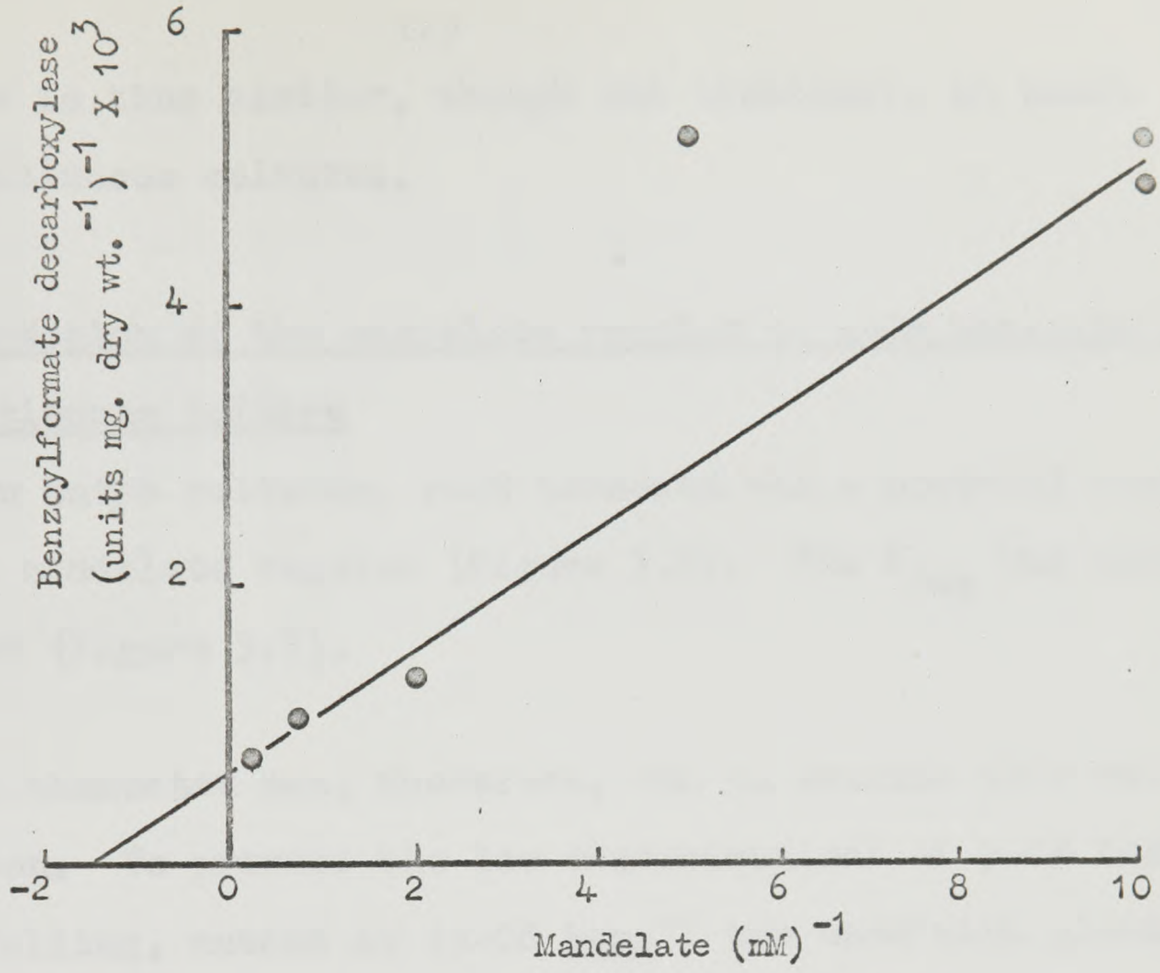


FIGURE 6.5

Induction constant for benzoylformate decarboxylase from continuous culture.

Mutant D 57 (md$^-$) was grown in a nitrogen-limited chemostat on glucose with increasing concentrations of mandelate (see legend to Fig. 4.5). The specific activity of benzoylformate decarboxylase was determined at steady state at each inducer concentration and the data plotted by the method of Lineweaver and Burk (1934). The K_{ind} is about 0.7 mM.

regulon is thus similar, though not identical, in batch and continuous cultures.

3. Repression of the mandelate regulon by p-OH benzoate in continuous culture

In batch cultures, p-OH benzoate was a powerful repressor of the mandelate regulon (Figure 3.6). The K_{rep} was about 0.02 mM (Figure 3.7).

A chemostat was, therefore, run to examine this repression. To prevent the low concentrations of p-OH benzoate from falling, mutant A2 (p-OH benz$^-$) was used with glucose as the carbon source. The specific activity of benzoylformate decarboxylase was determined at steady state with 5 or 10 mM mandelate as inducer. p-OH Benzoate (0.04 mM) was then added to the medium and the specific activity of the enzyme redetermined when the new steady state had been established. The concentration of p-OH benzoate was then increased to 0.08 mM and the measurement repeated.

The results are presented in Table 6.3. Significant repression was exerted by p-OH benzoate at these concentrations and an increased inducer concentration partly reversed the repression. A comparison between batch and continuous

TABLE 6.3

Repression of benzoylformate decarboxylase by p-OH benzoate in continuous culture.

Mutant A 2 (p-OH benz$^-$) was grown in a nitrogen-limited chemostat with excess glucose. The dilution rate was 0.45 vol. hr.$^{-1}$ (M.G.T. = 90 min.). The culture was induced with mandelate (5.0 or 10.0 mM) and 0, 0.04 or 0.08 mM p-OH benzoate added. The specific activity of benzoylformate decarboxylase was measured at steady state.

Mandelate (mM)	p-OH Benzoate (mM)	Benzoylformate decarboxylase (units mg.$^{-1}$)	Repression %
5.0	0	835	-
	0.04	285	66
	0.08	96	88.5
10.0	0	760	-
	0.04	329	57
	0.08	260	66

cultures can be made. With the inducer present at 5.0 mM, 0.04 mM p-OH benzoate caused 66% repression in the chemostat (Table 6.3) and 45% in batch culture (Figure 3.7). When present at 0.08 mM, p-OH benzoate caused 88.5% and 62% repression respectively.

This result, but not that of the two previous experiments, suggests that the growth conditions of the chemostat may well lead to a somewhat higher level of catabolite repression than those of batch cultures. This may have to be taken into account when interpreting the results of enzyme adaptation to mixed substrates.

B. RESULTS

1. Mandelate ——> Mandelate + p-OH benzoate

Wild-type cells were grown to steady state in the chemostat on mandelate (10 mM) and the specific activity of benzoylformate decarboxylase determined. The mandelate concentration in the growth vessel was measured by the spectrophotometric method.

The medium supply was then changed to mandelate + p-OH benzoate (both 10 mM) and the specific activities of benzoylformate decarboxylase and p-OH benzoate hydroxylase determined at intervals for approximately seven generations.

The outflowing medium was monitored for both substrates. By this time, the steady state appeared to have been reached. Further measurements after 20 generations confirmed this.

The results are presented in Figure 6.6. Following the changeover, the concentration of p-OH benzoate rapidly rose in the growth vessel and exceeded the K_{ind} for p-OH benzoate hydroxylase and the K_{rep} for the mandelate regulon. Induction of p-OH benzoate hydroxylase commenced immediately after the rise in inducer concentration and the enzyme reached a specific activity normally associated with cells grown solely on p-OH benzoate.

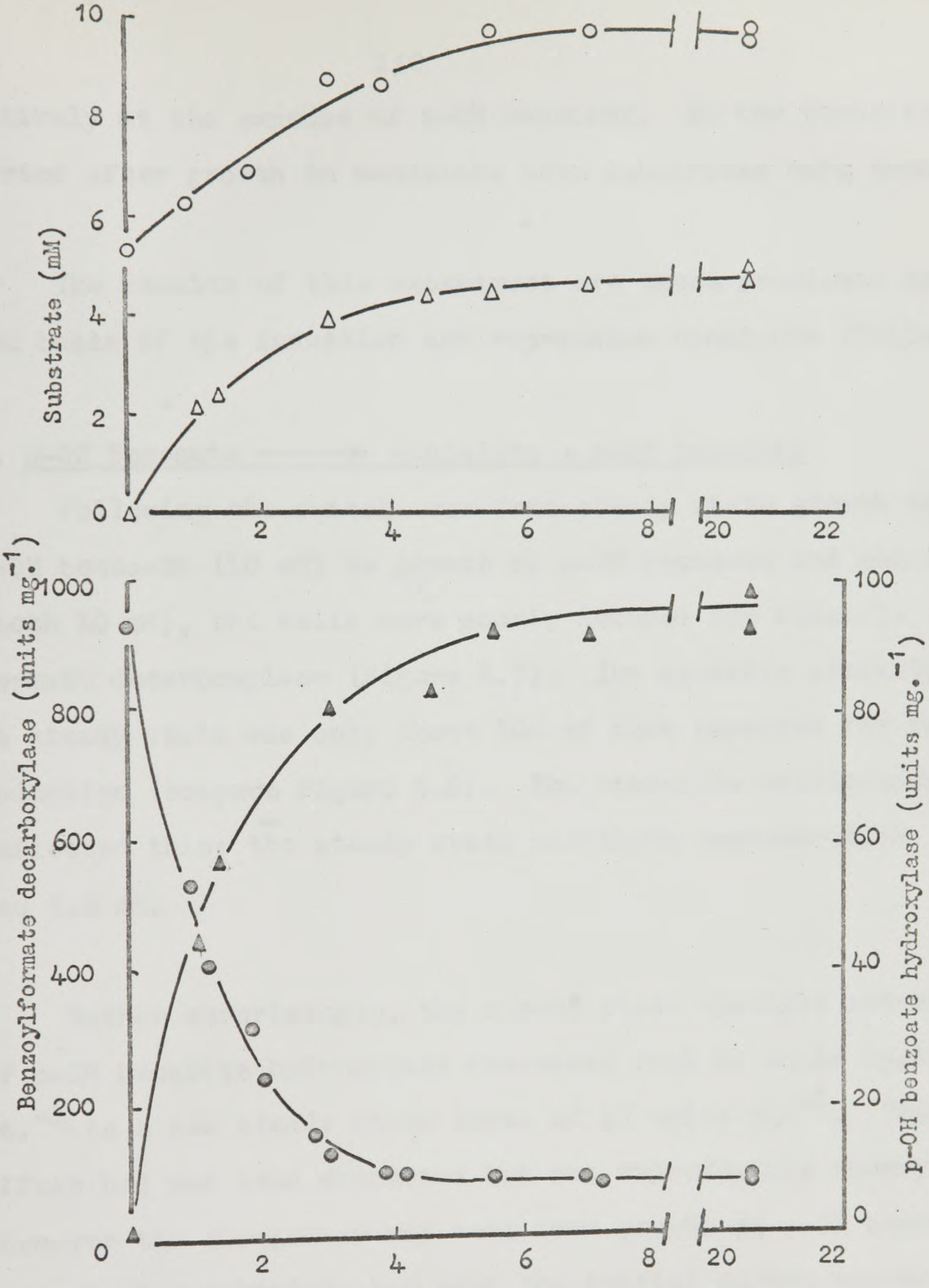
At the same time, repression of benzoylformate decarboxylase was observed as soon as the p-OH benzoate entered the growth vessel. At steady state, the specific activity of this enzyme had fallen to 8.5% of its initial value. There was a similar change in the utilisation of mandelate which decreased by about 95%. As a result the steady state concentration of mandelate in the growth vessel rose from 5.3 mM to 9.7 mM.

Thus at steady state in the presence of p-OH benzoate and mandelate, the cells are adapted to growth almost

FIGURE 6.6

Enzymic adaptation to mixed aromatic substrates in continuous culture : (1) Mandelate ⟶ Mandelate + p-OH benzoate.

Wild-type cells were grown in a nitrogen-limited chemostat on mandelate (10 mM) with a doubling time of 75 min. (D = 0.55 vol. $hr.^{-1}$). The specific activity of benzoylformate decarboxylase (◎) and the mandelate concentration in the growth vessel (O) were determined at steady state. p-OH Benzoate (10 mM) was then added to the inflowing medium and the specific activities of benzoylformate decarboxylase and p-OH benzoate hydroxylase (▲) and the mandelate and p-OH benzoate (Δ) concentrations were measured at intervals until the new steady state had been reached. The cell density was constant at 300 µg. dry wt. $ml.^{-1}$


Substrate (mM)
10
8
6
4
2
0
2
4
6
8
20
22
Benzoylformate decarboxylase (units mg.-1)
1000
800
600
400
200
0
p-OH benzoate hydroxylase (units mg.-1)
100
80
60
40
20
0
Generations after changeover to two substrates.

entirely at the expense of p-OH benzoate. In the transition period after growth on mandelate both substrates were used.

The results of this experiment are those predicted on the basis of the induction and repression constants (Table 5.1).

2. p-OH Benzoate ⟶ mandelate + p-OH benzoate

Following the switch-over from steady state growth on p-OH benzoate (10 mM) to growth on p-OH benzoate and mandelate (both 10 mM), the cells were poorly induced for benzoyl-formate decarboxylase (Figure 6.7). Its specific activity at steady state was only about 10% of that expected for full induction (compare Figure 6.6). The mandelate utilisation reflected this; the steady state mandelate concentration was 9.8 mM.

Rather surprisingly, the steady state specific activity of p-OH benzoate hydroxylase decreased from 83 units mg.dry wt.$^{-1}$ to a new steady state level of 47 units mg.$^{-1}$. This effect had not been predicted but was reproducibly observed whenever the changeover was made from growth on p-OH benzoate but not when mandelate had been the initial carbon source (Table 6.4). In the latter case, the specific activity of p-OH benzoate hydroxylase was that normally associated with

FIGURE 6.7

Enzymic adaptation to mixed aromatic substrates in continuous culture :

(2) p-OH Benzoate ⟶ Mandelate + p-OH benzoate.

Wild-type cells were grown in a nitrogen-limited chemostat on p-OH benzoate (10 mM) with a doubling time of 70 min. ($D = 0.6$ vol. $hr.^{-1}$). The specific activity of p-OH benzoate hydroxylase (▲) and the p-OH benzoate concentration in the growth vessel (△) were determined at steady state. Mandelate (10 mM) was then added to the inflowing medium and the specific activities of p-OH benzoate hydroxylase and benzoylformate decarboxylase (●) and the concentrations of p-OH benzoate and mandelate (○) were measured at intervals until the new steady state was established. The cell density was constant at 300 μg. dry wt. $ml.^{-1}$

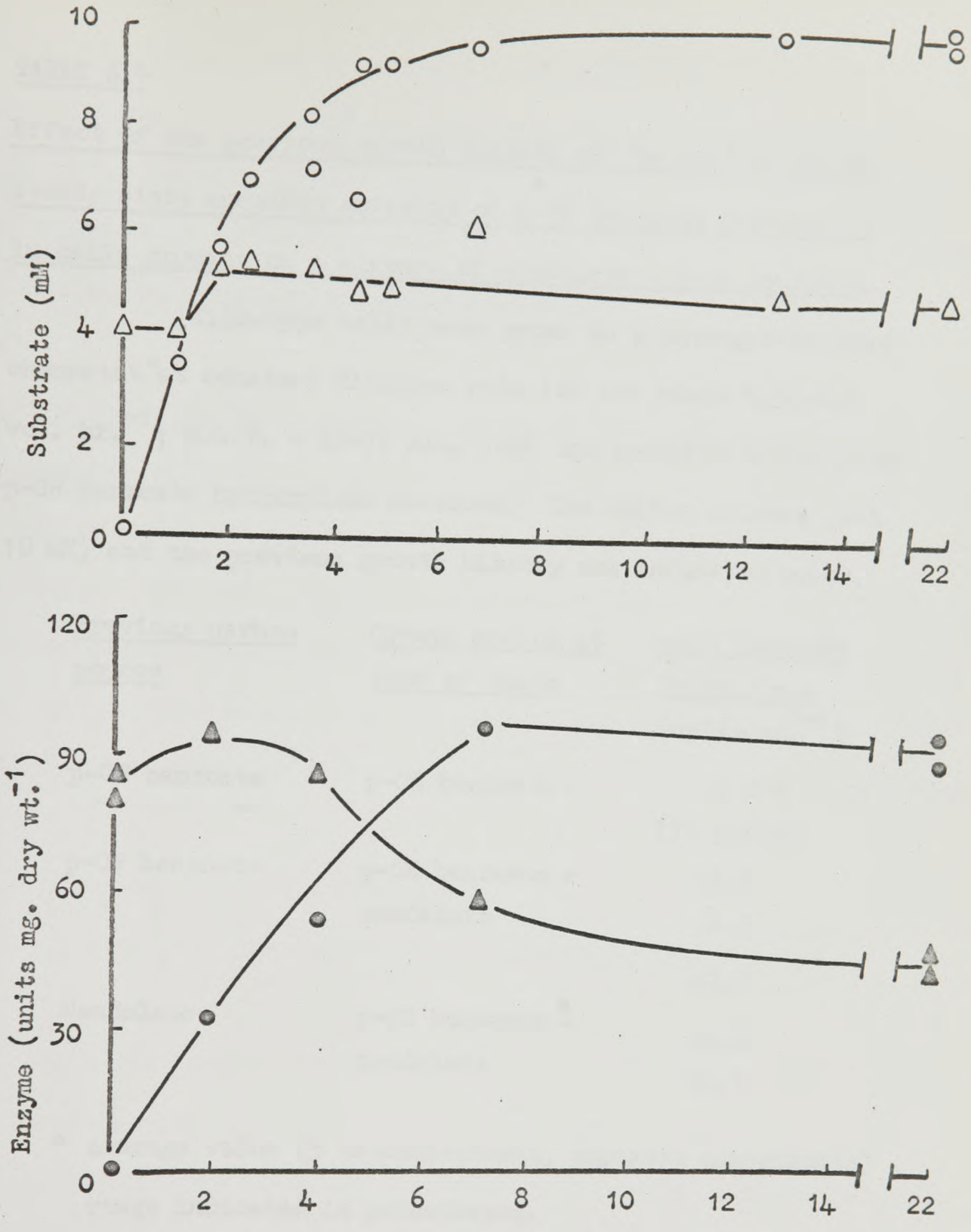

Substrate (ml)
10
8
6
4
2
0
2
4
6
8
10
12
14
22
Enzyme (units mg. dry wt.$^{-1}$)
120
90
60
30
0
2
4
6
8
10
12
14
22
Generations after changeover to two substrates

TABLE 6.4

Effect of the previous growth history of the culture on the steady state specific activity of p-OH benzoate hydroxylase in cells growing on a mixture of mandelate + p-OH benzoate.

Wild-type cells were grown in a nitrogen-limited chemostat at constant dilution rate (in the range 0.55-0.6 vol. $hr.^{-1}$; M.G.T. = 69-76 min.). and the specific activity of p-OH benzoate hydroxylase measured. The carbon sources (all 10 mM) and the previous growth history are indicated below.

Previous carbon source	Growth medium at time of assay	p-OH Benzoate hydroxylase (units $mg.^{-1}$)
p-OH benzoate	p-OH benzoate	88.0 * (77.5-115)
p-OH benzoate	p-OH benzoate + mandelate	53.5
		53.5
		47.0
Mandelate	p-OH benzoate + mandelate	94.0
		94.5

* average value (5 determinations, separate experiments) range indicated in parentheses.

cells grown on p-OH benzoate. When transferred from p-OH benzoate to the substrate mixture, the culture seemed to be able to maintain itself at its original cell density with a 15% reduction in its carbon utilisation and a 45% reduction in the corresponding enzyme activity.

Experiments to test other predictions about growth in media containing mandelate and p-OH benzoate. There were a number of other predictions concerning growth on mandelate + p-OH benzoate. It was suggested (Chapter 5) that, since the repression of mandelate enzymes by p-OH benzoate was partially reversible, it should be possible to induce the mandelate system in a mixture of mandelate and p-OH benzoate by:-

(i) reducing the p-OH benzoate concentration

(ii) increasing the mandelate concentration.

To test these possibilities, further chemostats were run in the same way as those above except that different inflow concentrations of mandelate and p-OH benzoate were used to provide either a high or a low ratio of mandelate to p-OH benzoate.

When the steady state concentration of mandelate was 0.91 mM and that of p-OH benzoate was 14.1 mM, benzoylformate

decarboxylase was undetectable. However, when the mandelate concentration was 17.0 mM and that of p-OH benzoate was 2.1 mM, the mandelate enzymes were about 25% induced (Table 6.5). There was no repression of the p-OH benzoate hydroxylase.

It was also predicted (Chapter 5) that the mandelate regulon should be fully induced in the presence of very low concentrations of p-OH benzoate ($< K_{rep}$). This was also tested experimentally in the following way. The wild-type was replaced by mutant A2 (p-OH benz$^-$) so that very low concentrations of p-OH benzoate (0.001 - 0.2 mM) could be used. The chemostat was run as before on mandelate (10 mM) and the specific activity of benzoylformate decarboxylase was determined at steady state. Then increasing concentrations of p-OH benzoate were added to the medium feed and, after each addition, the specific activity of benzoylformate decarboxylase was redetermined at the new steady state. The results are presented in Table 6.6.

As was predicted, very low concentrations of p-OH benzoate (0.001 and 0.01 mM) were insufficient to repress the mandelate regulon. A somewhat higher concentration (0.06 mM) caused 34% repression but the cells continued to

TABLE 6.5

Effect of the steady state concentration ratio of mandelate and p-OH benzoate on the induction of benzoylformate decarboxylase in continuous culture.

Wild-type cells were grown in nitrogen-limited chemostats on mixtures of mandelate + p-OH benzoate. The specific activity of benzoylformate decarboxylase and the concentrations of mandelate and p-OH benzoate in the growth vessel were determined at steady state (Methods). The doubling times of the cultures were in the range 70 - 80 min. (D = 0.53 - 0.60 vol. $hr.^{-1}$). The concentrations of the substrates in the feed are indicated in parenthesis.

p-OH Benzoate (mM)	Mandelate (mM)	Benzoylformate decarboxylase (units $mg.^{-1}$)
--	4.9 (10.0)	1020 *
2.1 (6.0)	17.0 (20.0)	250
14.1 (20.0)	0.91 (1.0)	0
4.3 (8.85)	9.5 (10.0)	107
4.8 (10.0)	9.8 (10.0)	80
5.1 (10.0)	9.8 (10.0)	95

* average of four determinations; range 930 - 1100 units $mg.^{-1}$

TABLE 6.6

Enzymic adaptation to mixed aromatic substrates in continuous culture : effect of low p-OH benzoate concentrations on the induction of benzoylformate decarboxylase in mandelate + p-OH benzoate.

Mutant A 2 (p-OH benz$^-$) was grown in a nitrogen-limited chemostat on mandelate (10 mM) with a doubling time of 90 min. ($D = 0.45$ vol. hr.$^{-1}$). The specific activity of benzoylformate decarboxylase was determined at steady state and then increasing concentrations of p-OH benzoate were added to the medium. After each steady state had been reached, the specific activity of the enzyme was redetermined. The cell density was 380 µg. dry wt. ml.$^{-1}$

p-OH Benzoate inflow (mM)	Benzoylformate decarboxylase (units mg. dry wt.$^{-1}$)	Repression %
0	1005	-
0.001	1022	0
0.010	1030	0
0.060	660	34
0.2	Culture diluted out	-

grow. An even higher concentration (0.2 mM) caused so much repression that the cells were no longer able to grow and were washed out. The medium flow was maintained and after two days the contents of the growth vessel again became turbid. A mutant that was still unable to utilise p-OH benzoate but in which the mandelate regulon was no longer subject to repression by p-OH benzoate had been selected. This mutant had similar properties to those obtained in a similar fashion by Mandelstam (1967).

3. Benzoate ——> Benzoate + mandelate

Since the spectrophotometric assay for mandelate was inapplicable to growth media from cultures grown on mandelate + benzoate, the radioactive assay had to be used for mandelate as well as for benzoate (see Methods). Both required carboxyl-labelled (^{14}C) acids, the metabolism of which resulted in the release of $^{14}CO_2$ and the consequent disappearance of the label from the medium. It was, therefore, necessary to run two identical chemostats in parallel so that each substrate concentration could be determined separately. In each chemostat, either the mandelate or the benzoate was labelled (0.002 $\mu Ci.ml.^{-1}$).

Wild-type cells were grown on benzoate (10 mM) and the steady state specific activity of benzoate oxidase and the benzoate concentration determined. The inflow medium was then changed to benzoate and mandelate (both at 10 mM) and the specific activities of benzoate oxidase and benzoylformate decarboxylase and the concentrations of the substrates were measured as the new steady state was approached (Figure 6.8).

The synthesis of benzoylformate decarboxylase was delayed for about 1.5 generations even though the concentration of the inducer in the growth vessel rose rapidly after the changeover. At steady state the enzyme was about 70-75% repressed. This delay in the induction and the final repressed state of the enzyme had both been predicted, as had been the constancy of the specific activity of benzoate oxidase (Figure 6.8).

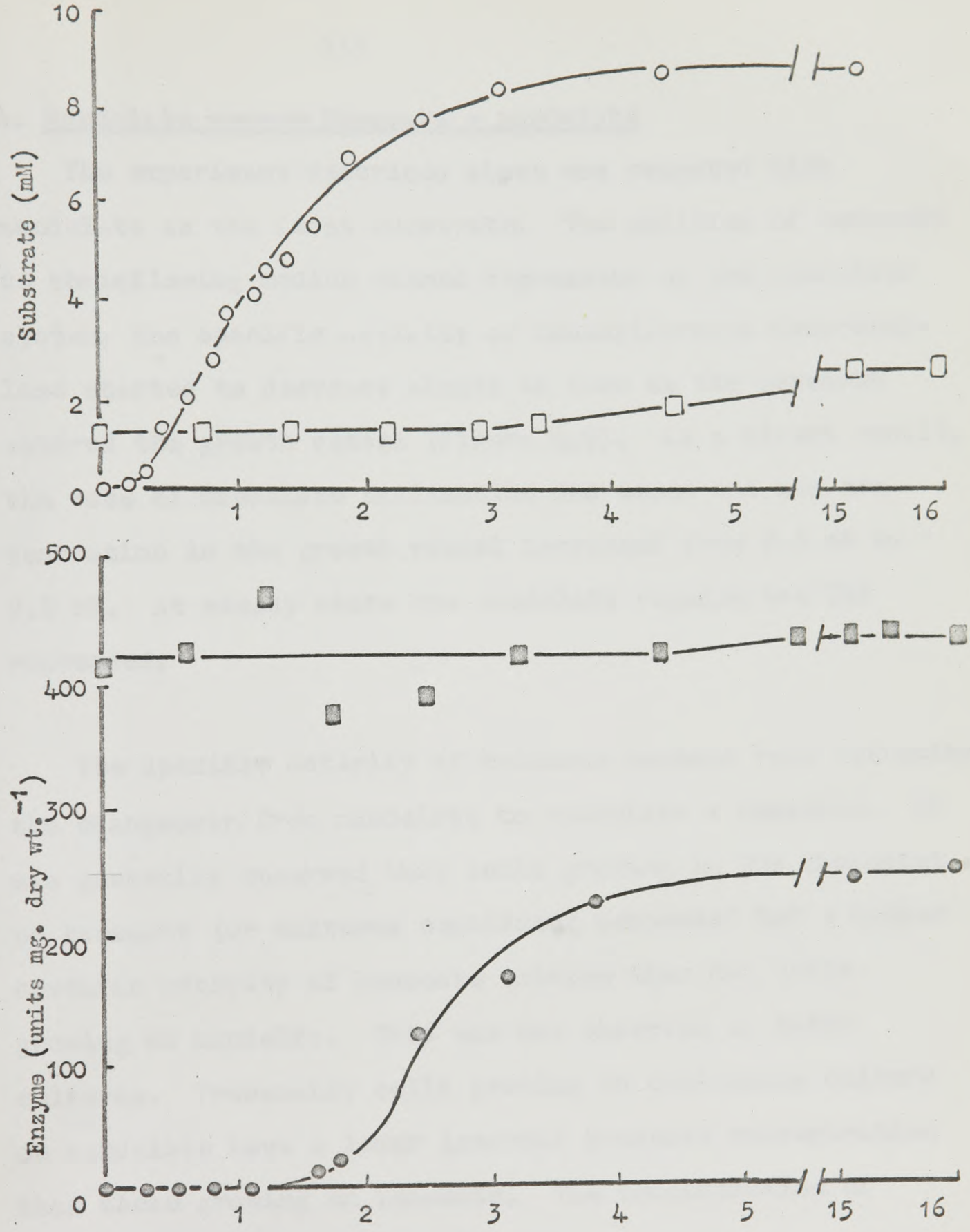
At steady state, the cells used a small amount of mandelate (concentration in the growth vessel = 9 mM) and grew mainly at the expense of benzoate (concentration = 2.5 mM).

FIGURE 6-8.

Enzymic adaptation to mixed aromatic substrates in continuous culture :

(3) Benzoate ⟶ Benzoate + mandelate.

Wild-type cells were grown in a nitrogen-limited chemostat on benzoate (10 mM) with a doubling time of 95 min. ($D = 0.44$ vol. $hr.^{-1}$). The specific activity of benzoate oxidase (■) was measured at steady state and then mandelate (also at 10 mM) was added to the inflowing medium. The changes in the specific activities of benzoate oxidase and benzoylformate decarboxylase (●) were measured while the new steady state was being established. The concentrations of benzoate (□) and mandelate (○) in the growth vessel were determined by running two parallel chemostats under identical conditions with either benzoate or mandelate labelled with 14-carbon (Methods). The cell density in both chemostats was 380 μg. dry wt. $ml.^{-1}$



Generations after changeover to two substrates.

4. Mandelate ——→ Benzoate + mandelate

The experiment described above was repeated with mandelate as the first substrate. The addition of benzoate to the inflowing medium caused repression of the mandelate system; the specific activity of benzoylformate decarboxylase started to decrease almost as soon as the benzoate entered the growth vessel (Figure 6.9). As a direct result, the rate of mandelate utilisation decreased and its concentration in the growth vessel increased from 5.0 mM to 9.0 mM. At steady state the mandelate regulon was 75% repressed.

The specific activity of benzoate oxidase rose following the changeover from mandelate to mandelate + benzoate. It was generally observed that cells growing in the chemostat on benzoate (or mixtures containing benzoate) had a higher specific activity of benzoate oxidase than had cells growing on mandelte. This was not observed in batch cultures. Presumably cells growing in continuous culture on mandelate have a lower internal benzoate concentration than those growing on benzoate. The concentration of benzoate in the vessel was 3.5 mM.

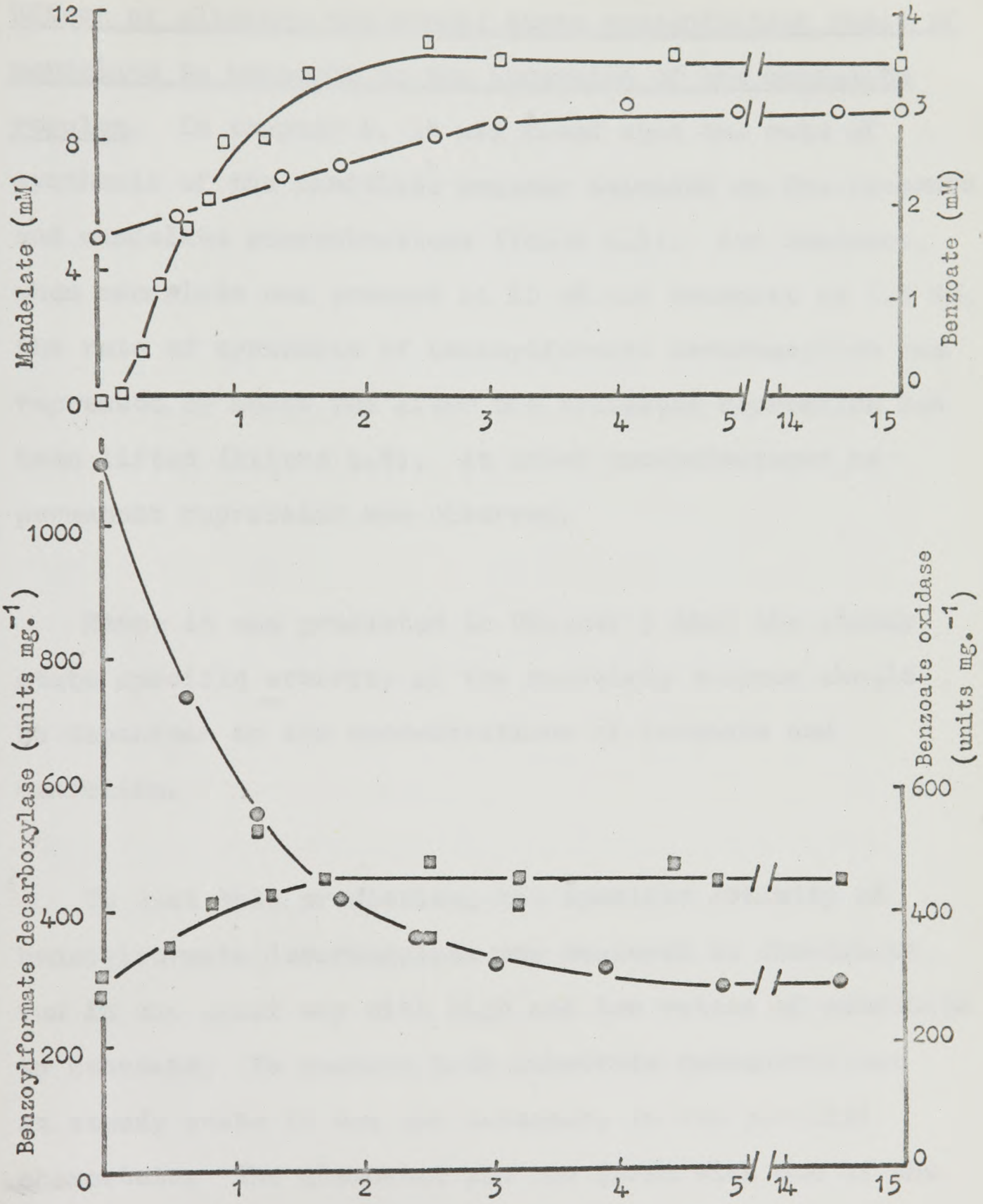
The results of this experiment also fitted the predictions (Table 5.1).

FIGURE 6-9.

Enzymic adaptation to mixed aromatic substrates in continuous culture :

(4) Mandelate ⟶ Benzoate + mandelate.

Wild-type cells were grown in a nitrogen-limited chemostat on mandelate (10 mM) with a doubling time of 97 min. ($D = 0.43$ vol. $hr.^{-1}$). The specific activities of benzoylformate decarboxylase (●) and benzoate oxidase (■) were determined at steady state and then benzoate (also at 10 mM) was added to the inflowing medium. The changes in the specific activities of these two enzymes were measured while the new steady state was being established. The concentrations of benzoate (□) and mandelate (○) in the growth vessel were determined by running two parallel chemostats under identical conditions with either mandelate or benzoate labelled with 14-carbon (Methods). The cell density in both chemostats was 350 μg. dry wt. $ml.^{-1}$


Mandelate (mM)
Benzoate (mM)
Benzoylformate decarboxylase (units mg.$^{-1}$)
Benzoate oxidase (units mg.$^{-1}$)
Generations after changeover to two substrates.

Effect of altering the steady state concentration ratio of mandelate to benzoate on the induction of the mandelate regulon. In chapter 4, it was found that the rate of synthesis of the mandelate enzymes depended on the benzoate and mandelate concentrations (Table 4.5). For instance, when mandelate was present at 10 mM and benzoate at 7.5 mM, the rate of synthesis of benzoylformate decarboxylase was repressed by about 70% after the transient repression had been lifted (Figure 4.9). At other concentrations no permanent repression was observed.

Hence it was predicted in Chapter 5 that the steady state specific activity of the mandelate enzymes should be dependent on the concentrations of benzoate and mandelate.

To test this prediction, the specific activity of benzoylformate decarboxylase was measured in chemostats run in the usual way with high and low ratios of mandelate to benzoate. To measure both substrate concentrations at steady state it was not necessary to run parallel chemostats. The chemostat was run first with one of the substrates in the mixture labelled. Its concentration was then determined at steady state and a small amount of

the second substrate, radioactively labelled, was then added to the reservoir. This did not significantly increase the concentration of the second substrate but the subsequent increase of label in the growth vessel was taken as a measure of the concentration of the second substrate.

Complete repression of the mandelate system was observed when a high concentration of benzoate (12.6 mM) was used with a low concentration of mandelate (0.93 mM) (Table 6.7). Conversely, a low benzoate concentration (1.2 mM) with a high mandelate concentration (18.9 mM) permitted a 50% induction of the mandelate system.

Although these results support the prediction that the steady state specific activity of the mandelate enzymes should be dependent on the concentration ratio of mandelate to benzoate, the degree of induction observed at the high ratio is considerably less than that which would be predicted for these concentrations using the data of Chapter 4.

TABLE 6.7

Effect of concentrations of mandelate and benzoate on the enzymic adaptation to mixtures of benzoate + mandelate in continuous culture.

Wild-type cells were grown to steady state in nitrogen-limited chemostats on mandelate and benzoate with a doubling time of about 90 min. ($D = 0.45$ vol. $hr.^{-1}$). The specific activities of benzoate oxidase and benzoylformate decarboxylase and the concentrations of benzoate and mandelate in the growth vessel were determined. In the Table the inflow concentrations of the substrates are in parentheses. A chemostat was run on mandelate alone (20 mM) for the determination of the fully induced activity of benzoylformate decarboxylase.

Mandelate (mM)	Benzoate (mM)	Benzoylformate decarboxylase (units $mg.^{-1}$)	Benzoate oxidase (units $mg.^{-1}$)
14.5 (20)	--	935	330
18.9 (20)	7.5 (1.2)	466	520
0.93 (1.0)	12.6 (20)	0	497

5. p-OH Benzoate ——→ p-OH benzoate + benzoate

The behaviour of cells growing in the chemostat on p-OH benzoate (10 mM) towards additional benzoate (10 mM) is illustrated in Figure 6.10.

The changeover was accompanied by a decrease in the specific activity of p-OH benzoate hydroxylase. There was a delay of about 1.0 generation before repression occurred but this could probably be accounted for by the fact that the benzoate took about this time to build up to a repressing concentration in the growth vessel. The steady state specific activity of p-OH benzoate hydroxylase was about 77% repressed. The concentration of p-OH benzoate increased as a result from 5.2 mM to 8.6 mM.

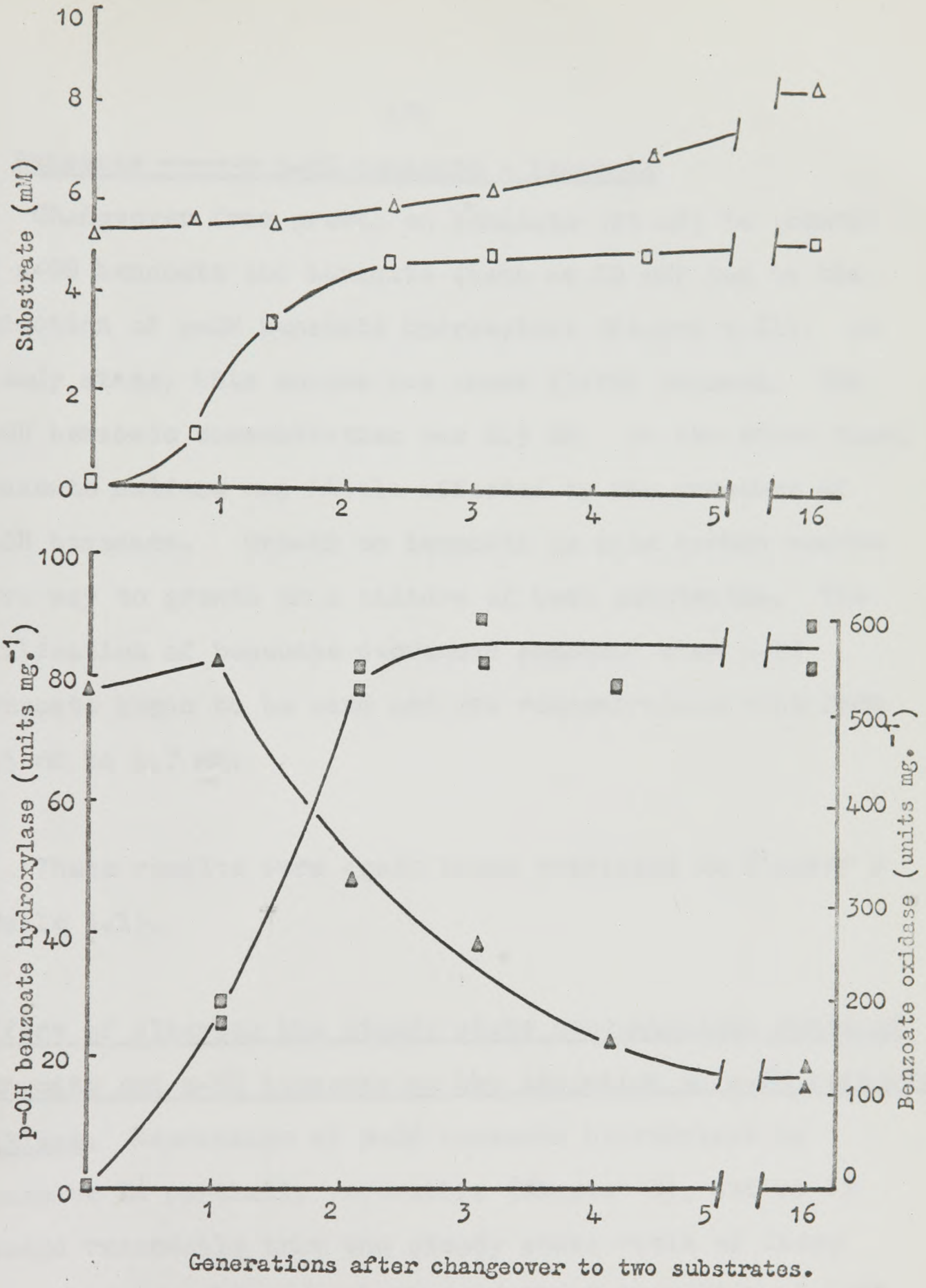
On the other hand, benzoate oxidase was rapidly induced following the changeover and its steady state specific activity was about the same as that observed in fully induced cells. The benzoate concentration was 5.5 mM.

These results again confirm the predictions of the previous chapter (Table 5.1).

FIGURE 6.10

Enzymic adaptation to mixed aromatic substrates in continuous culture : (5) p-OH Benzoate ——> p-OH Benzoate + benzoate.

Wild-type cells were grown in a nitrogen-limited chemostat on p-OH benzoate (10 mM) with a doubling time of 87 min. ($D = 0.48$ vol. $hr.^{-1}$). The specific activity of p-OH benzoate hydroxylase (Δ) and the p-OH benzoate concentration in the growth vessel (Δ) were determined at steady state. Benzoate (10 mM) was then added to the inflowing medium and the specific activities of p-OH benzoate hydroxylase and benzoate oxidase (■) and the concentrations of p-OH benzoate and benzoate (□) were measured at intervals until the new steady state had been reached. The cell density was constant at 360 μg. dry wt. $ml.^{-1}$


Substrate (ml)
10
8
6
4
2
0
1
2
3
4
5
16
p-OH benzoate hydroxylase (units mg.-1)
Benzoate oxidase (units mg.-1)
100
80
60
40
20
0
600
500
400
300
200
100
0
1
2
3
4
5
16
Generations after changeover to two substrates.

6. Benzoate ——→ p-OH benzoate + benzoate

Changeover from growth on benzoate (10 mM) to growth on p-OH benzoate and benzoate (both at 10 mM) led to the induction of p-OH benzoate hydroxylase (Figure 6.11). At steady state, this enzyme was about 25-35% induced. The p-OH benzoate concentration was 8.5 mM. On the other hand, benzoate oxidase was little affected by the presence of p-OH benzoate. Growth on benzoate as sole carbon source gave way to growth on a mixture of both substrates. The utilisation of benzoate decreased somewhat when p-OH benzoate began to be used and its concentration rose from 3.5 mM to 4.7 mM.

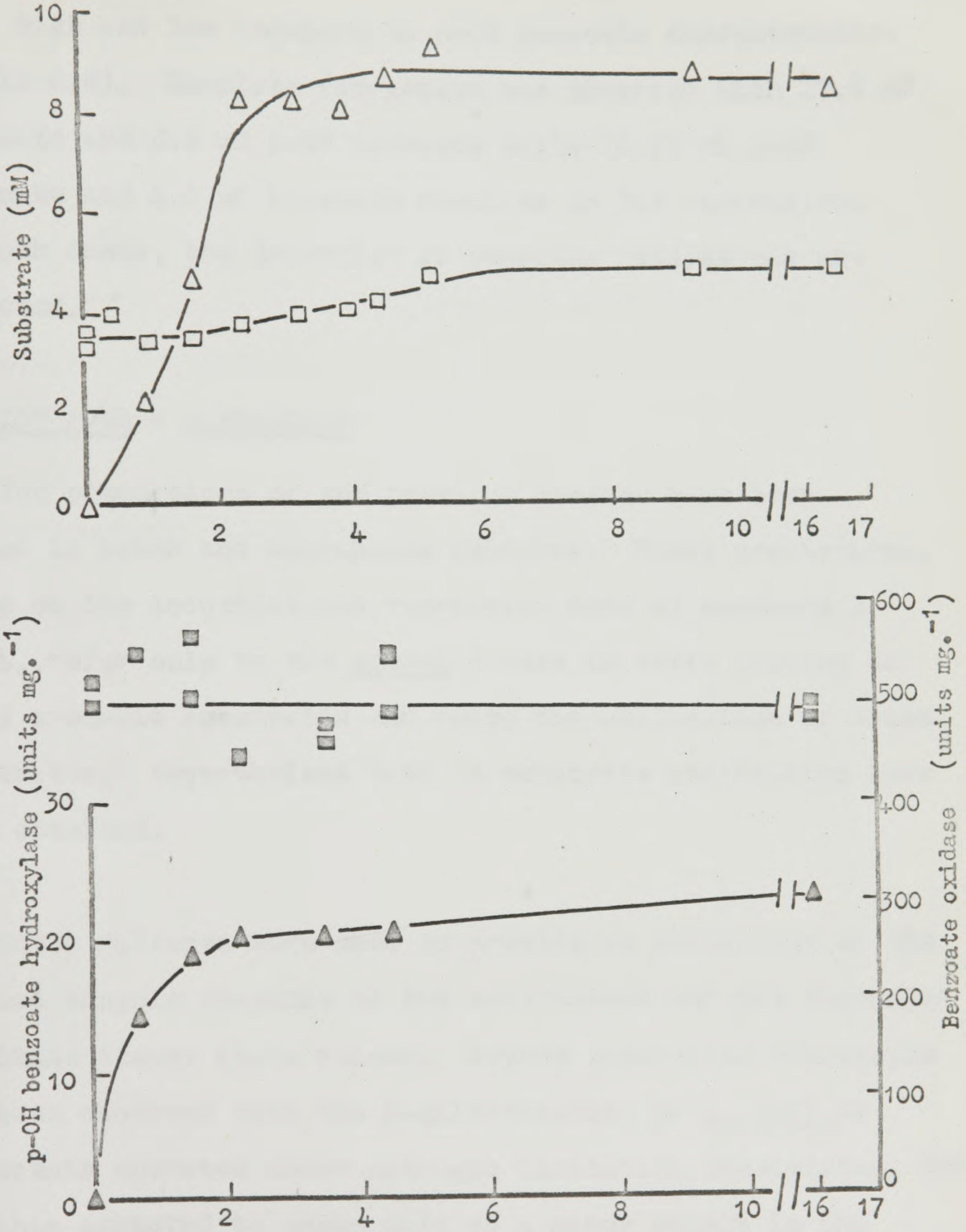
These results were again those predicted in Chapter 5 (Table 5.1).

Effect of altering the steady state concentration ratio of benzoate and p-OH benzoate on the induction of p-OH benzoate hydroxylase. Repression of p-OH benzoate hydroxylase by benzoate is partially reversible (Chapter 3), and so it seemed reasonable that the steady state ratio of these substrates should control the extent of induction of p-OH benzoate hydroxylase. This was tested by running chemostats

FIGURE 6.11

Enzymic adaptation to mixed aromatic substrates in continuous culture : (6) Benzoate —> p-OH Benzoate + benzoate.

Wild-type cells were grown in a nitrogen-limited chemostat on benzoate (10 mM) with a doubling time of 87 min. (D = 0.48 vol. $hr.^{-1}$). The specific activity of benzoate oxidase (■) and the benzoate concentration in the growth vessel (□) were determined at steady state. p-OH Benzoate (10 mM) was then added to the inflowing medium and the specific activities of benzoate oxidase and p-OH benzoate hydroxylase (▲) and the concentrations of benzoate and p-OH benzoate (△) measured at intervals until the new steady state had been established. The cell density was constant at 360 μg. dry wt. $ml.^{-1}$



Generations after changeover to two substrates.

with high and low benzoate to p-OH benzoate concentrations (Table 6.8). Complete repression was observed with 17.0 mM benzoate and 2.8 mM p-OH benzoate while 16.75 mM p-OH benzoate and 4.6 mM benzoate resulted in 30% repression. In both cases, the induction of benzoate oxidase was unaffected.

SECTION FOUR : DISCUSSION

The predictions of the previous chapter have been tested in batch and continuous cultures. These predictions, based on the induction and repression data of chapters 3 and 4, refer only to the enzyme levels in cells growing on mixed aromatic substrates and not to the utilisation of those substrates. Nevertheless data on substrate utilisation have been obtained.

Batch cultures were used to provide an indication of the initial enzymic response to the environment and the chemostat to obtain steady state values. Severe catabolite repression has been observed with the β-galactosidase of E. coli in chemostats operated under nitrogen limitation (Mandelstam, 1962) but this appeared to occur only to a minor extent in the aromatic acid system of P. putida and can be ignored in this discussion.

TABLE 6.8

Effect of concentrations of p-OH benzoate and benzoate on the enzymic adaptation to mixtures of p-OH benzoate + benzoate in continuous culture.

Wild-type cells were grown to steady state in nitrogen-limited chemostats on p-OH benzoate and benzoate with a doubling time of 90 min. (D = 0.45 vol. hr.$^{-1}$). The specific activities of p-OH benzoate hydroxylase and benzoate oxidase were determined. In the Table the inflow concentrations of the substrates are in parentheses.

p-OH Benzoate (mM)	Benzoate (mM)	p-OH Benzoate hydroxylase (units mg.$^{-1}$)	Benzoate oxidase (units mg.$^{-1}$)
16.75 (20)	4.8 (8.33)	53.5	500
2.8 (2.9)	17.0 (25)	5.0	480

In general, the predictions of Chapter 5 were experimentally validated in both batch and continuous cultures. The enzymic compositions of cells grown in p-OH benzoate + benzoate and in benzoate + mandelate were independent of which of the two alternative substrates had been the first growth substrate. This was predicted, but when a similar experiment was done with mandelate and p-OH benzoate, the result depended on the order of addition of the substrates. When p-OH benzoate was added to cells growing on mandelate, the specific activity of p-OH benzoate hydroxylase was about the same as that of cells growing solely on p-OH benzoate. On the other hand, cells grown first on p-OH benzoate lost about 50% of their p-OH benzoate hydroxylase when transferred to mandelate and p-OH benzoate. Benzoylformate decarboxylase was, as predicted, almost completely repressed in both cultures. No explanation of this decrease in p-OH benzoate hydroxylase can be offered.

A lag in the induction of the mandelate regulon was predicted when cells were transferred from benzoate to benzoate + mandelate. This was found in the chemostat studies, but inexplicably was not observed in the batch culture experiments. Again no explanation can be found for

this and the phenomenon was not investigated further. No period of intense, transient repression of the mandelate regulon was observed when cells growing on mandelate were transferred to mandelate + benzoate. This had been predicted on the basis of the difference in susceptibility to benzoate of mandelate-induced and uninduced cells (see Chapter 4).

In the other transition states, the specific activities of all the enzymes closely followed changes in the inducer or repressor concentrations. Again this was predicted, since, apart from the effect of benzoate on the mandelate regulon, no other transient repressor effects had been found with these substrates.

As expected, the extent of induction of the enzymes for the less preferred substrate in each case was dependent on, and could be varied by, the concentration ratio of the two substrates.

Measurement of the substrate concentrations in the growth vessel and the reservoir of the chemostat enabled substrate utilisation rates to be determined. The rate of utilisation of the less preferred substrate depended upon

the extent of induction of its enzyme system, but the relationship was not strictly proportional. This is consistent with the idea that catabolic enzymes may be synthesised in excess of the amount necessary to maintain growth (Miller, Beckwith and Müller-Hill, 1968). Substrate utilisation was negligible when its enzyme was more or less completely repressed.

Cases have been reported in which the addition of a second substrate to a growing culture immediatdy inhibited the metabolism of the first substrate even though its enzymes were still present (Gaudy, Gaudy and Komolrit, 1963; Stumm-Zollinger, 1966; McGinnis and Paigen, 1969). This catabolite inhibition may be due to feedback inhibition of key enzymes in the metabolic pathway (Zwaig and Lin, 1966; Wilson and Niederpruem, 1967; Hug, Roth and Hunter, 1968) or to the exclusion of the substrate from the cells (Horecker, Thomas and Monod, 1960; Adhya and Echols, 1966).

In this system, no evidence for any gross disparity of enzyme content and substrate utilisation was found, This is consistent with the statements by Stanier et al. (1965) and Hegeman (1966) that feedback inhibition does not occur in the aromatic system of P. putida.

In summary, therefore, the results of this chapter show that the extent to which different parts of the convergent pathways of aromatic acid metabolism are induced can be predicted from, and is largely determined by, the induction and repression constants of those enzymes immediately responsible for the metabolism of alternative substrates. The implications of this will be discussed in the next chapter.

CHAPTER SEVEN

SUMMARY AND

GENERAL DISCUSSION

SUMMARY AND GENERAL DISCUSSION

Induction constants of three inducing substrates - mandelate, benzoate and p-OH benzoate - have been obtained in Pseudomonas putida. Repression constants, describing the repressor effects of each substrate on the induction by either of the other two substrates, have also been measured. These constants have been used to predict the choice of enzyme pathways in cells growing on pairs of aromatic substrates (Table 5.1). The predictions were then tested in batch and continuous cultures. Since, in general, the enzymic behaviour of the cells conformed to the predictions, it seems that the induction and repression constants are in fact the parameters which determine the enzymic adaptation of this organism to mixed aromatic substrates. It is reasonable to suppose that induction and repression constants would determine the enzymic response to other inducing substrates in P. putida.

From the induction and repression constants alone, the substrate utilisation by cells growing in the presence of mixed substrates could not be predicted except when those constants indicated the complete or almost complete repression of an enzyme. Nevertheless, when substrate utilisation was measured experimentally in this study, it could be accounted for, at least qualitatively, by the enzyme induction.

Pardee (1961) has suggested that for microorganisms, an ability to grow fast is of immense selective advantage. Frequently, a fast growth rate is not associated with the most efficient utilisation of the carbon source. For instance, when E. coli is grown aerobically on glucose, it grows rapidly and produces acetate (Roberts et al., 1955), even though complete oxidation of the acetate to CO_2 and H_2O would yield much more energy. Only after the glucose is exhausted is the acetate used to any great extent. Similarly, when P. oxalaticus is grown on formate and oxalate, formate is metabolised preferentially and results in a faster growth rate than oxalate could support (Blackmore and Quayle, 1968). On the basis of ATP and NADH yield per mole of substrate, oxalate would appear to be the better substrate (Blackmore et al., 1968).

The growth rate in simple media is presumably determined by the rate at which monomers or energy can be supplied for protein synthesis (Pardee, 1961; Maynard-Smith, 1969). Ierusalimsky (1967) has shown that one reaction of the cell, be it an enzyme supplying energy or biosynthetic intermediates or the uptake of a substrate, can be considered as the growth-limiting process. Any other metabolic activity of the cell that reduces the efficiency of this rate-

limiting step, e.g. by depriving it of energy or substrate, reduces the overall rate of growth. Overproduction or over-activity of other enzymes are obvious examples of the uneconomic use of energy or metabolites which could be better used for overall protein synthesis and growth.

In laboratory conditions, economy of protein synthesis has been correlated with an increased growth rate and selection of the economic strain over less strictly controlled strains. Thus Novick (1961) showed that mutants of E. coli constitutive for β-galactosidase grew more slowly than inducible strains in media where the enzyme was not required for growth. Prototrophs grow more slowly than auxotrophs in media which meet the nutritional requirement (Roeptke, Libby and Small, 1944; Zamenhof and Eichhorn, 1967). Mutants lacking feedback inhibition also grow more slowly than the wild-type (Baich and Johnson, 1968). In all cases, the faster growing form established numerical superiority over the slower growing form in a comparatively small number of generations.

If this were generally true, one would expect that, in the presence of two substrates (one of which is able to support a faster growth rate than the other) there would

be a selective advantage for the strain which can ignore the poor substrate and devote all its 'metabolic attention' to the better substrate. Economy of protein synthesis would be gained by completely repressing the enzymes required to metabolise the poorer substrate.

Besides studying the growth of Pseudomonas oxalaticus on a mixture of formate and oxalate, Blackmore and Quayle (1968) also used mixtures of formate and other carbon sources. The carbon of formate is used autotrophically via two inducible enzymes, ribulose diphosphate carboxylase and phosphoribulokinase (Quayle and Keech, 1960). The other carbon sources are used heterotrophically. When the organism was grown on formate and another carbon source, e.g. succinate, glycerol, malonate, that carbon source which would normally have supported the faster growth rate on its own was used and the enzymes for the metabolism of the poorer substrate were repressed.

In contrast, the present study has shown that Pseudomonas putida, growing in the presence of alternative aromatic carbon sources, does not appear to select the one that supports the faster growth rate. This is particularly obvious in the case of mandelate and p-OH benzoate.

Mandelate supports a rate of growth about 30% faster than p-OH benzoate (Table 2.3), yet growth on a mixture of these two substrates leads to almost complete repression of the mandelate pathway. In the case of mandelate and benzoate, mandelate again is the substrate capable of supporting faster growth (Table 2.3), but growth in mixtures of mandelate and benzoate led to partial repression of the mandelate system and mixed substrate utilisation with benzoate predominating.

The third pair of aromatic substrates, p-OH benzoate and benzoate, did appear to result in the partial selection of the substrate capable of supporting the faster growth rate, i.e. benzoate. However, repression of the p-OH benzoate system was not complete and mixed substrate utilisation, with benzoate predominating, took place.

Without detailed investigation of the molecular concentrations of the enzymes of these pathways, it is not possible to say whether mandelate or p-OH benzoate requires the greater number of enzyme molecules for its metabolism. However, mandelate metabolism clearly involves more enzymes than the metabolism of benzoate and it has to be concluded that, in the case of these two substrates, protein economy rather

than growth rate is the factor determining the choice of substrate.

Virtually nothing is known about the natural habitat of Pseudomonas putida except that it is one of a large number of soil-living microorganisms metabolising soluble aromatic soil constituents arising from fungal degradation of plant aromatic polymers (principally lignin). It seems certain, however, that substrate concentrations in natural habitats, far from approaching those considered normal in laboratory experiments, are likely to be very low and growth-limiting. In addition, other species will be present, competing for those limited amounts of substrate. Tempest (1970) has argued that the ability to grow at sub-maximum rates on limiting substrates would be more likely to be of value to an organism in its natural environment than would the property of having a fast growth rate at high substrate concentrations.

This may be so, but data on the quantitative occurrence of soluble aromatic acids in the soil are restricted. However, some conclusions can be drawn. The data of Shorey (1914) and Walters (1917) suggest that the benzoate concentration of certain soils might be in the range of 0.5 - 0.05 mM. The

concentration of p-OH benzoate could be in the range 0.7 - 0.008 mM (Walters, 1917; Whitehead, 1964). Benzoate oxidase is likely, therefore, to be less than fully induced in cells of Pseudomonas putida living under natural conditions, whereas p-OH benzoate hydroxylase could be fully induced.

It is also clear that different soils vary quite widely in their aromatic acid content (Shorey, 1914; Walters, 1917; Waksman, 1938; Flaig, 1960; Whitehead, 1964; Martin-Martinez and de Petre, 1965). Since ruminant faeces are rich in benzoate [as hippurate] (Waksman, 1952; Brauns and Brauns, 1960), grazing land might have quite high local benzoate concentrations. The occurrence of mandelate is likely to be particularly restricted, since it occurs only as the glucoside in certain plants, e.g. Prunus spp.(Cross, Bunbury & Hey, 1965). Hence the soil water around particles of plant remains may be rich in specific aromatic acids. Soil microorganisms, including P. putida, that can colonise the hyphae of fungi actively engaged in degrading aromatic plant debris may also be exposed to locally different proportions and high concentrations of soluble aromatic substrates (Lockwood, 1968).

It is therefore very difficult to suggest what the environment of P. putida might originally have been, let alone define the selection pressures that have led to the control observed in the aromatic pathways. Presumably, protein economy, rather than fast growth rate, was the determining factor and must have been of some evolutionary advantage in the organism's ecological niche.

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