Induction and Activity of the Mandelate Pathway Enzymes in Bacterium NCIB 8250

by

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A C K N O W L E D G E M E N T S.

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A B B R E V I A T I O N S.

The standard abbreviations found in Biochem. J. (1970). 116, 1. are used throughout this thesis with the exception of:

o	^{o}C
Tris	2-amino-2-hydroxymethylpropane-1,3-diol

In addition the following abbreviations are used:

NCIB	National Collection of Industrial Bacteria
ATCC	American Type Culture Collection
Carboxy Q	N-ethyl-2-carboxy-7-chloro-4-quinolone
N.T.G.	N-methyl-N-nitroso-N′-nitroguanidine
Hepes	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
Tricine	N-tris (hydroxymethyl) methylglycine

No Enzyme Commission numbers are given for the enzymes under study since some of the enzymes have not been assigned numbers, and recent work has put in doubt the validity of the Enzyme Commission numbers of some of the other enzymes.

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S U M M A R Y.

(1) This thesis starts with a synopsis of the literature on the metabolism of mandelate and related compounds by micro-organisms. Present knowledge of the control of these pathways is also summarised.

(2) The experimental work described in this thesis is primarily concerned with the activity and control of the enzymes converting L-mandelate and benzyl alcohol to cis,cis-muconate in bacterium NCIB 8250 which is an Acinetobacter species.

(3) Techniques were worked out for mutagenesis with N-methyl-N-nitroso-N′-nitroguanidine and ultraviolet irradiation. Using the former treatment, "mandelate mutants", i.e. strains able to grow on benzoate or benzyl alcohol but not on L-mandelate, "benzyl alcohol mutants", i.e. strains able to grow on benzoate or L-mandelate but not on benzyl alcohol, and "benzoate mutants", i.e. strains unable to grow on L-mandelate, benzyl alcohol or benzoate, were isolated.

Three classes of mandelate mutants were obtained: those which lacked L-mandelate dehydrogenase; those which lacked benzoylformate decarboxylase; and those which lacked L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase.

The three benzyl alcohol mutants examined did not possess a functional benzyl alcohol dehydrogenase or labile benzaldehyde dehydrogenase.

All the benzoate mutants studied were able to grow on catechol but not on benzoate or its precursors.

Mutants meso-constitutive for L-mandelate dehydrogenase and benzoylformate decarboxylase and hyperinducible for the stable benzaldehyde dehydrogenase were obtained as a result of spontaneous mutation and incubation with N-methyl-N-nitroso-N′-nitroguanidine.

(4) An examination of the growth patterns of the blocked mutants provided supporting evidence for the postulates of Kennedy (1967) and Kennedy & Fewson (1968a,b) that the enzymes which convert L-mandelate, benzyl alcohol and their substituted analogues to the corresponding benzoates are non-specific in their activity and induction; that L-mandelate is metabolised to benzoate only through benzoylformate; and that 2-hydroxybenzoate is oxidised directly to catechol and not through benzoate.

(5) Heat denaturation experiments, the properties of the blocked mutants and the pattern of monovalent cation activation provided evidence for the existence of a heat-stable benzaldehyde dehydrogenase with a half-life of about 500min at 37°, and a heat-labile benzaldehyde dehydrogenase with a half-life of about 30min at 37°. Assays were devised in which the activity of the two enzymes could be differentiated.

(6) Benzoate oxidase activity was obtained in a cell-free extract. The enzyme requires FAD, an NADH generating system and Fe^{++} ions for optimal activity. The properties of the

enzyme were related to its possible mode of action. The activity obtained was low, and consequently the enzyme was measured in whole cells in the experiments on enzyme induction.

(7) A method was worked out for measuring L-mandelate dehydrogenase, benzoylformate decarboxylase, the stable and labile benzaldehyde dehydrogenase, benzyl alcohol dehydrogenase, catechol oxygenase and NADH oxidase activity in a single cell-extract. If the benzaldehyde dehydrogenase activity was very high, the activity of the labile and stable enzymes had to be measured in a separate extract.

(8) Experiments on the kinetics of induction showed that: L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase were coordinately induced (Regulon R_1); the labile benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase were coordinately induced (Regulon R_2); and benzoate oxidase (Regulon R_3) and catechol oxygenase (Regulon R_4) were independently regulated. Supporting evidence for these conclusions came from a study of the blocked and constitutive mutants and from an examination of the enzymes gratuitously induced by thiophenoxyacetate. The inductive properties of the constitutive mutants and the inductive pattern produced by thiophenoxyacetate indicated that Regulon R_1 can be divided into two subunits consisting of L-mandelate dehydrogenase and benzoylformate decarboxylase on the one hand and the stable benzaldehyde dehydrogenase on the other hand.

(9) From a study of the mandelate mutants, it was concluded that benzoylformate is the primary inducer of the Regulon R_1 enzymes. It was not possible to determine whether benzyl alcohol, benzaldehyde or both are inducers of the Regulon R_2 enzymes. Several suggestions were put forward to explain the induction of the Regulon R_2 enzymes by benzoylformate.

(10) It was concluded that the pathway for the formation of benzoate from L-mandelate and benzoylformate is physiologically and genetically distinct from the pathway which converts benzyl alcohol and benzaldehyde to benzoate. The pathways occurring in bacterium NCIB 8250 were compared with those in other organisms.

I N T R O D U C T I O N.

1. GENERAL ASPECTS OF DISSIMILATION OF AROMATIC COMPOUNDS BY MICRO-ORGANISMS.

All living organisms and especially the plant world synthesise a vast array of aromatic structures ranging from simple phenols to complex polymers such as tannin and lignin. It is now generally accepted that all biologically synthesised organic molecules must be degraded by some form of life to maintain the Biochemical Cycle of Nature. In the case of aromatic compounds this is largely accomplished by several bacterial and fungal species (e.g. Alexander, 1961; Dagley, 1967; Evans, 1968). These micro-organisms are able to metabolise a large variety of benzenoid structures either as sources of organic carbon for growth or by co-metabolism with other substrates (e.g. Raymond, Jamison & Hudson, 1967; Fewson, Kennedy & Livingstone, 1968). The disruption of benzene ring compounds by micro-organisms has attracted more and more attention in recent years as a means of destroying toxic aromatic by-products of industry (e.g. Lunt & Evans, 1970) and pesticides, which are frequently substituted aromatic hydrocarbons (e.g. Kearney & Kaufman, 1969).

The aromatic ring has to be opened to allow complete metabolism. Ring cleavage is effected through either ortho- or para-dihydroxyphenols so that the initial transformations of

aromatic compounds are aimed at producing one of these key compounds. Polynuclear compounds are degraded by a series of successive ring openings (Dagley, 1967).

The cleavage of ortho-dihydroxyphenols can be divided into two distinct types (Evans, 1968):
(1) the oxidative cleavage of the carbon-carbon bond between two adjacent hydroxylated carbon atoms ("ortho cleavage"); and (2) the oxidative cleavage of the carbon-carbon bond between a hydroxylated and a non-hydroxylated carbon atom ("meta cleavage").

These two methods of benzenoid ring cleavage produce a muconic acid and a muconic acid semialdehyde respectively. The muconic acid is then metabolised to β-oxoadipate which is cleaved to succinate and acetyl-CoA, and the muconic acid semialdehyde is converted to pyruvate and a carbonyl derivative whose structure depends on the substituents on the original benzenoid nucleus (Ribbons, 1965). This variety of ring opening mechanisms leads to the situation that the same compound may be degraded in different ways by different organisms; e.g. catechol is subjected to ortho cleavage by Pseudomonas putida to give cis,cis-muconate (Ornston & Stanier, 1964) and to meta cleavage by a pseudomonad examined by Dagley, Evans & Ribbons (1960) to give α-hydroxymuconic acid semialdehyde. Conversely one organism may possess several different enzymes for ring cleavage; e.g. Pseudomonas fluorescens possesses two specific enzymes for the ortho cleavage of

protocatechuate and caffeate, and two more enzymes for the meta cleavage of catechol and homoprotocatechuate (Seidman, Toms & Wood, 1969). A number of the enzymes responsible for ortho and meta cleavage has been purified and extensively examined (Nozaki, Kojima, Nakazawa, Fujisawa, Ono, Kotani, Hayaishi & Yamano, 1966; Hayaishi & Nozaki, 1969).

The cleavage of para-dihydroxyphenols is achieved by the rupture of the bond between the carbon atom bearing a hydroxyl group and an adjacent carbon atom carrying a hydrogen or carboxyl group, or a side-chain. Thus homogentisate is split by Vibrio O1 to give maleylacetoacetate (Chapman & Dagley, 1962).

Although the cleavage of the aromatic nucleus is generally an aerobic process involving the incorporation of atmospheric oxygen, Rhodopseudomonas palustris possesses a reductive pathway for the anaerobic metabolism of benzoate (Dutton & Evans, 1969; Guyer & Hegeman, 1969). The initial stages of this pathway involve the reduction of benzoate to cyclohex-1-ene-1-carboxylate and the conversion of this compound to pimelate.

2. METABOLISM OF MANDELATE AND RELATED COMPOUNDS.

Mandelate can be utilised as sole source of carbon and energy by a few bacteria belonging to the genus Pseudomonas (Stanier, Palleroni & Doudoroff, 1966) or to the genus Acinetobacter (Baumann, Doudoroff & Stanier, 1968). Recently Jamaluddin, Subba Rao & Vaidyanathan (1970) showed that the fungus, Aspergillus niger, can also metabolise mandelate. It is possible that this compound can be used as a growth substrate by a number of other organisms which have not yet been tested for growth on it. Mandelate is probably not often used as a possible growth substrate, and negative results are unlikely to be published. The one report in the literature of an organism being unable to grow on mandelate is that of Taylor & Hoare (1969) who found that Thiobacillus A2 did not grow on this compound.

The metabolism of mandelate and related compounds will be discussed in two parts, which are:

(1) the manipulation of the side chain and ring hydroxylation leading to the formation of the ortho-dihydroxyphenols, catechol and protocatechuate;

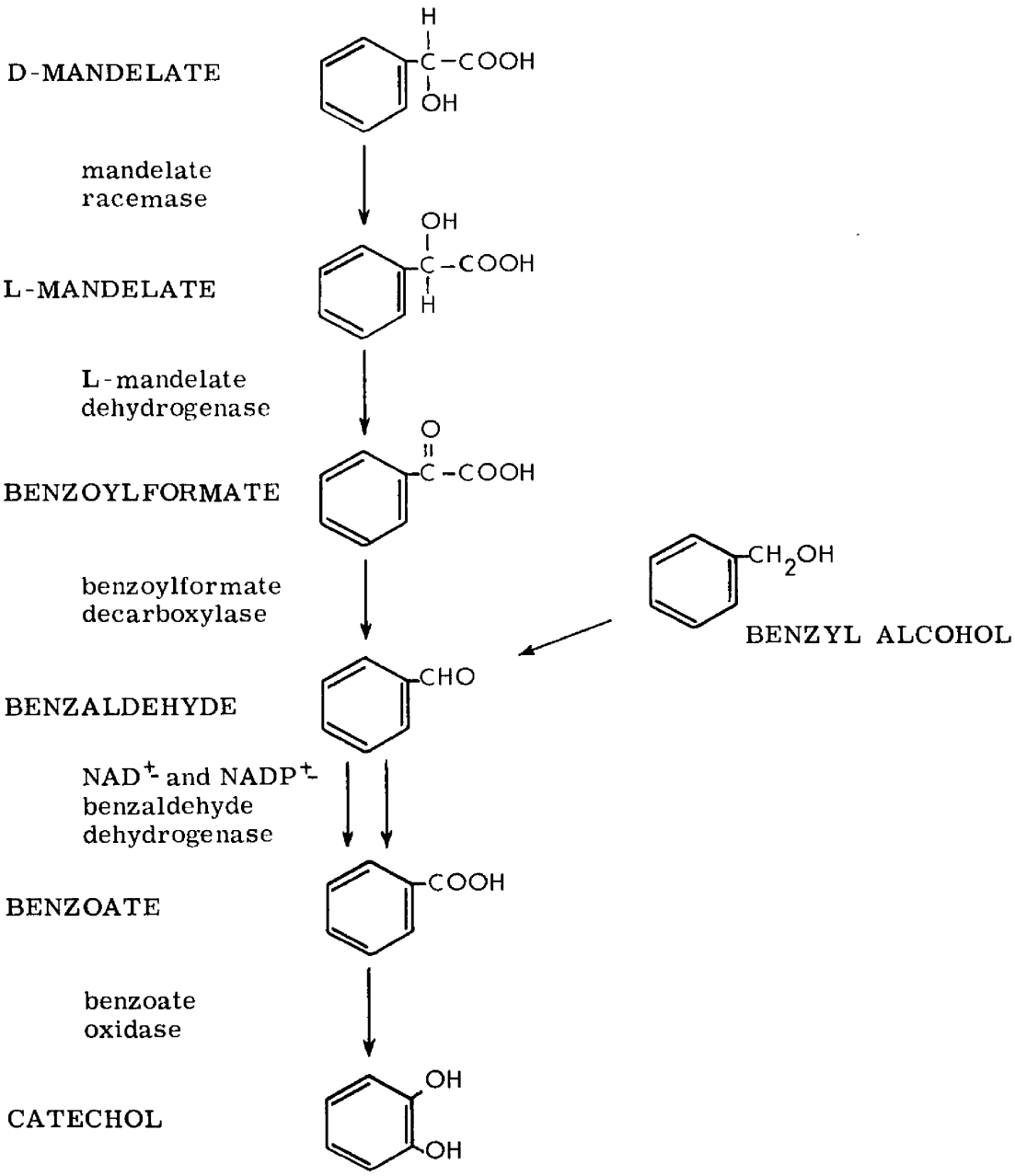
and (2) the ring cleavage of catechol and protocatechuate, and the subsequent metabolism of the aliphatic products formed.

2.1. Oxidation of mandelate to catechol.

2.1.1. The pathway operating in Pseudomonas species.

Stanier (1947 & 1948) proposed from growth and simultaneous adaptation experiments that in P. putida (ATCC 12633, then known as P. fluorescens A.3.12 (Stanier et al., 1966)) D,L-mandelate was oxidised to benzoate via benzoylformate and benzaldehyde (Fig.1). Confirmation of the suggested pathway was provided by Stanier, Gunsalus & Gunsalus (Gunsalus, Stanier & Gunsalus, 1953; Gunsalus, Gunsalus & Stanier, 1953; Stanier, Gunsalus & Gunsalus, 1953) who succeeded in chemically identifying the intermediates, and separating the enzymes which effected the conversion of D,L-mandelate to benzoate. These enzymes were mandelate racemase, L-mandelate dehydrogenase, benzoylformate decarboxylase, and an NAD^+- and an $NADP^+$-specific benzaldehyde dehydrogenase. The reason for this organism having two benzaldehyde dehydrogenases to carry out the one biochemical reaction is still not known. Three of these enzymes, mandelate racemase (Weil-Malherbe, 1966), benzoylformate decarboxylase (Sampson & Hunt, 1969) and the $NADP^+$-specific benzaldehyde dehydrogenase (Stachow, Stevenson & Day, 1967) have been extensively purified, and their catalytic properties have been examined. Stevenson & Mandelstam (1965) have subsequently obtained indirect evidence for the existence of a third benzaldehyde dehydrogenase

OXIDATION OF MANDELATE TO CATECHOL IN PSEUDOMONAS PUTIDA



induced by growth on benzaldehyde. This enzyme was detected in whole cells, but not in cell-free extracts as it was destroyed in the extraction procedure.

Stanier (1948) proposed that benzyl alcohol was converted to benzoate via benzaldehyde (Fig.1), but this initial idea based on growth experiments was never followed up. On the other hand, Claus & Walker (1964) appeared to exclude the possibility that benzyl alcohol was oxidised to benzoate in a soil pseudomonad on the basis of simultaneous adaptation studies.

The simultaneous adaptation experiments of Sleeper & Stanier (1950) suggested that catechol was produced by the oxidation of benzoate. This substantiated the original work of Evans (1947) who had shown that growth of Vibrio O1 on benzoate led to the accumulation of small amounts of catechol in the medium. Sleeper (1951) verified that benzoate was converted to catechol by the use of [carboxy-^{14}C]benzoate and [1-^{14}C]benzoate. From the distribution of the label in the products he deduced that one of the hydroxyl groups of catechol replaced the carboxyl group of benzoate and that the other hydroxyl group was attached in ortho juxtaposition. 2-Hydroxybenzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, 2,3-dihydroxybenzoate, 3,4-dihydroxybenzoate, 2-hydroxybenzaldehyde and phenol, which were thought to be likely intermediates between benzoate and catechol by Sleeper & Stanier (1950), were ruled out by growth and simultaneous adaptation

experiments. Although no intermediates between benzoate and catechol could be identified in P. putida, Voets (1958) and Walker & Harris (1970) showed that in some species of Azotobacter, 2-hydroxybenzoate is an intermediate in the conversion of benzoate to catechol. Mandelstam & Jacoby (1965), Kemp & Hegeman (1968) and Dr G.D. Hegeman (Department of Bacteriology and Immunology, University of California, Berkeley, U.S.A., private communication) have been unable to detect benzoate oxidase in cell-free extracts of Pseudomonas species.

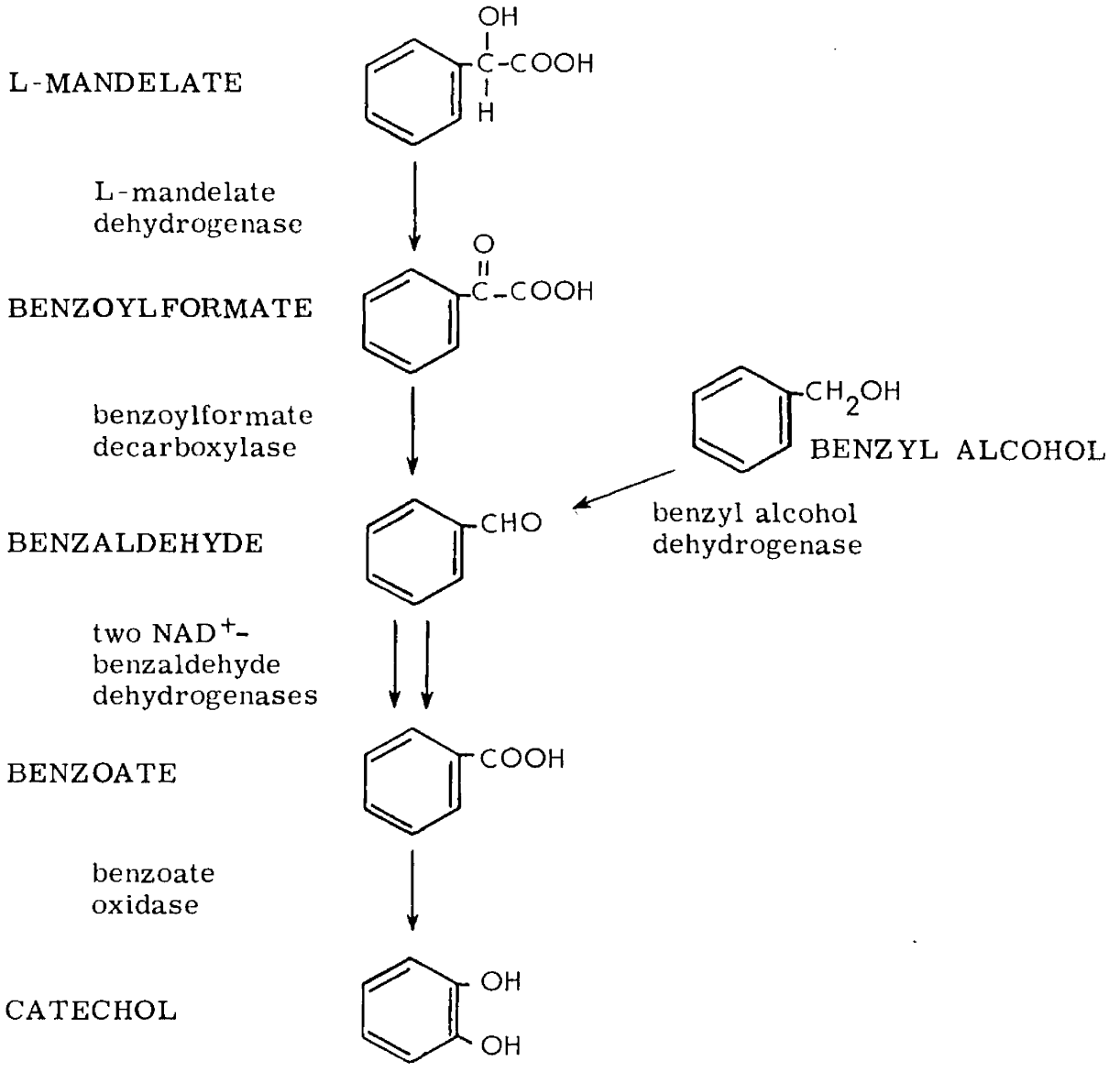
Mandelate is converted to benzoate by an analogous pathway in Pseudomonas aeruginosa (ATCC 15692) (Rosenberg & Hegeman, 1969) except that this organism appears to have only one benzaldehyde dehydrogenase and no mandelate racemase so that it can oxidise only the L-isomer of mandelate.

2.1.2. The pathway operating in Acinetobacter species.

A similar pathway for the oxidation of mandelate and benzyl alcohol to catechol (Fig.2) was shown to operate in bacterium NCIB 8250 (an Acinetobacter sp.; see p.44) by Kennedy & Fewson (1968a,b). This organism was unable to metabolise D-mandelate and contained two NAD^+- benzaldehyde dehydrogenases rather than both an NAD^+- and an $NADP^+$- benzaldehyde dehydrogenase. One of the benzaldehyde dehydrogenases was found to be heat-stable and the other heat-labile. Their evidence for the pathway came from simultaneous adaptation experiments and the detection of the enzymes in

Fig.2.

OXIDATION OF MANDELATE TO CATECHOL IN BACTERIUM NCIB 8250



cell-free extracts. Benzoate oxidase in this organism, like that in Pseudomonas species could not be detected in cell-free extracts (Fewson, private communication). Although Kennedy & Fewson (1968a) postulated that L-mandelate was metabolised via benzoylformate, they could not exclude the possibility that a proportion of the L-mandelate was decarboxylated to form benzyl alcohol. Stanier had apparently not considered this rather unlikely possibility in his work with P. putida.

2.1.3. The pathway operating in the fungus Aspergillus niger.

The pathway for the oxidation of mandelate in Aspergillus niger (Fig.3) (Jamaluddin et al., 1970) shows several differences from the bacterial system. There are two specific mandelate dehydrogenases with quite different properties, which convert L- and D-mandelate directly to benzoylformate. Benzoylformate is then metabolised to benzoate which undergoes successive hydroxylations to give 4-hydroxybenzoate and protocatechuate.

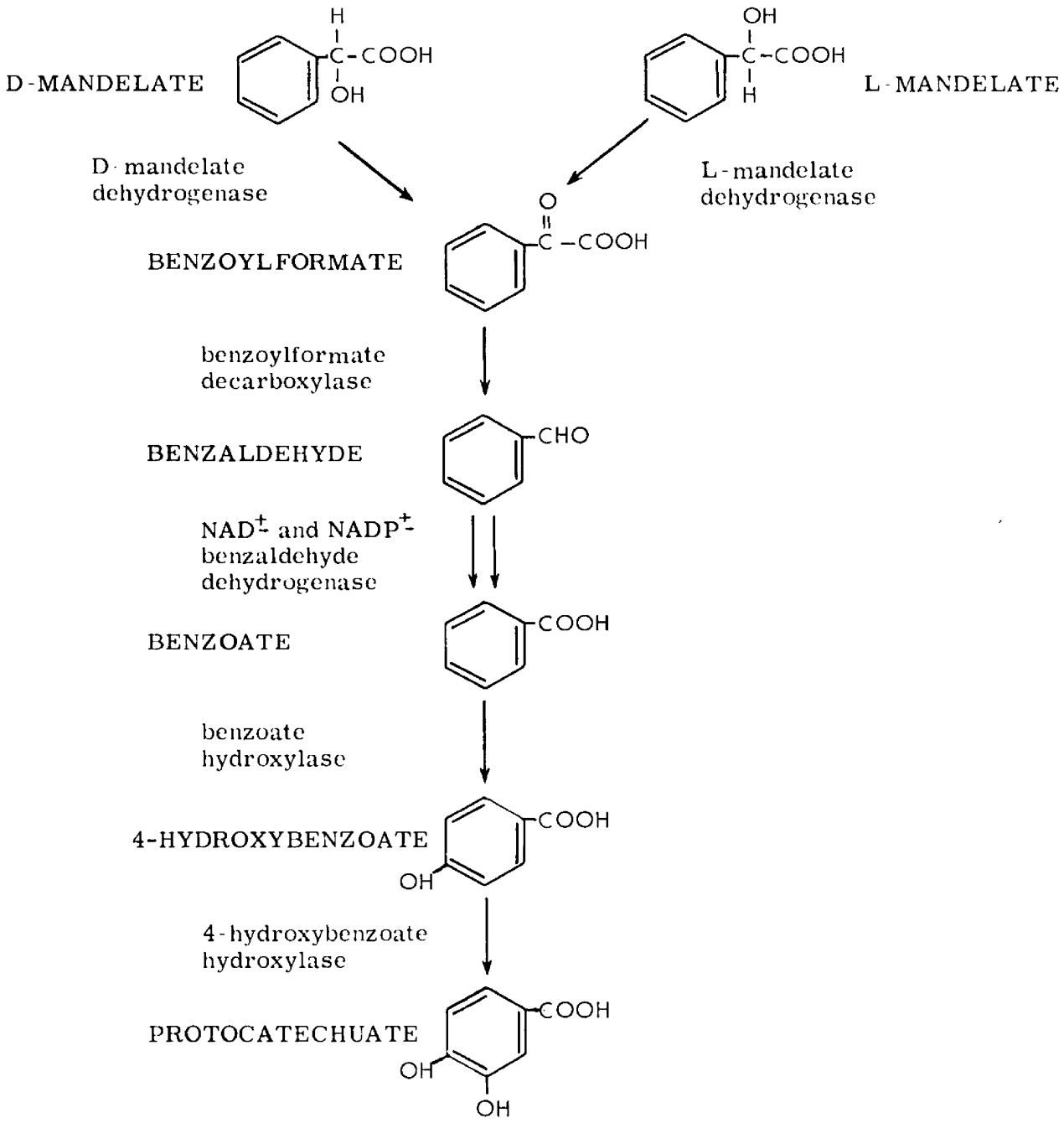
2.2. Oxidation of substituted mandelates to catechol or protocatechuate.

2.2.1. The pathway operating in Pseudomonas putida.

Gunter (1953) showed that 4-hydroxy-D,L-mandelate was metabolised to 4-hydroxybenzoate in P. putida by a pathway parallel to that for the metabolism of D,L-mandelate, except that the D-isomer of 4-hydroxymandelate was not oxidised. Stanier et al. (1953) suggested that a common set of enzymes

Fig.3.

OXIDATION OF MANDELATE TO PROTOCATECHUATE IN ASPERGILLUS NIGER



could be responsible for the conversion of both 4-hydroxy-mandelate and mandelate to the corresponding benzoates. This suggestion was substantiated by Stevenson & Mandelstam (1965) who isolated a mutant of this organism lacking L-mandelate dehydrogenase activity which was unable to grow on either mandelate or 4-hydroxymandelate.

The simultaneous adaptation experiments of Sleeper & Stanier (1950) indicated that 4-hydroxybenzoate was oxidised to protocatechuate. 4-Hydroxybenzoate hydroxylase, the enzyme which catalyses this reaction, has been purified by Hosokawa & Stanier (1966) and its reaction mechanism has been studied by Hesp, Calvin & Hosokawa (1969).

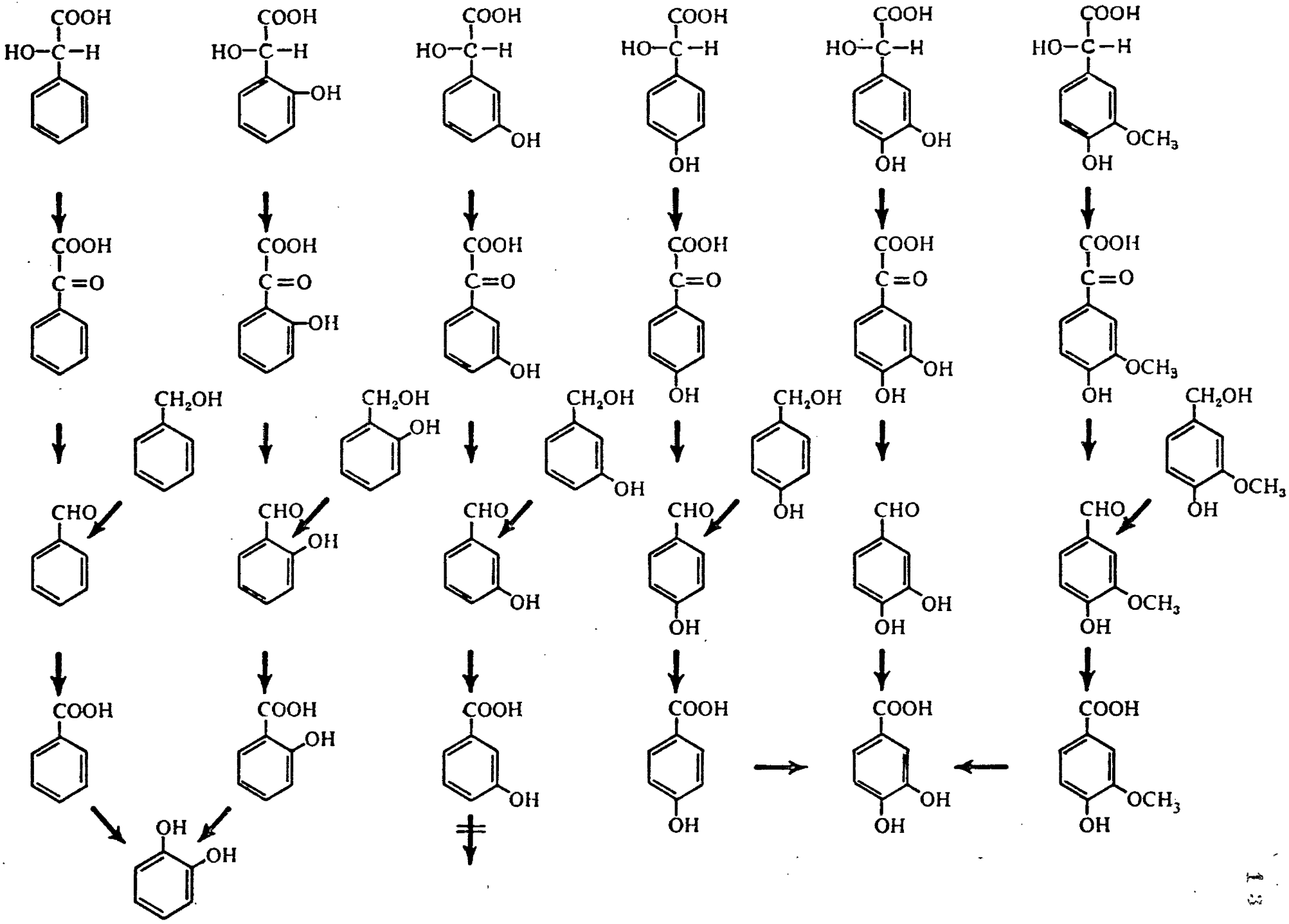
2.2.2. The pathway operating in bacterium NCIB 8250.

Kennedy & Fewson (1968a,b) postulated from simultaneous adaptation experiments and from an examination of the kinetic properties of the enzymes involved that bacterium NCIB 8250 oxidised L-mandelate, benzyl alcohol and their substituted analogues, which included the 2-hydroxy, 3-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy derivatives, to the corresponding benzoates by a series of parallel pathways whose enzymes were non-specific in their activity and induction.

Metabolism of the benzoates was brought about by specific enzymes which were generally produced only by growth on a homologous substrate, and which converted the benzoates to either catechol or protocatechuate (Fig.4). The exception to this observation was that growth on 2-hydroxybenzoate induced

Fig.4.

The metabolism of mandelate and related compounds by bacterium NCIB 8250.


COOH
HO–C–H
OH
C=O
CH2OH
CHO
OCH3

benzoate oxidase as well as 2-hydroxybenzoate hydroxylase (Kennedy & Fewson, 1968a, their Table 3). Consequently Kennedy & Fewson (1968a) could not exclude the possibility that benzoate was an intermediate in the conversion of 2-hydroxybenzoate to catechol.

The pathway for the metabolism of 4-hydroxy-3-methoxybenzoate was based on simultaneous adaptation experiments, but the enzymes involved were never examined. Cartwright & Buswell (1967), however, separated vanillate O-demethylase, the enzyme which converts 4-hydroxy-3-methoxybenzoate to protocatechuate, from protocatechuate 3,4-oxygenase in Pseudomonas T, and were able to show that the first intermediate in the dissimilation of 4-hydroxy-3-methoxybenzoate was protocatechuate.

3-Hydroxybenzoate is not oxidised by bacterium NCIB 8250 as the organism does not possess an enzyme capable of its further metabolism. A number of other substituted mandelates and benzyl alcohols; e.g. nitro, fluoro, bromo and chloro, can also be degraded to the corresponding benzoates by suitably induced cells (Kennedy & Fewson, 1968a). Only in the case of the fluoro-substituted compounds does metabolism proceed beyond the benzoate level (Kennedy & Fewson, 1968a; Fewson et al., 1968). None of these partially metabolised compounds supports growth by itself.

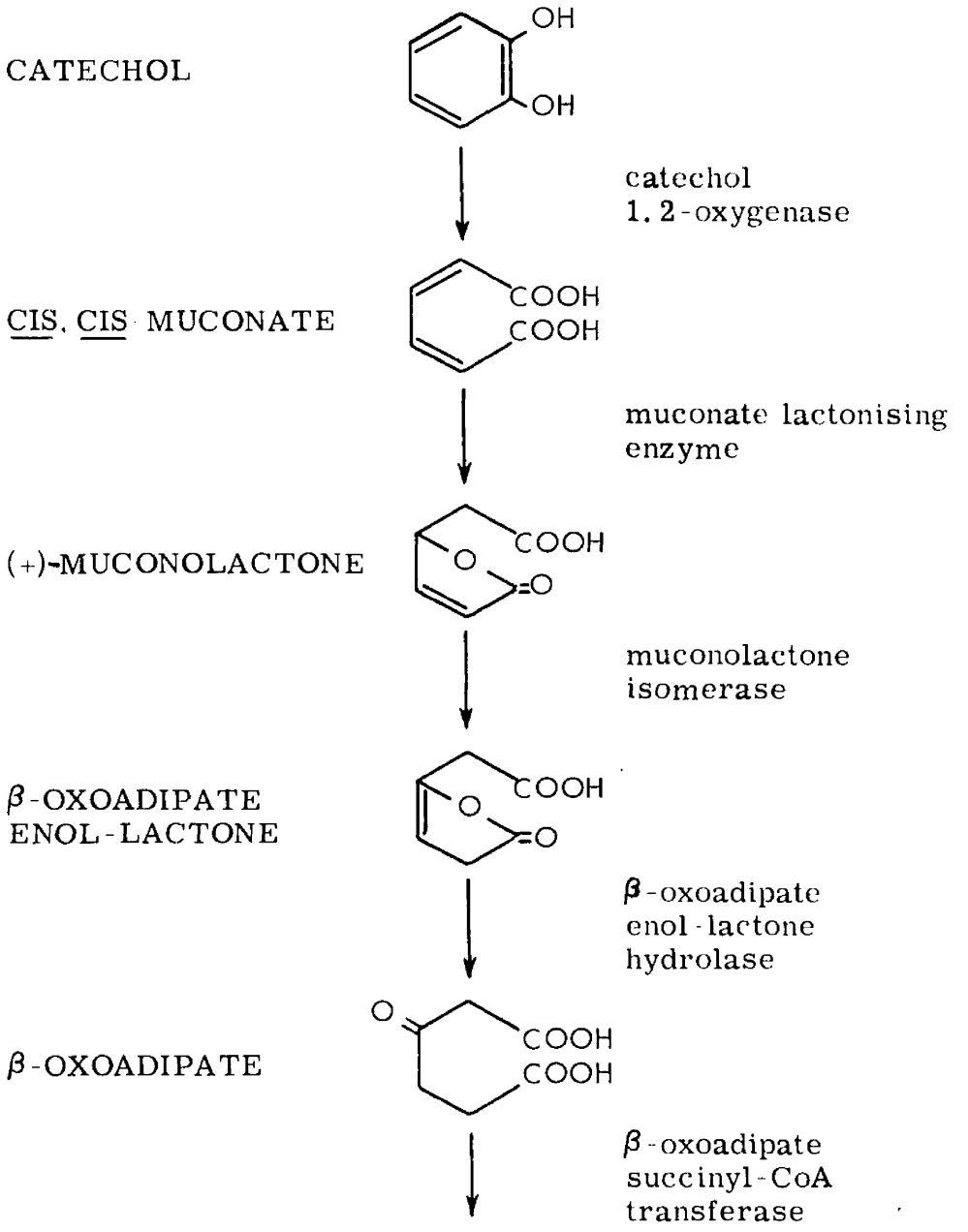
2.3. Conversion of catechol to β-oxoadipyl-CoA.

The reactions involved in the conversion of catechol to β-oxoadipyl-CoA (Fig.5) are identical in P. putida (ATCC 12633) and in Acinetobacter species (Cánovas, Ornston & Stanier, 1967). In both types of organism, ortho cleavage of catechol occurs. The individual intermediates and enzymes of the pathway were by and large identified using P. putida as the biological material, but all the enzymes of the pathway have since been detected in P. aeruginosa (ATCC 15692) (Kemp & Hegeman, 1968) and in Acinetobacter calcoaceticus (ATCC 23393) (Cánovas & Stanier, 1967), which is closely related to bacterium NCIB 8250, but which cannot grow on mandelate (Baumann et al., 1968).

Cis,cis-muconate, the product of ring opening and (+)-muconolactone were identified as intermediates of the pathway by Evans & Smith (1951) and Evans, Smith, Linstead & Elvidge (1951) who showed that freeze-dried cells of P. fluorescens and Vibrio 01 grown on catechol converted these compounds to β-oxoadipate. The next intermediate in the pathway, β-oxoadipate enol-lactone, was identified by Ornston & Stanier (1966). β-Oxoadipate was first implicated as a possible metabolite in the oxidation of aromatic compounds by Kilby (1948) who identified it as an excretion product in cultures of Vibrio 01 grown on phenol. Subsequently Stanier, Sleeper, Tsuchida & McDonald (1950) isolated and characterised β-oxoadipate as the product of catechol metabolism by freeze-

Fig.5.

THE CONVERSION OF CATECHOL TO β-OXOADIPYL-CoA IN PSEUDOMONAS PUTIDA AND ACINETOBACTER CALCOACETICUS



dried cells of P. putida grown on mandelate. The work of Ornston (1966c) implicated β-oxoadipyl-CoA as the terminal intermediate of the pathway although it has not yet been chemically identified. He found that cells of P. aeruginosa (ATCC 17503) and Pseudomonas multivorans (ATCC 17759) grown on adipate gratuitously synthesised those enzymes of the catechol pathway which are induced by β-oxoadipate. From this observation he concluded that β-oxoadipyl-CoA was an intermediate of the catechol pathway as it was the only compound likely to be common to both the catechol and adipate pathways.

With the exception of the β-oxoadipate succinyl-CoA transferase in P. putida, all the enzymes involved in the conversion of catechol to β-oxoadipyl-CoA in P. putida (Ornston, 1966c), in P. aeruginosa (Kemp & Hegeman, 1968) and in A. calcoaceticus (Cánovas & Stanier, 1967) have been detected in cell-free extracts, and in a number of cases have been extensively purified (Ornston, 1966a,b). In bacterium NCIB 8250, although not all the enzymes of the catechol pathway have been measured, three key enzymes, catechol 1,2-oxygenase (Kennedy & Fewson, 1968b), muconolactone isomerase (Ornston & Stanier, 1966) and β-oxoadipate enol-lactone hydrolase (Ornston, 1966c) have been assayed. Presumably, therefore, this organism metabolises catechol by the pathway shown in Fig.5.

Although the organisms discussed in this Section dissimilate catechol through the ortho cleavage pathway, there

are many reports in the literature of organisms employing the meta cleavage pathway; e.g. Vibrio 01 growing on naphthalene has been reported to synthesise a catechol 2,3-oxygenase (Griffiths, Rodrigues, Davies, & Evans, 1964), and P. putida (NCIB 10105) (Feist & Hegeman, 1969) and P. fluorescens (Seidman, Toms & Wood, 1969) growing on phenol induce the same enzyme. Only the ortho cleavage pathway, however, has been dealt with in detail as only this pathway has so far been implicated in the metabolism of mandelate and related compounds.

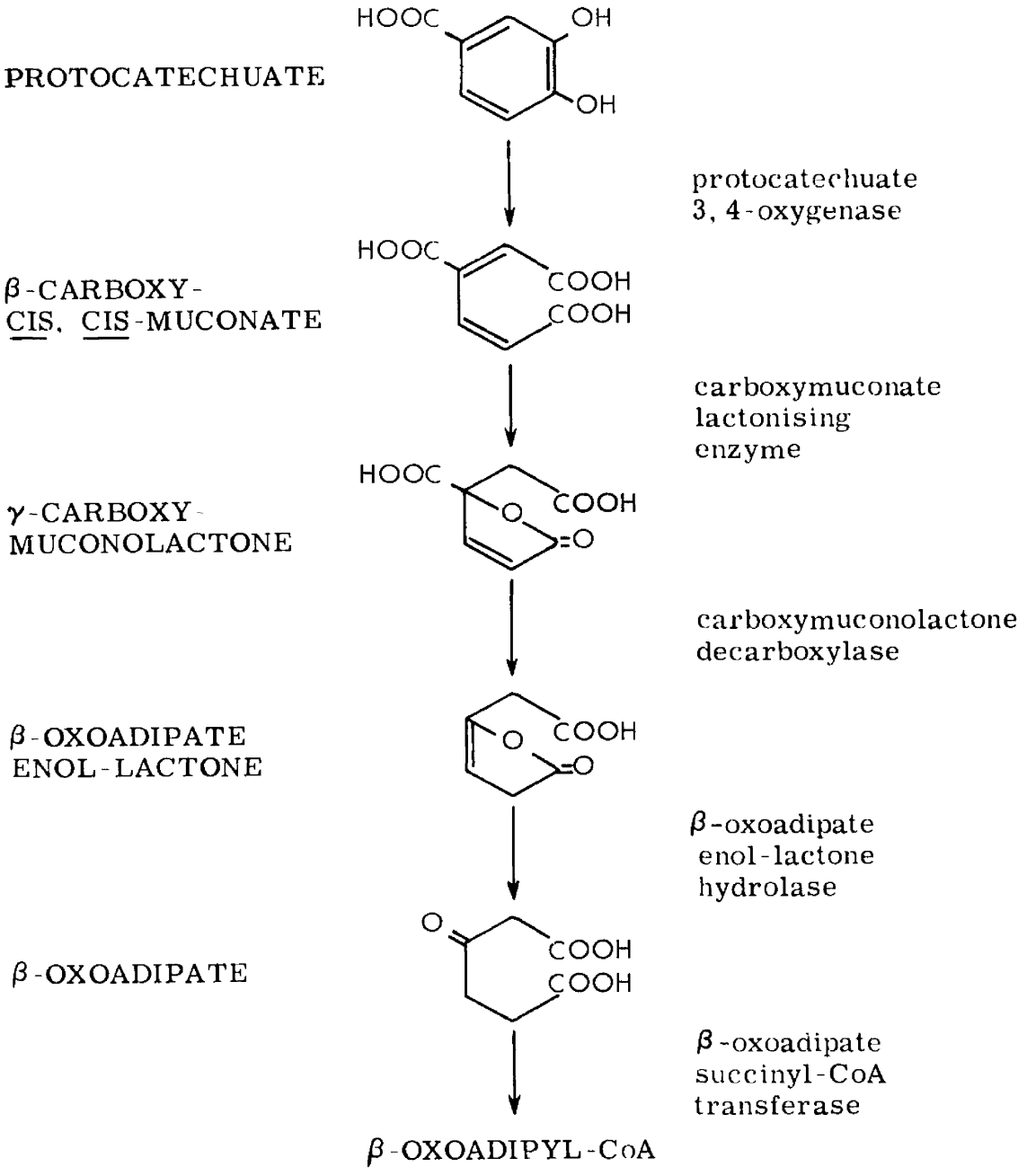
2.4. Conversion of protocatechuate to β-oxoadipyl-CoA.

The biochemical pathways for the conversion of protocatechuate to β-oxoadipyl-CoA (Fig.6) are chemically analogous to those of the catechol pathway, and are identical in P. putida (ATCC 12633) and in Acinetobacter species. In both types of organism, ortho cleavage of protocatechuate occurs. As with the catechol pathway, the individual enzymes and intermediates of the pathway were almost exclusively identified using P. putida as the biological material, but as before, all the enzymes of the pathway have since been detected in P. aeruginosa (ATCC 15692) (Kemp & Hegeman, 1968) and in A. calcoaceticus (ATCC 23393) (Cánovas & Stanier, 1967).

The first intermediate of the pathway to be identified was β-oxoadipate which was chemically characterised by Stanier, Sleeper, Tsuchida & McDonald (1950) as the product of protocatechuate metabolism by freeze-dried cells of P. putida that had been grown on 4-hydroxybenzoate. The next metabolite

Fig.6.

THE CONVERSION OF PROTOCATECHUATE TO β-OXOADIPYL-CoA IN PSEUDOMONAS PUTIDA AND ACINETOBACTER CALCOACETICUS



to be characterised was β-carboxy-cis,cis-muconate, the product of ring cleavage (McDonald, Stanier & Ingraham, 1954). Originally it was thought that the metabolism of β-carboxymuconate to β-oxoadipate was different in the two genera. This was because Cain (1961) found that (+)-muconolactone was the product of the metabolism of β-carboxymuconate by a heat-treated extract of bacterium NCIB 8250 grown on protocatechuate, and that this lactone was converted to β-oxoadipate by an unheated extract; whereas in P. putida (+)-muconolactone was not attacked by the enzyme system which converted β-carboxymuconate to β-oxoadipate. The subsequent work of Ornston & Stanier (1966), however, showed that the pathways in the two genera are in fact identical, and that the two intermediates between β-carboxymuconate and β-oxoadipate are γ-carboxymuconolactone and β-oxoadipate enol-lactone. These workers attributed the formation of (+)-muconolactone in bacterium NCIB 8250 to the conversion of β-oxoadipate enol-lactone to this compound by the basal amounts of muconolactone isomerase present in the extract; a phenomenon which does not occur in Pseudomonas spp.. β-Oxoadipate-enol lactone is produced by the spontaneous decarboxylation of γ-carboxymuconolactone, the product of the metabolism of β-carboxymuconate by the heat-treated extract.

With the exception of β-oxoadipate succinyl-CoA transferase in P. putida, all the enzymes involved in the conversion of protocatechuate to β-oxoadipyl-CoA in P. putida (Ornston

1966c), in P. aeruginosa (Kemp & Hegeman, 1968) and in A. calcoaceticus (Cánovas & Stanier, 1967) have been detected in cell-free extracts, and in a number of cases have been extensively purified (Ornston, 1966a). In bacterium NCIB 8250, all the enzymes of the protocatechuate pathway have been measured with the exception of β-oxoadipate succinyl-CoA transferase (Ornston, 1966c; Kennedy & Fewson, 1968b).

By means of radioactive techniques, the chemical characterisation of β-carboxymuconolactone and the isolation of the enzymes involved, Gross, Gafford & Tatum (1956) showed that β-carboxymuconolactone was an intermediate in the conversion of protocatechuate to β-oxoadipate in the fungus Neurospora crassa. Cain, Bilton & Darrah (1968) confirmed this observation, and by studying the pathway in several fungal species concluded that the protocatechuate pathway in fungi proceeds via β-carboxymuconolactone rather than via the γ-isomer which is the characteristic pathway in bacteria.

The organisms discussed in this Section dissimilate protocatechuate through the ortho cleavage pathway; but there are many reports in the literature of organisms employing the meta cleavage pathway; e.g. Pseudomonas testosteroni growing on 4-hydroxybenzoate synthesises a protocatechuate 4,5-oxygenase (Wheelis, Palleroni & Stanier, 1967). As with catechol, however, protocatechuate has so far always been found to be degraded by an ortho cleavage pathway when derived from substituted mandelates or related compounds.

2.5. A comparison between the catechol and protocatechuate pathways in *Pseudomonas* and *Acinetobacter* species.

Although the biochemical pathways are identical in the two genera, there are differences in their enzyme complements. *Acinetobacter* spp. synthesise two β-oxoadipate enol-lactone hydrolases and two β-oxoadipate succinyl-CoA transferases. One set of these enzymes is induced by growth on catechol, and the other set by growth on protocatechuate so that although β-oxoadipate enol-lactone represents the chemical point of convergence of the two pathways, β-oxoadipyl-CoA represents the enzymic point of convergence. On the other hand in *Pseudomonas* spp., β-oxoadipate enol-lactone represents both the chemical and enzymic point of convergence as this genus synthesises only one β-oxoadipate succinyl-CoA transferase and one β-oxoadipate enol-lactone hydrolase.

3. ENZYME REGULATION IN MICRO-ORGANISMS.

3.1. General aspects.

In recent years a great deal of interest has been shown in the regulation of metabolic pathways, and this has been reflected in the number of reviews which have appeared on the subject; e.g. Stadtman (1966), Buttin (1968), Cohen (1968), Clarke & Lilly (1969), Paigen & Williams (1970) and Sanwal (1970).

Metabolic pathways in micro-organisms are subject to both fine and coarse controls. The fine controls constitute very sensitive and rapidly adjusting control mechanisms which regulate enzyme activity. Both linear and branched metabolic pathways are regulated by this mechanism. In a linear pathway the biosynthesis of the product is often controlled by feedback inhibition of the first enzyme of the pathway by the product of the final enzyme of the sequence; e.g. 5-phosphoribosyl-ATP pyrophosphorylase is inhibited by histidine (Ames, Martin & Garry, 1961). In a branched diverging pathway the situation is more complex as the inhibition of an enzyme common to both pathways by the end-product of one pathway would result in the synthesis of an insufficient amount of the other products due to a reduction in the concentration of a common intermediate. Several mechanisms exist in micro-organisms to overcome this problem, and these include:

(1) synthesis of isofunctional enzymes, each of which is controlled by a different end-product; e.g. the three

aspartokinases of Escherichia coli, one of which is inhibited by lysine, the second by threonine, and the third by homoserine (Stadtman, Cohen, Le Bras & De Robichon-Szulmajster, 1961); (2) concerted feedback inhibition, which requires an excess of two or more end-products to achieve inhibition; e.g. the inhibition of the aspartokinase of Rhodopseudomonas capsulatus requires the presence of both threonine and lysine (Datta & Gest, 1964);
and (3) cooperative feedback inhibition in which the inhibition of the common enzyme is greater in the presence of a mixture of end-products than in the presence of a single end-product; e.g. the inhibition of the glutamine phosphoribosylpyrophosphate amidotransferase of Aerobacter aerogenes by AMP and GMP (Nierlich & Magasanik, 1965).

In a branched pathway, in addition to the inhibition of the first enzyme of the pathway, there is usually also inhibition of the first enzyme after each branch point; thereby increasing the flexibility of the pathway.

Monod, Changeux & Jacob (1963) described inhibition of this type as allosteric inhibition brought about by the combination of the inhibitor with the enzyme at a site other than the active site, and resulting in an altered conformation of the enzyme protein. Allosteric inhibition has been found to be a very general mechanism for the regulation of enzyme activity, and is important in maintaining the balance between anabolism and catabolism during growth; thereby eliminating an

unnecessary expenditure of energy when an adequate supply of end-product is available.

Coarse controls, on the other hand, are relatively insensitive and slow to take effect as they regulate the rate of enzyme synthesis. The main advantage of this control mechanism is that it allows only enzymes which are required for growth in a particular medium to be synthesised.

Most of the current ideas on the regulation of adaptive enzyme synthesis have developed from the model proposed by Jacob & Monod (1961) for the regulation of the lac system in E. coli. In their classical paper Jacob & Monod (1961) proposed that the three structural genes of the lac system are coordinately controlled by means of a cytoplasmic repressor produced by a regulator gene. In the absence of inducer the repressor binds to the operator site adjacent to the three closely linked structural genes, which form a contiguous section of DNA known as an operon, and prevents enzyme synthesis. The three genes are released from repression when an inducer combines with the repressor molecule and removes it from the operator site. This hypothesis has been strengthened by a great deal of experimental work, and especially by the isolation of the repressor (Gilbert & Müller-Hill, 1966). Subsequent work has shown the existence of a promoter region, adjacent to the operator but distal from the structural genes, and this promoter region is probably the binding site for RNA polymerase (Ippen, Miller, Scaife & Beckwith, 1968).

Close linkage of genes sharing a common repressor need not occur. The enzymes of the arginine pathway map at different sites on the E. coli chromosome (Gorini, Gundersen & Burger, 1961). Maas & Clark (1964) proposed the term "regulon" to describe such a system which was defined as a group of enzyme forming units which respond to a single repressor. Two types of regulon can be distinguished: those in which structural genes for the enzymes are adjacent to one another (e.g. lac); and those in which they are not (e.g. arg). Therefore, the term regulon can be used to describe any group of enzymes which form a regulatory unit irrespective of their genetic linkage.

The enzymes of the lac operon are defined as inducible enzymes as they are synthesised only in the presence of specific compounds. In comparison, the enzymes of biosynthetic pathways are frequently repressible since these enzymes are not formed in the presence of specific metabolites. For repressible systems the cytoplasmic repressor is thought to be produced in an inactive form, and can only bind to the operator region when combined with a low molecular weight corepressor molecule (Jacob & Monod, 1961). As with allosteric inhibition, both linear and branched pathways are subject to repression. An example of the former is the repression of histidine biosynthesis by histidine (Ames & Garry, 1959). In branched biosynthetic pathways the problem of an over-abundance of one end-product repressing the formation of the enzymes required for the synthesis of other end-products is solved by a series

of mechanisms analogous to those used to overcome the same problem in allosteric inhibition. These include:

(1) isofunctional enzymes each of which is repressed by a different end-product; e.g. the three aspartokinases of E. coli, one of which is repressed by threonine + isoleucine, the second by methionine and the third by lysine (Patte, Le Bras & Cohen, 1967);

and (2) concerted repression which requires an excess of two or more end-products to achieve repression; e.g. the repression of homoserine dehydrogenase I and aspartokinase I of E. coli requires the presence of both threonine and isoleucine (Cohen, 1968).

Although in the majority of cases, the regulation of enzyme synthesis involves a negative type of control similar to that postulated for the lac system by Jacob & Monod (1961), a few instances of positive control have been recorded. Englesberg, Irr, Power & Lee (1965) showed that the enzymes required for arabinose metabolism in E. coli were regulated by a system of positive control. Their results suggested that the regulator gene produced an activator which in combination with an inducing molecule was required for the expression of the structural genes.

In several cases, it has been found that the induction of an enzyme system can be inhibited by a number of compounds which prevent the permeation of the inducer into the cell. Thus, although the inducer of the enzyme system is present in

the growth medium, the enzymes themselves are not synthesised as the inducer does not gain entry to the cell. An example of this type of control mechanism has been reported by Adhya & Echols (1966) who found that the induction of the galactose enzymes by galactose in E. coli was inhibited by glucose or fructose.

A reduction in the rate of synthesis of certain enzymes has been found to occur in the presence of glucose or other readily metabolised carbon sources. This phenomenon known as catabolite repression has been fully described by Magasanik (1961). Magasanik proposed that catabolite repression was brought about by catabolites formed from the metabolism of glucose and related compounds accumulating in the cell, and repressing the formation of enzymes whose activity would augment the already large intracellular pool of these compounds. The nature of the catabolite(s) which serves as the corepressor is not yet known although a considerable amount of effort has been expended in trying to identify it (Paigen & Williams, 1970). Recent work has shown that cyclic 3′,5′-AMP overcomes the catabolite repression produced by glucose (Perlman, De Crombrugghe & Pastan, 1969).

Catabolite repression is only one of several effects on enzyme activity and synthesis that may be exerted by glucose. Others are:

(1) transient repression - this represents a period of intense repression lasting up to one generation which occurs immediately after cells are exposed to glucose (Moses & Prevost, 1966);

(2) interference with inducer transport (Adhya & Echols, 1966);

and (3) catabolite inhibition - this is a control exerted by glucose on enzyme activity rather than enzyme formation, and is analogous to feedback inhibition in biosynthetic pathways (McGinnis & Paigen, 1969).

This Section has summarised the basic types of control mechanism operating in micro-organisms. Many variations of these have been shown to occur; but only one, the control of converging pathways for the metabolism of mandelate and related compounds, will be discussed in detail in the next Section.

3.2. Control of converging metabolic pathways - the mandelate and 4-hydroxybenzoate pathways.

In his hypothesis of sequential induction, Stanier (1947) proposed that each metabolite induced the specific synthesis of the enzyme responsible for its conversion to the next intermediate of the metabolic pathway. At that time the hypothesis of sequential induction was widely accepted without rigorous proof because no plausible alternative was put forward. However, the enunciation of the operon theory (Jacob & Monod, 1961) coupled with the finding that groups of enzymes operative in both energy-yielding and biosynthetic pathways of bacteria can be synthesised coordinately, threw doubt on the original suggestion of complex sequential induction. Total coordinate induction can be excluded from a

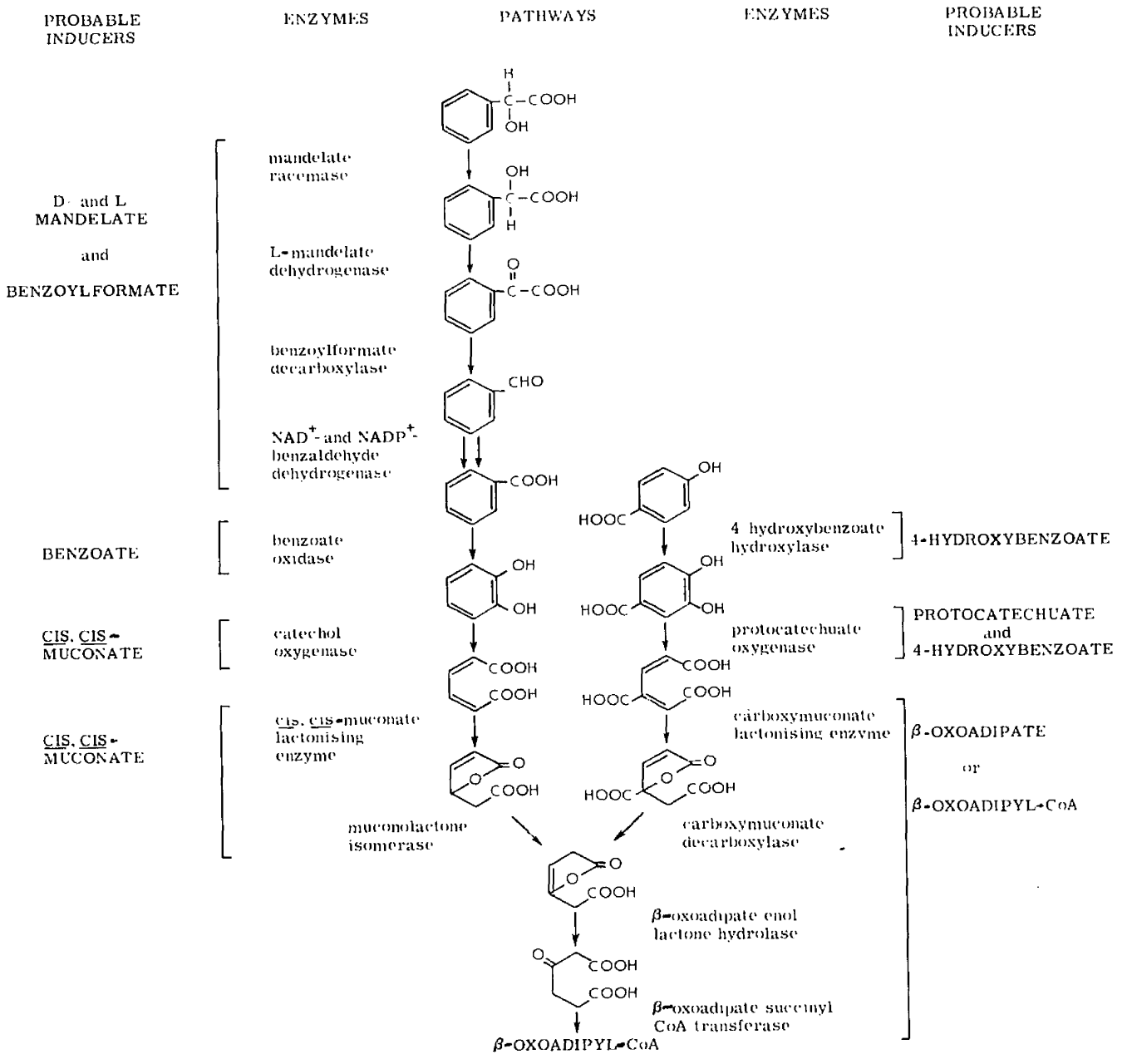
consideration of the sequential induction experiments. The alternatives are, accordingly, total sequential induction or coordinate inductions of blocks of enzymes, physiologically interconnected by sequential steps. In practice it was found that induction of the mandelate pathway was partly coordinate and partly sequential (Stanier, Hegeman & Ornston, 1965).

Hegeman (1966a,b,c) found from a study of the wild type, and blocked and constitutive mutants of P. putida that the first five enzymes of the mandelate pathway (Fig.7) were coordinately induced by D- and L-mandelate and benzoylformate. Chakrabarty, Gunsalus & Gunsalus (1968) showed by means of interstrain gene transfer that the genes responsible for the conversion of mandelate to benzoate were cotransducible. Stanier, Hegeman & Ornston (1965) showed that benzoate oxidase was regulated independently of the other enzymes of the pathway, and was induced by benzoate.

Ornston (1966c) studied the regulation of the protocatechuate and catechol pathways in P. putida and observed that catechol oxygenase formed an independent regulatory unit induced by cis,cis-muconate. Ornston could not rule out the possibility that catechol could also induce catechol oxygenase: but considered it unlikely as catechol was structurally dissimilar from cis,cis-muconate. However, Kemp & Hegeman (1968) and Bird & Cain (1968), by growing P. aeruginosa (ATCC 15692 and 8b2 respectively) anaerobically on lactate + catechol using nitrate as the terminal electron acceptor (i.e. using

Fig.7.

REGULATION OF THE MANDELATE AND 4 HYDROXYBENZOATE PATHWAYS IN PSEUDOMONAS PUTIDA



conditions under which catechol could not be converted to cis,cis-muconate), showed that cis,cis-muconate but not catechol induced catechol oxygenase.

Ornston (1966c) also found that:

(1) muconate lactonising enzyme and muconolactone isomerase constituted a second regulatory unit induced by cis,cis-muconate;

(2) protocatechuate oxygenase formed a third regulatory unit induced by protocatechuate;

and (3) β-carboxymuconate lactonising enzyme, γ-carboxymuconolactone decarboxylase and β-oxoadipate enol-lactone hydrolase formed a fourth regulatory unit induced by β-oxoadipate or its CoA derivative.

Ornston (1966c) did not study the regulation of β-oxoadipate succinyl-CoA transferase and 4-hydroxybenzoate hydroxylase; but Hosokawa (unpublished results in Kemp & Hegeman, 1968) showed that the former enzyme was synthesised coordinately with the other enzymes induced by β-oxoadipate, and that the latter enzyme was regulated independently of the other enzymes of the pathway, and was induced by 4-hydroxybenzoate. Hosokawa also confirmed the observation of Ornston (1966c) that protocatechuate oxygenase was independently regulated, but found that 4-hydroxybenzoate as well as protocatechuate served as an inducer of this enzyme.

The regulation of the enzymes which are responsible for the metabolism of D,L-mandelate and 4-hydroxybenzoate to β-oxoadipyl-CoA in P. putida is shown in Fig.7.

The mandelate pathway in P. aeruginosa is not only different from that in P. putida (p.8), but is also controlled quite differently. In this organism L-mandelate dehydrogenase is induced by L-mandelate. Benzoylformate decarboxylase and benzaldehyde dehydrogenase constitute a separate regulatory unit induced by benzoylformate (Rosenberg & Hegeman, 1969). The genes which code for the enzymes involved in the dissimilation of L-mandelate and 4-hydroxybenzoate have been mapped in P. aeruginosa (Kemp & Hegeman, 1968; Rosenberg & Hegeman, 1969). These workers found that the genes for enzymes which shared a common inducer were very closely linked.

Kennedy & Fewson (1968a,b) have examined the induction of the mandelate pathway enzymes in bacterium NCIB 8250. From a consideration of the enzymes synthesised by growth on various compounds (Table 1), they suggested that L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase formed a regulatory group (which they called Regulon R_1); that benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase constituted a second regulatory group (which they called Regulon R_2); and that benzoate oxidase formed a third regulatory group. These workers were unable to deduce the primary inducers of the regulatory units. An interesting feature of the inductive patterns observed by Kennedy & Fewson was the synthesis of the Regulon R_2 enzymes during growth on L-mandelate or benzoylformate. The results of Kennedy & Fewson (1968a,b) pointed to four possible

Table 1.

The pattern of the synthesis of the mandelate pathway enzymes observed in bacterium NCIB 8250 by Kennedy & Fewson (1968a, Table 3; 1968b, Tables 1 and 2, and Fig.1).

Enzyme	Growth Substrate					
	D,L-Mandelate	Benzoylformate	Benzyl alcohol	Benzaldehyde	Benzoate	Catechol
L-Mandelate dehydrogenase	+	+	-	-	-	-
Benzoylformate decarboxylase	+	+	-	-	-	-
Stable benzaldehyde dehydrogenase	+	+	-	-	-	-
Labile benzaldehyde dehydrogenase	+	+	+	+	-	-
Benzyl alcohol dehydrogenase	+	+	+	+	-	-
Benzoate oxidase	+	+	+	+	+	-
Catechol 1,2-oxygenase	+	+	+	+	+	+

explanations for this phenomenon:

(1) L-mandelate and benzoylformate were inducers of the Regulon R_2 enzymes;

(2) induction was brought about by benzaldehyde formed from the metabolism of benzoylformate;

(3) Regulons R_1 and R_2 were genetically linked;

and (4) part of the L-mandelate was metabolised to benzoate via benzyl alcohol.

Cánovas & Stanier (1967) studied the regulation of the catechol and 4-hydroxybenzoate pathways in A. calcoaceticus (ATCC 23393), and found that:

(1) catechol oxygenase formed a regulatory unit induced by cis,cis-muconate;

(2) muconate lactonising enzyme, muconolactone isomerase, β-oxoadipate enol-lactone hydrolase II and β-oxoadipate succinyl-CoA transferase II constituted a second regulatory unit induced by cis,cis-muconate;

(3) 4-hydroxybenzoate hydroxylase formed a regulatory unit induced by 4-hydroxybenzoate;

and (4) protocatechuate oxygenase, β-carboxy-cis,cis-muconate lactonising enzyme, γ-carboxymuconolactone decarboxylase, β-oxoadipate enol-lactone hydrolase I and β-oxoadipate succinyl-CoA transferase I constituted a regulatory unit induced by protocatechuate.

Farr & Cain (1968) showed that cis,cis-muconate and the structurally related compounds, 2-hydroxymuconic semialdehyde

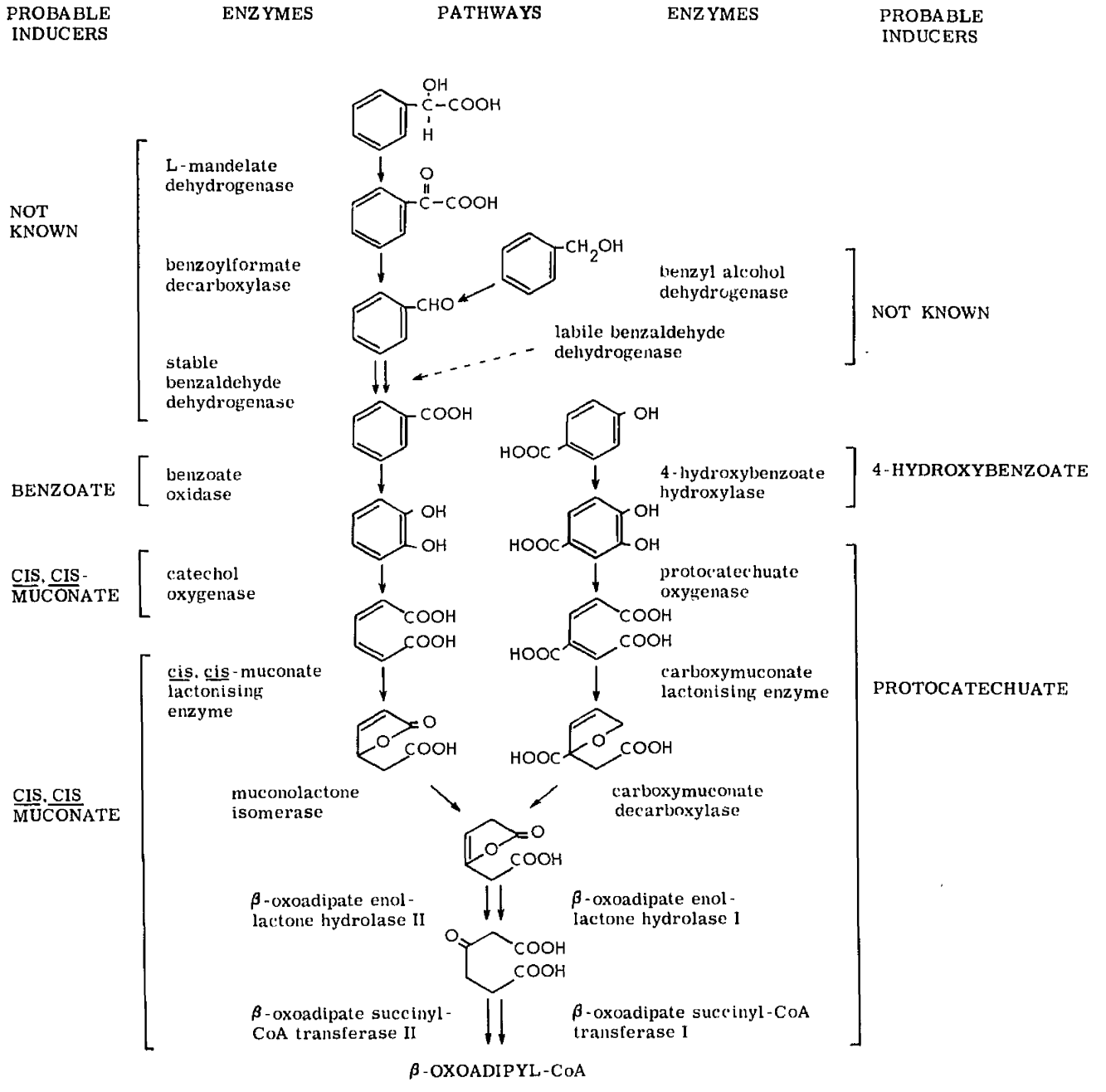
and 2-hydroxy-5-methylmuconic semialdehyde, induced catechol oxygenase in bacterium NCIB 8250. Neither Farr & Cain (1968) nor Cánovas & Stanier (1967) could exclude the possibility that catechol could also induce catechol oxygenase; but considered it unlikely owing to the structural differences between catechol and cis,cis-muconate. Nevertheless Beveridge & Tall (1969) observed that 2-fluorophenol, which they found not to be metabolised by bacterium NCIB 8250, induced catechol oxygenase. This finding suggests that an aromatic compound may after all be able to induce this enzyme.

The regulation of the enzymes which convert L-mandelate and 4-hydroxybenzoate to β-oxoadipyl-CoA in Acinetobacter species is summarised in Fig.8.

Mandelstam & Jacoby (1965) and Stevenson & Mandelstam (1965) showed that the mandelate pathway in P. putida was subject to repression. They found that the first coordinate group of enzymes, consisting of L-mandelate dehydrogenase, benzoylformate decarboxylase and benzaldehyde dehydrogenase, was inducible by mandelate and repressible by benzoate, catechol or succinate. The possibility that benzoate and catechol repressed enzyme synthesis by virtue of their metabolism to succinate was excluded by the use of mutants unable to grow on benzoate or catechol. These workers also showed that a second regulon consisting of benzoate oxidase was repressed by catechol, succinate or acetate, and a third regulon consisting of catechol oxygenase was repressed by

Fig.8.

REGULATION OF THE MANDELATE AND 4-HYDROXYBENZOATE PATHWAYS IN ACINETOBACTER SPECIES



succinate or acetate. The overall effect of these observations is that the enzymes of each regulatory group are repressed by their immediate products and also by the products of the succeeding groups of enzymes. These workers called this effect "multi-sensitive end-product repression". On the other hand Hegeman (1966a) was unable to detect end-product repression in the same strain of P. putida. The growth conditions used by the two groups of workers, however, were so different that there is not necessarily a contradiction between the two sets of observations. Hegeman (unpublished results in Hegeman, 1966c) was also unable to demonstrate feedback inhibition of several enzymes of the pathway.

3.3. General aspects of the metabolism of catechol and protocatechuate.

3.3.1. The differences in the regulation of the catechol and protocatechuate pathways in Pseudomonas and Acinetobacter species.

Stanier, Hegeman & Ornston (1965) found that sequential induction in the mandelate pathway occurred at sites where the particular intermediate was also a common naturally occurring compound or at points of convergence of two or more metabolic pathways. Thus, sequential induction usually permits the utilisation of a wide variety of carbon sources with minimal synthesis of nonfunctional enzymes. A sequential inductive step, however, does not occur at the site of convergence of the catechol and protocatechuate pathways in

Pseudomonas and Acinetobacter species. Ornston (1966c) suggested that this was because β-oxoadipate enol-lactone does not possess a chemical structure which permits it to function as an inducer. To circumvent this difficulty, Acinetobacter species synthesise two isofunctional β-oxoadipate enol-lactone hydrolases, each under different regulatory control, each synthesised coordinately with a block of enzymes specific to one of the two pathways, and each induced by a metabolic precursor specific to the branch in question. Hence in Acinetobacter species the control mechanisms permit strict economy of induced enzyme synthesis, but at the price of genetic redundancy. In comparison, the regulatory problem in Pseudomonas species is solved by synthesising β-oxoadipate enol-lactone hydrolase coordinately with two enzymes specific to the protocatechuate pathway. Since β-oxoadipate or its CoA derivative (which are intermediates of both pathways) is the inducer of this coordinate block, two enzymes specific to the protocatechuate pathway are gratuitously synthesised when cells are grown on precursors of the catechol pathway.

3.3.2. Evolution of the two pathways.

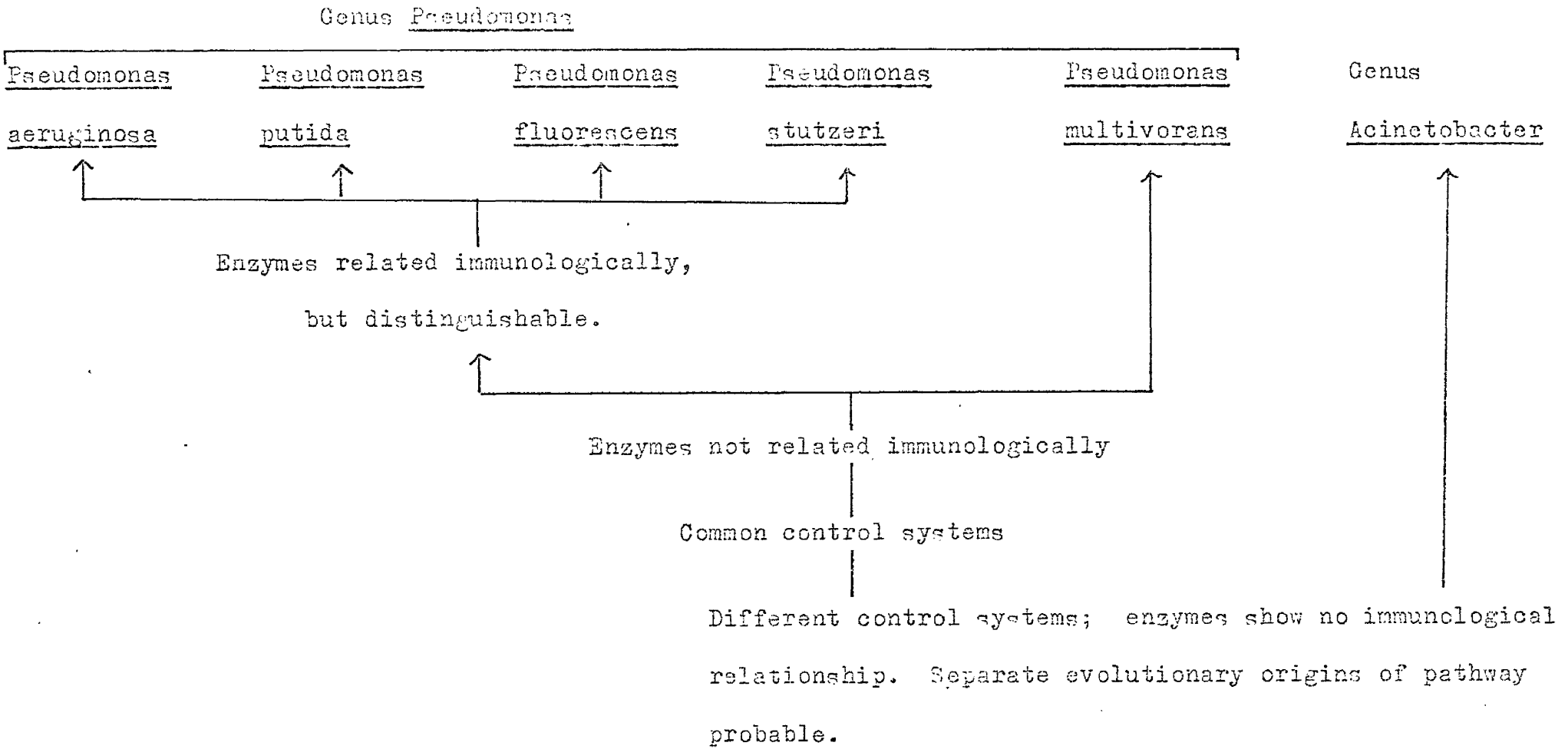
Groups of bacteria as diverse as Pseudomonas, Hydrogenomonas, Acinetobacter, Bacillus and Mycobacterium have been found to possess the β-oxoadipate pathway for the dissimilation of catechol and protocatechuate. As this represents a wide taxonomic distribution of this pathway, the

question arises of whether it had a single evolutionary origin or whether it had an independent evolution in a number of genera. In the case of at least some Pseudomonas and Acinetobacter species, current evidence, based on the regulation of the pathway and on the immunological homology of the enzymes concerned (Stanier, 1968), points to the independent origin of the pathway in the two genera. Stanier (1968) found that the regulation of the β-oxoadipate pathway was similar in all the Pseudomonas species examined, and that, with the exception of P. multivorans, extracts of these organisms grown on 4-hydroxybenzoate cross-reacted with antisera prepared against purified carboxymuconate lactonising enzyme and carboxymuconolactone decarboxylase of P. putida; whereas in A. calcoaceticus the regulation of the pathway was entirely different, and no cross-reacting material was obtained. The combined regulatory and immunological evidence for the separate evolution of the pathway in the two genera is summarised in Fig.9.

The control of the catechol and protocatechuate pathways in other groups of micro-organisms is different from that observed in Acinetobacter and Pseudomonas species. In Nocardia opaca all the enzymes of the protocatechuate pathway as well as muconolactone isomerase and the β-oxoadipate transferase of the catechol pathway were coordinately induced by β-oxoadipate or its CoA derivative (Rann & Cain, 1969). The remaining two enzymes, catechol oxygenase and cis,cis-muconate lactonising enzyme were non-coordinately induced by cis,cis-muconate. In

Fig.9.

An interpretation of the evolution of the β-ketoadipate pathway in two bacterial groups (Stanier, 1968, Fig.12).



the yeast Rhodotorula mucilaginosa, which lacks the catechol pathway, all the enzymes of the protocatechuate pathway were induced by 4-hydroxybenzoate (Cain, 1969).

3.3.3. Tangential pathways of metabolism.

As a general rule, the pathways for the dissimilation of aromatic compounds represent converging metabolic pathways in that there is only one pathway for the subsequent metabolism of the common intermediates. Occasionally, however, aromatic compounds are metabolised by means of tangential pathways in which the subsequent metabolism of the common intermediate depends on the nature of its precursor. The metabolism of benzoate and phenol by P. putida (NCIB 10105) represents an example of tangential pathways as the catechol formed from phenol is oxidised via a meta cleavage pathway (catechol 2,3-oxygenase), whilst the catechol formed from benzoate is oxidised via an ortho cleavage pathway (catechol 1,2-oxygenase) (Feist & Hegeman, 1969). Although catechol is an intermediate common to both pathways, their mode of regulation ensures the mutually exclusive occurrence of the meta or ortho pathway enzymes in cells grown on phenol or benzoate respectively. This is achieved by product-induction of catechol 1,2-oxygenase and precursor-induction of catechol 2,3-oxygenase so that when the organism is grown on benzoate or catechol, the inducer of the meta cleavage pathway enzymes is not formed; whereas when the organism is grown on phenol, the catechol is metabolised before it can be converted to the inducer of the ortho cleavage pathway enzymes.

4. TAXONOMY AND METABOLISM OF BACTERIUM NCIB 8250.

4.1. Taxonomy.

Happold & Key (1932) isolated an organism which they called Vibrio O1 from sewage effluent containing spent gas-work liquors. It was stated to be a Gram-negative vibrio with an oxidase system, without action on any of the common sugars, and not liquifying gelatin. Presumably the organism was motile, in view of the designation "vibrio", and because it was stated to resemble *Vibrio tyrosinatica* (Happold & Key, 1932) and *Vibrio cuneatus* (Evans, 1947; Kilby, 1951) in morphological appearance.

Much of the early work on the metabolism of aromatic compounds was done with this organism (e.g. Evans & Happold, 1939; Evans, 1947; Kilby, 1948; Evans & Smith, 1951; Dagley, Fewster & Happold, 1953), but the original strain appears to have been lost. Most subsequent work thought to have been done with Vibrio O1 has, in fact, been carried out with bacterium NCIB 8250. This organism was deposited as Vibrio O1 in the National Collection of Industrial Bacteria by Professor W.C. Evans (Department of Biochemistry and Soil Science, University College of North Wales, Bangor.) and is listed as an *Achromobacter* species. Bacterium NCIB 8250 is neither like the original Vibrio O1 nor characteristic of the genus *Vibrio* as *inter alia*; it is a non-motile, oxidase-negative coccobacillus (Fewson, 1967b).

Bacterium NCIB 8250 has been placed in the Moraxella-Acinetobacter group of bacteria by Sebald & Véron (1963), Véron (1966), Fewson (1967b) and Baumann et al.(1968). Experiments on DNA homologies by Johnson, Anderson & Ordal (1970) have provided supporting evidence for this classification of bacterium NCIB 8250. Although there now seems to be general agreement on the classification of this organism, the nomenclature of the Moraxella-Acinetobacter group is still the subject of debate. A number of generic names (e.g. Mima, Herellea, Moraxella and Acinetobacter) and specific names (e.g. lwoffi and calcoaceticus) are still in circulation (Baumann et al., 1968). The organism is therefore always referred to as bacterium NCIB 8250 in this laboratory. Members of the Moraxella-Acinetobacter group of bacteria occur ubiquitously in soil and water (Henderson, 1965; Baumann, 1968), and in many cases represent a sizable proportion of the bacteria present. They appear as Gram-negative non-motile rods, which characteristically occur in pairs. The dimensions of growing cells range from 0.9 to 1.6 by 1.5 to 2.5μ. The organisms are catalase-positive and oxidase-negative obligate aerobes which are generally unable to metabolise carbohydrates, but which can utilise a great variety or organic compounds as sole sources of carbon.

4.2. Metabolism of bacterium NCIB 8250.

The pathways for the dissimilation of aromatic compounds in bacterium NCIB 8250 represent complex converging metabolic pathways. In addition to benzoate and 2-hydroxybenzoate

(Fig.4), anthranilate (Fewson, unpublished results) and phenol (Beveridge & Tall, 1969) are also converted to catechol by bacterium NCIB 8250. Protocatechuate represents the point of convergence of the 4-hydroxybenzoate, 3,4-dihydroxybenzaldehyde and 4-hydroxy-3 methoxybenzoate pathways (Fig.4). Protocatechuate and catechol then undergo ring fission, and by a series of aliphatic interconversions give rise to β-oxoadipate enol-lactone, the site of convergence of the two pathways. β-Oxoadipate enol-lactone is metabolised to β-oxoadipyl-CoA which is the last intermediate specific to the metabolism of aromatic compounds as it is cleaved to acetyl-CoA and succinate (Kilby, 1951). The succinate and acetyl-CoA formed are then dissimilated by means of the Krebs cycle, the presence of which is well established in this organism (Dagley & Walker, 1956). A summary of these converging metabolic pathways is given in Fig.10, and there is little doubt that this could be extended since bacterium NCIB 8250 can metabolise a wide range of additional compounds (Fewson, 1967a). The pattern of control of this type of metabolic situation is clearly of interest.

A SUMMARY OF CONVERGING METABOLIC PATHWAYS IN BACTERIUM NCIB 8250

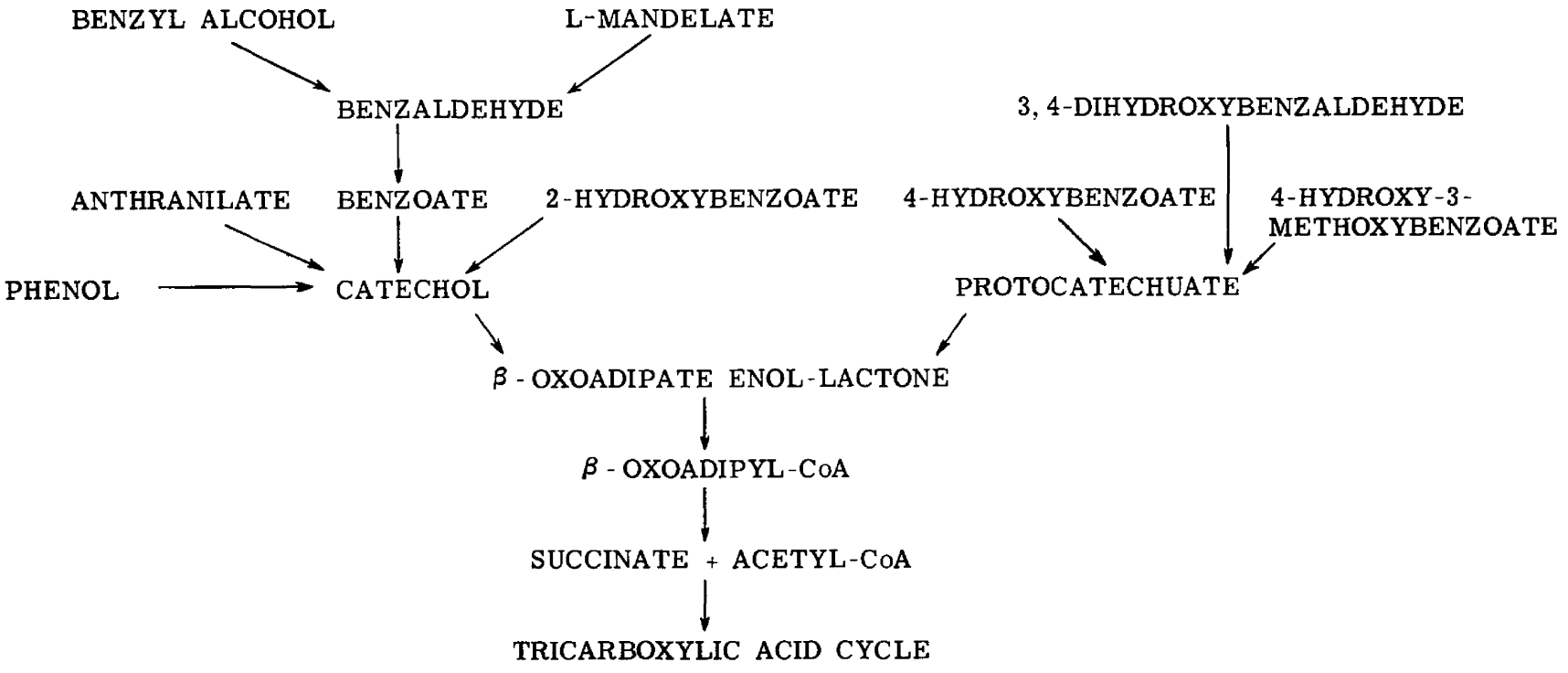


Fig.10.

5. SCOPE OF THIS THESIS.

The main aims of this project were:

(1) to fully investigate the regulatory groupings suggested by Kennedy & Fewson (1968a,b) for the enzymes involved in the conversion of L-mandelate and benzyl alcohol to *cis,cis*-muconate in bacterium NCIB 8250; i.e. the enzymes involved in the aromatic part of the mandelate pathway;

(2) to establish their primary inducers;

and (3) to examine other aspects of their regulation and activity

Before the work on enzyme regulation could be undertaken, however, a certain amount of development work had to be performed. The first job was to develop rapid, sensitive, accurate and reproducible spectrophotometric assays for the labile and stable benzaldehyde dehydrogenase, benzoylformate decarboxylase and benzoate oxidase - the four enzymes which had not previously been completely characterised (Kennedy & Fewson, 1968b; Fewson, private communication). In addition, the properties of the two benzaldehyde dehydrogenases were examined in some detail so as to establish beyond doubt the existence of two enzymes. This was necessary as biphasic heat inactivation curves (see Kennedy & Fewson, 1968b, Fig.1) do not constitute foolproof evidence for the occurrence of two enzymes since purified enzymes are sometimes known to give biphasic heat inactivation curves (e.g. Cline & Hu, 1965). Concurrently with the work undertaken on these enzymes in this project, Kennedy

& Zatman (Dr L.J. Zatman, Department of Microbiology, University of Reading, private communication) have purified the two benzaldehyde dehydrogenases. Once the enzyme assays had been developed, although the work with benzoate oxidase was only partially successful, the next task was to measure all the enzymes under study in the one extract. This was desirable as it enormously reduces the effort, time and chemicals required. The third piece of developmental work was the isolation of blocked and constitutive mutants. These mutants were used to check the postulated regulatory units, and the blocked mutants were also used to identify the primary inducers of the regulons. Before mutants could be isolated, conditions for mutagenesis had to be developed since there are no reports in the literature of the successful isolation of any type of mutant of bacterium NCIB 8250. Indeed Dr P.A. Whittaker (Department of Botany, University of Hull.) and Dr M. Jones (Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth.) (private communications) have independently failed to isolate desired mutants of this organism.

Throughout this work, L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase have been designated as the enzymes of Regulon R_1, and benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase as the enzymes of Regulon R_2 as suggested by Kennedy & Fewson (1968b). In addition benzoate oxidase has been designated as the enzyme of Regulon R_3, and catechol oxygenase as the enzyme of Regulon R_4.

METHODS.

1. BACTERIOLOGICAL TECHNIQUES.

1.1. Organisms.

Bacterium NCIB 8250 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, where it is maintained as an Achromobacter sp.. This organism was the parent of the various mutant strains isolated in this work.

Escherichia coli (NCIB 8545), Pseudomonas aeruginosa (NCIB 8704), Proteus vulgaris (NCIB 67), Achromobacter sp. (NCIB 5178) and Acinetobacter lwoffii (NCIB 5866) were obtained from the National Collection of Industrial Bacteria. Escherichia coli (ATCC 15223) was obtained from the American Type Culture Collection, Rockville, Maryland, U.S.A. Sarcina lutea was obtained from the private collection of Professor E.F. Gale, Department of Microbiology, Cambridge. Staphylococcus aureus was obtained from the private collection of Dr W.H. Holms.

Bacterium NCIB 8250 and the mutants obtained from it were characterised by a number of bacteriological tests (Table 2) carried out according to the methods detailed by Cowan & Steel (1965). The ability to grow on the compounds listed in Table 2 was also used for characterisation purposes as the results of Fewson (1967a) and Baumann et al.(1968) suggested

Table 2. Characterisation of bacterium NCIB 8250.

Bacterium NCIB 8250 and the mutants obtained from it were characterised by the bacteriological tests shown opposite, which are described in Cowan & Steel (1965). In addition the organisms were further characterised as bacterium NCIB 8250 on the basis of their ability to utilise a number of compounds as sole sources of carbon and energy. The growth of bacterium NCIB 8250 on these carbon and energy sources is described in Methods (p.59).

E. coli (ATCC 15223) was used as an example of a Gram-negative organism, as a negative control in the motility, urease and oxidase tests, and as a positive control in the glucose fermentation, nitrate reduction and acid/gas from glucose tests. E. coli (NCIB 8545) was used as a positive control in the test for motility. Staphylococcus aureus was used as an example of a Gram-positive organism and as a positive control in the catalase test. P. aeruginosa and Proteus vulgaris were used as positive controls in the oxidase and urease tests respectively. Achromobacter sp. was used as a negative control in the glucose oxidation and fermentation tests. Sarcina lutea and A. lwoffii were used as negative controls in the acid/gas from glucose and nitrate reduction tests respectively.

Test	Bacterium NCIB 8250
Shape	short rods, frequently in pairs
Gram stain	Gram-negative
Aerobic growth	+
Motility	-
Colony on nutrient agar	dull white, circular, slightly raised and intact
Oxidase (Kovacs)	-
Urease (Christensen)	-
Catalase	+
Glucose oxidation	-
Glucose fermentation	-
Acid/gas from glucose	-
Nitrate reduction	-
Growth on 5mM-L-mandelate	+
Growth on 5mM-benzyl alcohol	+
Growth on 2mM-benzoate	+
Growth on 1mM-2-hydroxybenzoate	+
Growth on 2mM-histamine	+
Growth on 5mM-arginine	-

that very few other known organisms would have this growth pattern. Some of the mutants differed from this pattern since they could not grow on L-mandelate, benzyl alcohol or benzoate (see Results, Section 3.2.).

1.2. Storage of organisms.

All organisms were maintained in Oxoid cooked-meat medium (CM 82; Oxoid Ltd., London, S.E.1.) stored at 4°. Subcultures were made into Oxoid nutrient broth (CM 1) at intervals of approximately 6-12 months. These were also kept at 4°, and were used to provide the starting inocula in all growth experiments.

1.3. Growth media.

All glassware used for growth of organisms was washed by boiling in either 1% (w/v) haemosol (Meinecke & Co. Inc., Baltimore, Maryland, U.S.A.) or 10% (v/v) nitric acid, followed by thorough rinsing in tap water and then in glass-distilled water.

1.3.1. Media.

The "basal medium" used throughout this work was taken from Fewson (1967a) and consisted of 2g KH_2PO_4 + 1g $(NH_4)_2SO_4$ in 1 l glass distilled water, adjusted to pH 7.0 with 5N-NaOH. "Salts medium" consisted of basal medium + 20ml/l sterile 2% (w/v) $MgSO_4.7H_2O$ added asceptically after sterilization of the basal medium.

In the preparation of growth media, if the carbon source could be autoclaved, the basal medium + carbon source was adjusted to pH 7.0 after addition of carbon source. If the carbon source was heat labile, its pH was adjusted to 7.0 before Millipore filtration and addition to sterile basal medium.

All flasks were plugged with non-absorbent cotton wool which was covered with aluminium foil prior to autoclaving.

1.3.2. Agar plates.

Nutrient agar plates used for the isolation and cloning of mutants, and for colony development in viable counting were made from Oxoid nutrient agar (CM 3). The molten medium was distributed into Petri dishes and dried by leaving inverted for 36h in the 30° hot room.

The agar plates used for the selection of blocked mutants were made by boiling 1.5% (w/v) Japanese agar with basal medium and carbon source, if heat stable, for 15min. The molten agar medium was autoclaved at 109°, and sterile 2% (w/v) $MgSO_4.7H_2O$ and sterile carbon source, if heat labile, were added. The complete medium was distributed into Petri dishes, and dried by leaving inverted for 36h in the 30° hot room.

1.3.3. Sterilization.

All media with the exception of the compounds listed below were sterilized by steam at 109° for periods of time

which varied with the volume of the medium. The efficacy of sterilization was originally determined by Fewson (unpublished results), and was always checked by the colour change of Browne's tubes (Albert Browne Ltd., Leicester.). The following compounds, because of their probable heat lability, were sterilised by filtration through Millipore filters (GSWP047 00, 0.22μ) prior to their addition to sterile basal media: benzoylformate; benzyl alcohol; 2-hydroxybenzyl alcohol; 4-hydroxybenzyl alcohol; benzaldehyde; 4-hydroxy-3-methoxybenzaldehyde; Carboxy Q; catechol; histamine; penicillin V and thiophenoxyacetate. L-Mandelate was Millipore filtered when it was used in the induction experiments.

1.3.4. Storage of media.

All media with a volume less than 100ml were stored in the cold room (nominally at 4°) until required. Media with a volume greater than 100ml were stored in the hot room at 30° until required.

1.4. Measurement of growth.

Turbidity of cell suspensions was measured at 500nm in a Spectronic 20 colorimeter (Bausch & Lomb Inc., Rochester, New York, U.S.A.). Suspensions were routinely measured in optically matched, 11.7mm light path, 4 x ½ inch Spectronic 20 test-tubes (Bausch & Lomb Inc., catalogue no. 33-29-27).

Samples having an E_{500} greater than 0.28 were diluted in basal medium so that the resultant E_{500} was between 0.050 and 0.20.

In experiments designed to measure the kinetics of induction of the mandelate pathway enzymes, the turbidity of cell suspensions was measured at 500nm in a Unicam SP.800 Ultraviolet Spectrophotometer (Pye Unicam Instruments Ltd., Cambridge.) connected to a Servoscribe Chart Recorder (Kelvin Electronics Co., Wembley, Middlesex.) in order to estimate their protein concentration. 1cm light path glass cuvettes were used and the samples were read against air. The value obtained was corrected by subtraction of the E_{500} of the basal medium. The protein concentration of the samples was estimated from the turbidity by means of a conversion curve (Fig. 11).

Growth rates were determined by plotting $\log_{10}(E_{500})$ against time: the slopes of the resulting straight lines gave a measure of the specific growth rate (Dawes, 1963).

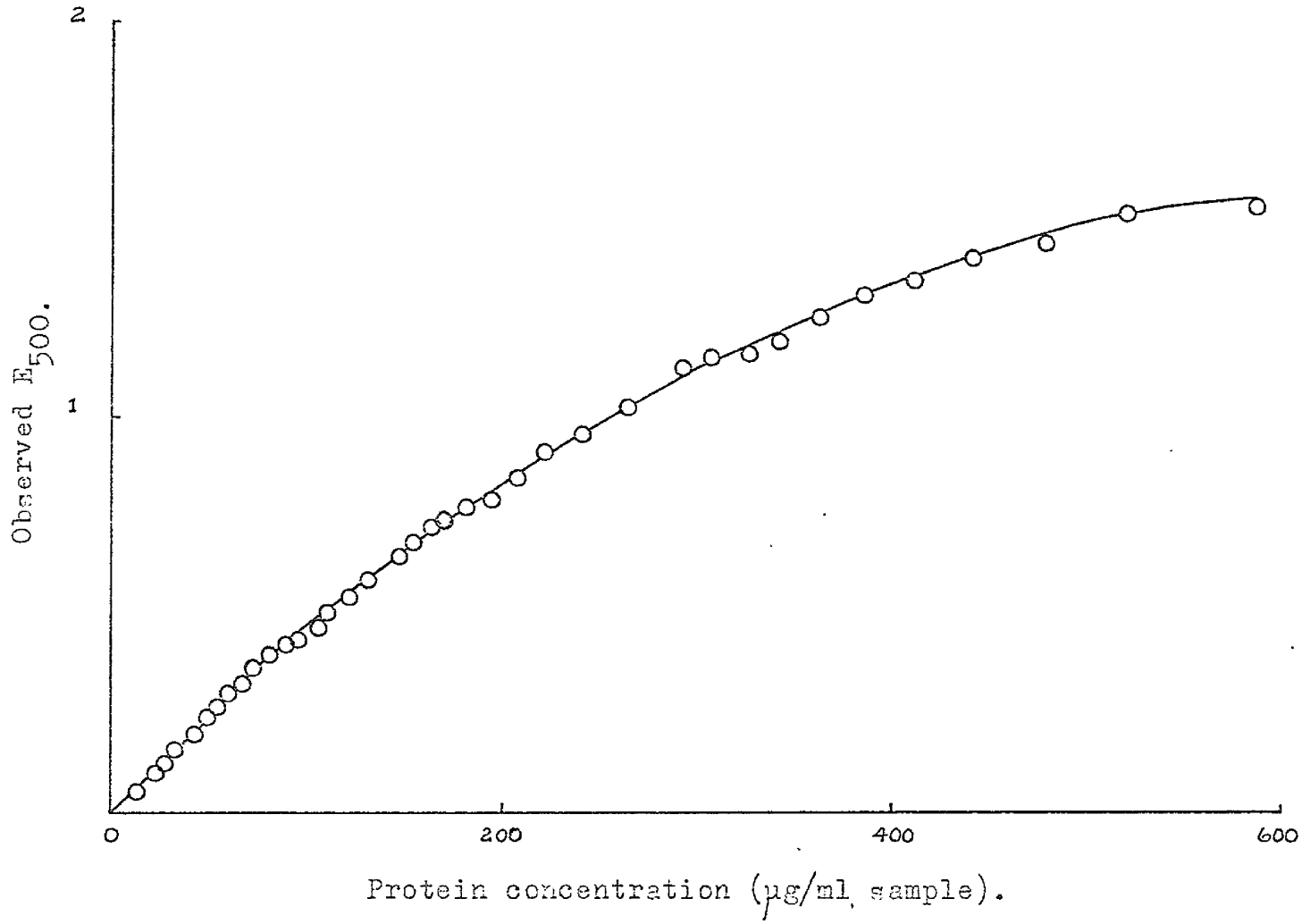
1.5. Growth of bacterium NCIB 8250.

The methods used for growing bacterium NCIB 8250 were based on those used by Fewson (1967a) and Kennedy & Fewson (1968a,b).

Inocula for growth of bacterium NCIB 8250 in defined media were prepared immediately before they were required. 0.1% inocula from the nutrient broth stock cultures were added to 100ml amounts of nutrient broth contained in 500ml Erlenmeyer

Fig.11. Turbidity - protein conversion curve for bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 10mM-glutamate-salts medium as described in Methods. The turbidity of the culture was measured at 500nm in the Unicam SP.800 Spectrophotometer and its protein concentration by means of the Lowry method as described in Section 5.2. The culture was progressively diluted in basal medium, and the protein concentration and turbidity were estimated at each dilution. This process was continued until the turbidity of the dilutions was directly proportional to their cell concentration. Turbidities which were not directly proportional to cell concentration were corrected for non-linearity. This was done by estimating what the turbidity would have been if it was directly proportional to cell concentration. The regression between these corrected turbidity values and the values for the protein concentration of the dilutions was calculated. The conversion curve which is the graph of observed turbidity against protein concentration was then drawn.


2
1
Observed E_{500}.
0
200
400
600
Protein concentration (µg/ml sample).

flasks, and the cultures were incubated without shaking for 24h at 30°.

1.5.1. Growth of bacterium NCIB 8250 for use in the experiments concerned with the development of experimental methods.

1.5.1.1. Cells grown on 5mM-L-mandelate, 5mM-benzyl alcohol, 10mM-glutamate + 1mM-thiophenoxyacetate, 10mM-glutamate or 10mM-succinate.

For these experiments 4l basal medium containing the appropriate carbon source was prepared in a 10l flat-bottomed flask containing a 45mm polypropylene coated stirring bar. 80ml sterile 2% (w/v) $MgSO_4.7H_2O$ and, as inoculum, 10ml of a 24h nutrient broth culture were added to the flask. The culture was grown for 12-15h at 30° under conditions of vigorous aeration on the apparatus described by Harvey, Pewsey & Holms (1968). This apparatus consists of magnet drive assemblies which effect the high speed rotation of magnetic stirring bars. An additional quantity of carbon and energy source equal to the amount present at inoculation and in a volume not greater than 200ml was then added to the culture. Three-quarters of a generation time later, the flask was removed from the growth apparatus, and immediately surrounded by ice. The cells were harvested as soon as possible by batch centrifugation at 15,500g for 20min at 4° (M.S.E. "High Speed 18" Refrigerator Centrifuge, M.S.E., London.). The supernatant solution was decanted and the pellet was washed by resuspension in ice-cold sterile distilled water. The

cells were recentrifuged at 12,000g for 25min at 4°, the supernatant solution was decanted, and the cells were weighed and stored at -60°.

1.5.1.2. Cells grown on 2mM-benzoate.

The procedure used to grow bacterium NCIB 8250 on 2mM-benzoate was similar to that used in Section 1.5.1.1. except that 40ml of a 24h nutrient broth culture was used as inoculum, and that the cells were harvested when the E_{500}, as measured in the Spectronic 20, reached 0.2. No additional substrate was added in this case.

1.5.2. Growth of bacterium NCIB 8250 for the measurement of the kinetics of induction of the mandelate pathway enzymes.

10ml of a 24h nutrient broth culture was added to 4l glutamate-salts medium in a 10l flat-bottomed flask containing a 45mm polypropylene coated stirring bar. The culture was grown for 12-15h at 30° on the apparatus described by Harvey et al.(1968). An additional quantity of glutamate equal to the amount present at inoculation, and in a volume of 200ml was added to the culture. Three-quarters of a generation time later, the flask was removed from the growth apparatus, and immediately surrounded by ice. The cells were harvested as soon as possible by batch centrifugation at 15,500g for 20min at 4°. The supernatant solution was decanted and the pellet was washed by resuspension in ice-cold basal medium. The cells were recentrifuged at 12,000g for 25min at

4°, the supernatant solution was decanted, and the cells were weighed and resuspended in basal medium at 30° to a concentration of 75mg wet wt./ml.

35ml of this suspension and 40ml sterile 2% (w/v) $MgSO_4.7H_2O$ were added to 1650ml 10.9mM-glutamate-basal medium, which gave a 10mM-glutamate-salts medium when all the additions had been made, in a 2l flask. The flask was fitted with a side arm to facilitate sampling. The culture was grown at 30° with rapid stirring and forced aeration (200cm^3/min) in the apparatus described by Harvey et al.(1968). Growth was followed by taking samples periodically for turbidity estimation in the Spectronic 20. In the later experiments, as will be discussed in Results, Section 2., the turbidity was also measured in the Unicam SP.800 Spectrophotometer. At the same time 4ml samples were added to 8ml 1N-NaOH, and used for protein estimation. After approximately a generation the inducer was added in a volume of 75ml, whereupon samples were taken for turbidity and protein estimation every 5min. 100ml samples for enzyme estimation were withdrawn from the growth flask at suitable time intervals onto 40g crushed distilled water ice. The cells were then harvested by batch centrifugation at 15,500g for 20min at 4° (M.S.E. "High Speed 18" Refrigerator Centrifuge). The supernatant solution was decanted and the pellet was washed by resuspension in ice-cold sterile distilled water. The cells were recentrifuged at 12,000g for 25min at 4°, the supernatant solution was

decanted, and the cells were weighed and stored at -60°.

1.5.3. Growth of bacterium NCIB 8250 for the isolation of mutants.

For these experiments 50ml basal medium containing 10mM-succinate was prepared in 250ml Erlenmeyer flasks. 1ml sterile 2% (w/v) $MgSO_4.7H_2O$ was added to each flask prior to inoculation.

A 0.2% inoculum from a 24h nutrient broth culture was added to the 10mM-succinate-salts medium, and the culture was grown for 12-15h at 30° on a rotary shaker (L.K. V; L.H. Engineering Co., Bells Hill, Stoke Poges, Bucks.) moving at about 180 oscillations/min. A second succinate culture was inoculated from this culture using a 7% inoculum, and grown to an E_{500} of 0.40 on the Spectronic 20. The cells were then harvested by batch centrifugation in sterile 50ml (Oak Ridge Type) poly-carbonate bottles (M.S.E.; catalogue no. 59466) at 12,000g for 10min at 4° (M.S.E. "13" Refrigerator Centrifuge). The supernatant solution was decanted, and the pellet was resuspended in either 10ml sterile basal medium for mutation by u.v. irradiation or in 10ml sterile 10mM-sodium citrate buffer pH 6.0 for mutation with N.T.G.

Subsequent growth of bacterium NCIB 8250 after mutagenesis is described in Section 2.

1.5.4. Growth of bacterium NCIB 8250 for the characterisation of mutants.

For these experiments 5ml salts medium containing 2mM-histamine, 1mM-2-hydroxybenzoate, 5mM-L-mandelate, 5mM-benzyl

alcohol, 2mM-benzoate or 5mM-arginine, 3ml salts medium containing 10mM-succinate, and 10ml peptone water medium (Oxoid CM 10) were prepared in capped 6 x 5/8 inch test-tubes (Oxoid aluminium caps). The peptone water medium was inoculated with 1 drop of a nutrient broth stock culture from a Pasteur pipette, and incubated without shaking for 48h at 30°. The salts media containing the carbon and energy sources were then inoculated with 1 drop of the peptone water culture from a Pasteur pipette, and incubated without shaking at 30°. A visual estimation of growth was recorded as "growth" or "no growth" at 48h, 72h and 96h after inoculation.

1.5.5. Growth of bacterium CIB 8250 to test the nutritional versatility of the mutants.

50ml of basal medium containing the appropriate carbon source at 1 and 2mM with the exception of 4-hydroxy-D, L-mandelate which was at 2 and 4mM was prepared in 250ml Erlenmeyer flasks. 1ml sterile 2% (w/v) $MgSO_4.7H_2O$ and, as inoculum, 0.1ml of a 24h nutrient broth culture were added to the flasks. The cultures were incubated at 30° on the rotary shaker. A visual estimation of growth was recorded as "growth" or "no growth" at 12h, 24h, 36h, 60h, 84h and 108h after inoculation.

1.5.6. Growth of the wild type and constitutive mutants of bacterium CIB 8250 for the measurement of the non-induced levels of the mandelate pathway enzymes.

1.5ml $MgSO_4.7H_2O$ and, as inoculum, 0.2ml of a 24h nutrient broth culture were added to 80ml 8mM-glutamate-basal

medium in a 250ml Erlenmeyer flask. The culture was grown for 15h at 30° on the rotary shaker. This culture and 20ml sterile 2% (w/v) $MgSO_4.7H_2O$ were added to 700ml 9.14mM-glutamate-basal medium, which gave an 8mM-glutamate-salts medium when the additions had been made, in a 1l flask. The flask was fitted with a side arm to facilitate sampling. The culture was grown at 30° in the apparatus described by Harvey et al. (1968). Growth was followed by taking samples at intervals for turbidity estimation in the Spectronic 20.

100ml samples for enzyme estimation were withdrawn onto 40g crushed distilled water ice at the beginning, the middle and the end of the last generation (see Fig. 47). The cells were then harvested by batch centrifugation at 15,500g for 20min at 4° (M.S.E. "High Speed 18" Refrigerator Centrifuge). The supernatant solution was decanted and the pellet was washed by resuspension in ice-cold sterile distilled water. The cells were recentrifuged at 12,000g for 25min at 4°, the supernatant solution was decanted, and the cells were weighed and stored at -60°.

1.5.7. Growth of the wild type and mutant strains of bacterium NCIB 8250 for the measurement of the induced levels of the mandelate pathway enzymes.

10ml $MgSO_4.7H_2O$ and, as inoculum, 5ml of a 24h nutrient broth culture were added to 500ml 10mM-glutamate-basal medium in a 1l flask. The culture was grown for 12-14h at 30° on the apparatus described by Harvey et al.(1968). 20ml of this

culture and 1.5ml sterile 2% (w/v) $MgSO_4.7H_2O$ were added to 60ml 13.3mM-glutamate-basal medium containing inducer in a 250ml Erlenmeyer flask. The culture was grown for 2½h at 30° on the rotary shaker. The cells were then harvested by batch centrifugation at 15,000g for 20min at 4° (M.S.E. "High Speed 18" Refrigerator Centrifuge). The supernatant solution was decanted and the pellet was washed by resuspension in ice-cold sterile distilled water. The cells were recentrifuged at 12,000g for 25min at 4°, the supernatant solution was decanted, and the cells were weighed and stored at -60°.

1.6. Measurement of viability.

The cultures were serially diluted 1/10 with basal medium. 0.1ml portions of the three dilutions most likely to contain approximately 100 viable cells were placed on 3 separate nutrient agar plates, and spread by means of a glass-spreader. The plates were incubated at 30° for 24h. The plates of the dilution which gave the nearest number of colonies to a hundred were counted and averaged.

2. ISOLATION OF MUTANTS.

2.1. Mutagenic treatments and the selection of mutants.

2.1.1. Ultraviolet irradiation followed by penicillin treatment and the replica plating selection technique (Table 20; A).

Mutagenesis by u.v. irradiation was adapted from the method of Demerec & Latarjet (1946).

9ml of the suspension of cells in basal medium prepared as described in Section 1.5.3. was irradiated for 140s with an unfiltered u.v. lamp (Hanovia Bactericidal Equipment Model 13; Engelhard Hanovia Lamps, Slough, Bucks.) at a distance of 84cm from the lamp. During mutagenesis the suspension was gently stirred with a magnet in a sterile glass Petri dish, 9cm in diameter. As will be discussed in Results, Section 3.1.1.1., this treatment gave approximately a 99.9% kill. In each experiment a viable count was made on the suspension before and after irradiation to check the efficiency of the treatment. 2.5ml of the irradiated cells was added to 50ml 10mM-succinate-salts medium in a 250ml Erlenmeyer flask, and the culture was grown overnight at 30° on a rotary shaker (MK.V; L.H. Engineering Co.) moving at about 180 oscillations/min. These manipulations were conducted in dim light using a sodium lamp (40 watt D.C. SOX lamp; Philips Electrical Ltd., London.) to prevent the possibility of photoreactivation.

This culture was harvested by batch centrifugation in sterile 50ml poly-carbonate bottles (M.S.E.; catalogue no.

59466) at 12,000g for 10min at 4° (M.S.E. "13" Refrigerator Centrifuge) to remove any residual carbon and energy source. The supernatant solution was decanted, and the pellet was washed twice by resuspension in ice-cold basal medium and centrifugation at 12,000g for 10min at 4°. The cells were then resuspended in 10ml basal medium at 30°, and 2.5ml of this suspension was added to 50ml 2mM-benzoate-, 5mM-L-mandelate- or 5mM-benzyl alcohol-salts medium. The benzoate and benzyl alcohol cultures were grown for 2h at 30° on the rotary shaker, at which time penicillin V (at a final concentration of 1mg/ml) was added, and the cultures were grown for a further 1½h. The L-mandelate culture because of the longer mean generation time was grown for 2½h before the addition of penicillin V, and for 2h after it. The cells were harvested and washed as above to remove penicillin V, and resuspended in 10ml basal medium. 0.1ml portions containing approximately 100 viable cells were placed on agar plates containing 10mM-succinate as sole source of carbon and energy, and spread by means of a glass-spreader.

The colonies on the succinate agar plates were grown for 36-40h at 30° at which time they were about 2mm in diameter. The colonies were then replicated by the method of Lederberg & Lederberg (1952) onto agar plates containing, in order of replication, 5mM-L-mandelate, 5mM-benzyl alcohol or 2mM-benzoate. A final replication onto nutrient agar plates was made to test the success of replication. Strains which failed to grow on either L-mandelate, benzyl alcohol or benzoate were cloned on nutrient agar.

2.1.2. Mutagenesis by treatment with N.T.G. followed by the replica plating selection technique (Table 20; B).

Mutagenesis by incubation with N.T.G. was adapted from the method of Adelberg, Mandel & Chen (1965).

9ml of the suspension of cells in sterile 10mM-sodium citrate buffer pH 6.0 prepared as described in Section 1.5.3. was added to 1ml 1mg/ml N.T.G. (dissolved in the same buffer). The resulting suspension was placed in a sterile 50ml Erlenmeyer flask plugged with cotton wool, and incubated for 30min at 30° in a shaking water bath. As will be discussed in Results, Section 3.1.1.2., this treatment gave approximately a 99.9% kill. In each experiment a viable count was made on the suspension before and after incubation to check the efficiency of the treatment.

After incubation the suspension was serially diluted 1/10 with basal medium. 0.1ml portions containing approximately 100 viable cells were placed on agar plates containing 10mM-succinate as sole source of carbon and energy, and spread by means of a glass-rod. The colonies on the succinate agar plates were grown for 36-40h at 30° at which time they were about 2mm in diameter. The colonies were then replicated by the method of Lederberg & Lederberg (1952) onto agar plates containing, in order of replication, 5mM-L-mandelate, 5mM-benzyl alcohol or 2mM-benzoate. A final replication onto nutrient agar plates was made to test the success of replication. Strains which failed to grow on either L-mandelate, benzyl alcohol or benzoate were cloned on nutrient agar.

2.1.3. Mutagenesis by treatment with N.T.G. followed by the limiting nutrient selection technique (Table 20; C).

9ml of the suspension of cells in sterile 10mM-sodium citrate buffer pH 6.0 prepared as described in Section 1.5.3. was added to 1ml 1mg/ml N.T.G. (dissolved in the same buffer). The resulting suspension was placed in a sterile 50ml Erlenmeyer flask plugged with cotton wool, and incubated for 30min at 30° in a shaking water bath. As will be discussed in Results, Section 3.1.1.2., this treatment gave approximately a 99.9% kill. In each experiment a viable count was made on the suspension before and after incubation to check the efficiency of this treatment.

After incubation the N.T.G. was removed by centrifugation at 12,000g for 10min at 4° (M.S.E. "13" Refrigerator Centrifuge) in sterile 50ml poly-carbonate bottles (M.S.E.; catalogue no. 59466). The supernatant solution was decanted and the pellet was washed twice by resuspension in ice-cold basal medium and centrifugation at 12,000g for 10min at 4°. The cells were then resuspended in 10ml basal medium at 30°.

2ml of this suspension was added to 50ml 10mM-succinate- or 2mM-benzoate-salts medium (depending on whether glutamate or benzoate respectively was the limiting nutrient in the selection procedure), and the culture was grown overnight at 30° on the rotary shaker. 3ml of the benzoate or succinate culture was inoculated into homologous medium and grown at 30° on the rotary shaker for the time interval which represented two generations.

The culture was then serially diluted 1/10 with basal medium. 0.1ml portions containing approximately 100 viable cells were placed on agar plates containing 5mM-L-mandelate + 0.2mM-benzoate or 2mM-benzoate + 0.3mM-glutamate as sole source of carbon and energy, and spread by means of a glass-rod. The colonies were grown for 36-40h at 30°. The smaller colonies (1mm in diameter) were then picked from among the more numerous larger ones (2mm in diameter), and streaked on agar plates containing 5mM-L-mandelate, 2mM-benzoate or 10mM-succinate as sole source of carbon. Strains which failed to grow on either L-mandelate or benzoate were cloned on nutrient agar.

2.1.4. Mutagenesis by treatment with N.T.G. followed by Carboxy penicillin treatment and the limiting nutrient selection technique (Table 20; D).

9ml of the suspension of cells in sterile 10mM-sodium citrate buffer pH 6.0 prepared as described in Section 1.5.3. was added to 1ml 1mg/ml N.T.G. (dissolved in the same buffer). The resulting suspension was placed in a sterile 50ml Erlenmeyer flask plugged with cotton wool, and incubated for 30min at 30° in a shaking water bath. As will be discussed in Results, Section 3.1.1.2., this treatment gave approximately a 99.9% kill. A viable count was made on the suspension before and after incubation to check the efficiency of the treatment.

After incubation the N.T.G. was removed by centrifugation at 12,000g for 10min at 4° (M.S.E. "13" Refrigerator Centrifuge) in sterile 50ml poly-carbonate bottles. The supernatant solution was decanted and the pellet was washed twice by

resuspension in ice-cold basal medium and centrifugation at 12,000g for 10min at 4°. The cells were then resuspended in 10ml basal medium at 30°. 2ml of this suspension was added to 50ml 10mM-succinate-salts medium, and the culture was grown overnight at 30° on the rotary shaker.

The culture was harvested by batch centrifugation at 12,000g for 10min at 4° (M.S.E. "13" Refrigerator Centrifuge) to remove any residual carbon and energy source. The supernatant solution was decanted and the pellet was washed twice by resuspension in ice-cold basal medium and centrifugation at 12,000g for 10min at 4°. The cells were then resuspended in 10ml basal medium at 30°, and 2.5ml of this suspension was added to 50ml 5mM-L-mandelate-salts medium. The culture was grown for 3h at 30° on the rotary shaker, at which time Carboxy Q (at a final concentration of 90μM) was added, and the culture was grown for a further 2h. The cells were harvested and washed as above to remove the Carboxy Q, and resuspended in 10ml basal medium at 30°. 3ml of the suspension was added to 50ml 10mM-succinate-salts medium, and the culture was grown to a turbidity of 0.09 on the Spectronic 20.

The culture was then serially diluted 1/10 with basal medium. 0.1ml portions containing approximately 100 viable cells were placed on agar plates containing 2mM-benzoate + 0.3mM-glutamate as sole source of carbon and energy, and spread by means of a glass-rod. The colonies were grown for 36-40h at 30°. The smaller colonies (1mm in diameter) were

then picked from among the more numerous larger ones (2mm in diameter), and streaked on agar plates containing 5mM-L-mandelate, 2mM-benzoate or 10mM-succinate as sole source of carbon and energy. Strains which failed to grow on either L-mandelate or benzoate were cloned on nutrient agar.

2.1.5. Isolation of spontaneous and mutagen induced constitutive mutants (Table 21; A and B).

The constitutive mutants were enriched by the alternate culture technique and selected by means of the L-mandelate dehydrogenase whole cell assay. The alternate culture technique was adapted from the method of Cohen-Bazire & Jolit (1953). The L-mandelate dehydrogenase whole cell assay was adapted from the method of Hegeman (1966c). This assay gives a simple qualitative test to screen organisms for L-mandelate dehydrogenase activity as the enzyme can be detected visually by the coupled reduction of the dye, 2,6-dichlorophenol-indophenol, to its leuco form.

For the isolation of spontaneous constitutive mutants the starting inoculum for the alternate culture enrichment procedure was 2ml of the unharvested succinate culture described on p.59. For the isolation of constitutive mutants induced by incubation with N.T.G., the starting inoculum was prepared as follows. 9ml of the suspension of cells in sterile 10mM-sodium citrate buffer pH 6.0 prepared as described in Section 1.5.3. was added to 1ml 1mg/ml N.T.G. (dissolved in the same buffer). The resulting suspension was placed in a

sterile 50ml Erlenmeyer flask plugged with cotton wool and incubated for 30min at 30° in a shaking water bath. As will be discussed in Results, Section 3.1.1.2., this treatment gave approximately a 99.9% kill. A viable count was made on the suspension before and after incubation to check the efficiency of the treatment. After incubation the N.T.G. was removed by centrifugation at 12,000g for 10min at 4° (M.S.E. "13" Refrigerator Centrifuge). The supernatant solution was decanted and the pellet was washed twice by resuspension in ice-cold basal medium and centrifugation at 12,000g for 10min at 4°. The cells were then resuspended in 10ml basal medium at 30° and 2ml of this suspension was used as the starting inoculum in the alternate culture enrichment procedure.

The starting inocula described above were grown in 50ml 10mM-glutamate-salts medium in 250ml Erlenmeyer flasks for 16h at 30° on the rotary shaker. 5ml of these cultures was added to 50ml 5mM-L-mandelate-salts medium in 250ml Erlenmeyer flasks, and the cultures were grown for 8h at 30° on the rotary shaker. 0.5ml of these L-mandelate cultures was added to 50ml 10mM-glutamate-salts medium in 250ml Erlenmeyer flasks, and the cultures were grown for 16h at 30° on the rotary shaker. This procedure for growing alternately on glutamate- and L-mandelate-salts media was repeated a further 9 times.

The cultures were then serially diluted 1/10 with basal medium. 0.1ml portions containing approximately 100 viable cells were placed on nutrient agar plates, and spread by means

of a glass-rod. The colonies were grown for 24h at 30°. One half of an individual colony on a nutrient agar plate was spread on another nutrient agar plate, and the other half was added to 3ml 5mM-glutamate-salts medium in a capped 6 x 5/8 inch test-tube (Oxoid aluminium caps). The cultures in the test-tubes were grown for 48h without shaking.

The cells grown on 5mM-glutamate were centrifuged for 15min at 4,000g at room temperature (M.S.E. "Super Medium" Centrifuge) to remove any residual carbon source. The supernatant solution was decanted and the pellet was resuspended in 3ml basal medium. The permeability barriers of the cells were destroyed by mixing the cells for 8-10s on a Whirlimix mixer (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire) with 0.5ml 2% (v/v) toluene in ethanol added from a Zippette dispenser (2ml capacity Zippette; Jencons Ltd., Hemel Hempstead, Herts.), and letting the resulting suspension stand for 8-20min. 2,6-Dichlorophenol-indophenol at a final concentration of 67μM and L-mandelate at a final concentration of 500μM were then added by means of 100μl Eppendorf pipettes (Eppendorf Larburg Micropipette; Eppendorf Geräetebau, Hamburg, West Germany.). Decolourisation was followed visually. The other half of those colonies which gave rise to cells that decolourised the dye were cloned on nutrient agar.

2.2. Storage of mutants.

After cloning, the mutant strains were inoculated into 10ml nutrient broth medium in a McCartney bottle. The culture was grown for 24h at 30° without shaking. 0.1ml of this culture was then used to inoculate 10ml cooked-meat medium in a McCartney bottle. This culture was grown for 48h at 30° without shaking, and then stored at 4°. Subcultures were made into nutrient broth at intervals of approximately 6-12 months, and these were also kept at 4°. These nutrient broth stock cultures were used to prepare inocula for growth of bacterium NCIB 8250 in defined media.

3. CELL EXTRACTION.

The enzymes were extracted by modifications of the method of Kennedy & Fewson (1968b) involving ultrasonic disruption with the 13mm probe of the Dawe "Soniprobe" (Dawe Instruments Ltd., London; Type 1130A).

3.1. Ultrasonic disruption of cells used in the initial experiments of Results, Section 1.2.

Cells were resuspended to 40 or 50mg wet wt./ml in 0.04M- or 0.08M-Tris-HCl buffer pH 8.5, and a 12ml amount was pipetted into an 8 dram Trident container (Johnsen & Jorgensen Ltd., London.). The Trident container was then placed in an ice-water slurry, and the Dawe Soniprobe was lowered 7mm into the suspension. The total time of sonication was 15min at a current of 5A, but the current was switched off during every alternate minute to aid cooling, thus giving 8min actual disruption. The extract was centrifuged at 12,000g for 25min at 4° (M.S.E. Refrigerated "13" Centrifuge) to remove whole cells and debris, and the supernatant fluid was used to measure enzyme activity and protein concentration.

3.2. Ultrasonic disruption of non-induced cells whose extracts were used to dilute extracts containing induced levels of the mandelate pathway enzymes.

Cells were resuspended to 50mg wet wt./ml in 0.04M-Tris-HCl buffer pH 8.5. A 20-25ml amount was pipetted into a "rosette" which is a cone-shaped glass vessel equipped with 4

cooling vanes as described by Kennedy (1967). The rosette was placed in an ice-water slurry, and the Dawe Soniprobe was lowered 7mm into the suspension. The total time of sonication was 7min at a current of 5A, but the current was switched off during every alternate minute to aid cooling, thus giving 4min actual disruption. The extract was centrifuged at 12,000g for 25min at 4° (M.S.E. Refrigerated "13" Centrifuge) to remove whole cells and debris, and the supernatant fluid was used to measure enzyme activity and protein concentration.

2.3. Ultrasonic disruption of cells containing the coupling enzyme for the benzoylformate decarboxylase assay.

Cells grown on benzyl alcohol were resuspended to 50mg wet wt./ml in 0.08M-Tricine buffer pH 9.5, and a 20-25ml amount was pipetted into the rosette. The rosette was placed in an ice-water slurry, and the Dawe Soniprobe was lowered 7mm into the suspension. The total time of sonication was 7min at a current of 5A, but the current was switched off during every alternate minute to aid cooling, thus giving 4min actual disruption time. The extract was centrifuged at 12,000g for 25min at 4° (M.S.E. Refrigerated "13" Centrifuge) to remove whole cells and debris, and the supernatant fluid was used to couple the benzoylformate decarboxylase reaction.

2.4. Ultrasonic disruption of cells used in the benzoate oxidase assay.

Cells were resuspended to 100mg wet wt./ml in 0.05M-Tris-HCl buffer pH 7.5, and a 4-6ml amount was pipetted into

a 1 dram Trident container. The container was then placed in a chilled brass holder (Fig. 12), which is similar to the Branson sealed atmosphere treatment chamber (Dawe Instruments Ltd., catalogue no. 7530/5), and was designed by Dr W.H. Holms. It was specially built to take a 1 dram Trident container so that there was very little movement of the container inside the holder.

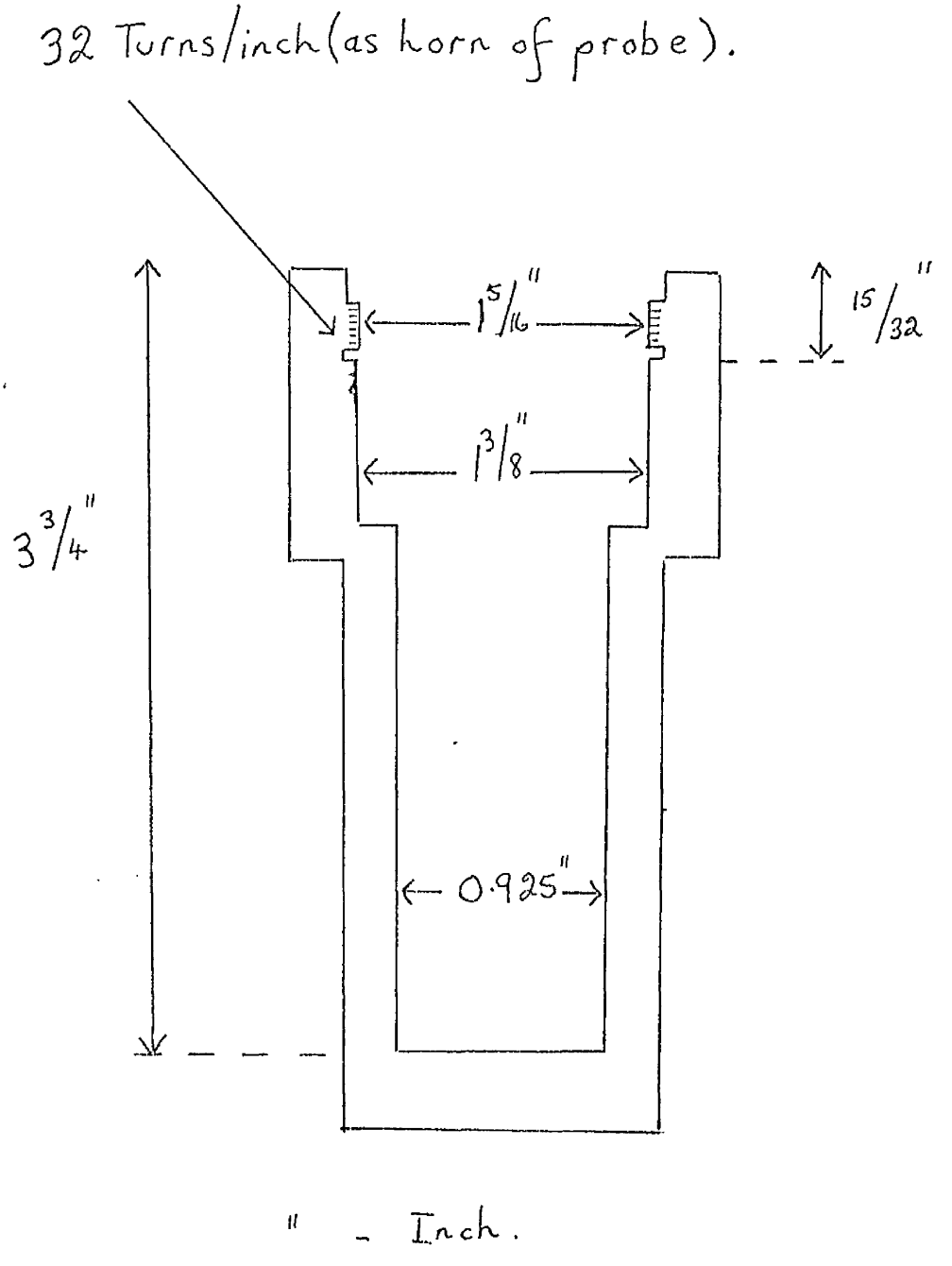
The brass holder was screwed onto the horn of the Soniprobe, and was lowered into an ice-water slurry. The cells were sonicated for ½min at a current of 2.5-3.0A. The extract was centrifuged at 12,000g for 25min at 4° (M.S.E. Refrigerated "13" Centrifuge) to remove whole cells and debris, and the supernatant fluid was used to measure enzyme activity and protein concentration.

3.5. Ultrasonic disruption of cells used for all other purposes.

Cells were resuspended to 50mg wet wt./ml in 0.04M-Tris-HCl buffer pH 8.5, and a 5ml amount was pipetted into a 1 dram Trident container. The brass holder was screwed onto the horn of the Soniprobe, and was lowered into an ice-water slurry. The total time of sonication was 6½min at a current of 2.5A, but the current was switched off during every alternate half-minute to aid cooling, thus giving 3½min actual disruption. The extract was centrifuged at 12,000g for 25min at 4° (M.S.E. Refrigerated "13" Centrifuge) to remove whole cells and debris, and the supernatant fluid was used to measure enzyme activity and protein concentration.

Fig.12.

The brass holder used in the sonication procedure.



" - Inch.

Part of the extract was titrated with the amount of 0.2N-NaOH required to raise the pH of a 0.04M-Tris-HCl buffer containing no protein from 8.5 to 10.3. The treated extract was used to assay benzaldehyde dehydrogenase. When benzaldehyde dehydrogenase was the only enzyme to be assayed, the extraction was performed in 0.04M-Tris-HCl buffer pH 10.3. When the total benzaldehyde dehydrogenase activity exceeded 80 units/mg protein, a second extract was prepared in 0.08M-sodium pyrophosphate buffer pH 9.5, and was used to assay benzaldehyde dehydrogenase activity.

4. ENZYME ASSAYS.

1 unit of enzyme activity corresponds to the disappearance of 1nmol substrate/min. Specific activity is defined as units/mg protein.

4.1. Spectrophotometric assays.

All spectrophotometric assays were conducted in 1cm path-length silica cuvettes containing a total volume of 3.0ml. Measurements of extinction were made with a Unicam SP.800 Ultraviolet Spectrophotometer (Pye Unicam Instruments Ltd.) connected to a Servoscribe Chart Recorder (Kelvin Electronics Co.). With the exception of L-mandelate dehydrogenase, the initial linear period of the time-course was taken as a measure of enzyme activity. The initial rate was linear for the four minute duration of the experiment, and the assays were performed four at a time using the automatic sample changer on the Unicam SP.800. In the case of L-mandelate dehydrogenase, the non linear rate over the first 45s was ignored when estimating enzyme activity. Occasionally, however, when very high enzyme activity was present, the initial rate was linear for a shorter period of time. When this occurred, the assays were carried out singly, and changes in extinction were measured continuously.

All reactions were carried out at 27°. No reference cuvettes were used as the extinctions were read against air.

All additions in a volume of 1ml or less were made by means of Eppendorf pipettes (Eppendorf Marburg Micropipette). The reagents in the reaction mixture were mixed with plungers (Calbiochem, Los Angeles, California, U.S.A.; catalogue no. 85019). The assays performed in Results, Sections 2 and 3 were carried out in duplicate.

4.1.1. L-Mandelate dehydrogenase.

L-Mandelate dehydrogenase was measured by a modification of the method of Hegeman (1966a). The assay depends on the reduction of 2,6-dichlorophenol-indophenol to the leuco form concomitant with the oxidation of L-mandelate to benzoylformate. The reaction was measured at 600nm, near the maximal extinction of the oxidised form of the dye. In addition to enzyme the reaction mixture contained:

200μmol of potassium dihydrogen phosphate-dipotassium hydrogen phosphate buffer pH 7.0.

200nmol of 2,6-dichlorophenol-indophenol.

1.5μmol of L-mandelic acid.

The reaction was initiated by the addition of L-mandelate. The extinction coefficient of the dye at 600nm and pH 7.0 is $20.6 \times 10^6 cm^2/mol$, and therefore a decrease in extinction of 6.87 units corresponds to the oxidation of 1μmol of L-mandelate to benzoylformate.

4.1.2. Benzoylformate decarboxylase.

Benzoylformate decarboxylase was measured by a direct assay and a coupled assay. The direct assay was a modification of the method of Hegeman (1966a). The assay makes use of the fact that the substrate, benzoylformate, has an extinction coefficient of $8.1 \times 10^4 cm^2/mol$ at 334nm and pH 6.0, whilst benzaldehyde and subsequent products have negligible extinction at this wavelength. Thus a decrease in extinction of 0.0272 units corresponds to the decarboxylation of 1μmol of benzoylformate. In addition to enzyme the reaction mixture contained:

100μmol of potassium dihydrogen phosphate-dipotassium hydrogen phosphate buffer pH 6.0.

100μg of thiamine pyrophosphate chloride (dissolved in 0.05M-phosphate buffer pH 6.0).

2.5μmol of benzoylformic acid.

The reaction was initiated by the addition of benzoylformate.

The coupled assay was a modification of the method of Kennedy & Fewson (1968b). The assay involves coupling the formation of benzaldehyde to the reduction of NAD^+ with an excess of benzaldehyde dehydrogenase. The appearance of NADH was measured at 340nm. Since NADH oxidase interfered with the reduction of NAD^+ at low levels of benzoylformate decarboxylase activity, the extract in which benzoylformate

decarboxylase was to be assayed was heated at 37° for 60min to remove any NADH oxidase activity except for the experiments described in Results, Section 1.1.2.2. In addition to enzyme the reaction mixture contained:

200μmol of Tris-HCl buffer pH 9.0.

100μg of thiamine pyrophosphate chloride (dissolved in 0.05M-Tris-HCl buffer pH 9.0).

3μmol of NAD^+.

600μg protein of an extract prepared from cells grown on benzyl alcohol as described in Section 3.3.

2.5μmol of freshly prepared benzoylformic acid.

The reaction was initiated by the addition of benzoylformate. The extinction coefficient of NADH at 340nm is $6.22 \times 10^6 cm^2/mol$; and therefore an increase in extinction of 2.07 units corresponds to the decarboxylation of 1μmol of benzoylformate. Under the conditions of the assay, the extinction change due to disappearance of benzoylformate was insignificant in proportion to the increase in extinction due to NADH formation.

The coupled assay was used to measure benzoylformate decarboxylase activity except when the use of the direct assay is specifically stated in the Text.

4.1.3. Labile and stable benzaldehyde dehydrogenase.

The two enzymes were measured by a modification of the method of Gunsalus et al.(1953). The assays measure the benzaldehyde-dependent reduction of NAD^+ at 340nm. The extinction coefficient of NADH at 340nm is $6.22 \times 10^6 cm^2/mol$;

and therefore an increase in extinction of 2.07 units corresponds to the oxidation of 1μmol of benzaldehyde. The activity of the two enzymes was measured in Tris buffer if the enzymes were extracted in Tris buffer, and in the majority of cases this occurred when the total benzaldehyde dehydrogenase activity was less than 80 units/mg protein. The two enzymes were assayed in sodium pyrophosphate buffer if the enzymes were extracted in sodium pyrophosphate buffer, and this usually occurred when the total benzaldehyde dehydrogenase activity was greater than 80 units/mg protein.

(1) Tris buffer assay (total activity less than 80 units/mg protein).

The activity of benzaldehyde dehydrogenase was measured before and after heating for 2h at 37°. The heat inactivation was carried out in a stoppered 3 dram Trident container (Johnsen & Jorgensen Ltd., London.). The activity after 2h heating was taken as the activity of the stable benzaldehyde dehydrogenase, and the difference between this activity and the activity before heating as the labile benzaldehyde dehydrogenase activity. In addition to enzyme the reaction mixture contained:

200μmol of Tris-HCl buffer pH 9.5.

150μmol of K_2HPO_4.

1.5μmol of NAD^+.

300nmol of benzaldehyde.

The reaction was initiated by the addition of benzaldehyde.

(11) Sodium pyrophosphate assay (total activity greater than 80 units/mg protein).

The heat inactivation of benzaldehyde dehydrogenase was followed kinetically at 37° by withdrawing samples from a 6 x 5/8 inch stoppered test-tube at intervals for enzyme assay. The graph of benzaldehyde dehydrogenase activity against time of heating was drawn, and as explained in Results, Section 1.1.1.3., the activity of the labile and stable enzymes was estimated from it. In addition to enzyme the reaction mixture contained:

200μmol of sodium pyrophosphate buffer pH 9.0.

150μmol of K_2HPO_4.

1.5μmol of NAD^+.

300nmol of benzaldehyde.

The reaction was initiated by the addition of benzaldehyde.

4.1.4. Benzyl alcohol dehydrogenase.

Benzyl alcohol dehydrogenase was measured by a modification of the method of Kennedy & Fowson (1968b). The assay measures the benzyl alcohol-dependent reduction of NAD^+ at 340nm. The extinction coefficient of NADH at 340nm is $6.22 \times 10^6 cm^2/mol$; and therefore an increase in extinction of 2.07 units corresponds to the oxidation of 1μmol of benzyl alcohol. In addition to enzyme the reaction mixture contained:

200µmol of sodium pyrophosphate buffer pH 9.0.

1.5µmol of NAD^+.

300nmol of benzyl alcohol.

The reaction was initiated by the addition of benzyl alcohol.

4.1.5. Catechol 1,2-oxygenase.

Catechol 1,2-oxygenase was measured by the method of Hegeman (1966a). The assay measures the accumulation of cis,cis-muconate in the presence of EDTA which inhibits the subsequent enzyme in the pathway. The reaction is measured at 260nm at which wavelength catechol absorbs weakly and cis,cis-muconate strongly. The extinction coefficient of cis,cis-muconate at 260nm is $16.8 \times 10^6 cm^2/mol$, and therefore an increase in extinction of 5.60 units corresponds to the accumulation of 1µmol of cis,cis-muconate. In addition to enzyme the reaction mixture contained:

200µmol of potassium phosphate-dipotassium hydrogen phosphate buffer pH 7.0.

4µmol of EDTA.

300nmol of catechol.

The reaction was initiated by the addition of catechol.

4.1.6. NADH oxidase.

NADH oxidase was measured by a modification of the method of Kennedy & Fewson (1968b). The assay measures the oxidation of NADH at 340nm. The extinction of NADH at

340nm is 6.22 x $10^6 cm^2/mol$; and therefore a decrease in extinction of 2.07 units corresponds to the oxidation of 1μmol of NADH. In addition to enzyme the reaction mixture contained:

200μmol of potassium dihydrogen phosphate-dipotassium hydrogen phosphate buffer pH 7.0.

200μg of NADH (dissolved in 0.2M-phosphate buffer).

The reaction was initiated by the addition of NADH.

4.2. Warburg assay of benzoate oxidase.

Benzoate oxidase was measured manometrically in cell-free extracts by following oxygen uptake in the Warburg apparatus (B. Braun, Apparatebau, Melsungen, West Germany; Model VL85).

Each 15ml single side-armed Warburg vessel was set up as follows:

Side arm: 2μmol of benzoate or water.

Main compartment: 200μmol of Tris-HCl buffer pH 7.5.
1μmol of $FeSO_4$.
100μmol of ethanol.
1μmol of NAD^+.
10nmol of FAD.
4U of alcohol dehydrogenase.
4-5mg of protein.

The contents of the side-arm and main compartment were contained in a volume of 2ml.

Centre well: 0.2ml 20% KOH and pleated filter paper (Whatman no. 52).

Atmosphere: Air.

Temperature: 27°.

The reagents were added in the order shown above. The contents of the Warburg vessels were equilibrated for 5min in 27° water baths, the manometers were sealed, and the air-tightness of the joints checked 5min later. The reaction was started by tipping the benzoate or water from the side arm into the main compartment.

4.3. Whole cell assay of benzoate oxidation.

Benzoate oxidase activity was determined in whole cells by measuring the disappearance of benzoate. This assay was developed by P.J. Roach (Fewson, Livingstone & Roach, 1970). The assay was conducted in a 50ml Erlenmeyer flask in a shaking water bath at 30°. The components of the reaction mixture were contained in a volume of 15ml, and consisted of:

200nmol of Tris-HCl buffer pH 7.5.

7.5μmol of benzoic acid.

14-20mg wet wt. of cells suspended in 0.05M-Tris-HCl buffer pH 7.5.

The reaction was initiated by the addition of the cell suspension after all the other components had been preincubated for 10min at 30°. 2ml samples were taken after 10, 20, 40 and 80min, added to 3ml chilled 16.7% (w/v) perchloric acid, mixed

and stood in an ice-water slurry for at least 15min. 15ml 0.1N-HCl was added and the contents of the test-tubes were mixed. The samples were then centrifuged at 4,000g for 20min at room temperature (M.S.E. "Supermedium" Centrifuge). The supernates were decanted, and their spectra were recorded against a water blank from 200 to 350nm. The extinctions at 230nm were read, and the amounts of benzoate which had disappeared were calculated from a standard curve of E_{230} against benzoate concentration. The rate of benzoate disappearance was calculated from the graph of the amount of benzoate which had disappeared against time of incubation.

5. ANALYTICAL METHODS.

5.1. Protein estimation in cell-free extracts.

Total protein in cell-free extracts was estimated in the Unicam SP.800 Spectrophotometer by measuring the E_{280} of samples which had been diluted in water to give an approximate E_{280} of 1. Calculations of total protein (mg/ml) were made by dividing the values of E_{280} by 4.9. Fewson (unpublished results) had previously established this relationship for cell-free extracts of bacterium NCIB 8250 as a result of experiments in which protein content was determined by E_{280} measurements and by mears of the Folin-Ciocalteau reagent (Lowry, Rosebrough, Farr & Randall, 1951) using bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.) as the standard.

5.2. Protein estimation of whole cells.

The whole cells were solubilized by standing for 24h at 30° in 0.66N-NaOH. Protein was then estimated by the Method of Lowry *et al.*(1951) as adapted for the Technicon Auto Analyser (Sheet 3/61 Ic; Technicon Instruments Ltd., Chertsey, Surrey.). Protein estimations were performed by Mr R. Blackie.

5.3. Rothera test.

1g solid $(NH_4)_2SO_4$ was added to a 2ml amount of the sample to be tested, and was dissolved by mixing. 0.1ml 5% (w/v) sodium nitroprusside was added and mixed. Finally 0.4ml ammonia (sp. gr. 0.88) was added and mixed. In the presence of a β-oxoacid the solution turned purple.

5.4. Statistical methods.

Analysis of variance was determined with the aid of programme ST1002 of the Programma 101 desk computer (British Olivetti Ltd., London W.1.). The coefficients of linear regression and correlation were determined with the aid of Programme 132 of the Programma 101 desk computer.

5.5. Measurement of pH.

The pH values of solutions having a volume less than 25ml were determined with a microsampling electrode, Type SMS 23 (Electronic Instruments Ltd., Richmond, Surrey.) attached to a Pye Dynacap pH meter (W.G. Pye & Co. Ltd., Cambridge.). The pH values of solutions having a volume greater than 25ml were determined by means of an E.I.L. direct reading pH meter (Electronic Instruments Ltd.; Model 23A). Both pH meters were calibrated daily with standard pH buffer tablets (Burroughs' Wellcome & Co. London.).

6. MATERIALS

All reagents were the best grade which could be obtained commercially. With the exception of the chemicals listed below, all compounds were supplied by British Drug Houses Ltd., Poole, Dorset.

Boehringer Corporation (London) Ltd., London W.5.
$NADP^+$.

Calbiochem, Los Angeles, California, U.S.A.
2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterine, Cleland's Reagent, Hepes and Tricine.

Fluka A.G., Buchs, Switzerland.
Benzoylformic acid, 4-hydroxybenzyl alcohol, 4-hydroxy-D,L-mandelic acid and L-mandelic acid.

K & K Laboratories Inc., Plainsview, New York, U.S.A.
Thioglycerol, riboflavin and thiophenoxyacetic acid.

Koch-Light Laboratories Ltd., Colnbrook, Bucks.
Mercaptoethanol and N.T.G.

Mann Research Laboratories, New York, U.S.A.
Tris and Tris-HCl

T.J. Sas & Son Ltd., London W.C.1.
Arginine, cysteine and glutamic acid-HCl.

Serravac Laboratories Ltd., Maidenhead, Berks.

Cytochrome c.

Sigma London Chemical Co. Ltd., London.

D-alanine, D-amino acid oxidase, ATP, CoA, FAD, FMN, D-glucose oxidase, glucose-6-phosphate dehydrogenase, GSH, lipoic acid, NAD^+, NADH, tetrahydrofolic acid and thiamine pyrophosphate chloride.

Carboxy Q and penicillin V-potassium salt were personal gifts to Dr J.H. Holms from I.C.I. (Pharmaceutical Division) Ltd., Alderley Park, Cheshire.

All bacteriological media with the exception of Seller's Differential Agar Medium (Difco Laboratories, Detroit 1, Michigan, U.S.A.) were supplied by Oxoid Ltd., London S.E.1.

Benzaldehyde was redistilled under nitrogen immediately before use in the experiments of Section 2, and benzyl alcohol was redistilled before use in the experiments of Section 2 and 3.

All solutions were made up in glass-distilled water.

R E S U L T S.

1. DEVELOPMENT OF EXPERIMENTAL METHODS.

1.1. Enzyme assays.

1.1.1. Benzaldehyde dehydrogenase.

Heat inactivation experiments by Kennedy & Fewson (1968b) indicated that cells grown on L-mandelate contained a heat-stable and a heat-labile benzaldehyde dehydrogenase, whereas cells grown on benzyl alcohol contained only the heat-labile enzyme. The first object of the present work was to confirm this observation. The next stage was to examine some of the properties of the two enzymes, and the third stage was to devise an assay system for their measurement. As explained in the Introduction, it was desirable to be able to measure the amounts of the two enzymes in the same extract.

Although there were some quantitative variations depending on the conditions of denaturation, early experiments showed that the observations of Kennedy & Fewson (1968b; their Fig.1) were readily repeatable (e.g. Fig.15).

In order to study the properties of the two enzymes, it was desirable to have cells containing each enzyme in the absence of the other. This had not been achieved by Kennedy & Fewson who obtained the stable enzyme only in the presence of the labile enzyme after growth on L-mandelate or benzoylformate. Using the gratuitous inducer thiophenoxyacetate (see Table 33),

however, cells were obtained which contained just the stable enzyme. Cells grown on benzyl alcohol, as observed by Kennedy & Fewson, contained only the labile enzyme. The early experiments were done with these two systems.

Benzaldehyde dehydrogenase extracted in Tris-HCl buffer was assayed in Tris-HCl buffer pH 9.5, whilst the same enzyme extracted in sodium pyrophosphate buffer was assayed in sodium pyrophosphate buffer pH 9.0 as maximum enzyme activity was obtained using these buffers.

1.1.1.1. Effect of cations on the activity of benzaldehyde dehydrogenase.

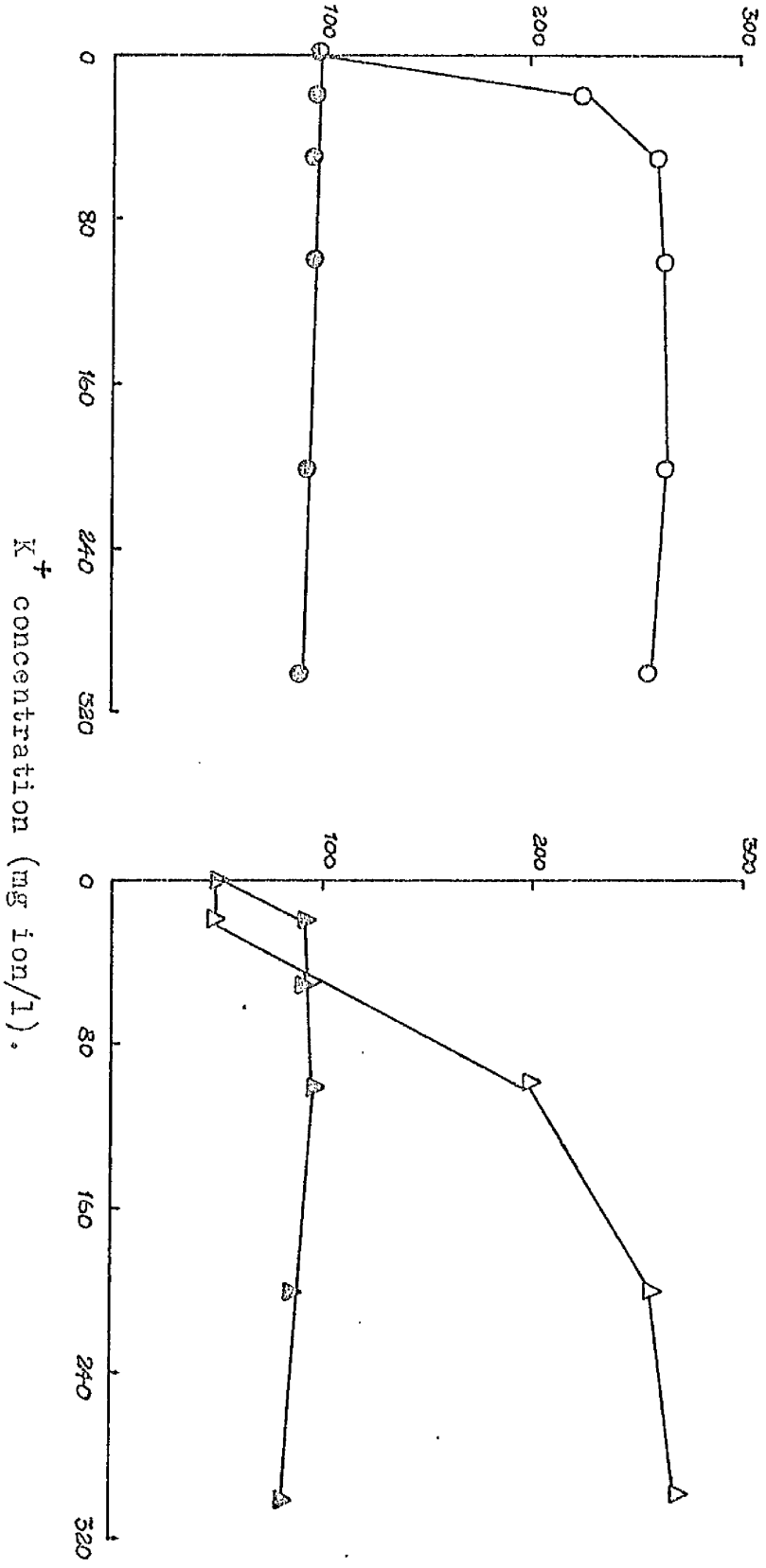
The effect of potassium ions on the activity of the benzaldehyde dehydrogenases found in cells grown on glutamate + thiophenoxyacetate or benzyl alcohol is shown in Fig.13. Since the sodium pyrophosphate buffer used in the extraction procedure is more concentrated than the Tris buffer used (0.08M compared to 0.04M), the Tris buffer is at a higher pH than the pyrophosphate buffer (pH 10.3 compared to pH 9.5) so that the actual pH of the extract is the same (pH 9.24 $\pm$ 0.01). This was presumably necessary because of the buffering effect of the extract. In both cases extracts prepared in Tris buffer show less activity than extracts prepared in pyrophosphate buffer. The benzaldehyde dehydrogenase induced by thiophenoxyacetate is activated several fold by potassium ions when the cells are extracted in Tris or pyrophosphate buffer. On the other hand the enzyme induced by benzyl alcohol is not

Fig. 13. The effect of potassium ions on the activity of benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol or 5mM-glutamate + 1mM-thiophenoxyacetate as sole source of carbon, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 10.3 or 0.08M-sodium pyrophosphate buffer pH 9.5 and sonicated in 5ml amounts as described in Methods (p.75). The extracts were assayed in the presence of various potassium ion concentrations as described in Methods (p.81) except that the enzyme was not heated.

- ○ – Extract from cells grown on glutamate + thiophenoxyacetate and resuspended in sodium pyrophosphate buffer.
- ● – Extract from cells grown on benzyl alcohol, and resuspended in sodium pyrophosphate buffer.
- △ – Extract from cells grown on glutamate + thiophenoxyacetate, and resuspended in Tris-HCl buffer.
- ▲ – Extract from cells grown on benzyl alcohol, and resuspended in Tris-HCl buffer.

% of the specific activity observed in cells extracted in sodium pyrophosphate buffer.



activated by potassium ions when extracted in pyrophosphate buffer, and the slight activation after extraction in Tris buffer brings the activity up only to the value obtained after extraction in pyrophosphate buffer.

The same type of experiment was repeated using other monovalent cations, and the maximum activity obtained with them is shown in Table 3. The Table also shows the ion concentration at which maximum activation occurs (where the activity of the enzyme is not increased by any concentration of the ion, a zero value is given for the ion concentration). The benzaldehyde dehydrogenase induced by thiophenoxyacetate is activated by all the ions tested ($NH_4^+ > K^+ > Rb^+ > Cs^+ > Na^+$) when extracted in pyrophosphate buffer, whereas when it is extracted in Tris buffer, NH_4^+, Cs^+ and Na^+ ions do not activate. The enzyme induced by benzyl alcohol, on the other hand, is not activated by any of these ions when extracted in pyrophosphate buffer, and the activation when extracted in Tris buffer brings the activity up only to the value obtained by extraction in pyrophosphate buffer.

Although a number of other cations, including Mg^{++}, Al^{+++}, Ca^{++}, Mn^{++}, Fe^{++}, Fe^{+++}, Co^{++}, Ni^{++}, Cu^{++} and Zn^{++}, each at final concentrations ranging from 3μM to 33mM, was tested, none was found to activate either of the enzymes.

1.1.1.2. Effect of thiols on the activity of benzaldehyde dehydrogenase.

Since Stachow, Stevenson & Day (1967) assayed the $NADP^+$-specific benzaldehyde dehydrogenase of *P. putida* in the presence

Table 3. The effect of cations on the activity of benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol or 10mM-glutamate + 1mM-thiophenoxyacetate as sole source of carbon, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 10.3 or 0.08M-sodium pyrophosphate buffer pH 9.5, and sonicated in 5ml amounts as described in Methods (p.75). The extracts were assayed in the presence of various ammonium, sodium, caesium, rubidium, or potassium ion concentrations as described in Methods (p.81) except that the enzyme was not heated. The ion concentration given refers to the concentration at which maximum activity occurred.

† The activity is expressed as a % of the specific activity observed in cells extracted in sodium pyrophosphate buffer.

* In the assay of an extract made in sodium pyrophosphate buffer, there is, in addition to the quoted ion concentration, a sodium ion concentration of 267mg ion/l as a result of the use of sodium pyrophosphate as the assay buffer.

n.a. - not applicable.

Ion	Benzyl alcohol cells				Glutamate + thiophenoxyacetate cells			
	Tris extraction		Na pyrophosphate* extraction		Tris extraction		Na pyrophosphate* extraction	
	max.† activity	ion concn. (mg ion/l)	max.† activity	ion concn. (mg ion/l)	max.† activity	ion concn. (mg ion/l)	max.† activity	ion concn. (mg ion/l)
0	50	n.a.	100	n.a.	67	n.a.	100	n.a.
NH_4^+	70	20	102	50	90	300	310	200
Na^+	97	100	100	0	89	300	125	20
Cs^+	109	50	100	0	93	100	198	300
Rb^+	103	50	105	20	231	300	261	200
K^+	99	100	100	0	266	300	264	100

of Cleland's Reagent, it was decided to test the effect of thiols on the activity of the two benzaldehyde dehydrogenases of bacterium NCIB 8250. Cleland's Reagent, mercaptoethanol, thioglycerol, GSH and cysteine, each at final concentrations ranging from 3μM to 33mM, did not activate either of the enzymes.

1.1.1.3. Heat inactivation of benzaldehyde dehydrogenase.

The heat inactivation of benzaldehyde dehydrogenase in cells induced by benzyl alcohol or thiophenoxyacetate is shown in Fig.14. In this experiment the half-lives of the stable enzyme induced by thiophenoxyacetate and of the labile enzyme induced by benzyl alcohol are 520 and 30min respectively. A slight complication is caused by the fact that the activity of the stable enzyme rises by about 35% (23 units/mg protein to 32 units/mg protein) during the first 10min inactivation at 37°, and then falls exponentially. This activation is independent of the presence of potassium in the reaction mixture since the same percentage activation occurs after heating for 10min in the absence or presence of potassium in the assay mixture. In this experiment the extraction buffer was 0.04M-Tris-HCl pH 10.3. When 0.08M-sodium pyrophosphate pH 9.5 was used as the extraction buffer, similar results were obtained except that there was no activation of the stable enzyme during the first few minutes of heating at 37°, and the activity declined exponentially all the time.

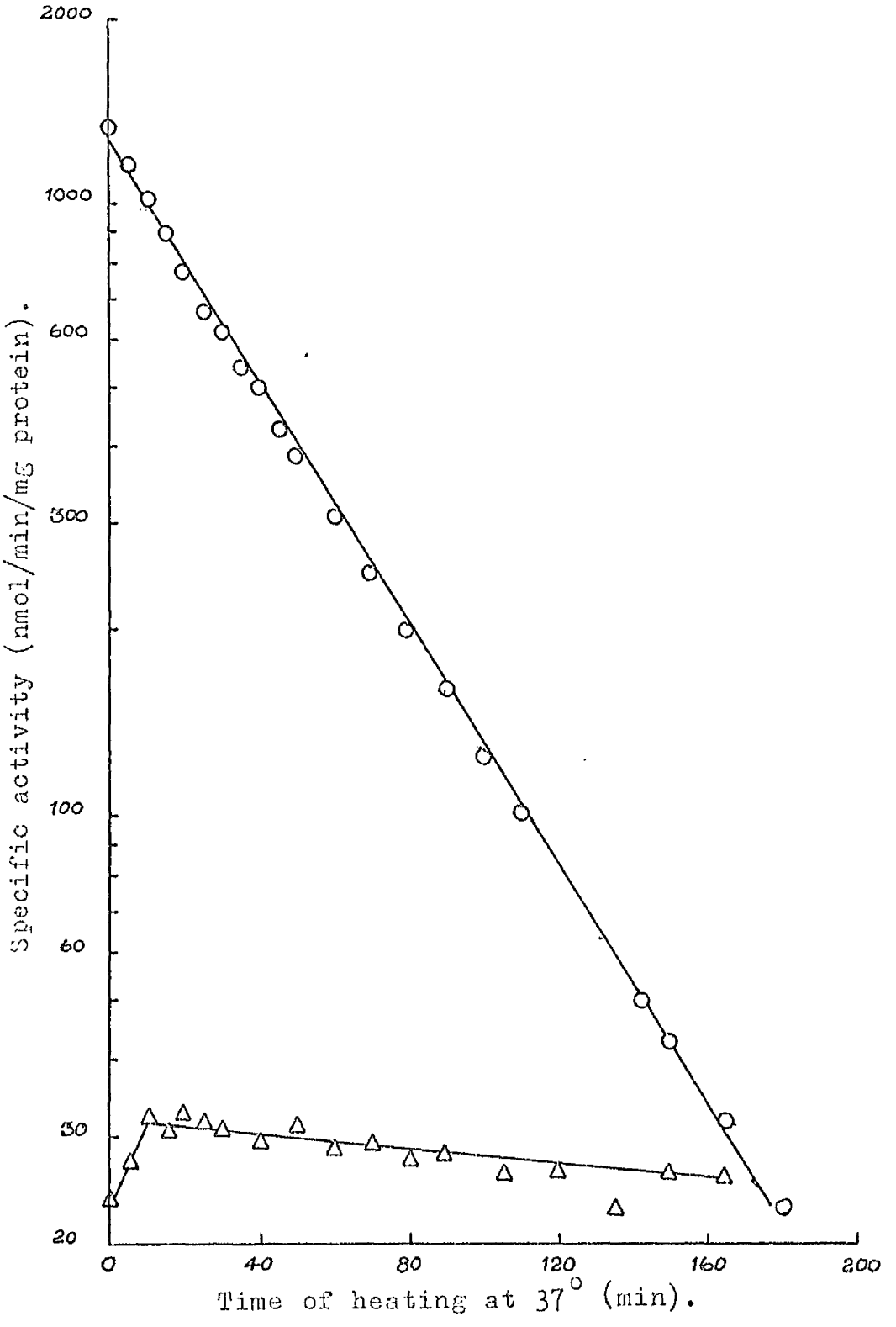
Kennedy & Fewson (1968b) have shown that cells grown on L-mandelate contain both the heat-stable and heat-labile

Fig.14. Kinetics of heat inactivation of benzaldehyde dehydrogenase of bacterium NCIB 8250 induced with 5mM-benzyl alcohol or 10mM-glutamate + 1mM-thiophenoxy-acetate.

Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol or 10mM-glutamate + 1mM-thiophenoxyacetate, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 10.3, and sonicated in 5ml amounts as described in Methods (p.75). The extracts were heated at 37° and samples were withdrawn at intervals for determination of benzaldehyde dehydrogenase activity.

○ - Benzyl alcohol cells.

△ - Glutamate + thiophenoxyacetate cells.


2000
1000
600
300
100
60
30
20
Specific activity (nmol/min/mg protein).
0
40
80
120
160
200
Time of heating at 37° (min).

enzymes, and this observation has been confirmed as shown in Fig.15. Extrapolation of the part of the graph which has a slow decay shows that there were 80 units/mg protein of the stable enzyme before the heat treatment started. By extrapolating the initial part of the curve to the axis, it can be seen that there were originally 200 units/mg protein of total benzaldehyde dehydrogenase activity. So by subtraction (200-80) there must have been 120 units/mg protein of the labile enzyme. Calculations were then made assuming that the original extract contained two enzymes having half-lives of 30 and 500min and specific activities of 120 and 80 units/mg protein respectively, and that the stable enzyme was activated by 35% over the first 10min of heating. (The two half-lives were, of course, chosen to give the best fit; as will be explained later, the half-lives did in fact vary slightly from experiment to experiment depending on the precise pH of the extract). The theoretical denaturation curve for this hypothetical mixture was drawn. The simulated curve (Fig.15) agrees well with the experimental curve which indicates that the heat inactivation curve for benzaldehyde dehydrogenase from cells grown on L-mandelate is consistent with the presence of two enzymes.

Although the heat inactivation curve in Fig.15 is due to the presence of two enzymes having half-lives of 30 and 500min, the apparent half-life of the labile enzyme, as calculated from the initial part of the graph, is 59min. This

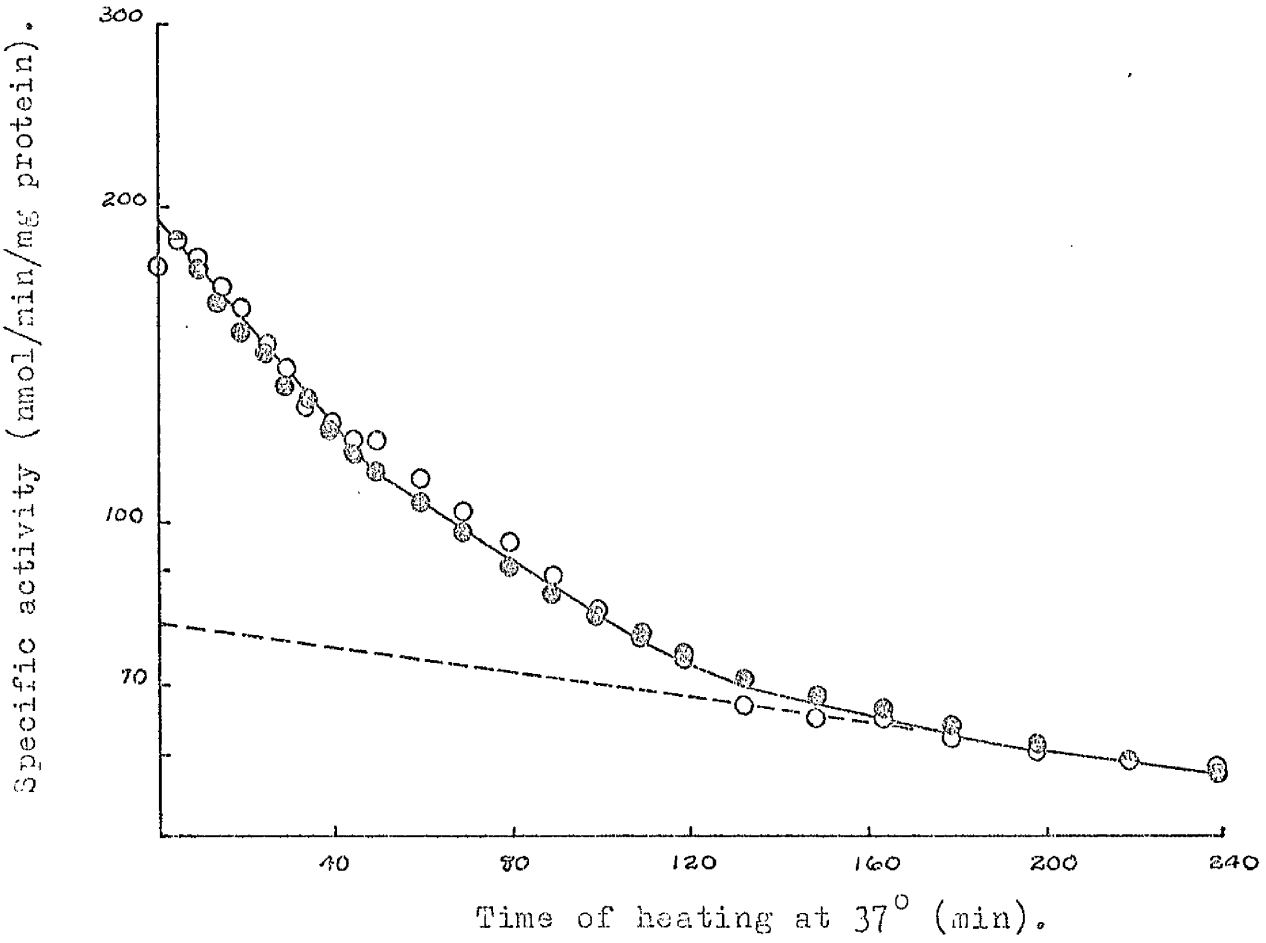
Fig.15. Kinetics of heat inactivation of benzaldehyde dehydrogenase of bacterium NCIB 8250 grown on 5mM-L-mandelate-salts medium and of a mixture of two simulated enzymes.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate-salts medium, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 10.3, and sonicated in 5ml amounts as described in Methods (p.75). The extract was heated at 37° and samples were withdrawn at intervals for determination of benzaldehyde dehydrogenase activity. The graph of specific activity of benzaldehyde dehydrogenase against time was drawn, and by extrapolation from the graph as described on p.99 it was calculated that there were 120 units/mg protein of the labile enzyme, and 80 units/mg protein of the stable enzyme present in the extract. The theoretical curve for the kinetics of heat inactivation of two enzymes having half-lives of 30min and 500min, and specific activities of 120 units/mg protein and 80 units/mg protein respectively was then drawn.

○ - L-Mandelate grown cells.

◎ - Theoretical curve.

The dotted line on the graph represents the extrapolation of the specific activity of the stable enzyme to zero time.


Specific activity (nmol/min/mg protein).
300
200
100
70
40
80
120
160
200
240
Time of heating at 37° (min).

apparent increase in the half-life of the labile enzyme is due to the fact that the observed decay rate is the vector sum of two independent and simultaneous decay rates corresponding to the decay of the heat-stable and heat-labile enzymes.

Fig.16 shows the results of a more rigorous experiment in that the heat inactivation curve for two simulated enzymes, having half-lives of 23 and 500min, and specific activities of 300 and 30 units/mg protein respectively, was drawn taking into account a 35% activation of the stable enzyme over the first 10min of heating. (As above, the half-lives were chosen to give the best fit). An extract was then made from a mixture of cells grown on glutamate + thiophenoxyacetate or benzyl alcohol so that it contained 300 and 30 units/mg protein of the labile and stable enzymes respectively. This extract was denatured at 37°, and the heat inactivation curve obtained is almost identical to the curve for the two simulated enzymes. This indicates that the heat inactivation curve for benzaldehyde dehydrogenase from a mixture of cells follows its theoretical pattern.

1.1.1.4. Development of an assay for the two enzymes.

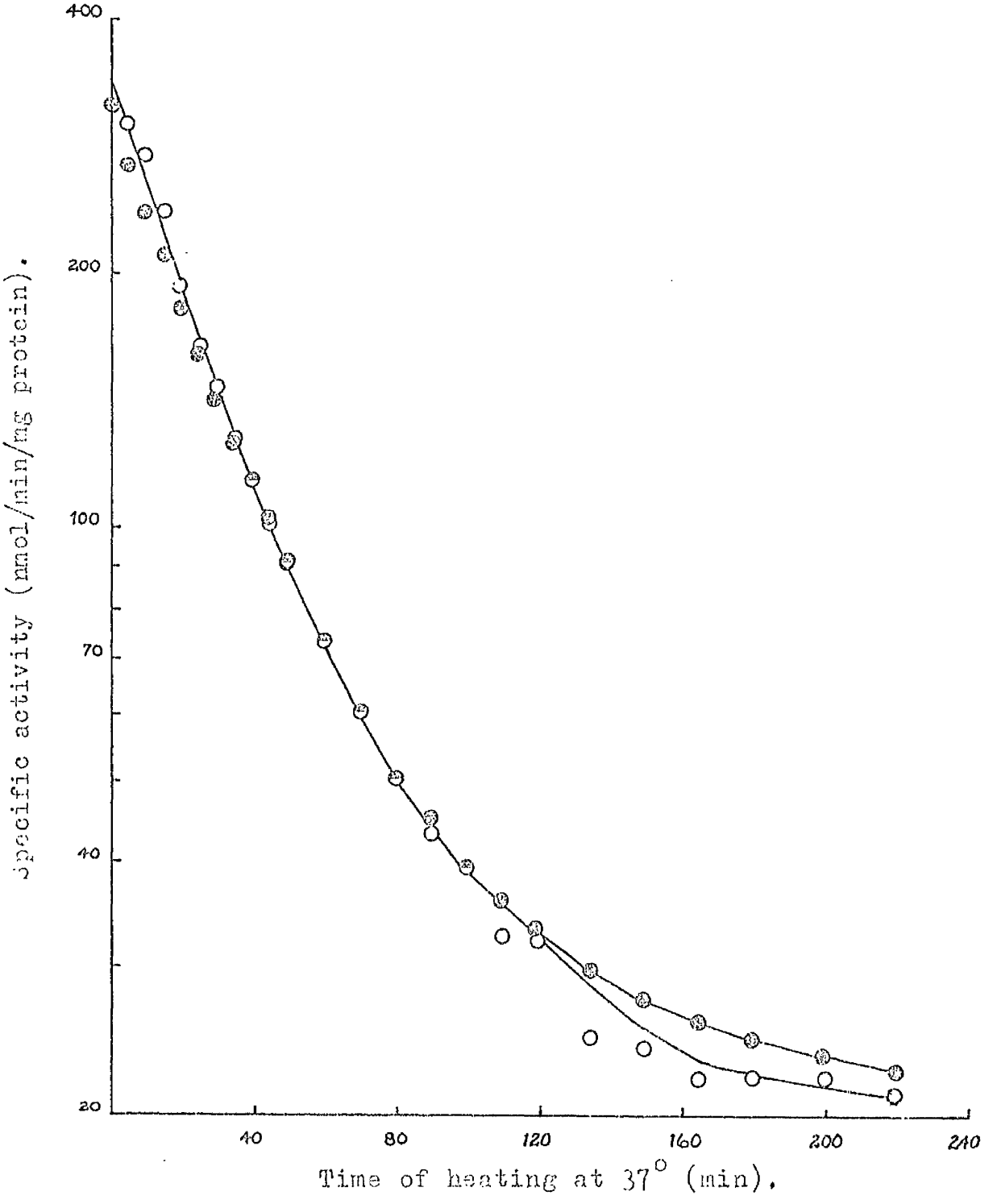
Since the half-life of the heat-labile enzyme is far less than that of the heat-stable enzyme, it has been possible to devise assays in which the individual activities of the two enzymes can be differentiated. In principle, this involves destroying the activity of the labile enzyme by heating it at 37°.

Fig.16. Kinetics of heat inactivation of benzaldehyde dehydrogenase from a mixture of cells grown on 5mM-benzyl alcohol or 10mM-glutamate + 1mM-thiophenoxyacetate, as sole source of carbon, and of a mixture of two simulated enzymes.

The theoretical curve for the kinetics of heat inactivation of two enzymes, having half-lives of 23min and 500min, and specific activities of 300 units/mg protein and 30 units/mg protein respectively, was drawn. Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol or 10mM-glutamate + 1mM-thiophenoxyacetate as sole source of carbon, harvested, washed, and stored as described in Methods. A mixture of cells of both types was resuspended in 0.04M-Tris-HCl buffer pH 10.3, and sonicated in 5ml amounts as described in Methods (p.75) so that the final extract contained 300 units/mg protein and 30 units/mg protein of the labile and stable enzymes respectively. The extract was heated at 37^{o} and samples were withdrawn at intervals for determination of benzaldehyde dehydrogenase activity.

O - Mixture of benzyl alcohol and glutamate + thiophenoxyacetate cells.

◎ - Theoretical curve.


400
200
100
70
40
20
40
80
120
160
200
240
Specific activity (nmol/min/mg protein).
Time of heating at 37° (min).

The residual activity is taken as the activity of the stable enzyme. The difference between this activity and the total benzaldehyde dehydrogenase activity is a measure of the labile enzyme.

As these assays depend on the selective destruction of the heat-labile benzaldehyde dehydrogenase, the effect of changing the buffer and pH of extraction on the rate of heat inactivation of this enzyme was determined. This was necessary because preliminary experiments had shown that quite different half-lives were obtained if slightly different buffers were used. Typical results are shown in Table 4. The data illustrate that the half-life of the labile enzyme is less in sodium pyrophosphate buffer than in Tris buffer; and as the actual pH of the enzyme extract in Tris buffer increases, the half-life of the enzyme decreases. In practice it was found that the actual pH of the extract depended on the concentration of the cell suspension and the efficiency of extraction; and that the higher the protein concentration of the extract, the lower the pH. As a result the actual half-life of the labile enzyme extracted from cells grown on benzyl alcohol (which contains only the heat-labile enzyme) varied between 23 and 33min when the extraction buffer was 0.04M-Tris pH 10.3. On the other hand the rate of heat inactivation of the heat-stable enzyme does not vary greatly with the buffer and pH of extraction, and this is shown in Table 5.

Using all the results obtained in this Section, it was possible to measure the activities of both benzaldehyde dehydrogenases in extracts which contained any relative or

Table 4. The effect of the buffer of extraction, and the pH of the extract on the rate of inactivation of benzaldehyde dehydrogenase of bacterium NCIB 8250 grown on benzyl alcohol as sole source of carbon.

Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol as sole source of carbon, harvested, washed, and stored as described in Methods. The cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5, pH 9.5, or pH 10.3, or 0.08M-sodium pyrophosphate buffer pH 9.5, and sonicated in 5ml amounts as described in Methods (p.75). Since the buffering capacity of the extraction buffer was not sufficient to maintain the pH of the extract at the pH of the buffer, the actual pH of the extract was determined by means of a micro-electrode. The extracts were heated at 37° and samples were withdrawn at intervals for determination of benzaldehyde dehydrogenase activity. The graph of specific activity of benzaldehyde dehydrogenase against time was drawn, and the half-life of benzaldehyde dehydrogenase was calculated from it.

* The pH 8.5 extract was titrated with the amount of 0.2N-NaOH required to alter the pH of a 0.04M-Tris-HCl buffer pH 8.5 containing no protein to pH 9.5.

Buffer of extraction	Benzaldehyde dehydrogenase ½ life (min)	Actual pH of the enzyme extract
0.04M-Tris-HCl buffer pH 8.5*	113	8.88
0.04M-Tris-HCl buffer pH 9.5	60	8.95
0.04M-Tris-HCl buffer pH 10.3	33	9.18
0.08M-sodium pyrophosphate buffer pH 9.5	19	9.16

Table 5. The effect of the buffer of extraction, and the pH of the extract on the rate of inactivation of benzaldehyde dehydrogenase of bacterium NCIB 8250 grown on mandelate, as sole source of carbon.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate, as sole source of carbon, harvested, washed, and stored as described in Methods. The cells were resuspended in 0.04M-Tris-HCl buffer pH 9.5, pH 9.9, or pH 10.3, or 0.08M-sodium pyrophosphate buffer pH 9.5, and sonicated in 5ml amounts as described in Methods (p.75). Since the buffering capacity of the extraction buffer was not sufficient to maintain the pH of the extract at the pH of the buffer, the actual pH of the extract was determined by means of a microelectrode. The extracts were heated at 37^{o} and samples were withdrawn at intervals for determination of benzaldehyde dehydrogenase activity. The graph of specific activity of benzaldehyde dehydrogenase against time was drawn, and the half-lives of benzaldehyde dehydrogenase was calculated from it.

Buffer of extraction	Benzaldehyde dehydrogenase ½ life (min)		Actual pH of the enzyme extract
	1st	2nd	
0.04M-Tris-HCl buffer pH 9.5	56	504	9.07
0.04M-Tris-HCl buffer pH 9.9	43.5	492	9.21
0.04M-Tris-HCl buffer pH 10.3	44	540	9.25
0.08M-sodium pyrophosphate buffer pH 9.5	31	541	9.23

absolute amount of each. Two approaches were used depending on the total benzaldehyde dehydrogenase activity.

(1) When the total benzaldehyde dehydrogenase activity was below 80 units/mg protein, the enzymes were extracted in 0.04M-Tris buffer pH 10.3, and inactivated at 37° for 120min. The residual activity was taken as a measure of the stable benzaldehyde dehydrogenase activity since the amount of the stable enzyme denatured in 120min approximately equals the increase in activity caused by the initial activation (Fig.14), and since the activity of the labile enzyme is reduced to negligible amounts on heating for 120min. The difference between this activity and the total benzaldehyde dehydrogenase activity before denaturation was taken as a measure of the labile benzaldehyde dehydrogenase activity.

(11) When the total enzyme activity was over 80 units/mg protein, there may have been detectable amounts of the labile enzyme left after 120 min denaturation, and thus any residual activity could be due to either the stable or labile enzyme. Therefore, if preliminary experiments showed that cells contained more than 80 units/mg protein of benzaldehyde dehydrogenase activity, the inactivation was followed kinetically in 0.08M-sodium pyrophosphate buffer pH 9.5 (in which buffer the half-life of the labile enzyme is shorter (Table 4)). The activities of the two enzymes were calculated by extrapolation of the heat inactivation curve; an example of this is given in Fig.15.

Full details of the final assay procedures developed along the lines described in this Section, and used in subsequent experiments, are given in Methods.

1.1.2. Benzoylformate decarboxylase.

The two methods quoted in the literature for measuring benzoylformate decarboxylase activity are those of Hegeman (1966a) and Kennedy & Fewson (1968b). Hegeman's assay measures the disappearance of benzoylformate, which has an extinction coefficient of $8.1 \times 10^4 cm^2/mol$ at 334nm. That of Kennedy & Fewson involves coupling the formation of benzaldehyde to the reduction of NAD^+, which has an extinction coefficient of $6.22 \times 10^6 cm^2/mol$ at 340nm, with an excess of benzaldehyde dehydrogenase. Hegeman's assay is too insensitive for routine use since it requires the addition of too much protein to observe significant changes in optical density at low enzyme specific activity. It was therefore decided to use the coupled assay of Kennedy & Fewson as it is 75 times more sensitive than Hegeman's assay. Fewson (personal communication), however, stated that Kennedy and he had not developed optimal conditions for measuring this enzyme in bacterium NCIB 8250, and consequently this had to be done before the assay could be used routinely.

1.1.2.1. Development of optimal assay conditions for the direct assay.

It was decided to find the optimal conditions for measuring the enzyme by the direct assay in bacterium

NCIB 8250 before developing an assay for the more complex coupled reaction so that information, uncomplicated by effects on the coupling enzyme, could be obtained about the enzyme itself.

Using the assay conditions of Kennedy & Fewson (1968b) except that the concentration of thiamine pyrophosphate was four times higher, the effect of varying the pH and buffer of extraction was determined. Table 6 shows that optimal activity (317 units/mg protein) is obtained using 0.08M-K_2HPO_4-KH_2PO_4 buffer pH 8.0 as the extraction buffer. Further experiments showed that benzoylformate at the concentration used produced a precipitate in the reaction mixture; but this was overcome by lowering the benzoylformate concentration from 8.3mM to 830μM. Examination of the effect of thiamine pyrophosphate concentration on benzoylformate decarboxylase activity indicated that thiamine pyrophosphate at a final concentration of 33.3μg/ml gave optimal enzyme activity; this was twice the concentration used by Kennedy & Fewson (1968b). The activity of the enzyme was optimal between pH 5.9 and 6.1 with the activity at pH 5.0 and 8.0 being 70 and 65% respectively of the optimal value.

1.1.2.2. Development of the coupled assay.

The coupled assay depends on the activity of benzaldehyde dehydrogenase being greater than that of benzoylformate decarboxylase. Kennedy (1967) showed that benzaldehyde dehydrogenase possessed little activity at pH 6.0, the pH at

Table 6. Effect of buffer and pH of extraction on the activity of benzoylformate decarboxylase in the direct assay.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, and stored as described in Methods. Cells were resuspended in the various buffers to 40mg wet wt./ml and sonicated in 12ml amounts as described in Methods (p.73). The extracts were centrifuged at 12,000g for 25 min at 4° and benzoylformate decarboxylase was assayed by the direct assay as described in Methods (p.80) with the exception of the amount of benzoylformate (25 μmol) and thiamine pyrophosphate (200 μg) used. Values for the specific activity of the enzyme are expressed as nmol substrate converted/min/mg protein.

Buffer and pH of extraction	Enzyme specific activity
0.08M-K_2HPO_4-KH_2PO_4 pH 6.0	precipitate
0.08M-K_2HPO_4-KH_2PO_4 pH 7.0	261
0.08M-K_2HPO_4-KH_2PO_4 pH 8.0	317
0.08M-Sodium pyrophosphate pH 6.0	221
0.08M-Sodium pyrophosphate pH 7.0	177
0.08M-Sodium pyrophosphate pH 8.0	94
0.08M-Hepes pH 6.0	precipitate
0.08M-Hepes pH 7.0	precipitate
0.08M-Hepes pH 8.0	312

which benzoylformate decarboxylase is measured in the direct assay. Consequently the buffers used in the coupled assay were at pH 8.0 or 9.0, pHs at which there is considerable benzaldehyde dehydrogenase activity.

Using the assay conditions developed for benzoylformate decarboxylase in the previous Section except for the use of Tricine pH 8.0 as the assay buffer, the effect of coupling the decarboxylation reaction to the reduction of NAD^+ in the presence of an excess of benzaldehyde dehydrogenase was determined. Benzaldehyde dehydrogenase was contained in crude extracts prepared from cells grown on benzyl alcohol and extracted in 0.08M-sodium pyrophosphate buffer pH 7.0 or 9.5 or 0.08M-Tricine buffer pH 7.0 or 9.5. These "coupling enzyme" extracts were used to couple the decarboxylation reaction to the reduction of NAD^+. The results showed that only the time-course of the reactions in which a pH 9.5 extract was used had an appreciable linear portion.

Once the choice of a pH 9.5 buffer for the extraction of benzaldehyde dehydrogenase had been decided, the effect of varying the buffer and pH of assay was determined. Table 7 shows that the presence of sodium pyrophosphate buffer in the assay reduces the activity of the enzyme by at least 50%. Maximum enzyme activity was obtained using Tricine pH 9.0 as the assay buffer and Tricine pH 9.5 as the extraction buffer of the coupling enzyme.

Once the choice of assay and extraction buffer had been decided, the effect of varying the coupling enzyme

Table 7. The effect of buffer and pH of assay, and buffer of extraction of the coupling enzyme on the activity of benzoylformate decarboxylase in the coupled assay.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate or 5mM-benzyl alcohol as sole source of carbon, harvested, washed, and stored as described in Methods. Benzoylformate decarboxylase was extracted by resuspending cells grown on mandelate in 0.08M-K_2HPO_4-KH_2PO_4 buffer pH 8.0 to 40mg wet wt./ml, and sonicating 12ml of this suspension as described in Methods (p.73). The coupling enzyme, benzaldehyde dehydrogenase, was extracted by resuspending cells grown on benzyl alcohol in the various buffers to 50mg wet wt./ml, and sonicating 12ml of this suspension as described in Methods (p.73). All the extracts were centrifuged at 12,000g for 25min at 4°, and benzoylformate decarboxylase was assayed as described in Methods (p.80) with the exception of the assay buffer, and the amount of coupling protein (270-310µg) and NAD^+ (1.5µmol) used. Values for the specific activity of the enzyme are expressed as nmol substrate converted/min/mg protein.

Coupling enzyme extraction buffer	Assay buffer	Enzyme Specific activity
0.08M-Sodium pyrophosphate pH 9.5	0.05M-Sodium pyrophosphate pH 7.5	135
	0.05M-Sodium pyrophosphate pH 9.0	80
	0.05M-Tricine pH 8.0	160
	0.05M-Tricine pH 9.0	252
0.08M-Tricine pH 9.5	0.05M-Sodium pyrophosphate pH 7.5	139
	0.05M-Sodium pyrophosphate pH 9.0	107
	0.05M-Tricine pH 8.0	519
	0.05M-Tricine pH 9.0	530

concentration in the assay mixture was determined. Fig.17 shows that over a wide range of benzoylformate decarboxylase activity a saturating amount of coupling enzyme is present except when the lowest concentration of coupling enzyme (52µg protein/ml) is used at the highest activity of benzoylformate decarboxylase. It was observed, however, that the initial linear portion of the time-course of the reaction was longer when the higher concentrations of coupling enzyme were used. A similar effect was observed when the NAD^+ concentration was increased. In practice a NAD^+ concentration of 1mM and a coupling enzyme concentration of 200µg protein/ml were sufficient to maintain a linear reaction for the four minute duration of the experiment.

It was found that storage of the coupling enzyme extract at -60° gave the same reaction profile as the unfrozen extract. The coupling protein extract was therefore routinely prepared in large quantities and stored at -60° until required.

1.1.2.3. Effect of protein from non-induced cells on the activity of benzoylformate decarboxylase.

It was possible that protein from non-induced cells of bacterium NCIB 8250 could interfere with the assay of low levels of benzoylformate decarboxylase activity resulting in the inaccurate measurement of low specific activities of the enzyme. The effect of diluting extracts containing benzoylformate decarboxylase with extracts of non-induced cells was therefore determined. Table 8 shows the results of the

Fig.17. The effect of concentration of coupling enzyme on the activity of benzoylformate decarboxylase in the coupled assay.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate or 5mM-benzyl alcohol as sole source of carbon, harvested, washed, and stored as described in Methods. Benzoylformate decarboxylase was extracted by resuspending cells grown on mandelate in 0.08M-K_2HPO_4-KH_2PO_4 buffer pH 8.0 to 40mg wet wt./ml, and sonicating 12ml of this suspension as described in Methods (p.73). The coupling enzyme, benzaldehyde dehydrogenase, was extracted by resuspending cells grown on benzyl alcohol in 0.08M-Tricine buffer pH 9.5 to 50mg wet wt./ml, and sonicating 18ml of this suspension in the 50ml rosette as described in Methods (p.74). Both extracts were centrifuged at 12,000g for 25 min at 4°. Different amounts of the extract containing benzoylformate decarboxylase were added to the reaction mixture, and the activity of benzoylformate decarboxylase was measured as described in Methods (p.80) using 50µl (○), 100µl (●), 200µl (△), or 500µl (▲) coupling enzyme, which corresponds to a final concentration of 52µg/ml, 105µg/ml, 209µg/ml or 523µg/ml respectively of coupling enzyme in the reaction mixture.

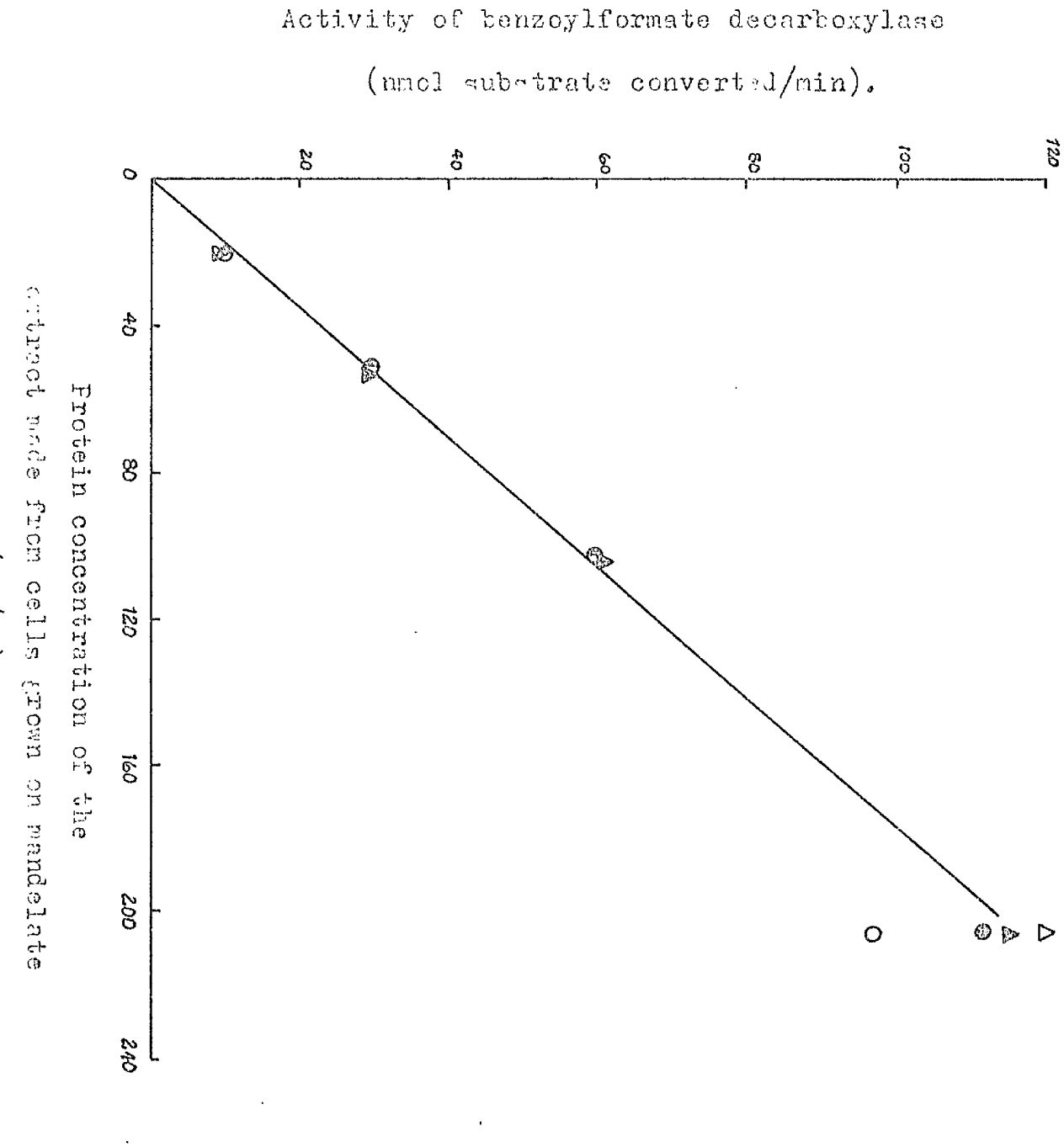

Activity of benzoylformate decarboxylase
(nmol substrate converted/min).
0
20
40
60
80
100
120
0
40
80
120
160
200
240
Protein concentration of the
extract made from cells grown on mandelate
(µg/ml).

Table 8. The effect of dilution with extracts made from cells grown on succinate on the activity of benzoylformate decarboxylase.

Bacterium NCIB 8250 was grown on four different occasions on 5mM-L-mandelate or 10mM-succinate as sole source of carbon, harvested, washed and stored as described in Methods. Both types of cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5. The cells grown on mandelate were sonicated in 5ml amounts, and cells grown on succinate in 25ml amounts as described in Methods (p.73). After both types of extract had been centrifuged, the succinate extract was used to dilute the mandelate extract 1/10 or 1/100. Benzoylformate decarboxylase was assayed as described in Methods (p.80), except that the extract was not heated, and NADH oxidase was assayed using 200µmol Tris-HCl buffer pH 9.0, and NADH dissolved in 0.2M-Tris-HCl buffer pH 9.0. Values for the specific activity of NADH oxidase are expressed as nmol substrate converted/min/mg protein. Values for the specific activity of benzoylformate decarboxylase are expressed as percentages of the specific activity of benzoylformate decarboxylase in the undiluted extracts made from cells grown on mandelate. The benzoylformate was stored at -10° until required for the benzoylformate decarboxylase assay.

Succinate extract NADH oxidase specific activity	Age of the benzoylformate (days)	Benzoylformate decarboxylase specific activity		
		No dilution	1/10 dilution	1/100 dilution
6.0	0	100	9.7	0.80
19.0	1	100	8.2	not tested
14.1	16	100	6.8	0
not tested	31	100	6.7	0

first experiments performed along these lines. Fortunately the NADH oxidase activity of the non-induced extract was also measured. Clearly, dilution of the enzyme leads to loss of activity. It is, however, impossible to decide from these data whether the effect is due to the age of the benzoylformate or the activity of NADH oxidase. (NADH oxidase could interfere by oxidising the NADH formed in the coupled system). Experiments were undertaken to separate these two effects. Table 9 shows that storage of the benzoylformate decreases the activity of the diluted extracts; but the activity of the diluted extracts in which fresh benzoylformate is used is still relatively lower than that of the undiluted extracts.

Table 10 shows that most of the NADH oxidase activity is lost on heating for 60min at 37°. Furthermore if the extracts are heated for 60min at 37°, benzoylformate decarboxylase activity in the diluted extract is approximately equivalent to that in the concentrated extract. Removal of NADH oxidase activity from the coupling protein extract by ultracentrifugation (heating the extract at 37° destroys the benzaldehyde dehydrogenase activity) had no effect on the activity of low levels of benzoylformate decarboxylase.

Both storage of the benzoylformate and the NADH oxidase activity of non-induced extracts reduced the activity of low levels of the enzyme. Consequently in subsequent assays, extracts in which benzoylformate decarboxylase activity was to be estimated were heated for 60min at 37°, and fresh

Table 9. The effect of storage of benzoylformate on the activity of benzoylformate decarboxylase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate or 10mM-succinate as sole source of carbon, harvested, washed and stored as described in Methods. Both types of cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5. The cells grown on mandelate were sonicated in 5ml amounts, and the cells grown on succinate in 25ml amounts as described in Methods (p.73). After both types of extract had been centrifuged, the succinate extract was used to dilute the mandelate extract 1/10, 1/20 or 1/100. Benzoylformate decarboxylase was assayed as described in Methods (p.80), using freshly prepared benzoylformate or benzoylformate which had been stored at -10°, except that the extract was not heated. Values for the specific activity of benzoylformate decarboxylase are expressed as percentages of the specific activity of benzoylformate decarboxylase in the undiluted extracts made from mandelate grown cells.

EXPERIMENT A.

Time of benzoylformate storage (days)	Benzoylformate decarboxylase specific activity			
	No dilution	1/10 dilution	1/20 dilution	1/100 dilution
0	100	8.0	4.1	0.55
14	98.9	7.7	3.9	0.36

EXPERIMENT B.

Time of benzoylformate storage (days)	Benzoylformate decarboxylase specific activity			
	No dilution	1/10 dilution	1/20 dilution	1/100 dilution
0	100	8.4	4.0	0.71
69	100	7.6	3.6	0.47

Table 10. The effect of heat inactivation on the activity of benzoylformate decarboxylase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate or 10mM-glutamate as sole source of carbon, harvested, washed, and stored as described in Methods. Both types of cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5, and sonicated in 5ml amounts as described in Methods (p.75). After both types of extract had been centrifuged, the glutamate extract was used to dilute the mandelate extract 1/40, or 1/10. All the extracts were inactivated at 37° for 60min. Benzoylformate decarboxylase and NADH oxidase were assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

EXPERIMENT A

Time of inactivation at 37°(min)	NADH oxidase specific activity	Benzoylformate decarboxylase specific activity	
		No dilution	1/40 dilution
0	91	252	0
60	4	230	7.2

EXPERIMENT B

Time of inactivation at 37°(min)	NADH oxidase specific activity	Benzoylformate decarboxylase specific activity	
		No dilution	1/10 dilution
0	18.7	197	13.7
60	1.8	192	19.7

benzoylformate was used in the enzyme assay. The complete assay procedure for measuring benzoylformate decarboxylase activity, based on the results described in the preceding Sections, is given in Methods.

1.1.3. Benzoate oxidase.

Of the enzymes of the mandelate pathway in bacterium NCIB 8250 only benzoate oxidase had not yet been detected in cell-free extracts. The only known cell-free assay for this enzyme was devised by Ichihara, Adachi, Hosokawa & Takeda (1962). These workers assayed the enzyme from P. fluorescens Mb-15, P. aeruginosa B-23 and Micrococcus ureae Et by measuring oxygen uptake in the Warburg apparatus. Since the manometric assay was the only known assay for this enzyme, it was decided to develop a similar assay for the benzoate oxidase of bacterium NCIB 8250; and then, if possible, adapt this assay so that the enzyme could be measured spectrophotometrically.

1.1.3.1. The assay of benzoate oxidase using the method of Ichihara et al.(1962).

The assay of Ichihara et al. (1962) involves measuring the oxygen uptake in the presence and absence of benzoate, and taking the difference between these two values as the benzoate oxidase activity. This procedure is necessary as there is a high endogenous uptake of oxygen. In their assay an NADPH generating system, which consisted of $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase, or an NADH generating system, which consisted of NAD^+, ethanol and alcohol dehydrogenase, Fe^{++} ions and GSH were required in addition to benzoate for enzyme activity.

Using the assay conditions of Ichihara et al. (1962) with

a cell-free extract of bacterium NCIB 8250 prepared by ultrasonic disruption, very low activity (0.5-2.5 units/mg protein) was obtained using the NADH generating system, and no activity was obtained using the NADPH generating system. This activity using the NADH generating system was abolished in the absence of Fe^{++} ions or ethanol; but was not altered by changing the concentration of GSH or by the presence of Fe^{++} ions, GSH or ethanol during the ultrasonic disruption of the cells. Ultrasonic disruption under nitrogen did not give any activity.

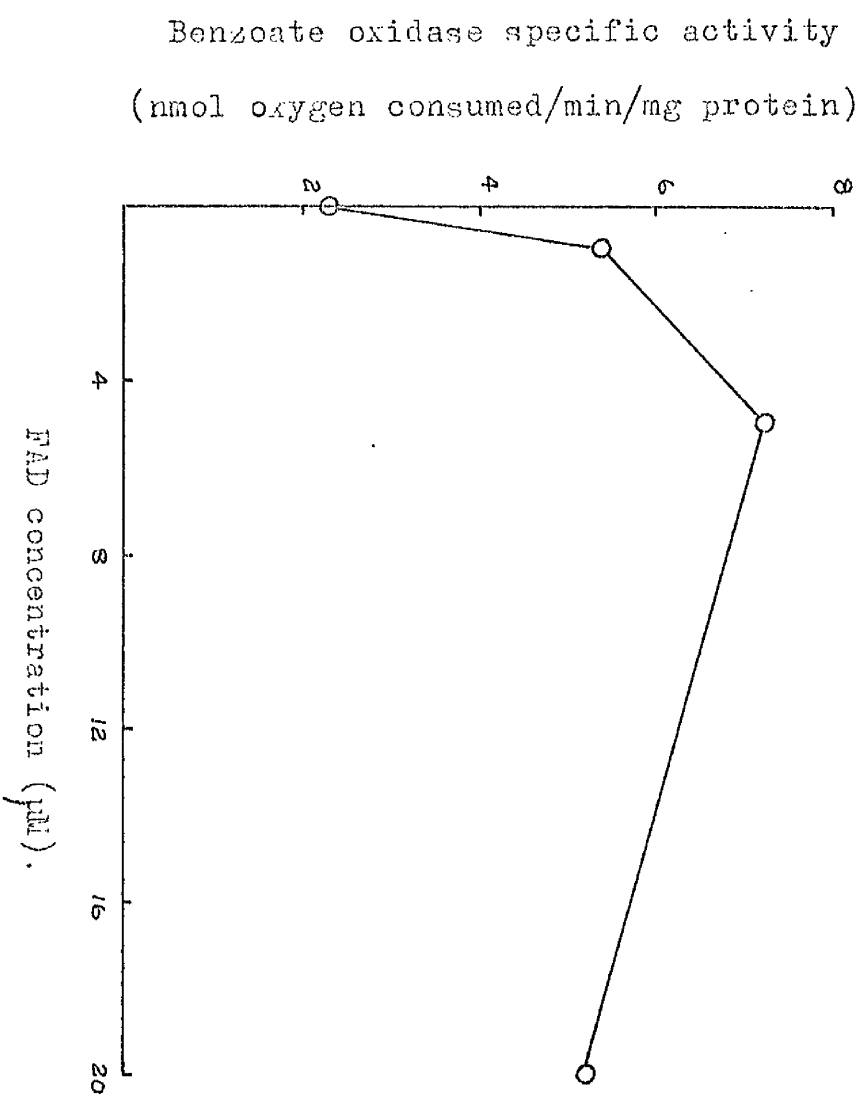
1.1.3.2. Effect of electron acceptors on the activity of benzoate oxidase.

Although benzoate oxidase is a dioxygenase, it requires NADH for activity as do several monooxygenases. Monooxygenases commonly require the presence of a flavin nucleotide cofactor for optimal activity, and so it was decided to see whether the enzyme activity could be increased by the presence of FAD. Fig.18 shows that the activity of the enzyme is three fold higher in the presence of optimal amounts of FAD (5μM).

Once it had been established that FAD was required for enzyme activity, optimal conditions for the enzyme assay were developed. By successive approximations it was found that optimal activity was obtained under the following conditions. The cells grown on benzoate were resuspended to 100mg wet wt./ml in 0.05M-Tris-HCl buffer pH 7.5, and a 4-6ml amount was

Fig.18. The effect of FAD on the activity of benzoate oxidase.

Bacterium NCIB 8250 was grown on 2mM-benzoate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Benzoate oxidase was measured manometrically as described in Methods (p.85) except that the reagents used were Tris-HCl buffer pH 7.5 (200μmol), $FeSO_4$ (1μmol), ethanol (50μmol), GSH (10μmol), NAD^+ (500nmol), alcohol dehydrogenase (16U), extract (10mg protein), and either benzoate (2μmol) or distilled water.


Benzoate oxidase specific activity
(nmol oxygen consumed/min/mg protein).
2
4
6
8
4
8
12
16
20
FAD concentration (μM).

pipetted into a 1 dram Trident container. The container was then placed in a chilled brass holder, and the brass holder was screwed onto the horn of the Soniprobe and lowered into an ice-water slurry. The cells were sonicated for ½min at a current of 2.5-3.0A. The extract was centrifuged at 4° for 25min at 12,000g, and the supernate was assayed for benzoate oxidase activity. The reaction mixture contained in addition to enzyme, Tris-HCl buffer pH 7.5 (200μmol), $FeSO_4$ (1μmol), ethanol (100μmol), NAD^+ (1μmol), FAD (10nmol), alcohol dehydrogenase (4U) and either benzoate (2μmol) or distilled water.

Fig.19 shows the time-course of oxygen uptake in the benzoate oxidase assay under optimal conditions. The difference between the oxygen uptake in the presence and absence of benzoate is taken as a measure of benzoate oxidase activity, which in this experiment corresponds to a specific activity of 18 units/mg protein. Table 11 shows that there is an absolute requirement for Fe^{++} ions and ethanol for enzyme activity, and in the absence of NAD^+, FAD or alcohol dehydrogenase the enzyme activity is reduced by at least 50%. GSH is not required for enzyme activity.

Even under these conditions, however, the activity of the enzyme in cell-free extracts is only 5-15% of that in whole cells. It was therefore decided to see whether the activity of the enzyme could be increased by the use of other electron acceptors. When the following compounds at a final

Fig.19. Time-course of oxygen uptake in the benzoate oxidase assay.

Bacterium NCIB 8250 was grown on 2mM-benzoate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Benzoate oxidase was measured manometrically by subtracting the initial rate of oxygen uptake in the absence of benzoate (⊗) from the rate in the presence of benzoate (○). Each Warburg flask contained, in a volume of 2ml, Tris-HCl buffer pH 7.5 (200μmol), $FeSO_4$ (1μmol), ethanol (100μmol), NAD^+ (1μmol), FAD (10nmol), alcohol dehydrogenase (4U), extract (4-5mg protein), and either benzoate (2μmol) or distilled water. The reaction was started by tipping the benzoate or distilled water into the main compartment from the side arm.

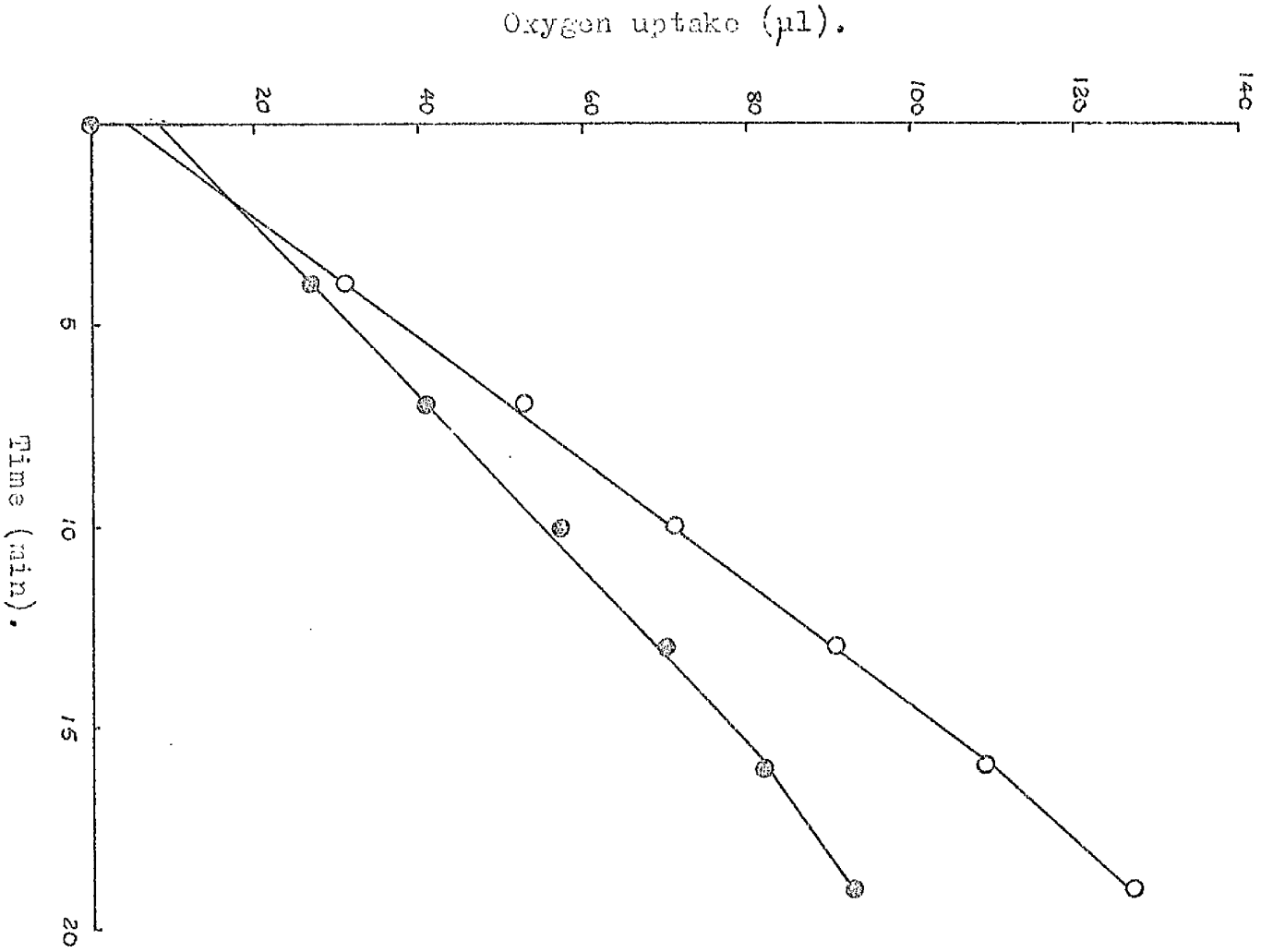

Oxygen uptake (µl).
20
40
60
80
100
120
140
5
10
15
20
Time (min).

Table 11. The effect of omitting reagents from the benzoate oxidase assay on the activity of the enzyme.

Bacterium NCIB 8250 was grown on 2mM-benzoate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Benzoate oxidase was measured manometrically as described in Methods (p.85) except that the reagents used were Tris-HCl buffer pH 7.5 (200μmol), $FeSO_4$ (1μmol), ethanol (50μmol), GSH (10μmol), NAD^+ (500nmol), FAD (10nmol), alcohol dehydrogenase (16U), extract (10mg protein) and either benzoate (2μmol) or distilled water.

Values for the specific activity of benzoate oxidase are expressed as percentages of the specific activity obtained in the presence of all the reaction components.

Reaction component omitted	Benzoate oxidase relative specific activity
None	100
GSH	107
NAD	39
Ethanol	0
FAD	39
Fe^{++} ions	0
Alcohol dehydrogenase	50

concentration of 5μM or 500μM were substituted for FAD in the reaction mixture, there was no or negligible benzoate oxidase activity: ascorbate; cytochrome c; 2,6-dichlorophenol-indophenol; 6,7-dimethyl-5,6,7,8-tetrahydropterine; FMN; methylene blue; potassium ferricyanide; riboflavin or tetrahydrofolic acid. The addition of ascorbate or cytochrome c, at the above concentrations, to the reaction mixture containing FAD did not increase the activity of the enzyme.

1.1.3.3. Effect of metal ions on the activity of benzoate oxidase.

The effects of Fe^{+++}, Mn^{++}, Mg^{++}, Cu^{++}, Zn^{++} or Ni^{++} ions, at a final concentration of 50μM, 500μM or 5mM, on the activity of benzoate oxidase were tested in the presence or absence of Fe^{++} ions. Only Fe^{+++} ions gave any activity in the absence of Fe^{++} ions, and in the presence of Fe^{++} ions, no ion gave an increase in enzyme activity.

1.1.3.4. Effect of other enzyme cofactors on the activity of benzoate oxidase.

Benzoate oxidase catalyses the decarboxylation of benzoate so there is the possibility that the enzyme requires the presence of cofactors which are frequently associated with decarboxylases. Consequently the effect of lipoate (5μM), CoA (5μM), ATP (500μM), thiamine pyrophosphate (5μM), ATP + Mg^{++} ions (500μM), CoA + ATP, CoA + Mg^{++} ions, CoA + ATP + Mg^{++} ions, Mg^{++} ions + ATP + CoA + thiamine pyrophosphate, lipoate + CoA + Mg^{++} ions + ATP or lipoate + CoA + Mg^{++} ions + ATP + thiamine pyrophosphate on the enzyme assay was tested. In no case was there an increase in enzyme activity observed.

1.1.3.5. Effect of altering the NADH oxidase activity on the activity of benzoate oxidase.

It is possible that endogenous NADH oxidase activity diverts electrons, which would normally have gone from NADH to the benzoate oxidase system, to the NADH oxidase system. Attempts were therefore made to by-pass or preferentially inhibit the NADH oxidase system. Many different approaches were tried; but in no case was an increase in benzoate oxidase activity observed, and in many cases the enzyme was in fact inactivated. These included: extraction and/or centrifugation at 27°; the direct formation of $FADH_2$ using D-alanine and D-amino acid oxidase, or glucose and glucose oxidase; delayed addition of benzoate; and the addition of cupferron (10μM, 100μM or 300μM), salicylaldehyde oxime (10μM or 300μM), sodium diethyldithiocarbamate (1μM or 30μM) or 2,2-bipyridyl (5μM, 100μM or 1mM).

1.1.3.6. Evidence for the metabolism of benzoate.

Evidence that the increase in oxygen uptake in the presence of benzoate is due to the disappearance of benzoate, and not to a non-specific stimulation of NADH oxidase, is: (1) the vessels which contained benzoate gave a positive Rothera test for ketones (presumably β-oxoadipate); and (2) extraction and measurement of the benzoate from the reaction mixture after 45min incubation showed that over half the benzoate had disappeared.

1.1.3.7. Conclusion.

Although it is possible to measure benzoate oxidase activity in cell-free extracts, the assay developed is unsuitable for routine measurement of enzyme activity. This is mainly because a large amount of protein (8mg) is required to measure induced levels of the enzyme, and consequently the measurement of the kinetics of benzoate oxidase induction would require an exorbitant amount of protein as the original uninduced enzyme levels are less than 1% of the induced level. As a result it was decided to stop working on this enzyme although a spectrophotometric assay had not been developed. P.J. Roach (Fewson *et al.*, 1970), however, developed a whole cell assay for the enzyme which measured benzoate disappearance, and this assay was used in the experiments on the kinetics of induction.

1.1.4. L-Mandelate dehydrogenase, benzyl alcohol dehydrogenase, catechol oxygenase and NADH oxidase.

These enzymes were extracted and assayed according to the methods of Kennedy & Fewson (1968b). Satisfactory reaction rates were obtained for each enzyme and as a result no alterations were made in these enzyme assays at this stage of the project. Except for L-mandelate dehydrogenase, the initial time-course of the reactions was linear. In the case of this enzyme, a varying rate was obtained over the initial period of the reaction owing to the endogenous reduction of the dye by the enzyme extract. This situation lasted for up to 45s, whereupon the reaction rate became linear. Consequently the rate over the first 45s was ignored when the activity of this enzyme was measured.

1.2. Measurement of the activity of the mandelate pathway enzymes in the one extract.

As described in the Introduction, it is advantageous to be able to measure all the mandelate pathway enzymes in the one extract. Thus, having worked out satisfactory methods for assaying all the enzymes (except benzoate oxidase), the next priority was to measure them in the one extract.

1.2.1. Choice of buffer and pH of extraction and assay.

Experiments of Kennedy (1967) showed that benzyl alcohol dehydrogenase activity could only be measured in extracts prepared in pH 8.5 buffer. Sodium pyrophosphate could not be used as the extraction buffer since Tables 6 and 7 show that this buffer inhibits benzoylformate decarboxylase. The extraction buffer, Tris-HCl, was therefore chosen because it buffered well in the range pH 8.0 to 9.0 (pKa = 8.3).

The extraction of the mandelate pathway enzymes in 0.08M-Tris-HCl buffer pH 8.0, 8.5 or 9.0 is shown in Table 12. In this experiment Tris-HCl buffer was used to assay the two benzaldehyde dehydrogenases, benzoylformate decarboxylase and benzyl alcohol dehydrogenase rather than the buffers used by Kennedy & Fewson (1968b) so as to maintain the homology between the assay and extraction buffer. Phosphate buffer pH 7.0 was used to assay L-mandelate dehydrogenase, catechol oxygenase and NADH oxidase as Kennedy (1967) and Fewson (unpublished results) had obtained optimal activity of these enzymes at pH 7.0, a pH at which Tris-HCl does not buffer well.

Table 12. The effect of pH of extraction on the activity of the enzymes of the mandelate pathway.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.08M-Tris-HCl buffer pH 8.5, pH 9.0 or pH 9.5 to 50mg wet wt./ml, and sonicated in 12ml amounts as described in Methods (p.73). The extracts were centrifuged at 12,000g for 25 min at 4°, and the supernates were assayed as described in Methods with the exception of the assay buffer used which is indicated in the Table. After the reaction was complete the actual pH of the reaction mixture was determined by means of microelectrodes. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Extraction buffer	Assay buffer	Enzyme specific activity	Actual pH of reaction mixture
L-Mandelate dehydrogenase	Tris pH 8.0	Phosphate pH 7.0	279	7.1
	Tris pH 8.5		298	7.0
	Tris pH 9.0		326	7.0
Benzoylformate decarboxylase	Tris pH 8.0	Tris pH 9.0	366	8.7
	Tris pH 8.5		382	8.7
	Tris pH 9.0		392	8.7
Stable benzaldehyde dehydrogenase	Tris pH 8.0	Tris pH 9.5	98	9.2
	Tris pH 8.5		100	9.3
	Tris pH 9.0		89	9.3
Labile benzaldehyde dehydrogenase	Tris pH 8.0	Tris pH 9.5	2	9.2
	Tris pH 8.5		14	9.3
	Tris pH 9.0		31	9.3
Benzyl alcohol dehydrogenase	Tris pH 8.0	Tris pH 9.0	17	8.7
	Tris pH 8.5		11	8.8
	Tris pH 9.0		6	8.8
Catechol oxygenase	Tris pH 8.0	Phosphate pH 7.0	675	7.1
	Tris pH 8.5		735	7.1
	Tris pH 9.0		764	7.1
NADH oxidase	Tris pH 8.0	Phosphate pH 7.0	135	7.1
	Tris pH 8.5		117	7.1
	Tris pH 9.0		107	7.1

The levels of L-mandelate dehydrogenase, benzoylformate decarboxylase, catechol oxygenase and NADH oxidase present in the three extracts are comparable with the levels obtained by Kennedy & Fewson (1968b), and represent a satisfactory amount of enzyme extracted from the cells. The activity of benzyl alcohol dehydrogenase, however, is extremely low (approximately 1/5 of the amount found by Kennedy & Fewson), and decreases with increasing pH of extraction. The labile benzaldehyde dehydrogenase activity is also very low especially at the lower pHs of extraction. This is because the half-life of the labile enzyme is too high for its complete heat inactivation in 2h at 37^{o} (Table 4), and thus some of the measured activity of the stable enzyme is really that of the labile enzyme.

Since the activity of benzyl alcohol dehydrogenase was low when it was extracted and assayed in Tris buffer, a comparison of its activity when extracted and assayed in different buffers was made, and this is shown in Table 13. The activity of benzyl alcohol dehydrogenase is higher when the enzyme is extracted in sodium pyrophosphate rather than Tris-HCl buffer; but the use of Tris-HCl buffer pH 8.5 as the extraction buffer and sodium pyrophosphate buffer pH 9.0 as the assay buffer produces an acceptable level of enzyme activity.

In this Section benzaldehyde dehydrogenase was extracted in Tris-HCl buffer as preliminary experiments had shown that

Table 13. The effect of pH and buffer of extraction and assay on the activity of benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.08M-Tris-HCl buffer pH 8.5 or pH 9.5, or 0.08M-sodium pyrophosphate buffer pH 8.5 to 50mg wet wt./ml, and sonicated in 12ml amounts as described in Methods (p.73). The extracts were centrifuged at 12,000g for 25min at 4°, and the supernates were assayed as described in Methods with the exception of the assay buffer used which is indicated in the Table. After the reaction was complete the actual pH of the reaction mixture was determined by means of microelectrodes. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

*After extraction the pH 8.5 extract was titrated with the amount of 0.2N-NaOH required to alter the pH of a 0.08M-Tris-HCl buffer containing no protein from 8.5 to 9.5 as described in Text p.133.

Enzyme	Extraction buffer	Assay buffer	Enzyme specific activity	Actual pH of reaction mixture
Benzyl alcohol dehydrogenase	Sodium pyrophosphate pH 8.5	Pyrophosphate pH 8.5	44	8.5
		Pyrophosphate pH 9.0	45	9.0
		Pyrophosphate pH 9.5	41	9.4
		Tris pH 8.5	28	8.5
		Tris pH 9.0	55	8.9
		Tris pH 9.5	42	9.5
	Tris-HCl pH 8.5	Pyrophosphate pH 8.5	28	8.5
		Pyrophosphate pH 9.0	37	9.0
		Pyrophosphate pH 9.5	14	9.4
		Tris pH 8.5	13	8.5
		Tris pH 9.0	13	8.8
		Tris pH 9.5	8	9.2
	Tris-HCl pH 9.5	Pyrophosphate pH 8.5	5	8.6
		Pyrophosphate pH 9.0	7	9.0
Stable benzaldehyde dehydrogenase	Tris-HCl pH 8.5	Tris-HCl pH 9.5	105	9.4
	Tris-HCl pH 8.5*		75	9.4
	Tris-HCl pH 9.5		82	9.4
Labile benzaldehyde dehydrogenase	Tris-HCl pH 8.5	Tris-HCl pH 9.5	25	9.4
	Tris-HCl pH 8.5*		39	9.4
	Tris-HCl pH 9.5		43	9.4

although the total benzaldehyde dehydrogenase activity was greater than 80 units/mg protein, that of the labile benzaldehyde dehydrogenase did not exceed 50 units/mg protein. Thus only negligible activity of the labile enzyme remained after 2h heating at 37°. As extraction of the labile benzaldehyde dehydrogenase in pH 8.5 buffer was too low for its complete inactivation in 2h at 37°, the pH of part of the extract was raised to 9.5 with 0.2N-NaOH. The benzaldehyde dehydrogenase activity of the treated extract was then compared with that of untreated extracts. Table 13 shows that although the activity of the two enzymes is slightly lower in the alkali treated extract, the remaining activity still represents an acceptable percentage of the activity in the pH 9.5 extract.

Since none of the enzymes is extracted at the pH at which it is assayed, it is essential that the buffering capacity of the assay buffer is adequate to maintain the actual pH of the reaction mixture at a constant value. It was found, however, that the addition of 500µl of protein extracted in 0.08M-Tris buffer pH 8.5 to the reaction mixture altered the pH of the reaction mixture by up to 0.2 units. Consequently the concentration of the extraction buffer was halved. This was sufficient to maintain the pH of the reaction mixture at a constant value, had no adverse effect on any of the enzyme activities, and actually increased the activity of benzyl alcohol dehydrogenase. In subsequent experiments, therefore, all the enzymes were extracted in 0.04M-Tris-HCl buffer pH 8.5.

As a consequence of lowering the concentration of the extraction buffer from 0.08M to 0.04M, the pH of the part of the extract used for the benzaldehyde dehydrogenase assay was raised to pH 10.3 rather than 9.5 with concentrated alkali. This ensured complete inactivation of the labile enzyme on heating for 2h at 37°.

1.2.2. Choice of sonication conditions.

A prime necessity in measuring enzyme activity of cells is to have a reproducible method of extracting the enzymes from the whole cells so that comparisons of enzyme activity can be made. This was provided by ultrasonic disruption of the cells. In the initial experiments cells were resuspended to 40 or 50mg wet wt./ml, and a 12ml amount was pipetted into an 8 dram Trident container. The Trident container was then placed in an ice-water slurry, the Dawe Soniprobe was lowered 7mm into the suspension, and the cells were sonicated for 8min at a current of 5A. This procedure has two disadvantages: (1) it is very wasteful because a large volume of cells has to be grown in order to obtain the necessary yield of cells; and (2) the sonication is not very reproducible as it is almost impossible to place the soniprobe by eye at the same depth in the cell suspension in two successive experiments. These disadvantages, however, were overcome by a suitable choice of sonication conditions which are described below.

The smallest volume in which the basal levels of all the mandelate pathway enzymes can be measured is 3.0ml. This

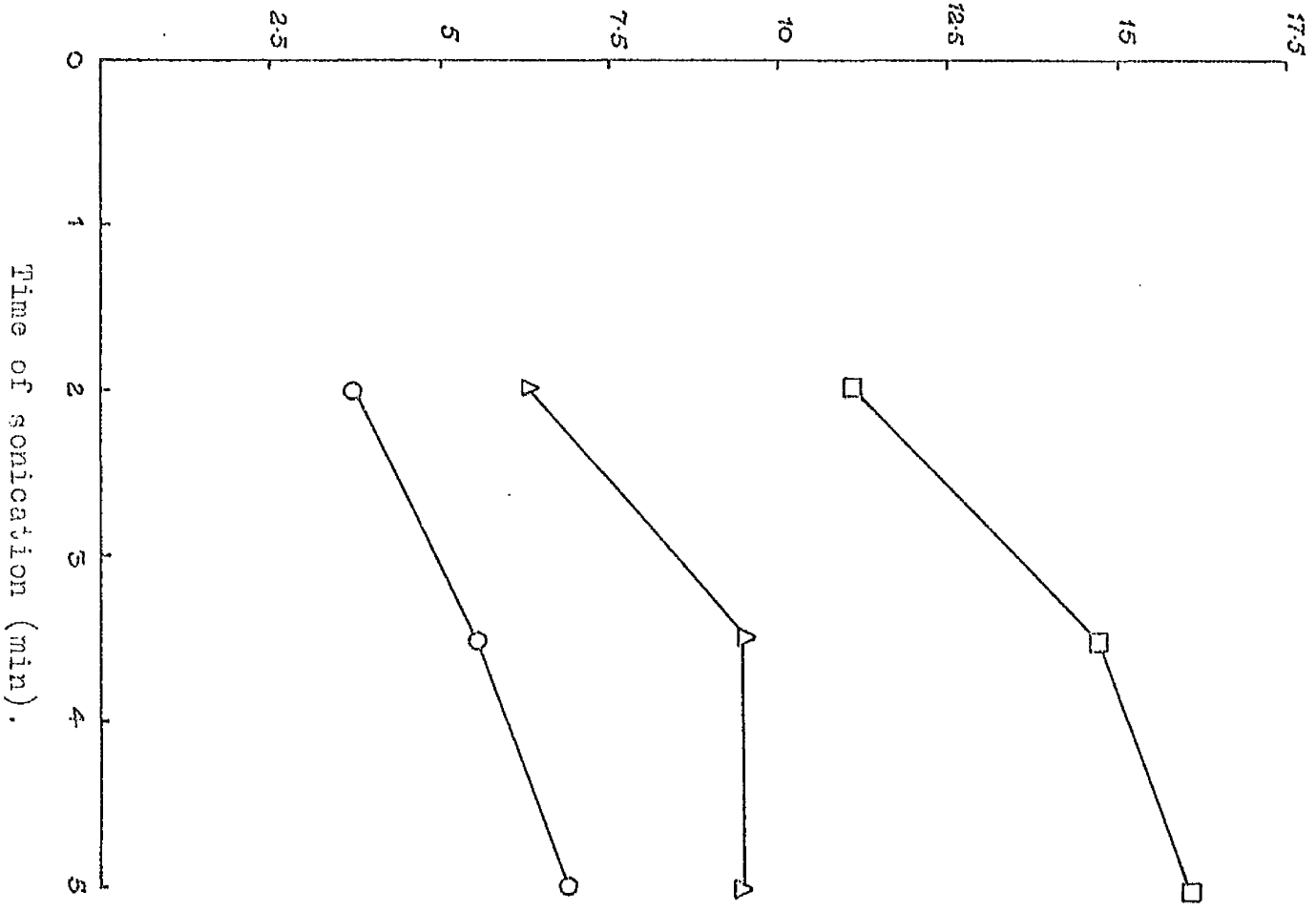
requires the sonication of 5.0ml amounts of suspensions in order to be able to recover 3ml of supernate after centrifugation. 5ml amounts were sonicated in 1 dram Trident containers which were placed in a chilled brass holder. The brass holder was screwed onto the horn of the Soniprobe and was lowered into an ice-water slurry. Screwing the brass holder onto the horn of the Soniprobe allows reproducible positioning. Thus the two disadvantages of the initial sonication procedure were overcome.

1.2.2.1. Effect of cell concentration and probe depth on the specific activity of the enzymes released by ultrasonic disruption.

Once the method of sonication had been decided, conditions which released optimal amounts of the enzymes had to be determined. A current of 2.5A was used to sonicate the 5ml amounts as this was the maximum current which could be obtained without frothing. Fig.20 shows that the specific activity of benzyl alcohol dehydrogenase released by ultrasonic disruption decreases with decreasing cell concentration of the resuspended cells. The specific activity released by sonicating a 12.5mg wet wt./ml cell suspension is only approximately 40% of the specific activity released by sonicating a 50mg wet wt./ml cell suspension. More dense cell suspensions were not sonicated since their subsequent use would have been impracticable. The reduction in specific activity released by sonicating a 12.5mg wet wt./ml cell suspension compared with a 50mg wet wt./ml cell suspension was

Fig. 20. Effect of cell concentration of the resuspended cells on the activity of benzyl alcohol dehydrogenase released by ultrasonic disruption of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate-salts medium, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 12.5 (O), 25 (△), or 50 (□) mg wet wt./ml and disrupted with the Dawe Soniprobe for various times. The extracts were centrifuged at 12,000g for 25 min at 4°, and the supernatants were assayed as described in Methods.


Specific activity of benzyl alcohol dehydrogenase
(nmol substrate converted/min/mg protein).
2.5
5
7.5
10
12.5
15
17.5
0
1
2
3
4
5
Time of sonication (min).

not so great for the other mandelate pathway enzymes with the exception of the stable benzaldehyde dehydrogenase. The specific activity of L-mandelate dehydrogenase, benzoylformate decarboxylase, the stable benzaldehyde dehydrogenase, the labile benzaldehyde dehydrogenase, catechol oxygenase and NADH oxidase was lowered by 0%, 10%, 70%, 30%, 10% and 25% respectively.

Fig.21 shows that about a 10% increase in the specific activity of L-mandelate dehydrogenase is achieved by using a probe depth of 1.5cm as opposed to 0.75cm. Although the graphs obtained for the other mandelate pathway enzymes are not shown, the specific activity of these enzymes was likewise increased by approximately 10% by using the deeper probe.

1.2.2.2. Effect of time of sonication on the release of enzyme specific activity.

Once the probe depth and cell concentration of the resuspended cells had been chosen, the time of sonication which gave satisfactory amounts of all the mandelate pathway enzymes had to be determined. Figs.22 to 24 show the time-course of release of the mandelate pathway enzymes. The release of L-mandelate dehydrogenase, NADH oxidase and benzyl alcohol dehydrogenase specific activity follows an approximately hyperbolic function; that of the two benzaldehyde dehydrogenases increases to a maximum and then declines; that of catechol oxygenase slowly increases; and that of benzoylformate decarboxylase remains constant and then declines.

Fig. 21. Effect of the Dawe Soniprobe depth on the activity of L-mandelate dehydrogenase released by ultrasonic disruption of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate-salts medium, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 50mg wet wt./ml, and disrupted with the Dawe Soniprobe for various times at a probe depth of 1.5cm (○) or 0.75cm (△). The extracts were centrifuged at 12,000g for 25 min at 4°, and the supernatants were assayed as described in Methods.

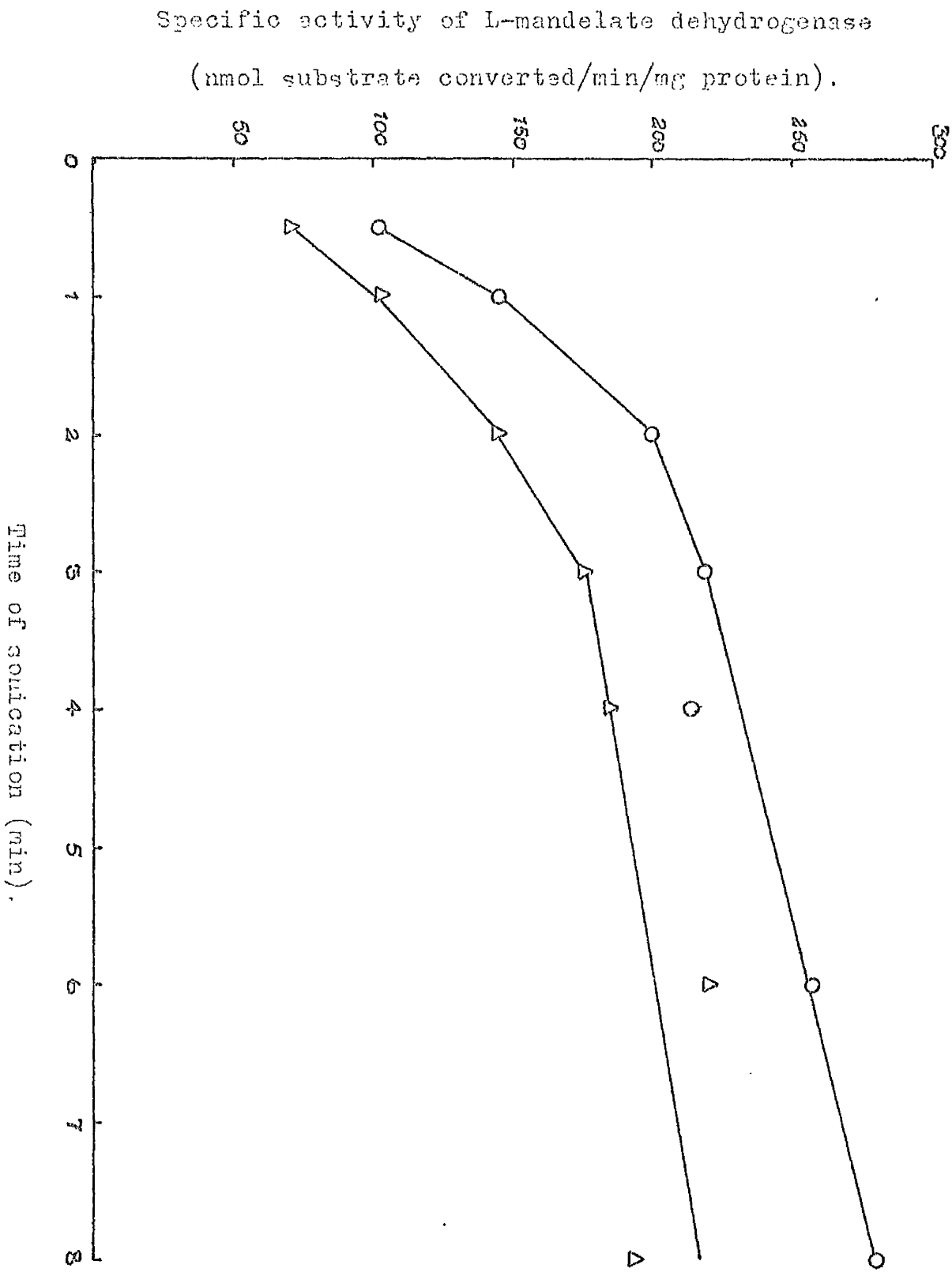

Specific activity of L-mandelate dehydrogenase
(nmol substrate converted/min/mg protein).
50
100
150
200
250
300
0
1
2
3
4
5
6
7
8
Time of sonication (min).

Fig.22. Enzyme activity of L-mandelate dehydrogenase and NADH oxidase released by ultrasonic disruption of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate-salts medium, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 50mg wet wt./ml, and disrupted with the Dawe Soniprobe for various times. The extracts were centrifuged at 12,000g for 25 min at 4^{o}, and the supernatants were assayed as described in Methods.

○ - L-Mandelate dehydrogenase.

△ - NADH oxidase.

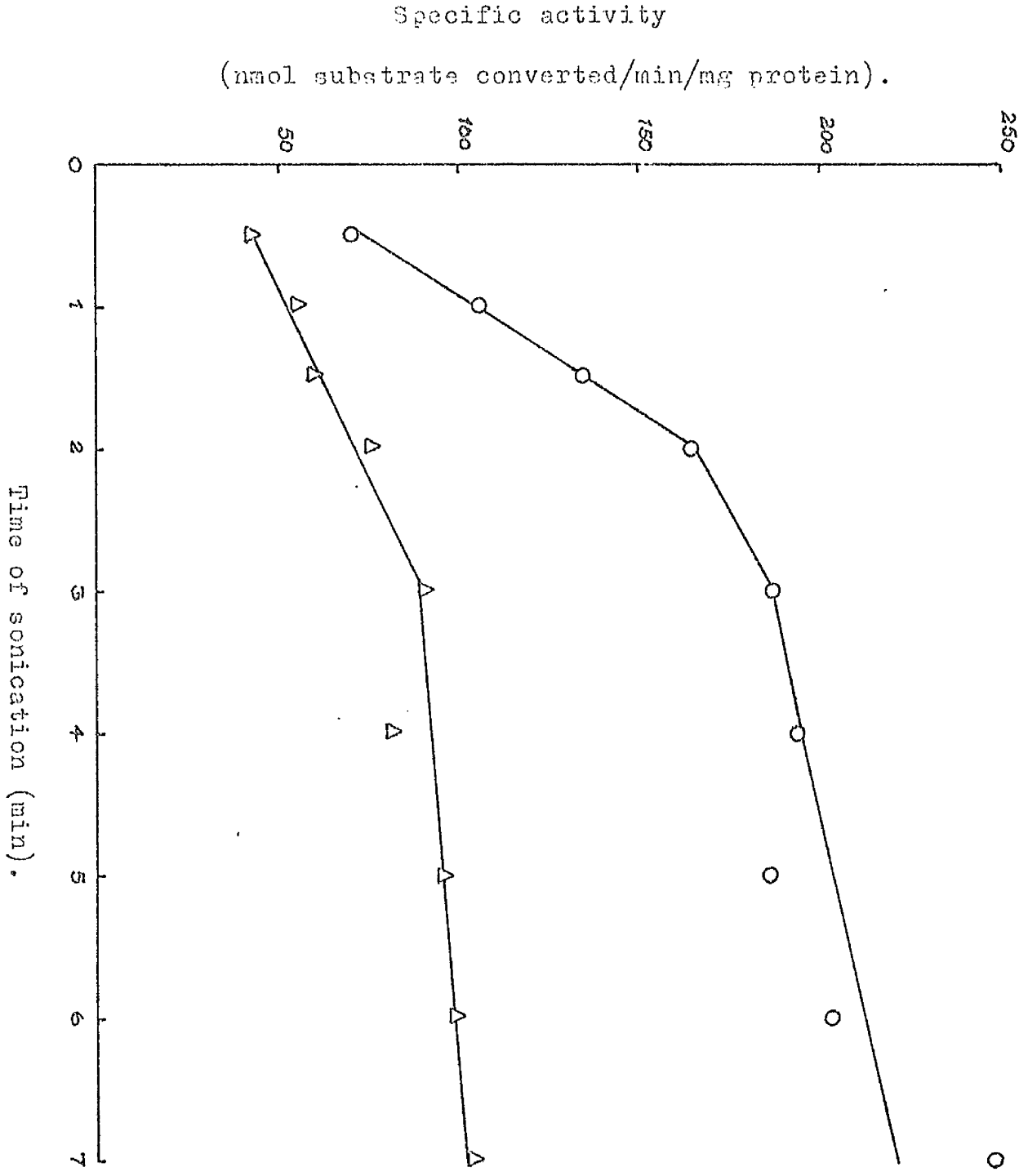

Specific activity
(nmol substrate converted/min/mg protein).
50
100
150
200
250
0
1
2
3
4
5
6
7
Time of sonication (min).

Fig.23. Enzyme activity of benzoylformate decarboxylase and catechol oxygenase released by ultrasonic disruption of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate-salts medium, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 50mg wet wt./ml and disrupted with the Dawe Soniprobe for various times. The extracts were centrifuged at 12,000g for 25 min at 4°, and the supernatants were assayed as described in Methods.

O - Benzoylformate decarboxylase.

△ - Catechol oxygenase.

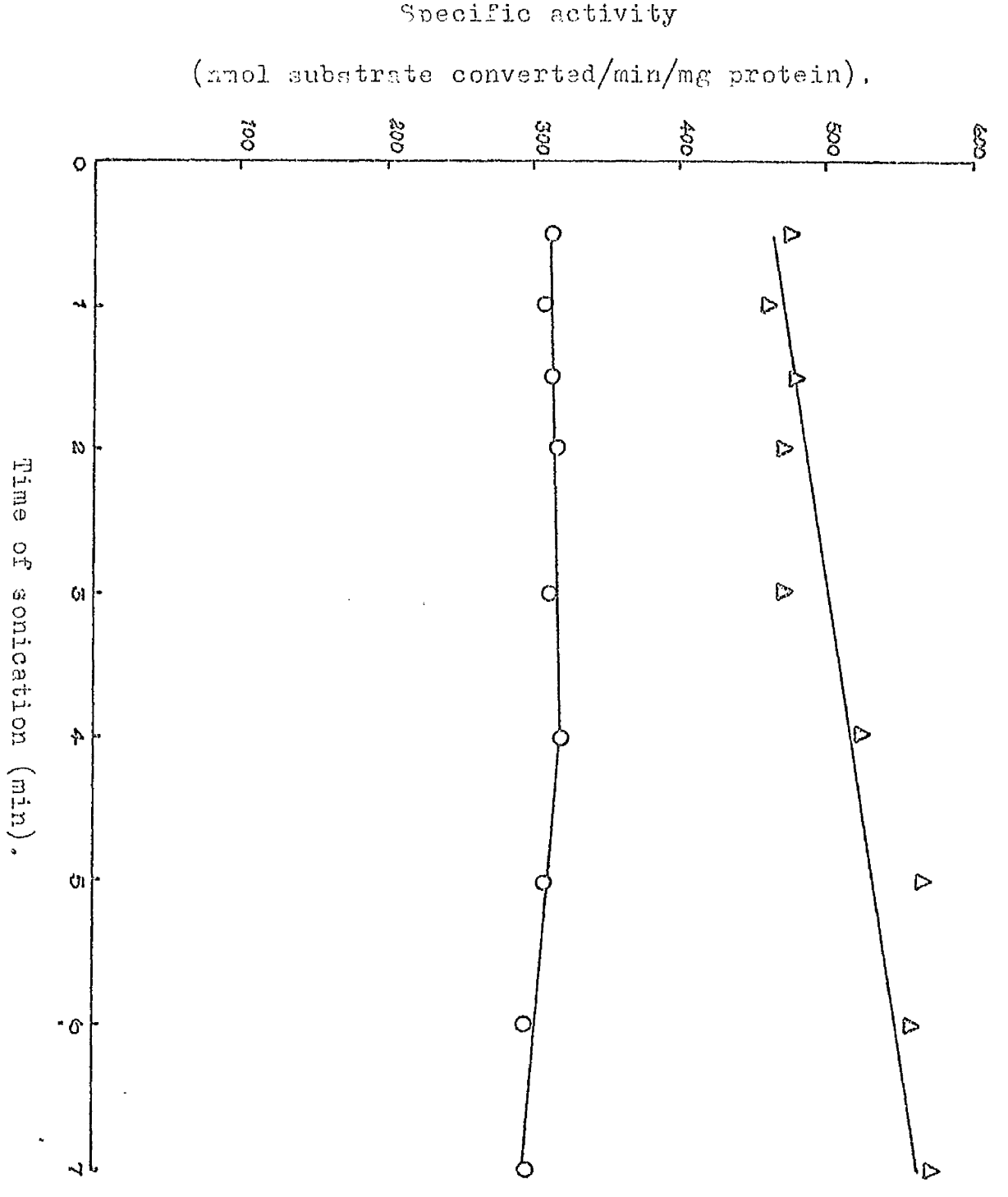

Specific activity
(nmol substrate converted/min/mg protein).
100
200
300
400
500
600
0
1
2
3
4
5
6
7
Time of sonication (min).

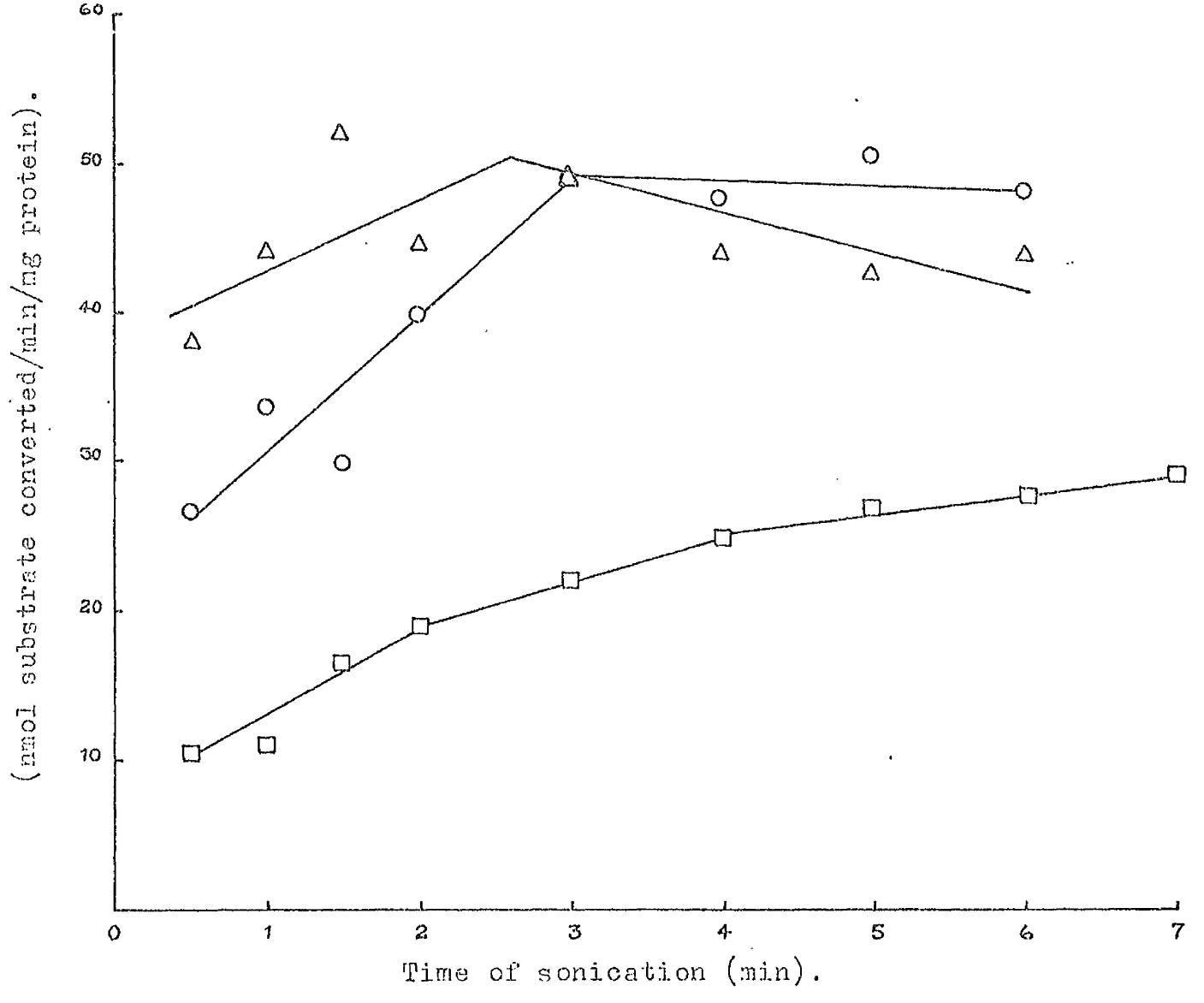
Fig.24. Enzyme activity of the two benzaldehyde dehydrogenases and benzyl alcohol dehydrogenase released by ultrasonic disruption of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate-salts medium, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 50mg wet wt./ml and disrupted with the Dawe Soniprobe for various times. The extracts were centrifuged at 12,000g for 25 min at 4^{o}, and the supernatants were assayed as described in Methods.

○ - Stable benzaldehyde dehydrogenase.

△ - Labile benzaldehyde dehydrogenase.

□ - Benzyl alcohol dehydrogenase.


60
50
40
30
20
10
0
1
2
3
4
5
6
7
(nmol substrate converted/min/mg protein).
Time of sonication (min).

An examination of Figs.22 to 24 shows that sonication for 3½min releases almost optimal amounts of all the enzymes.

From a consideration of all the results in this Section, the sonication conditions chosen for the extraction of the mandelate pathway enzymes in subsequent experiments were: 5ml of a 50mg wet wt./ml cell suspension sonicated for 3½min in a 1 dram Trident container placed in a chilled brass holder at a probe depth of 1.5cm using a current of 2.5A.

1.2.3. Effect of varying the protein concentration in the reaction mixture on the activity of the mandelate pathway enzymes.

As the levels of the mandelate pathway enzymes vary over 200 fold between induced and non-induced cells, different amounts of bacterial protein had to be added to the reaction mixture in order to obtain measurable rates of enzyme activity. The enzymes were added in 5µl-500µl amounts by means of Eppendorf pipettes. The advantage of using these pipettes is that the enzyme never has to be diluted in order to measure its activity, thereby cutting out errors caused by the possible loss of enzyme activity on dilution. The initial linear portion of the time-course was taken as a measure of enzyme activity. With the exception of L-mandelate dehydrogenase, the initial rate of the reaction was linear for the four minute duration of the experiment except when very large amounts of enzyme were used; and when this occurred, the initial rate was linear for at least a minute, which represented a sufficiently long period of time in which to

measure enzyme activity. In the case of L-mandelate dehydrogenase, the rate over the first 45s was ignored when measuring enzyme activity.

Because different amounts of enzyme were added to the assay mixture, it was essential that the enzyme activity was proportional to protein concentration in the reaction mixture in order to make meaningful comparisons between different enzyme levels. Figs.25 to 31 show that for all the mandelate pathway enzymes, enzyme activity is proportional to protein concentration. Volumes of extracts ranged from 5µl to 500µl.

1.2.4. Effect of dilution of the mandelate pathway enzymes with protein from non-induced cells.

In experiments designed to measure the initial kinetics of enzyme induction, low levels of enzyme specific activity had to be assayed. It was therefore decided to see whether diluting the mandelate pathway enzymes with protein from non-induced cells interfered with the assays. Two approaches were used. The first involved diluting the cells grown on L-mandelate or benzyl alcohol 1/10 with cells grown on succinate, and then sonicating the mixtures; and the second involved diluting mandelate or benzyl alcohol extracts 1/10 with a succinate extract after sonication.

The results of this experiment are shown in Table 14. Protein from succinate cells does not appear to interfere with the assay of low amounts of L-mandelate dehydrogenase, benzoylformate decarboxylase or catechol oxygenase as the

Fig.25. The effect of varying the protein concentration in the reaction mixture on the activity of L-mandelate dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture, and the activity of L-mandelate dehydrogenase was measured as described in Methods (p.79).

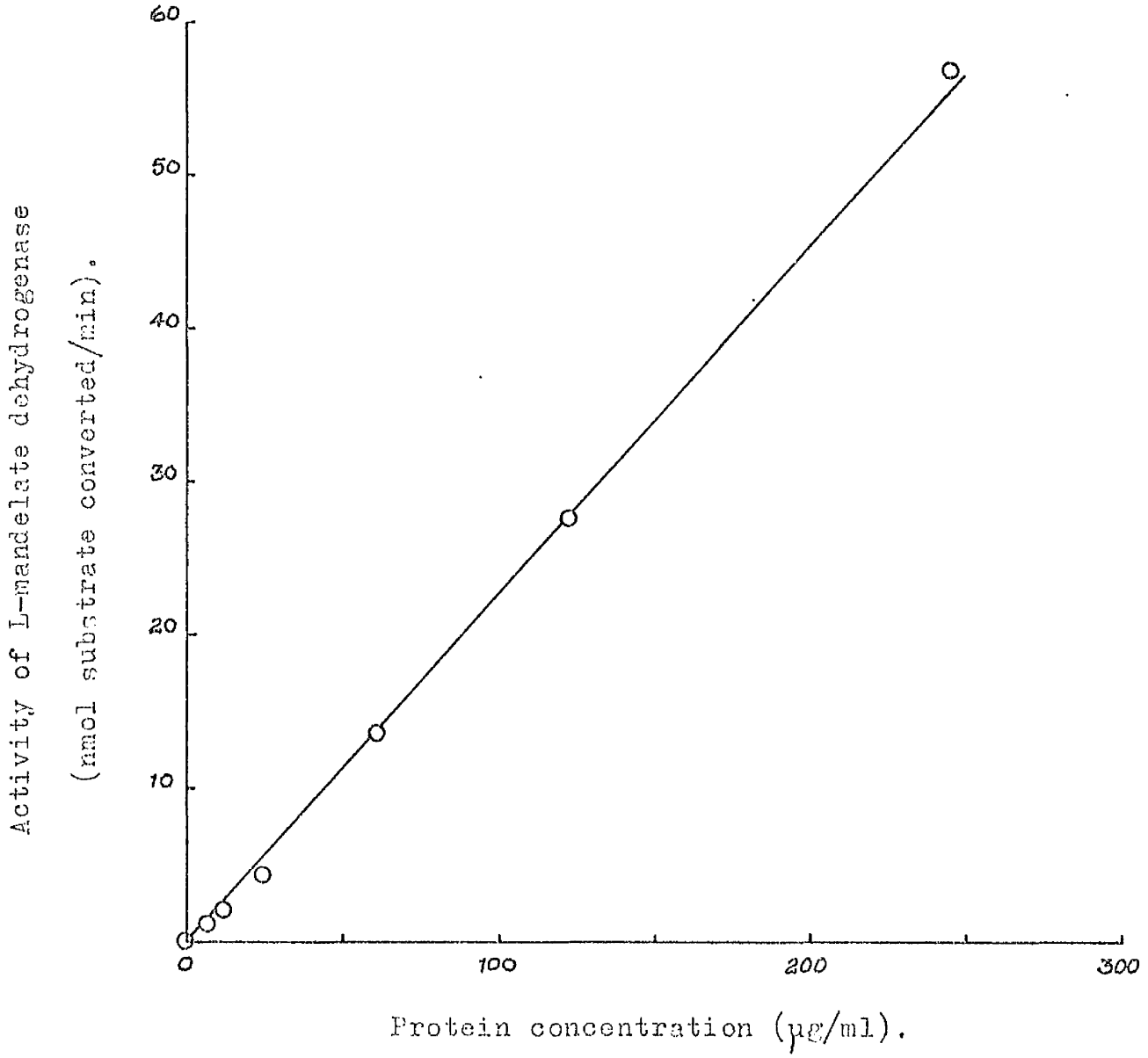

Activity of L-mandelate dehydrogenase
(nmol substrate converted/min).
60
50
40
30
20
10
0
100
200
300
Protein concentration (µg/ml).

Fig. 26. The effect of varying the protein concentration in the reaction mixture on the activity of benzoylformate decarboxylase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture and the activity of benzoylformate decarboxylase was measured as described in Methods (p.80).

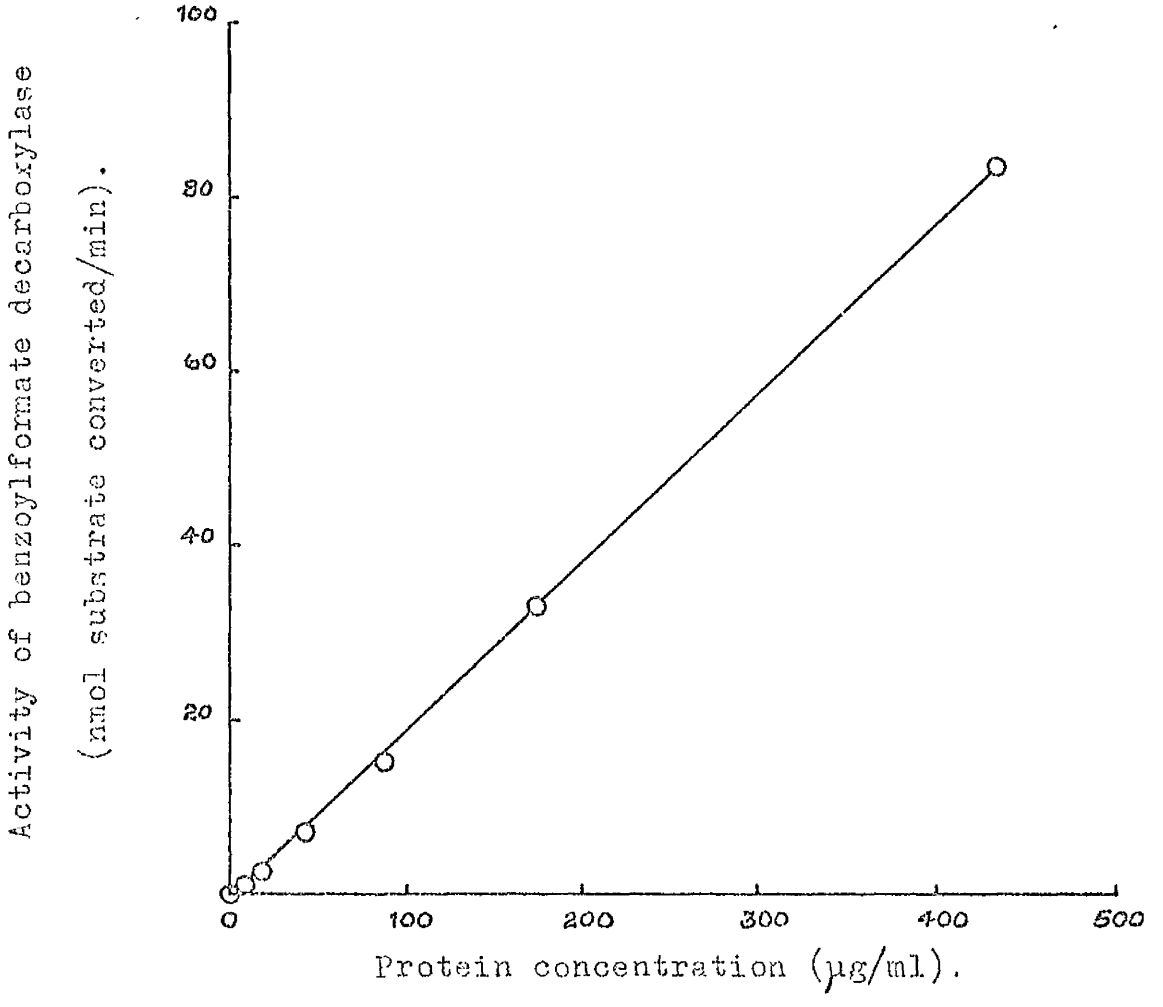

Activity of benzoylformate decarboxylase
(nmol substrate converted/min).
100
80
60
40
20
0
100
200
300
400
500
Protein concentration (µg/ml).

Fig.27. The effect of varying the protein concentration in the reaction mixture on the activity of the stable benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture, and the activity of the stable benzaldehyde dehydrogenase was measured as described in Methods (p.81).

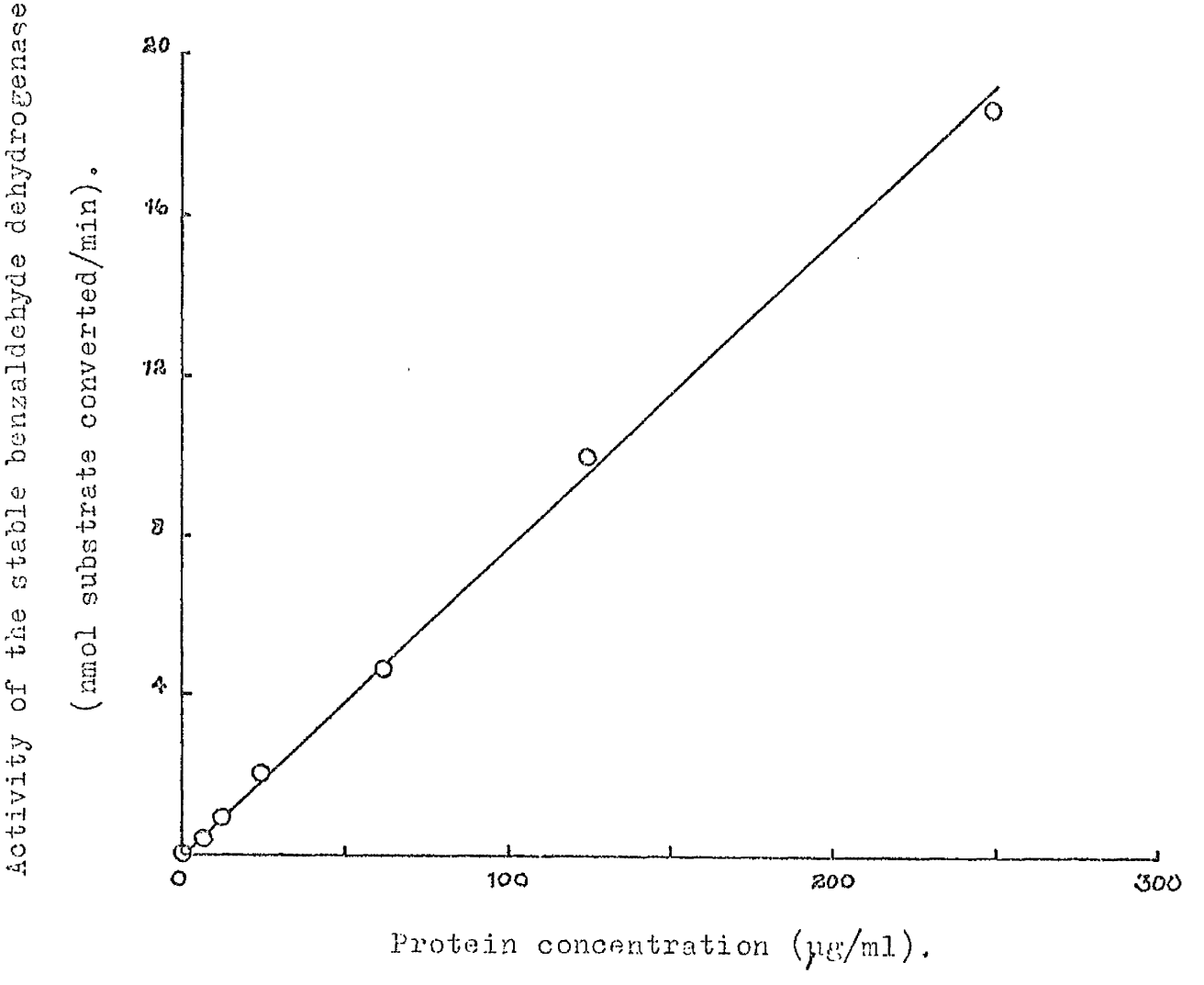

Activity of the stable benzaldehyde dehydrogenase
(nmol substrate converted/min).
20
16
12
8
4
0
100
200
300
Protein concentration (µg/ml).

Fig.28. The effect of varying the protein concentration in the reaction mixture on the activity of the labile benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture, and the activity of the labile benzaldehyde dehydrogenase was measured as described in Methods (p.81).

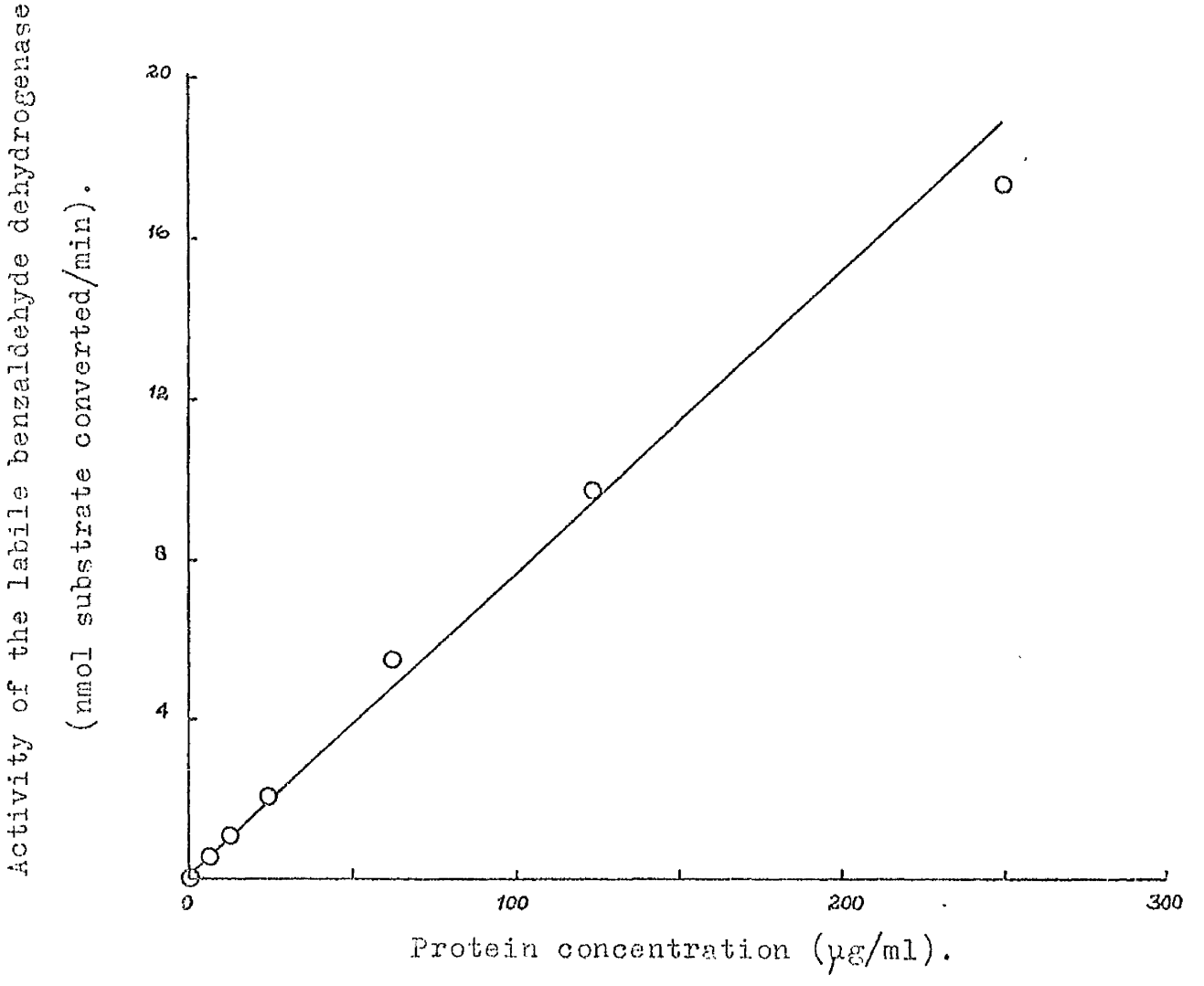

Activity of the labile benzaldehyde dehydrogenase
(nmol substrate converted/min).
20
16
12
8
4
0
100
200
300
Protein concentration (µg/ml).

Fig.29. The effect of varying the protein concentration in the reaction mixture on the activity of benzyl alcohol dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture, and the activity of benzyl alcohol dehydrogenase was measured as described in Methods (p.83).

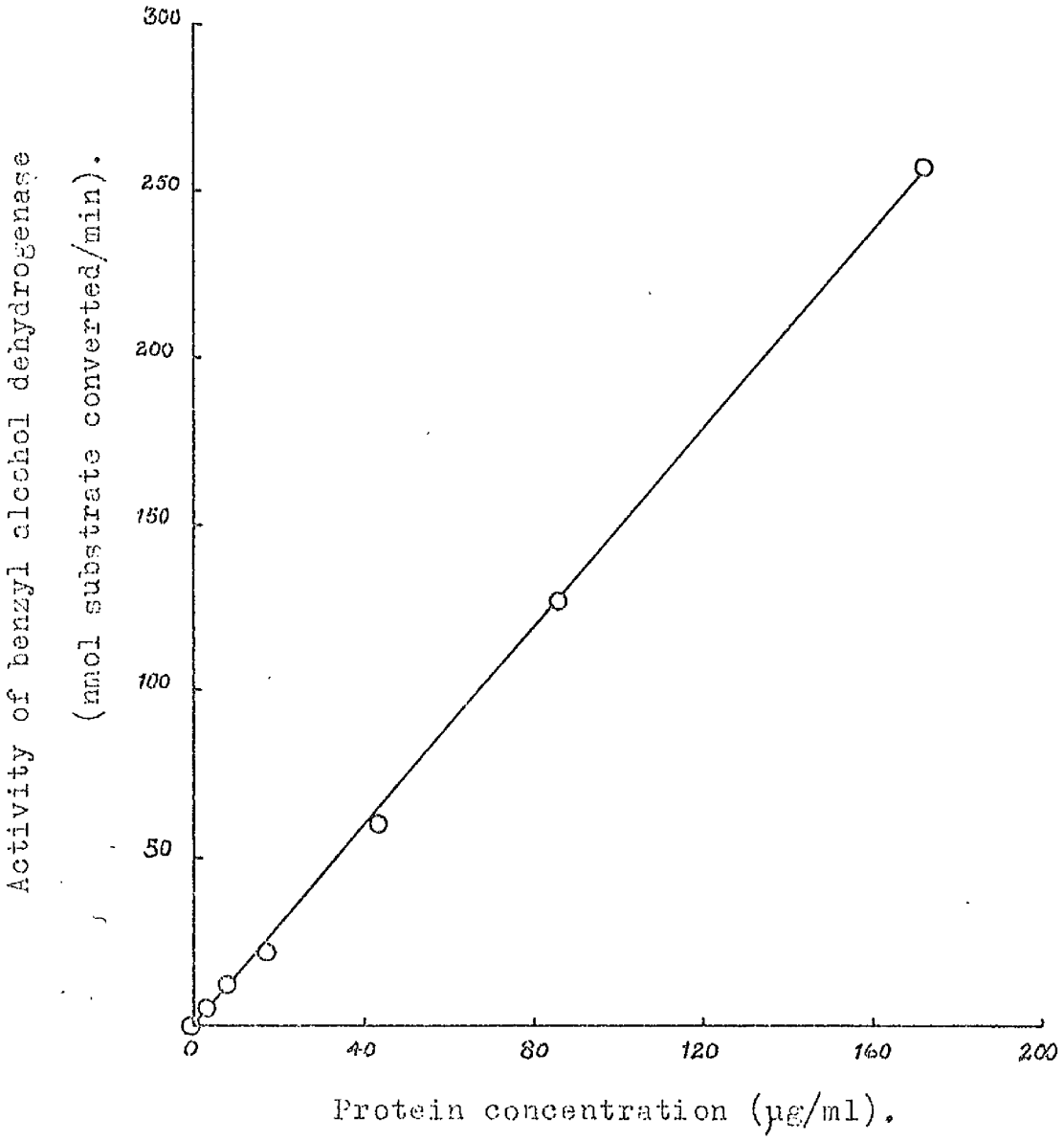

Activity of benzyl alcohol dehydrogenase
(nmol substrate converted/min).
300
250
200
150
100
50
0
40
80
120
160
200
Protein concentration (µg/ml).

Fig.30. The effect of varying the protein concentration in the reaction mixture on the activity of catechol oxygenase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture, and the activity of catechol oxygenase was measured as described in Methods (p.84).

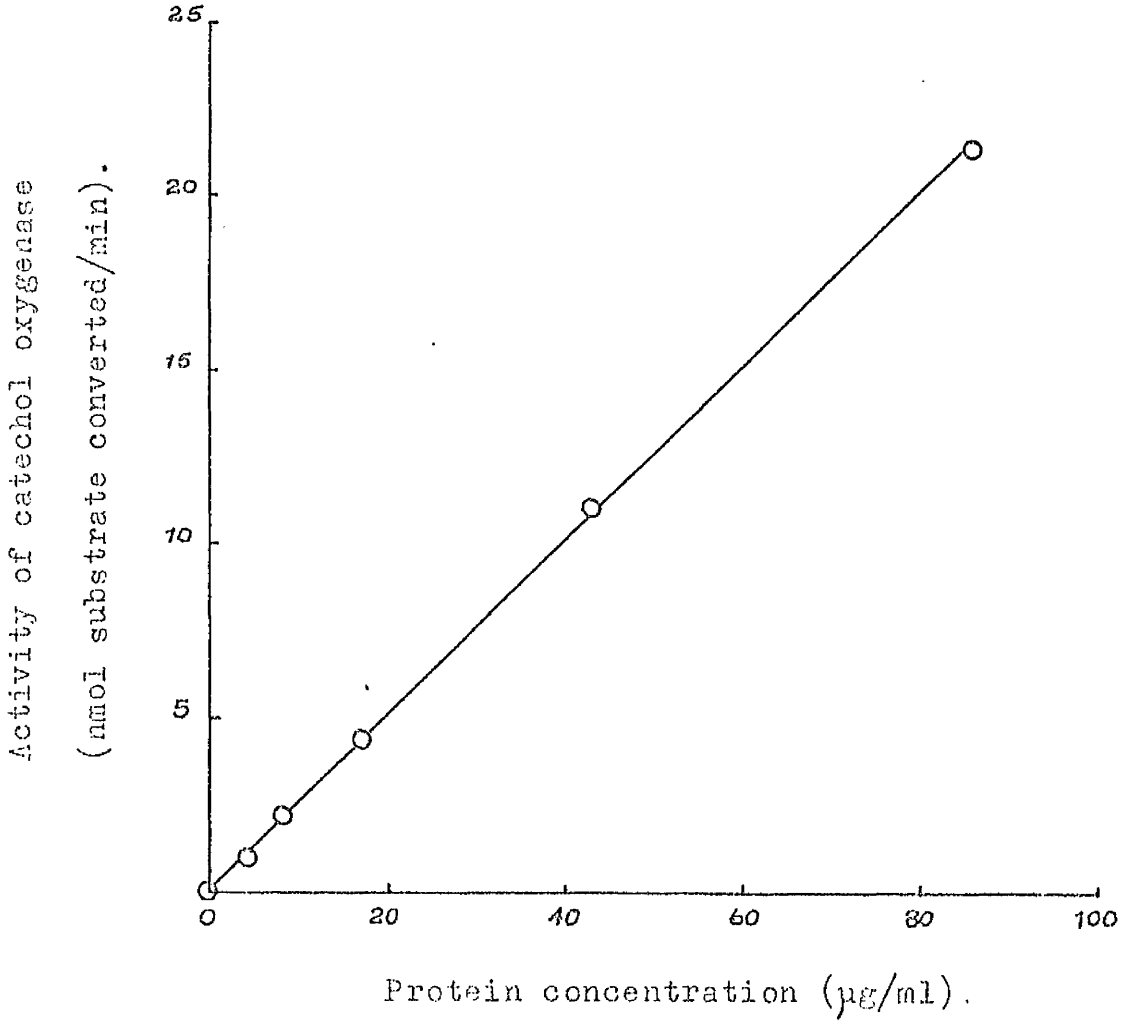

Activity of catechol oxygenase
(nmol substrate converted/min).
25
20
15
10
5
0
20
40
60
80
100
Protein concentration (µg/ml).

Fig.31. The effect of varying the protein concentration in the reaction mixture on the activity of NADH oxidase.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. Different amounts of protein were added to the reaction mixture, and the activity of NADH oxidase was measured as described in Methods (p.84).

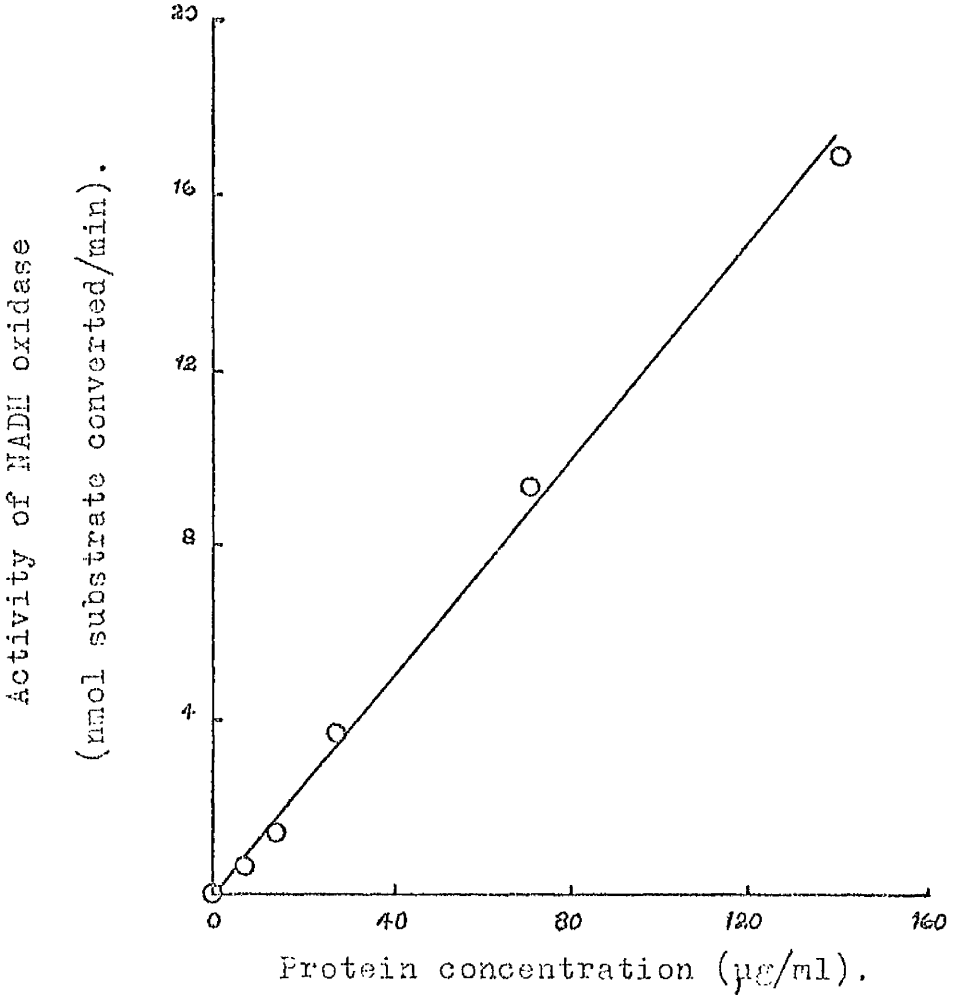

Activity of NADH oxidase
(nmol substrate converted/min).
20
16
12
8
4
0
40
80
120
160
Protein concentration (µg/ml).

Table 14. The effect of dilution with cells grown on succinate on the activity of the enzymes of the mandelate pathway.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate, 5mM benzyl alcohol, or 10mM-succinate as sole source of carbon, harvested, washed, and stored as described in Methods. The three types of cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5, and the cells grown on mandelate or benzyl alcohol were diluted 1/10 with cells grown on succinate, and sonicated and centrifuged as described in Methods. The remainder of the cells grown on succinate was sonicated and centrifuged as described in Methods, and was used to dilute the mandelate and benzyl alcohol extracts 1/10. The mandelate and benzyl alcohol extracts were also diluted 1/10 with 0.04M-Tris-HCl buffer pH 8.5. All the extracts were then assayed as described in Methods. The benzyl alcohol extracts were used to assay catechol oxygenase and benzyl alcohol dehydrogenase; the mandelate extracts were used to assay all the other enzymes. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	No dilution	cells diluted 1/10 with succinate cells before sonication	extracts diluted 1/10 with succinate extract after sonication	extracts diluted with 0.04M-Tris-HCl buffer pH 8.5 x 10
L-Mandelate dehydrogenase	228	23.2	21.9	21.6
Benzoylformate decarboxylase	191	19.7	17.5	15.6
Stable benzaldehyde dehydrogenase	61	5.4	5.0	6.5
Labile benzaldehyde dehydrogenase	30	3.8	4.8	3.3
Benzyl alcohol dehydrogenase	467	36.8	38.2	7.0
Catechol oxygenase	248	27.4	24.6	30.0

specific activities of the enzymes in the diluted extracts are approximately 1/10 of those in the undiluted extract. Of the diluted samples of the labile and stable benzaldehyde dehydrogenase, the cell-diluted extracts give satisfactory results; but the specific activities of the enzymes in the protein-diluted extracts are relatively 15% higher in the case of the labile enzyme and relatively 15% lower in the case of the stable enzyme than the corresponding specific activities of the enzymes in the undiluted extract. Since 15% of the specific activity in this case represented less than 2 enzyme units/mg protein, it was assumed that the relative differences in the specific activities were not meaningful, and consequently it was concluded that protein from succinate cells had no effect on the enzyme activities.

The activity of benzyl alcohol dehydrogenase in the 1/10 extract and cell dilutions is only 80% of the relative activity in the undiluted extract. As it was found that the protein concentration of both dilutions was lower than that of the undiluted extract (1.80 and 1.84mg protein/ml compared to 2.20mg/ml respectively), one explanation of this result could be that as the enzyme protein concentration of the extract falls relative to its Tris buffer concentration, the Tris buffer inhibits enzyme activity. This suggestion is supported by the observations that the specific activity of the enzyme in the 1/10 dilution in Tris buffer is only 15% of the relative activity in the undiluted extract (Table 14), and that the

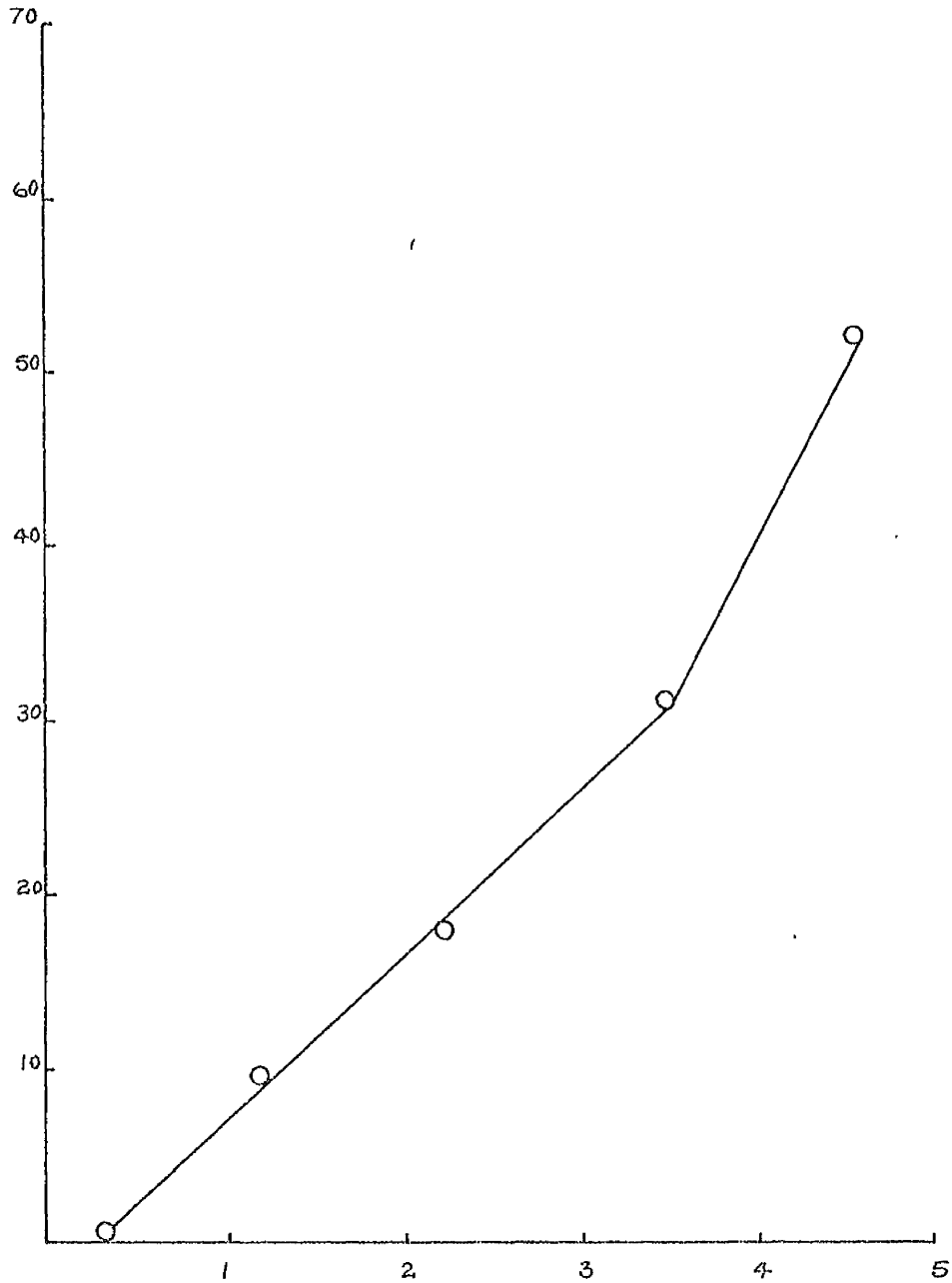
activity of the enzyme is higher when it is extracted in 0.04M-Tris-HCl rather than 0.08M-Tris-HCl buffer (see p.133). Additional evidence comes from a consideration of Fig.32. This shows that as the protein concentration of the extract increases so does its benzyl alcohol dehydrogenase specific activity. The slight variation in the final Tris buffer concentration of the reaction mixture caused by the addition of different amounts of extract has no effect on enzyme activity as the graph of enzyme activity against assay protein concentration is linear (Fig.29). The linearity of this graph also indicates that the relative concentration of sodium pyrophosphate, which is the assay buffer, has no effect on enzyme activity. Thus care must be taken to extract cells under the standard conditions so that the protein concentration of the extract remains constant. If this is done, a correction for changes in benzyl alcohol dehydrogenase activity due to changes in protein concentration need not be made.

Similar experiments using 1/100 dilutions rather than 1/10 dilutions were performed, and the results obtained in each case indicated that protein from non-induced cells did not interfere with the enzyme assays.

Since these experiments showed that protein from non-induced cells did not interfere with the enzyme assays, it follows that NADH oxidase did not interfere with the measurement of the reduction of NAD^+ by low levels of benzyl alcohol dehydrogenase or the stable or labile benzaldehyde

Fig.32. The effect of dilution of an extract made from cells grown on benzyl alcohol with extracts made from cells grown on succinate on the activity of benzyl alcohol dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-benzyl alcohol or 10mM-succinate as sole source of carbon, harvested, washed, and stored as described in Methods. The cells grown on benzyl alcohol were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 50mg wet wt./ml, and the cells grown on succinate to 25, 50, or 100mg wet wt./ml. The suspension of cells grown on benzyl alcohol was sonicated and centrifuged as described in Methods. The three suspensions of cells grown on succinate were sonicated and centrifuged as described in Methods, and each extract was used to dilute the benzyl alcohol extract 1/10. The benzyl alcohol extract was also diluted 1/10 with 0.04M-Tris-HCl buffer pH 8.5. All the extracts were then assayed as described in Methods (p.83). The specific activity of benzyl alcohol dehydrogenase was plotted against the protein concentration of the extract before its addition to the reaction mixture.


Specific activity of benzyl alcohol dehydrogenase
(nmol substrate converted/min/mg protein).
70
60
50
40
30
20
10
1
2
3
4
5
Protein concentration of the extract before
its addition to the reaction mixture (mg/ml).

dehydrogenase. This is in contrast to the finding with the coupled benzoylformate decarboxylase assay in which interference was found to occur in unheated extracts (Table 10), but not in heated extracts (Tables 10 and 14). This apparent lack of interference in the former group of assays could well be due to the fact that NADH oxidase activity as assayed in Tris-HCl buffer pH 9.5 (the assay buffer for the two benzaldehyde dehydrogenases) and sodium pyrophosphate buffer pH 9.0 (the assay buffer for benzyl alcohol dehydrogenase) is only 4% and 2% respectively of the optimal enzyme activity, whereas in Tris-HCl buffer pH 9.0 (the assay buffer for benzoylformate decarboxylase) it is 14% of the optimal activity.

These experiments also showed that the lowest measurable specific activity of L-mandelate dehydrogenase and catechol oxygenase was approximately 0.5 units/mg protein, and of benzoylformate decarboxylase, the two benzaldehyde dehydrogenases, benzyl alcohol dehydrogenase and NADH oxidase 0.8-1.0 units/mg protein. These values were based on a protein concentration of 2.20mg/ml in the cell extract, and the maximum addition of 500µl of extract to the reaction mixture.

1.2.5. Effect of storage on the activity of the mandelate pathway enzymes.

Since enzymes often lose activity on storage, the effect of storing a cell extract at 0° for various time intervals was determined so that the order, in which the enzymes should be assayed to minimise the loss of activity, could be decided.

From a consideration of the results in Table 15, the enzymes were assayed in the following order in subsequent experiments: NADH oxidase; benzyl alcohol dehydrogenase; L-mandelate dehydrogenase; benzaldehyde dehydrogenase; benzoylformate decarboxylase and catechol oxygenase.

Because it was inconvenient to assay the enzymes on the same day as the cells were harvested, the cells were stored at -60° until they were sonicated as preliminary experiments showed that storage of the cells at -60° did not result in the loss of any enzyme activity.

1.2.6. The statistical error in measuring the activity of the mandelate pathway enzymes.

The statistical error in the extraction and assay procedures was determined (Table 16). This experiment was performed before the experiments in Sections 2 and 3. All assays were subsequently done in duplicate, and there is little doubt that familiarity and practice with the technique progressively reduced the errors in the latter experiments since duplicate values for enzyme activity were always within 5% of the mean value, and were usually much closer than this.

Table 15. The effect of storing the cell extracts on the activity of the enzymes of the mandelate pathway.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, stored, and sonicated as described in Methods. The extract was stored for various times at 0^{o}, and then assayed as described in Methods (p.78). Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Time of storage (h)					
	0	1	2	4	8	23
L-Mandelate dehydrogenase	227	241	249	279	289	218
Catechol oxygenase	657	657	621	656	621	641
NADH oxidase	107	99	94	84	72	54
Benzoylformate decarboxylase	421	424	432	444	442	427
Benzyl alcohol dehydrogenase	22	22	20	16	12	7
Total Benzaldehyde dehydrogenase	106	106	110	98	93	82

Table 16. The statistical error in measuring the activity of the enzymes of the mandelate pathway.

Bacterium NCIB 8250 was grown on 5mM-L-mandelate as sole source of carbon, harvested, washed, and stored as described in Methods. Cells were resuspended in 0.04M-Tris-HCl buffer pH 8.5 to 50mg wet wt./ml, and four 5ml amounts of this suspension were sonicated and centrifuged as described in Methods. Each extract was assayed four times, and the mean values, which are expressed as nmol substrate converted/min/mg protein, for the sixteen specific activities of the enzymes were calculated. The S.E.M. as a percentage of the mean for the assay and extraction procedures was then calculated using an analysis of variance.

Enzyme	Average specific activity of 16 assays	S.E.M. of the assay % of the mean	S.E.M. of the extraction % of the mean
L-mandelate dehydrogenase	187	6.8	8.1
Benzoylformate decarboxylase	392	1.7	1.7
Stable benzaldehyde dehydrogenase	74	2.1	9.1
Labile benzaldehyde dehydrogenase	42	7.4	16.6
Benzyl alcohol dehydrogenase	15	4.3	6.3
Catechol oxygenase	558	4.1	14.8
NADH oxidase	81	8.5	6.1
Protein concentration	4 extractions 2.83mg/ml	not done	5.1

2. KINETICS OF INDUCTION OF THE MANDELATE PATHWAY ENZYMES.

The kinetics of induction of L-mandelate dehydrogenase, benzoylformate decarboxylase, the stable and labile benzaldehyde dehydrogenase, benzyl alcohol dehydrogenase, benzoate oxidase and catechol oxygenase were measured in order to help determine the regulatory patterns operating in the aromatic part of the mandelate pathway.

The inducer, in all cases, was added to a culture growing on 10mM-glutamate as sole source of carbon and energy. Glutamate was used as the carbon and energy source since preliminary experiments showed that samples taken from the penultimate generation of a culture growing on glutamate + L-mandelate contained similar amounts of L-mandelate dehydrogenase as samples taken from the penultimate generation of a culture growing on L-mandelate alone.

Samples taken from the growth flask for enzyme assay were withdrawn onto 40g crushed ice since preliminary experiments showed that this was as effective at stopping enzyme synthesis as the presence of chloramphenicol at a final concentration of 20μM (e.g. the specific activity of L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and catechol oxygenase in a sample withdrawn onto crushed ice was 47, 35 and 334 units/mg protein respectively; whilst the specific activity of the same enzymes in a sample withdrawn at the same time onto chloramphenicol was 48, 34 and 350 units/mg protein

respectively). This concentration of chloramphenicol was found to inhibit protein synthesis as measured by the Lowry method.

In the earlier experiments it was found that the values for protein as measured by the Lowry method showed a great deal of scatter on the growth curves. This scatter was caused by the errors in having to dilute the samples before estimating their protein content, and in having to measure a small change in growth rate over a short period of time. In the later experiments, protein was estimated indirectly by measuring turbidity in the Unicam SP.800 Spectrophotometer, and converting these readings to µg protein by means of a calibration curve (Fig.11).

2.1. Growth of bacterium NCIB 8250 before and after the addition of inducer.

Two examples of the growth obtained in these experiments are shown in Figs.33 and 34. The addition of benzyl alcohol decreases the growth rate on 10mM-glutamate, whereas the addition of L-mandelate has no effect. Different inducers had a range of effects of this type. Nevertheless any effects on growth rate were taken into account when measuring the kinetics of induction since the final results were calculated as differential rates of enzyme synthesis (Section 2.3.).

2.2. Time-course of induction of the mandelate pathway enzymes.

Fig.35 shows the time-course of appearance of benzoylformate decarboxylase, benzyl alcohol dehydrogenase,

Fig. 33. Growth of bacterium NCIB 8250 on 10mM-glutamate-salts medium before and after the addition of L-mandelate.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. Growth was determined by taking samples for protein estimation as described in Methods. L-Mandelate, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation (↑).

The samples for protein estimation taken before the addition of L-mandelate were corrected for the change in volume produced by the addition of inducer.

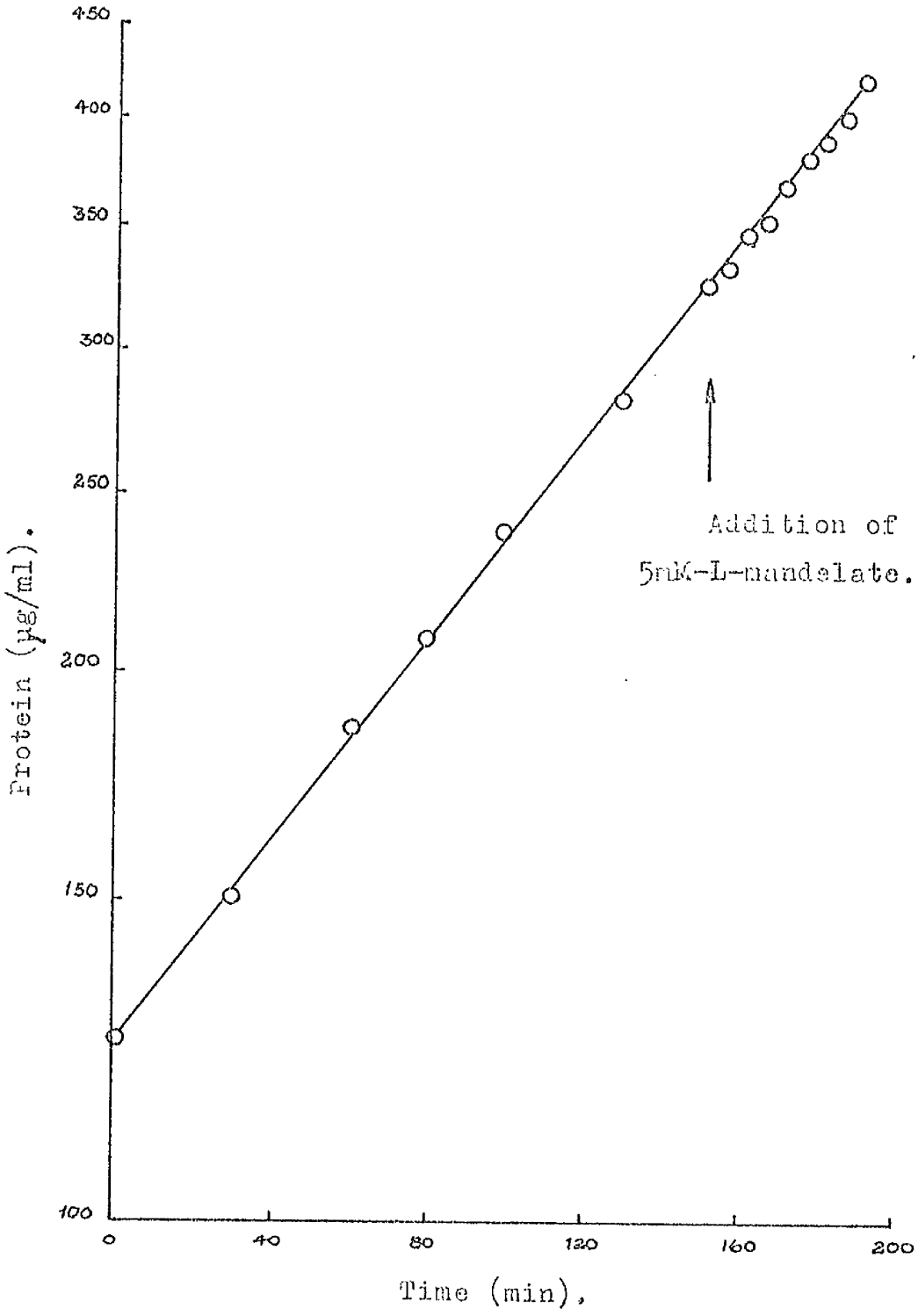

450
400
350
300
250
200
150
100
Protein (µg/ml).
0
40
80
120
160
200
Time (min).
Addition of
5mM-L-mandelate.

Fig. 34. Growth of bacterium NCIB 8250 on 10mM-glutamate-salts medium before and after the addition of benzyl alcohol.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. Growth was determined by taking samples for protein estimation as described in Methods. Redistilled benzyl alcohol, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation (↑).

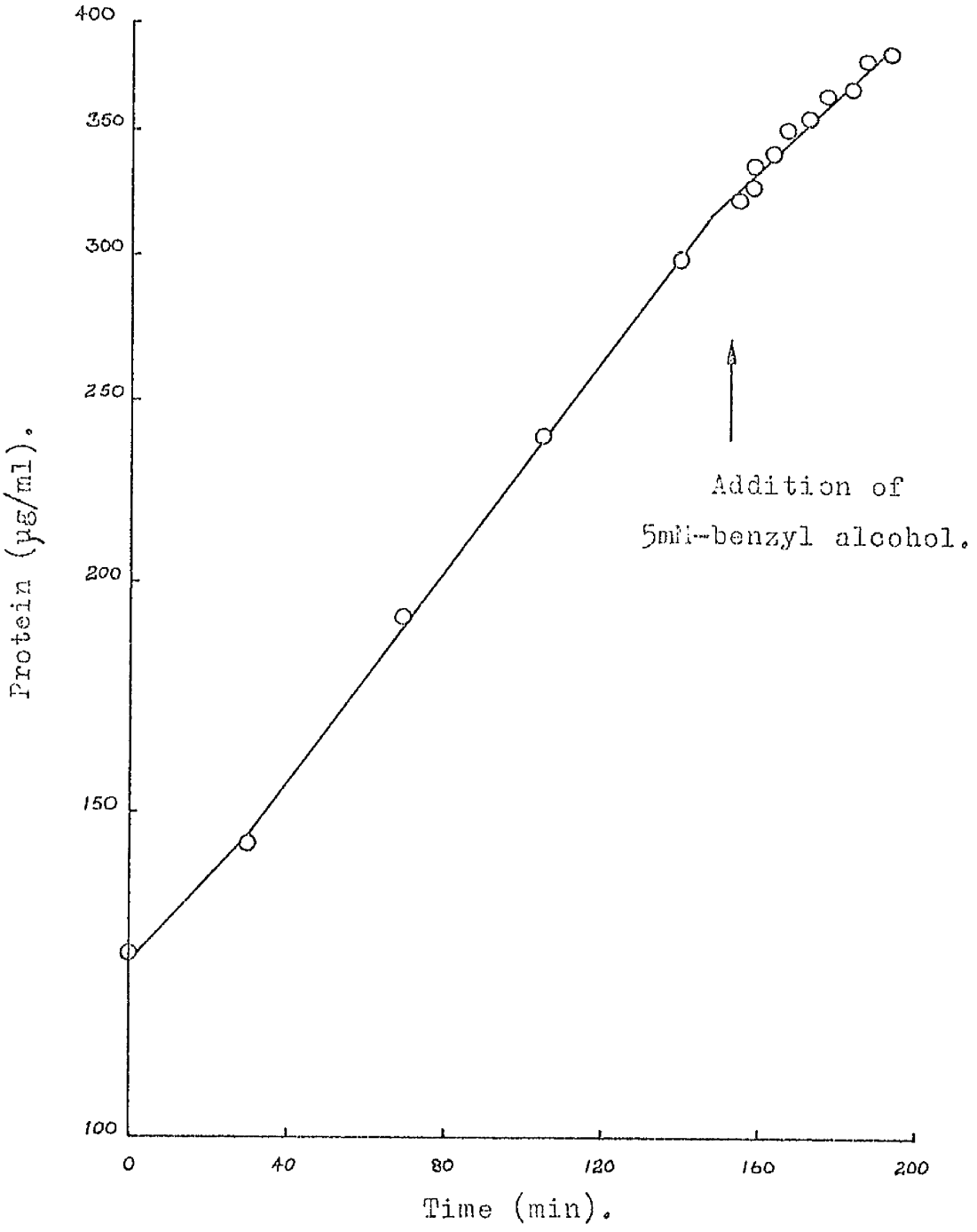

400
350
300
250
200
150
100
Protein (µg/ml).
0
40
80
120
160
200
Time (min).
Addition of
5mM-benzyl alcohol.

Fig.35. Time-course of appearance of four enzymes of the mandelate pathway after addition of L-mandelate to a culture growing on 10mM-glutamate-salts medium.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. L-Mandelate, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation. Samples were withdrawn from the growth flask, harvested, washed, stored, and sonicated, and the enzymes were assayed as described in Methods.

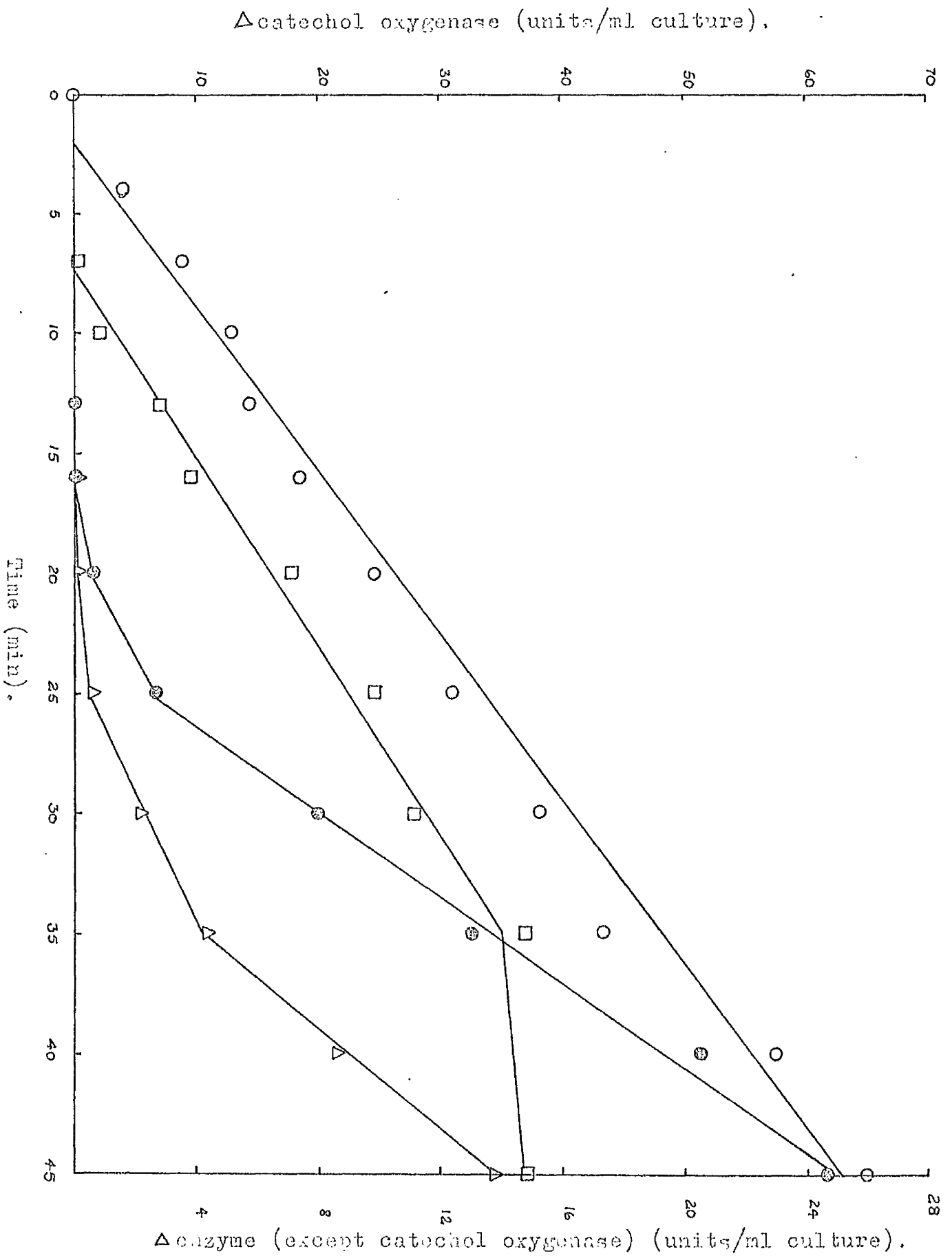
○ - Benzoylformate decarboxylase.

□ - Benzyl alcohol dehydrogenase.

△ - Benzoate oxidase.

o - Catechol oxygenase.

Enzyme units are expressed as nmol substrate converted/min.


Δcatechol oxygenase (units/ml culture).
0
10
20
30
40
50
60
70
Time (min).
0
5
10
15
20
25
30
35
40
45
Δenzyme (except catechol oxygenase) (units/ml culture).
4
8
12
16
20
24
28

benzoate oxidase and catechol oxygenase. L-Mandelate dehydrogenase and the stable benzaldehyde dehydrogenase follow the same general pattern as that of benzoylformate decarboxylase. Likewise, the time-course of appearance of the labile benzaldehyde dehydrogenase is similar to that of benzyl alcohol dehydrogenase. The flattening-off in the rate of formation of benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase was always observed with L-mandelate or benzoylformate as inducer.

An attempt was made to measure the time of appearance of all the mandelate pathway enzymes after the addition of L-mandelate, benzoylformate, benzyl alcohol and benzaldehyde, and this is shown in Table 17. The time of enzyme appearance after addition of inducer was taken as the intercept on the time-axis of the straight line representing the maximum rate of enzyme synthesis in the graph of Δenzyme against time (Fig.35). Strictly speaking, this method only indicates the time at which the maximum rate of enzyme synthesis occurs, and not the time of first appearance of enzyme. No account is taken of early periods of slow enzyme synthesis.

2.3. Differential plots for the induction of the mandelate pathway enzymes.

A convenient and valuable way of describing the kinetics of induction of an enzyme is the P value, which was defined by Monod, Pappenheimer & Cohen-Bazire (1952) as the gradient of the graph of increase in enzyme units/ml culture against

Table 17. Time of appearance of the mandelate pathway enzymes after addition of inducer to a culture growing on 10mM-glutamate-salts medium.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. The inducer, to give a final concentration of 5mM in the glutamate-salts medium in the case of L-mandelate, benzoylformate, or benzyl alcohol, or 1mM in the case of benzaldehyde, was added after approximately 1 generation. Samples were withdrawn from the growth flask, harvested, washed, stored, and sonicated, and the enzymes were assayed as described in Methods. The time of enzyme appearance after addition of inducer was taken as the intercept on the time-axis of the straight line representing the maximum rate of enzyme synthesis in a graph of Δ enzyme against time (e.g. Fig.35).

The figures in parenthesis represent the number of experiments performed.

Enzyme	Time of enzyme appearance after addition of inducer (min)			
	L-Mandelate	Benzoylformate	Benzyl alcohol	Benzaldehyde
L-Mandelate dehydrogenase	5 (3)	5 (2)	no induction	no induction
Benzoylformate decarboxylase	1 (2)	0 (1)	no induction	no induction
Stable benzaldehyde dehydrogenase	5 (3)	3 (2)	no induction	no induction
Labile benzaldehyde dehydrogenase	7 (3)	8 (2)	8 (3)	9 (1)
Benzyl alcohol dehydrogenase	8 (3)	8 (2)	8 (3)	14 (1)
Benzoate oxidase	29 (1)	not tested	24 (1)	not tested
Catechol oxygenase	25 (2)	21 (2)	18 (2)	15 (1)

increase in bacterial protein/ml culture after the addition of inducer. This graph is known as the differential plot. Examples of differential plots for the induction of L-mandelate dehydrogenase, benzoylformate decarboxylase, the stable and labile benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase are shown in Figs.36 to 40. These graphs cover a time span of approximately 25min.

The differential plot for the induction of L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase by thiophenoxyacetate (Fig.38) follows a different pattern from that obtained by induction with L-mandelate (Fig.36) or L-mandelate + benzyl alcohol (Fig.37). The plot for induction with thiophenoxyacetate is biphasic and gives two distinct P values for the induction of the Regulon R_1 enzymes.

Figs.39 and 40 show the induction of benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase by L-mandelate and benzyl alcohol respectively. L-Mandelate induces less of these enzymes than does benzyl alcohol. Thiophenoxyacetate was found to induce neither of these two enzymes, nor did it induce benzoate oxidase or catechol oxygenase. Using L-mandelate as inducer (Fig.39), the rate of formation of the labile benzaldehyde dehydrogenase begins to fall off after 20min induction. This falling-off in the rate of enzyme synthesis is analogous to the flattening-off in the rate of benzyl alcohol dehydrogenase synthesis observed in

Fig.36. Differential plot for the induction of L-mandelate dehydrogenase, benzoylformate decarboxylase, and the stable benzaldehyde dehydrogenase by L-mandelate.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. L-Mandelate, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated and assayed as described in Methods. Samples for protein were also withdrawn from the growth flask, and estimated as described in Methods.

O - L-Mandelate dehydrogenase.

Δ - Benzoylformate decarboxylase.

◎ - Stable benzaldehyde dehydrogenase.

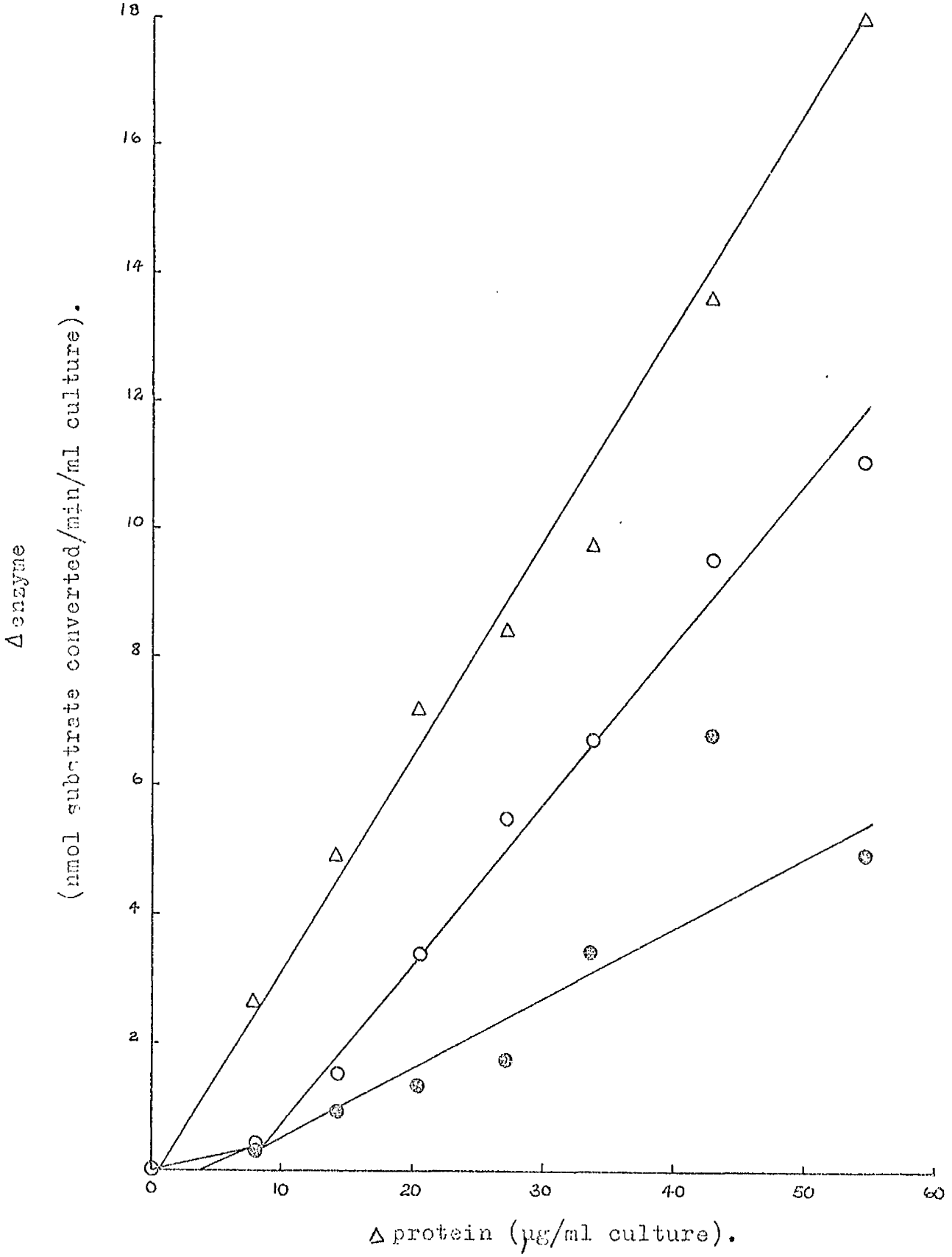

Δ enzyme
(nmol substrate converted/min/ml culture).
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
60
Δ protein (μg/ml culture).

Fig.37. Differential plot for the induction of L-mandelate dehydrogenase, benzoylformate decarboxylase, and the stable benzaldehyde dehydrogenase by L-mandelate + benzyl alcohol.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. L-Mandelate + benzyl alcohol, each to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated, and assayed as described in Methods. Samples for protein were also withdrawn from the growth flask, and estimated as described in Methods.

○ - L-Mandelate dehydrogenase.

△ - Benzoylformate decarboxylase.

⊕ - Stable benzaldehyde dehydrogenase.

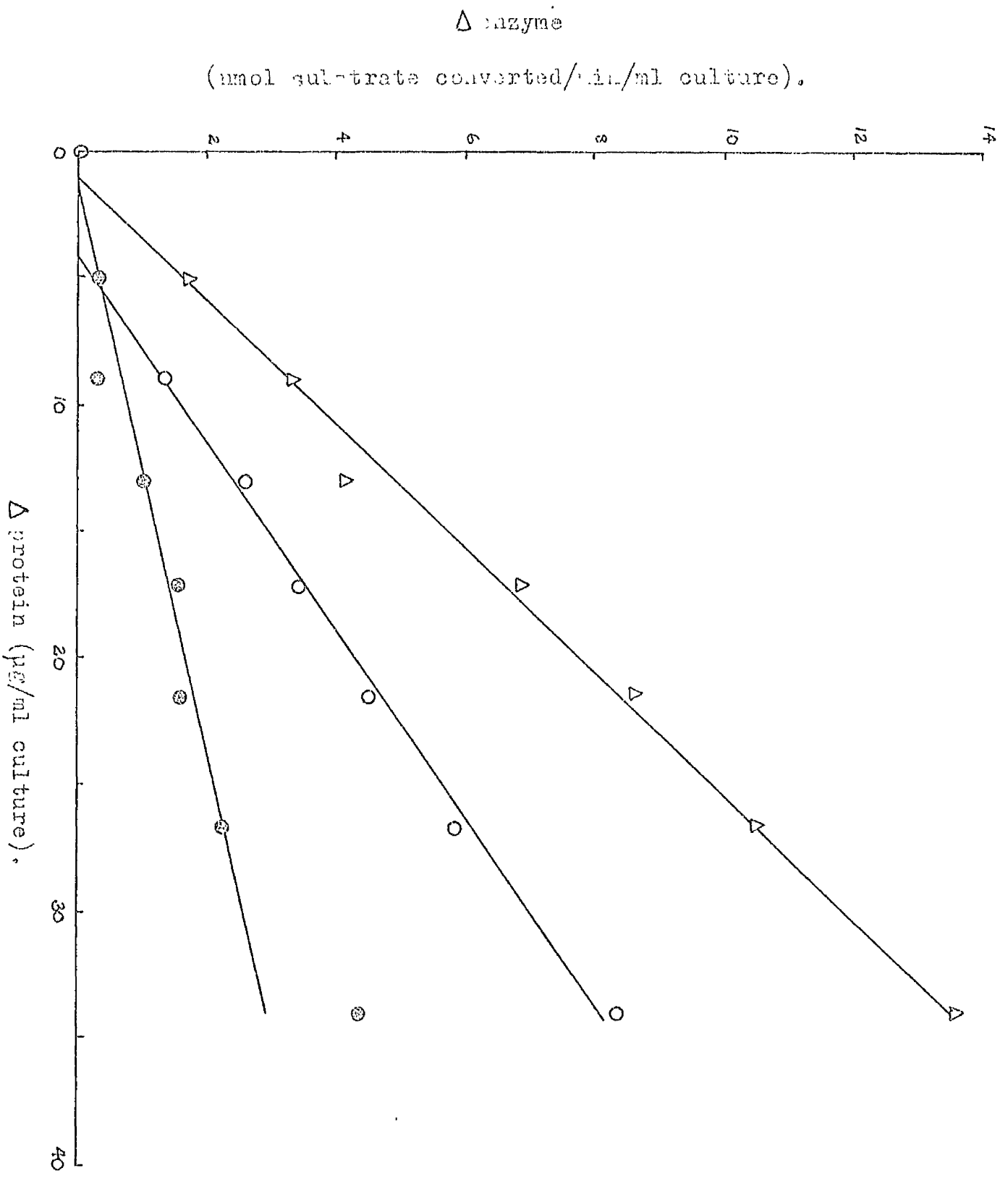

Δ enzyme
(umol substrate converted/min./ml culture).
0
2
4
6
8
10
12
14
0
10
20
30
40
Δ protein (μg/ml culture).

Fig.38. Differential plot for the induction of L-mandelate dehydrogenase, benzoylformate decarboxylase, and the stable benzaldehyde dehydrogenase by thiophenoxyacetate.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. Thiophenoxyacetate, to give a final concentration of 1mM in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated, and assayed as described in Methods. Samples for protein were also withdrawn from the growth flask, and estimated as described in Methods.

○ - L-Mandelate dehydrogenase.

△ - Benzoylformate decarboxylase.

◎ - Stable benzaldehyde dehydrogenase.

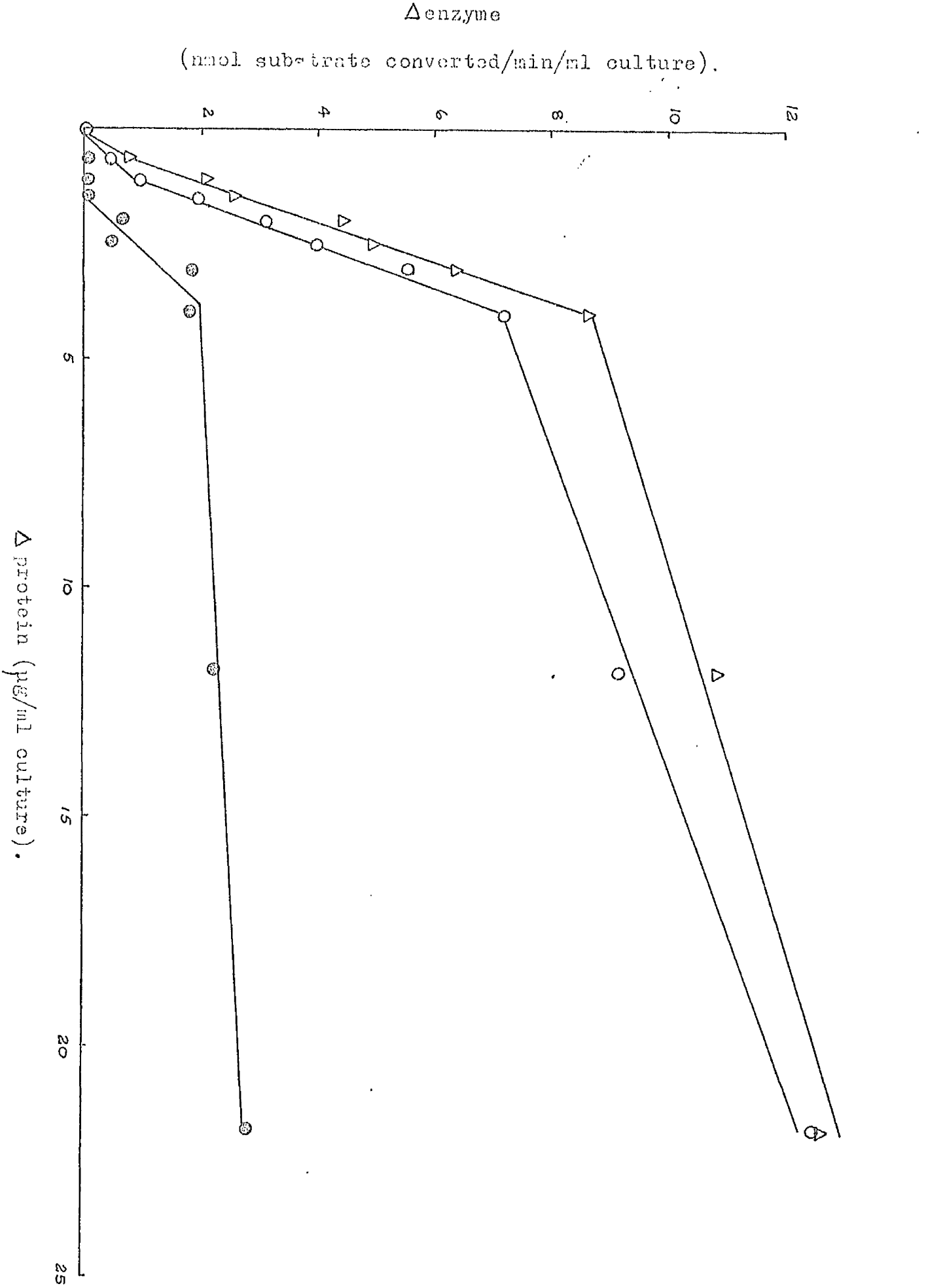

Δ enzyme
(nmol substrate converted/min/ml culture).
2
4
6
8
10
12
5
10
15
20
25
Δ protein (µg/ml culture).

Fig. 39. Differential plot for the induction of benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase by L-mandelate.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. L-Mandelate, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated, and assayed as described in Methods. Samples for protein were also withdrawn from the growth flask, and estimated as described in Methods.

○ - Labile benzaldehyde dehydrogenase.

◎ - Benzyl alcohol dehydrogenase.

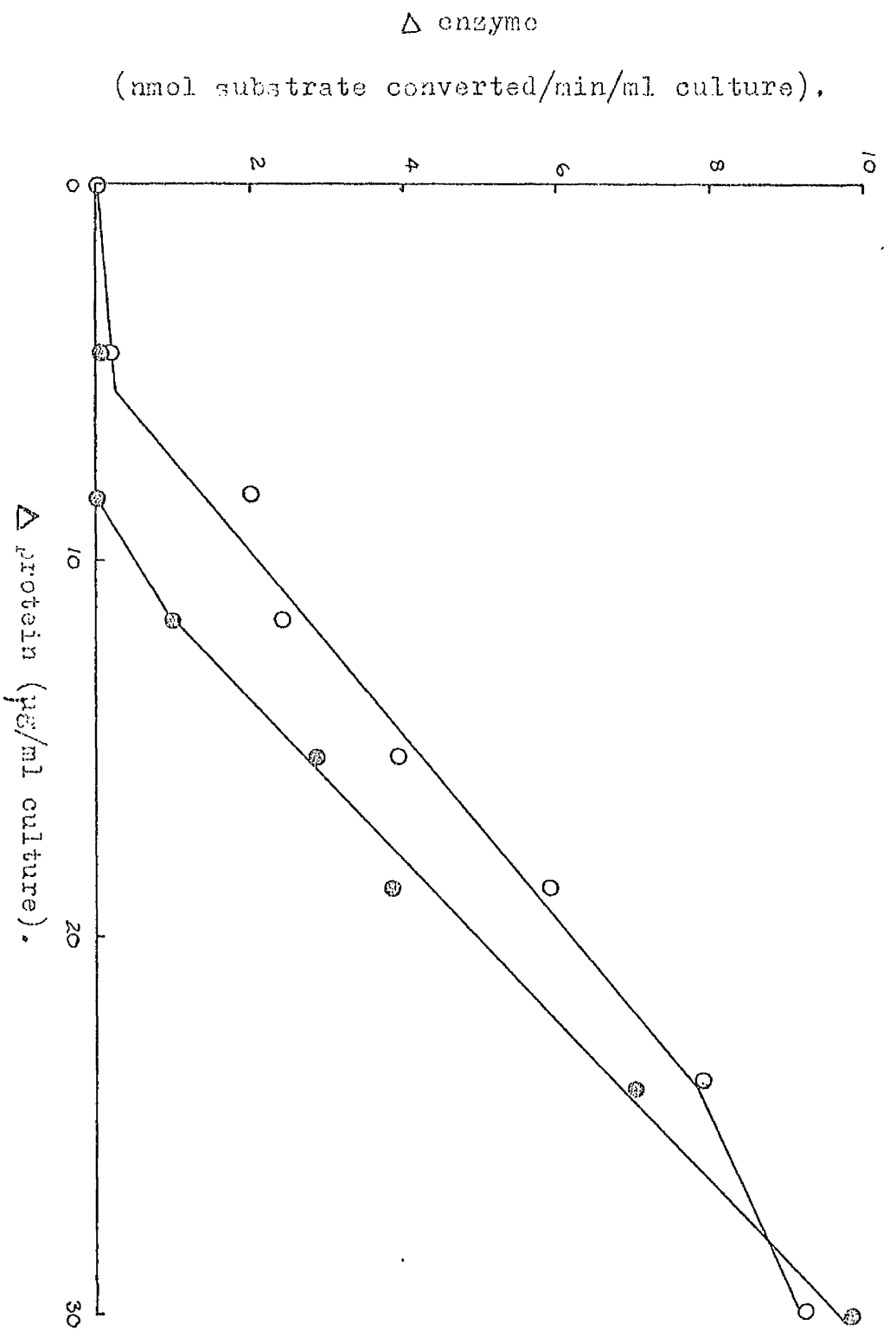

Δ enzyme
(nmol substrate converted/min/ml culture).
0
2
4
6
8
10
10
20
30
Δ protein (μg/ml culture).

Fig.40. Differential plot for the induction of benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase by benzyl alcohol.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. Benzyl alcohol, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated, and assayed as described in Methods. Samples for protein were also withdrawn from the growth flask, and estimated as described in Methods.

○ - Labile benzaldehyde dehydrogenase.

◎ - Benzyl alcohol dehydrogenase.

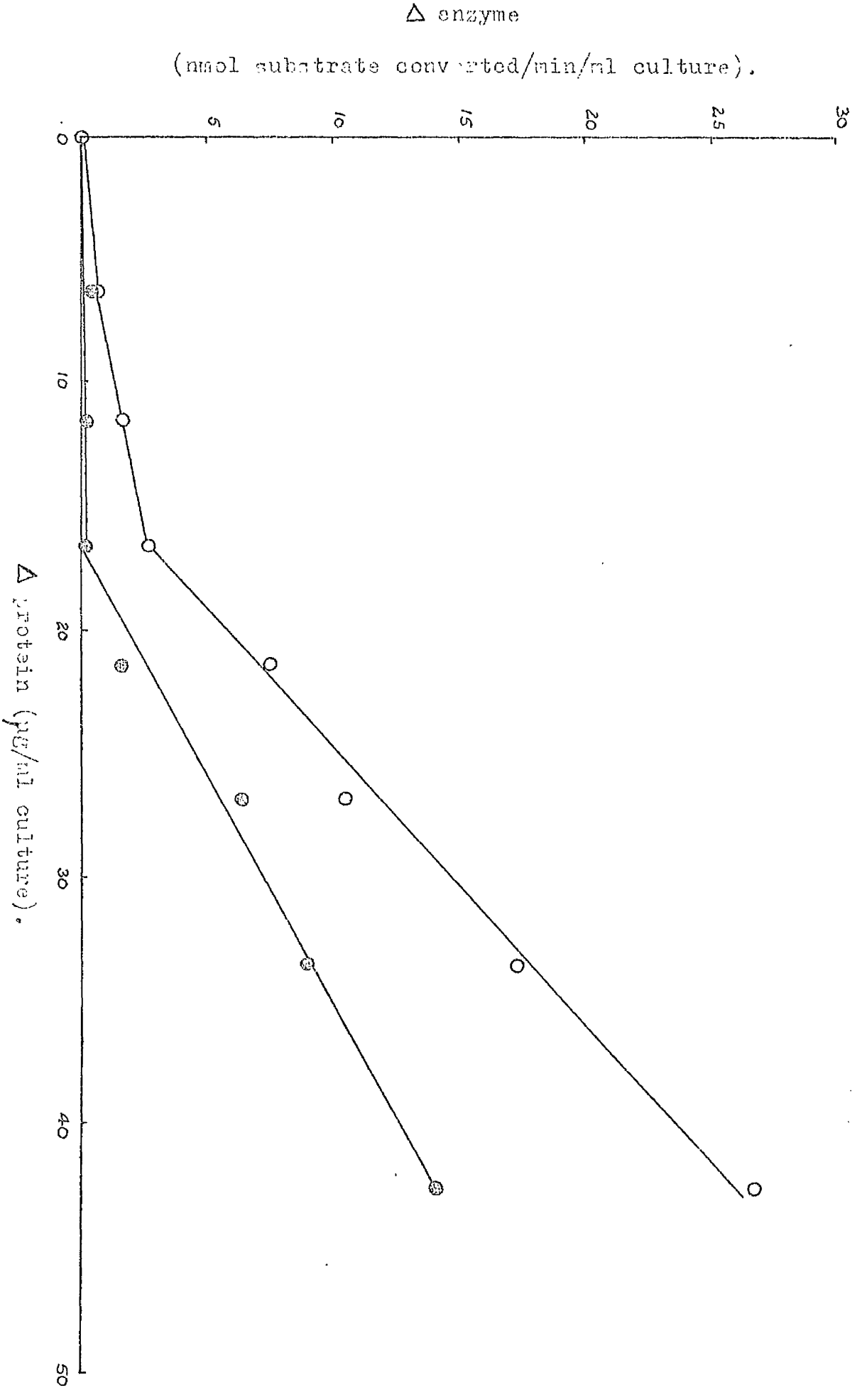

Δ enzyme
(nmol substrate converted/min/ml culture).
0
5
10
15
20
25
30
Δ protein (μg/ml culture).
0
10
20
30
40
50

Fig.35 although in the case of the labile benzaldehyde dehydrogenase this phenomenon is observed earlier (after 20min compared to 35min in the case of benzyl alcohol dehydrogenase).

Fig.41 shows the differential plot for the induction of catechol oxygenase and benzoate oxidase. The induction of these two enzymes occurs later than that of the enzymes of Regulons R_1 and R_2 with catechol oxygenase being induced slightly before benzoate oxidase.

According to Jacob & Monod (1961), if the induction of two enzymes is coordinately controlled, P values for their induction should be proportional to one another. Consequently the P values for the induction of the mandelate pathway enzymes were determined for various inducers which gave a range of P values. A large number of experiments was performed to give graphs of the type shown in Figs.36 to 41. The P values were calculated in each case. In order to determine the relationship between the various P values for the different enzymes, graphs were plotted of the P values for each enzyme against the P values for the other enzymes. In this way it was possible to see in which cases there was a positive correlation between the induction of pairs of enzymes.

The P values for the induction of benzoylformate decarboxylase, the stable and labile benzaldehyde dehydrogenase, benzyl alcohol dehydrogenase, benzoate oxidase and catechol oxygenase plotted against those of L-mandelate dehydrogenase are shown in Fig.42. In some cases there is relatively poor

Fig.41. Differential plot for the induction of benzoate oxidase and catechol oxygenase by L-mandelate.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. L-Mandelate, to give a final concentration of 5mM in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated, and assayed as described in Methods. Samples for protein were also withdrawn from the growth flask, and estimated as described in Methods.

O - Benzoate oxidase.

● - Catechol oxygenase.

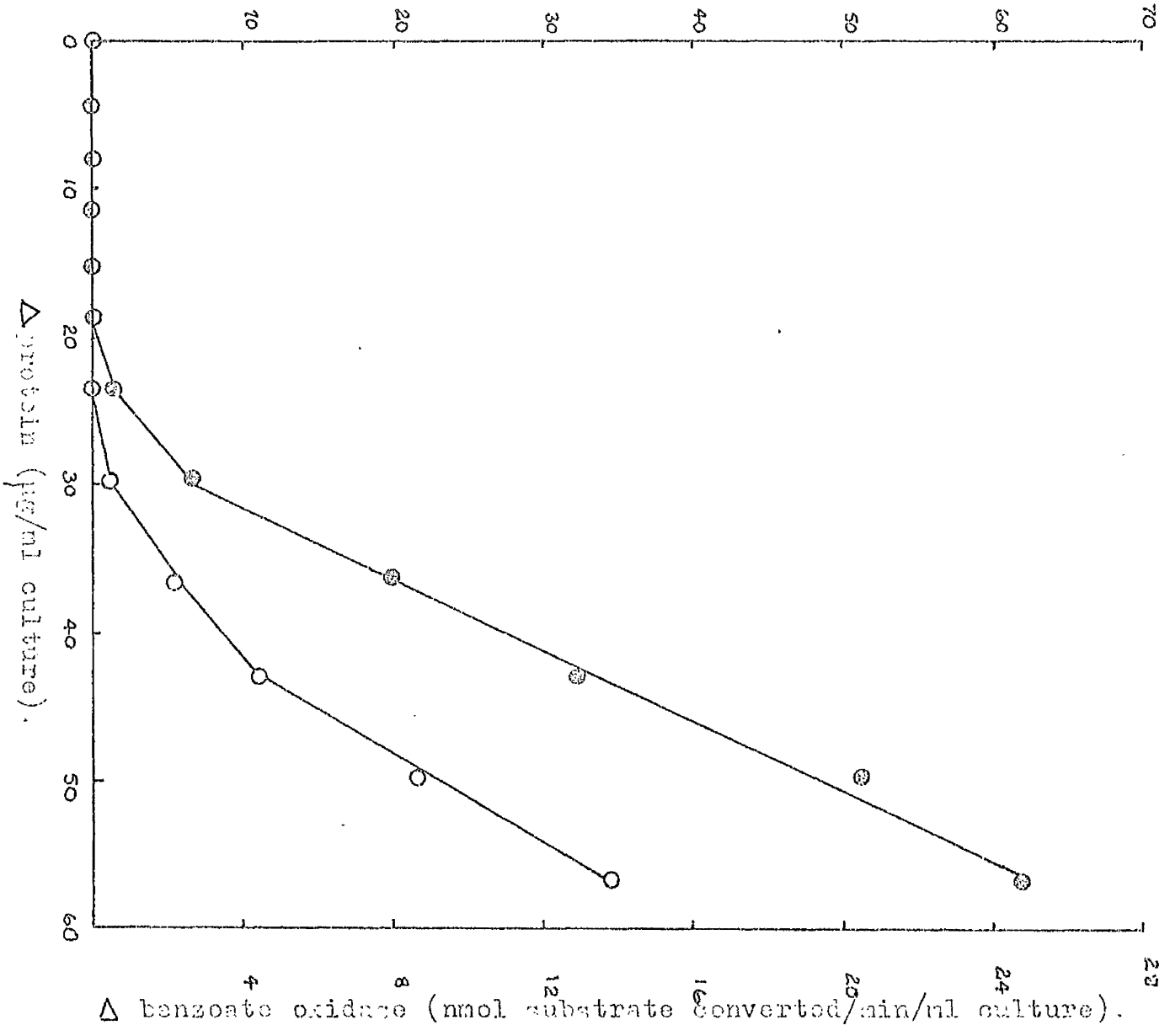
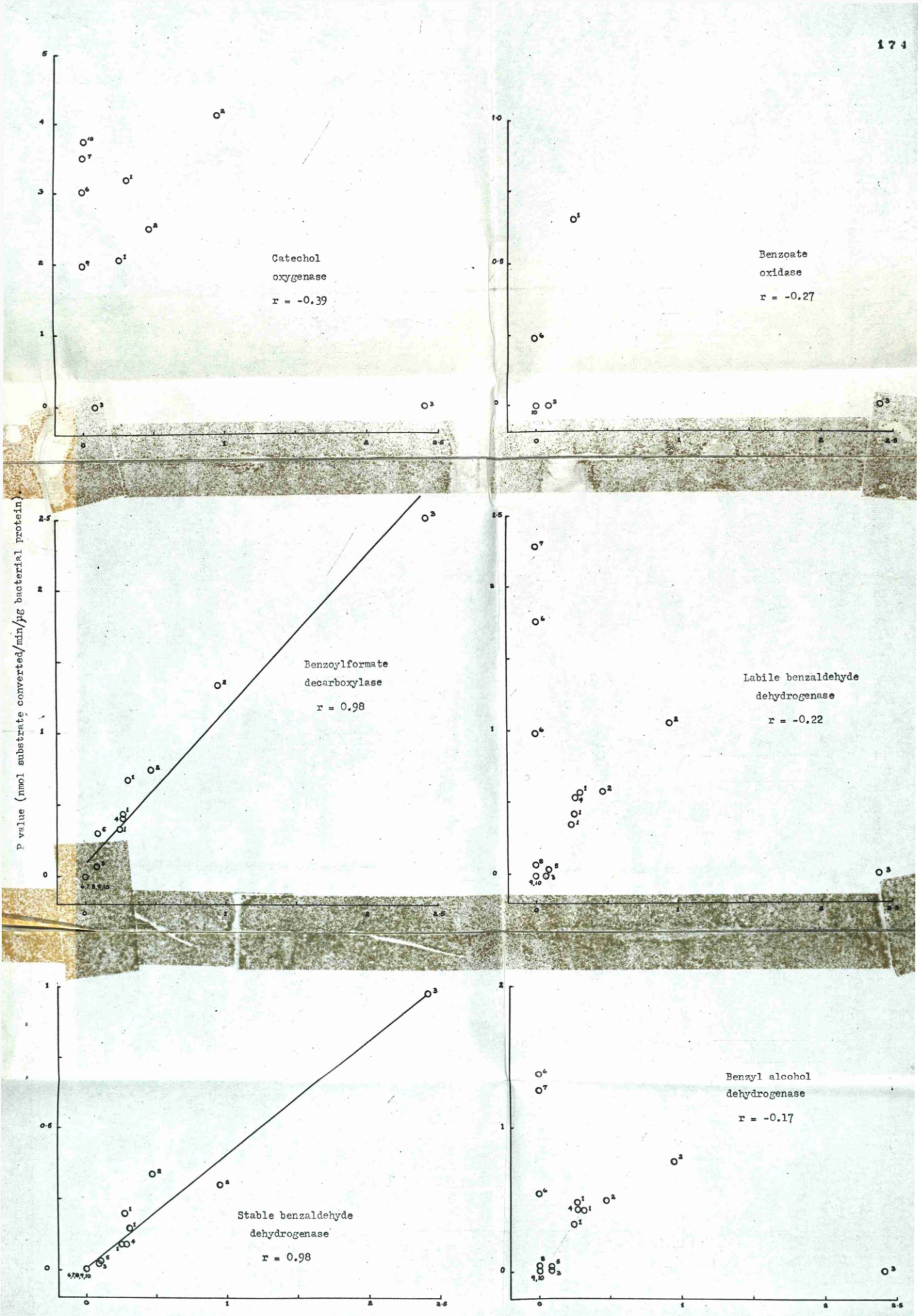

Δ catechol oxygenase (nmol substrate converted/min/ml culture).
10
20
30
40
50
60
70
0
10
20
30
40
50
60
Δ protein (μg/ml culture).
4
8
12
16
20
24
28
Δ benzoate oxidase (nmol substrate converted/min/ml culture).

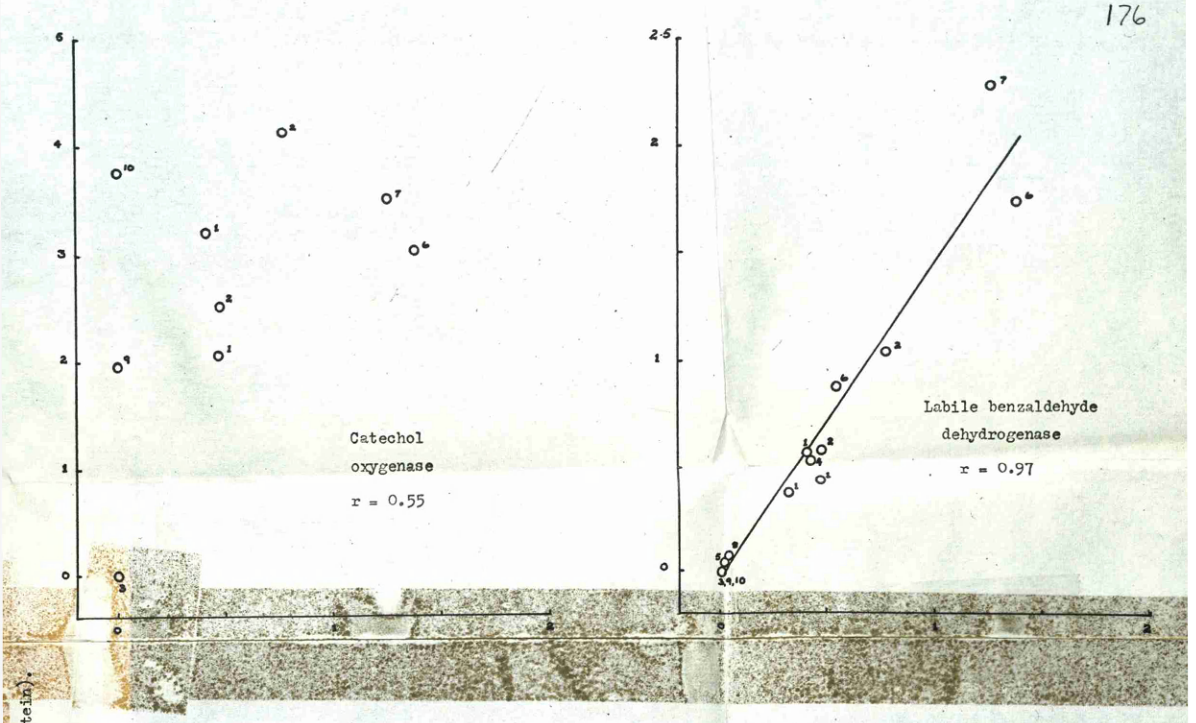
Fig.42. P values for the induction of the other mandelate pathway enzymes plotted against P values for the induction of L-mandelate dehydrogenase.

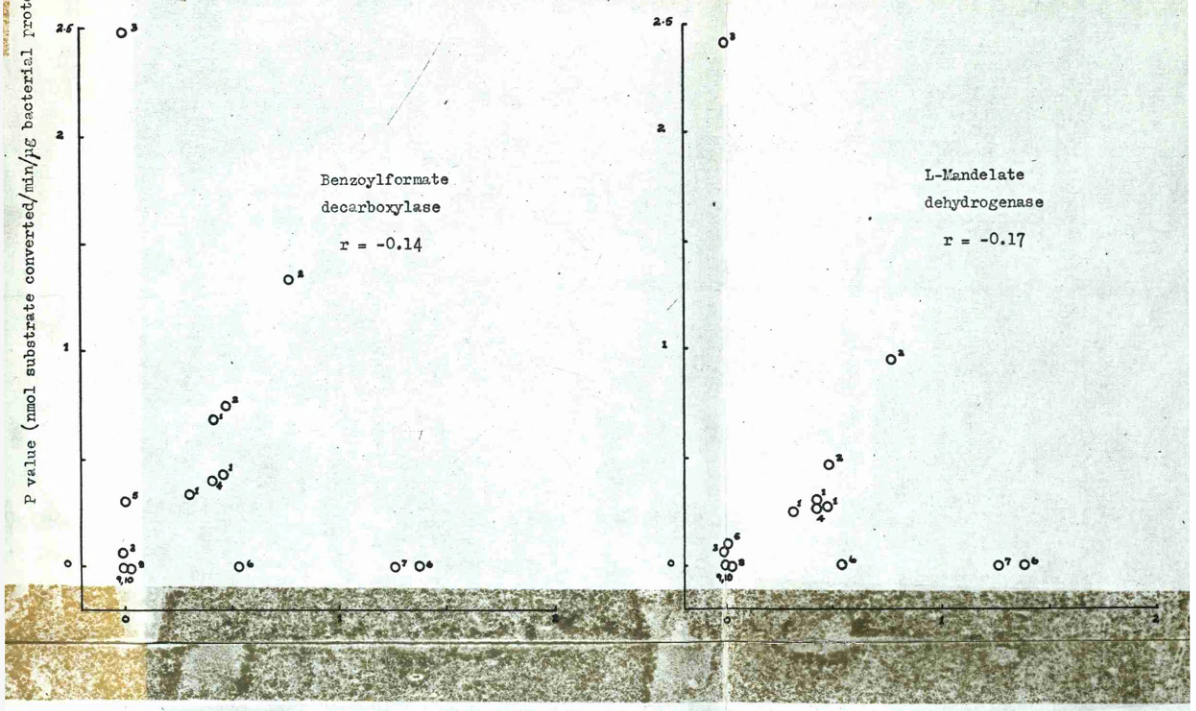
In a series of independent experiments similar to Fig.36, p.167, the mandelate pathway enzymes of bacterium NCIB 8250 were induced by a number of compounds, which are listed below, and some of which, as indicated by the numbers in brackets, were used more than once. In each experiment the differential plot was drawn for each enzyme, and from the gradient of the graph the P value for the induction of the enzyme was calculated and used in the graph opposite.

1 - 5mM-L-Mandelate (3).
2 - 5mM-Benzoylformate (2).
3 - 1mM-Thiophenoxyacetate.
4 - 5mM-L-Mandelate + 5mM-benzyl alcohol.
5 - 5mM-L-Mandelate + 5mM-acetate + 5mM-succinate.
6 - 5mM-Benzyl alcohol (2).
7 - 1mM-Benzaldehyde.
8 - 5mM-Benzyl alcohol + 5mM-acetate + 5mM-succinate.
9 - 1mM-Benzoate.
10 - 1mM-Catechol.

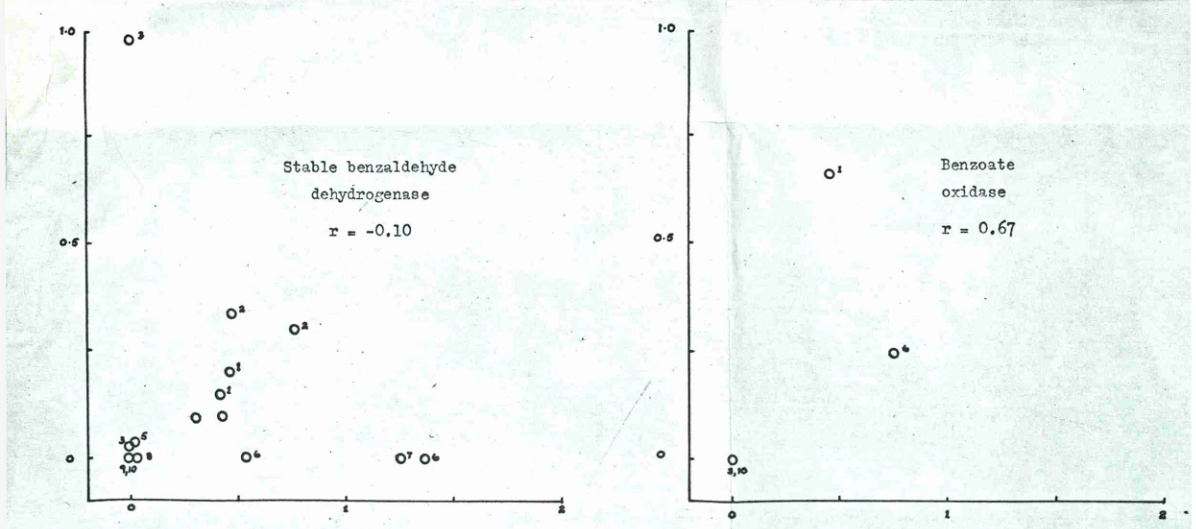
The correlation coefficients (r) for the various graphs are recorded on the graph to which they relate.


Catechol
oxygenase
r = -0.39
Benzoate
oxidase
r = -0.27
Benzoylformate
decarboxylase
r = 0.98
Labile benzaldehyde
dehydrogenase
r = -0.22
Stable benzaldehyde
dehydrogenase
r = 0.98
Benzyl alcohol
dehydrogenase
r = -0.17
P value (nmol substrate converted/min/µg bacterial protein).
L-Mandelate dehydrogenase P value
(nmol substrate converted/min/µg bacterial protein).





P value (nmol substrate converted/min/μg bacterial protein).



Benzyl alcohol dehydrogenase P value
(nmol substrate converted/min/μg bacterial protein).

agreement between the P values determined on two different occasions with the same inducer. This is largely because of errors in the determination of protein in the earlier experiments. However, although the absolute P values may vary, the same proportional error (if any) will be introduced into all the P values in the one experiment since the same protein values were used as a basis for calculating the P values of all the enzymes. Thus the ratio between P values for different enzymes will not vary, and therefore the test for coordinacy is unaffected.

The correlation coefficient is shown for each separate correlation in Fig.42. If there was a significant correlation between the P values ($r > 0.95$), the regression was calculated, and this is the line which is drawn in a number of cases. The correlation coefficients show that only benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase are synthesised coordinately with L-mandelate dehydrogenase.

Fig.43 shows a similar set of graphs for the P values of the other enzymes plotted against the P values of benzyl alcohol dehydrogenase. Only the labile benzaldehyde dehydrogenase is induced coordinately with benzyl alcohol dehydrogenase.

Benzoate oxidase and catechol oxygenase are not coordinately synthesised (Fig.44).

2.4. Repression of the mandelate pathway enzymes.

Mandelstam & Jacoby (1965) and Stevenson & Mandelstam (1965) observed repression of the initial enzymes of the

Fig.43. P values for the induction of the other mandelate pathway enzymes plotted against P values for the induction of benzyl alcohol dehydrogenase.

In a series of independent experiments similar to Fig.36, p.167, the mandelate pathway enzymes of bacterium NCIB 8250 were induced by a number of compounds, which are listed below, and some of which, as indicated by the numbers in brackets, were used more than once. In each experiment the differential plot was drawn for each enzyme, and from the gradient of the graph the P value for the induction of the enzyme was calculated and used in the graph opposite.

1 - 5mM.-L-Mandelate (3).
2 - 5mM.-Benzoylformate (2).
3 - 1mM.-Thiophenoxyacetate.
4 - 5mM.-L-Mandelate + 5mM.-benzyl alcohol.
5 - 5mM.-L-Mandelate + 5mM.-acetate + 5mM.-succinate.
6 - 5mM.-Benzyl alcohol (2).
7 - 1mM.-Benzaldehyde.
8 - 5mM.-Benzyl alcohol + 5mM.-acetate + 5mM.-succinate.
9 - 1mM.-Benzoate.
10 - 1mM.-Catechol.

The correlation coefficients (r) for the various graphs are recorded on the graph to which they relate.

Fig.44. P values for the induction of catechol oxygenase plotted against P values for the induction of benzoate oxidase.

In a series of independent experiments similar to Fig.36, p.167, benzoate oxidase and catechol oxygenase of bacterium .CIB 8250 were induced by a number of compounds which are listed below. In each experiment the differential plot was drawn for each enzyme, and from the gradient of the graph the P value for the induction of the enzyme was calculated and used in the graph opposite.

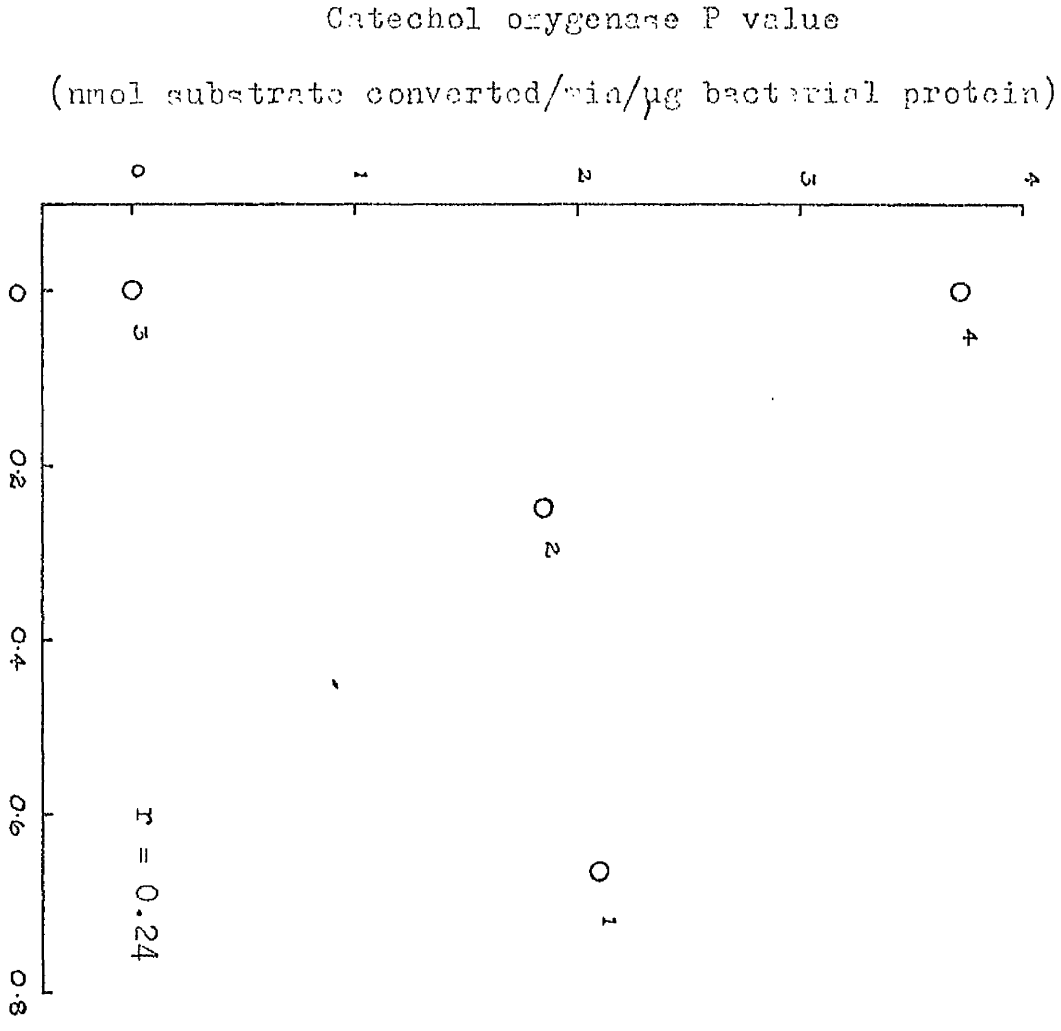
1 - 5mM-L-Mandelate.

2 - 5mM-Benzyl alcohol.

3 - 1mM-Thiophenoxyacetate.

4 - 1mM-Catechol.

The correlation coefficient (r) is recorded on the graph.


Catechol oxygenase P value
(nmol substrate converted/min/µg bacterial protein).
0
1
2
3
4
0
0·2
0·4
0·6
0·8
Benzoate oxidase P value
(nmol substrate converted/min/µg bacterial protein).
r = 0.24

mandelate pathway by intermediates of the pathway in P. putida. Although the present work was not intended to include a study of repression, it was decided to perform one experiment to see if similar effects could be produced in bacterium NCIB 8250. The prime aim of this experiment was to obtain additional sets of P values for the correlation experiments (Section 2.3.) and, as a bonus, to see if repression was operating in the mandelate pathway.

Table 18 shows the results of an experiment in which L-mandelate, L-mandelate + acetate + succinate, L-mandelate + benzyl alcohol, benzyl alcohol and benzyl alcohol + acetate + succinate were used as inducers of the mandelate pathway enzymes. The growth rate in the presence of benzyl alcohol is lower than in its absence, and the presence of acetate + succinate only marginally increases the growth rate. The presence of 5mM-acetate + 5mM-succinate reduces the P value for the induction of benzyl alcohol dehydrogenase by approximately 95%. This repression by acetate + succinate is less marked for the induction of L-mandelate dehydrogenase as the P value for this enzyme is only reduced by 60%. The presence of L-mandelate slightly represses the induction of benzyl alcohol dehydrogenase by benzyl alcohol.

Table 18. The effect of the presence of intermediates of the mandelate pathway on the P values for the induction of the mandelate pathway enzymes by benzyl alcohol or L-mandelate.

Bacterium NCIB 8250 was grown on 10mM-glutamate as sole source of carbon, harvested, washed, resuspended in basal medium, and inoculated into 10mM-glutamate-salts medium as described in Methods. The inducer, with and without potential repressor, to give a final concentration of 5mM in all cases in the glutamate-salts medium, was added after approximately 1 generation. Samples for enzyme assay were withdrawn from the growth flask, harvested, washed, stored, sonicated, and assayed as described in Methods. Growth was determined by taking samples for protein estimation as described in Methods, and from it the specific growth rate was calculated. The graph of increase in enzyme units/ml culture against increase in bacterial protein/ml culture was drawn for each enzyme and from the gradient of the graph the P value for the induction of the enzyme was calculated.

The specific growth rates are expressed as h^{-1}.
The P values for the induction of the enzymes are expressed as umol substrate converted/min/µg bacterial protein.

Enzyme	Inducer				
	L-Mandelate	L-Mandelate + Acetate + Succinate	L-Mandelate + Benzyl alcohol	Benzyl alcohol	Benzyl alcohol + Acetate + Succinate
L-Mandelate dehydrogenase	0.25	0.10	0.28	0	0
Benzyl alcohol dehydrogenase	0.32	0.01	0.43	0.55	0.03
Growth rate	0.38	0.43	0.29	0.30	0.33

3. ISOLATION, NUTRITIONAL VERSATILITY AND LEVELS OF THE MANDELATE PATHWAY ENZYMES IN MUTANT STRAINS OF BACTERIUM NCIB 8250.

3.1. Isolation of mutant strains of bacterium NCIB 8250.

The initial experiments were concerned with developing techniques for the isolation of mutants of bacterium NCIB 8250. Having achieved this, the next step was to obtain specific mutants of the mandelate pathway. To solve the various problems outlined in the Introduction, the specific mutants required were:

(1) constitutive mutants;

(2) organisms able to grow on benzoate or benzyl alcohol, but not on L-mandelate ("mandelate mutants");

(3) organisms able to grow on benzoate or L-mandelate, but not on benzyl alcohol ("benzyl alcohol mutants");

and (4) mutants unable to grow on benzoate, benzyl alcohol or L-mandelate ("benzoate mutants").

3.1.1. Development and application of mutagenic techniques.

3.1.1.1. Mutagenesis by ultraviolet irradiation followed by enrichment with penicillin V.

Since ultraviolet irradiation is one of the most common mutagenic treatments, it was chosen to induce mutation in bacterium NCIB 8250. Before using this technique, however, experimental conditions which were likely to give an optimal number of mutants had to be developed. Hegeman (1966b) and

Gorini & Kaufman (1960) showed that a 99.9% kill produced a satisfactory number of mutants in P. putida and E. coli respectively. In the absence of other information, it was decided to use the same kill in bacterium NCIB 8250.

A kill curve for ultraviolet irradiation using the Hanovia bactericidal lamp at a distance of 84cm from the culture was constructed (Fig.45). It shows that a 99.9% kill was achieved after 140s irradiation. Subsequently the cell suspension was irradiated for this time interval for the isolation of mutants.

It was decided to treat the irradiated culture with penicillin V in order to increase the percentage of the desired mutants in the bacterial population although penicillin V, as it interferes with cell wall synthesis, is chiefly applicable to Gram-positive bacteria, and has never been tested with bacterium NCIB 8250. However it has proved to be effective in the enrichment of Gram-negative organisms such as E. coli (Gorini & Kaufman, 1960) and P. aeruginosa (Loutit, 1955). The effect of penicillin concentration on the growth of bacterium NCIB 8250 is shown in Fig.46. From these data it was decided to use 1mg/ml penicillin in the enrichment procedure.

In three separate experiments using mutagenesis by ultraviolet irradiation followed by penicillin enrichment and the replica plating selection technique, an attempt was made to isolate blocked mutant strains of bacterium NCIB 8250. As shown in Table 20, although 9,600 organisms were screened, no mutants of the desired type were isolated. Mutants, however,

Fig. 45. Bactericidal effect of ultraviolet irradiation on bacterium NCIB 8250.

Bacterium NCIB 8250 was grown as described in Methods (p. 59). A viable count was made on the cell suspension in basal medium, 9ml of which was then irradiated with the ultraviolet lamp at a distance of 84cm from the lamp as described in Methods (p. 63). At various time intervals 0.1ml samples were withdrawn from the cell suspension for viable count determinations.

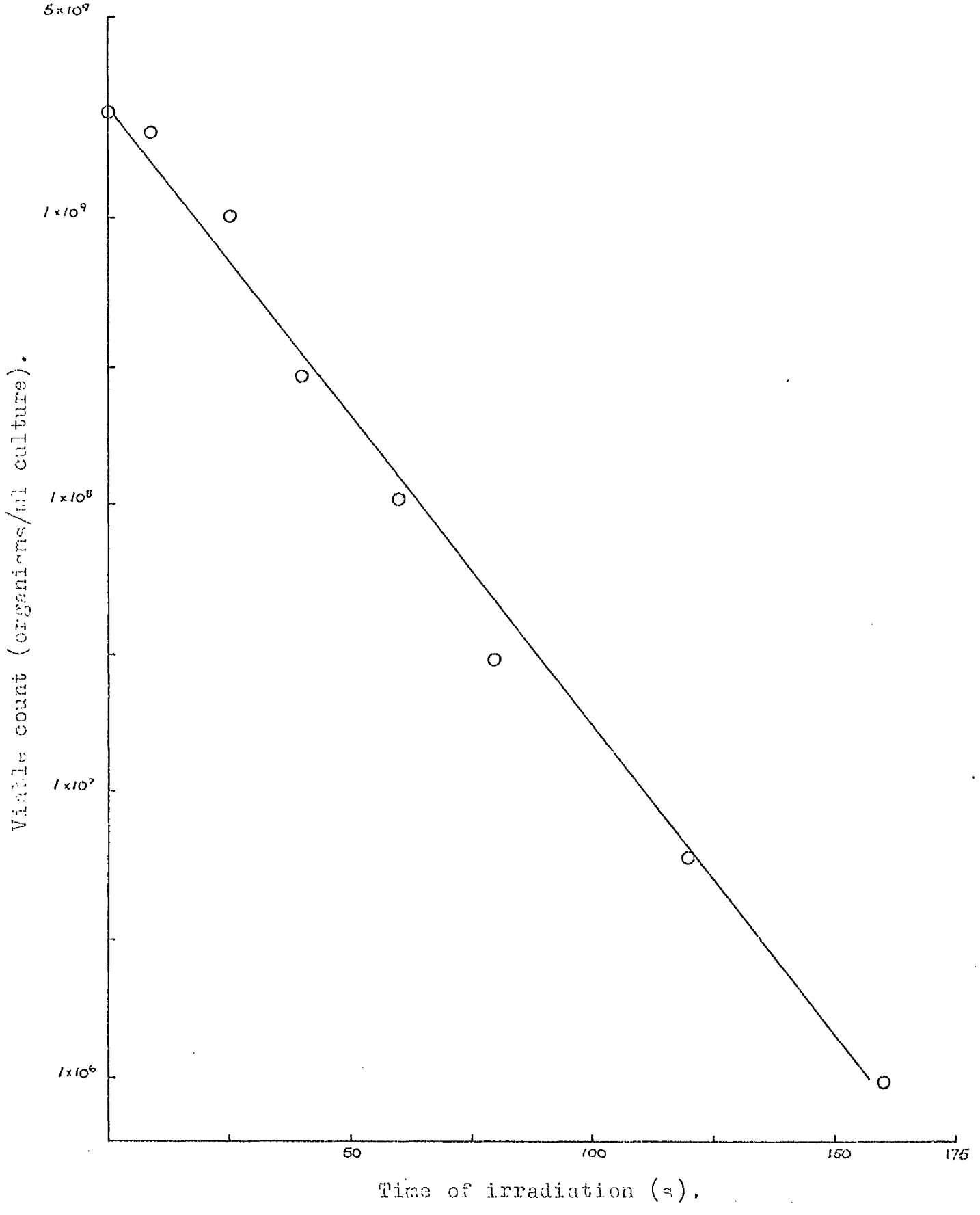
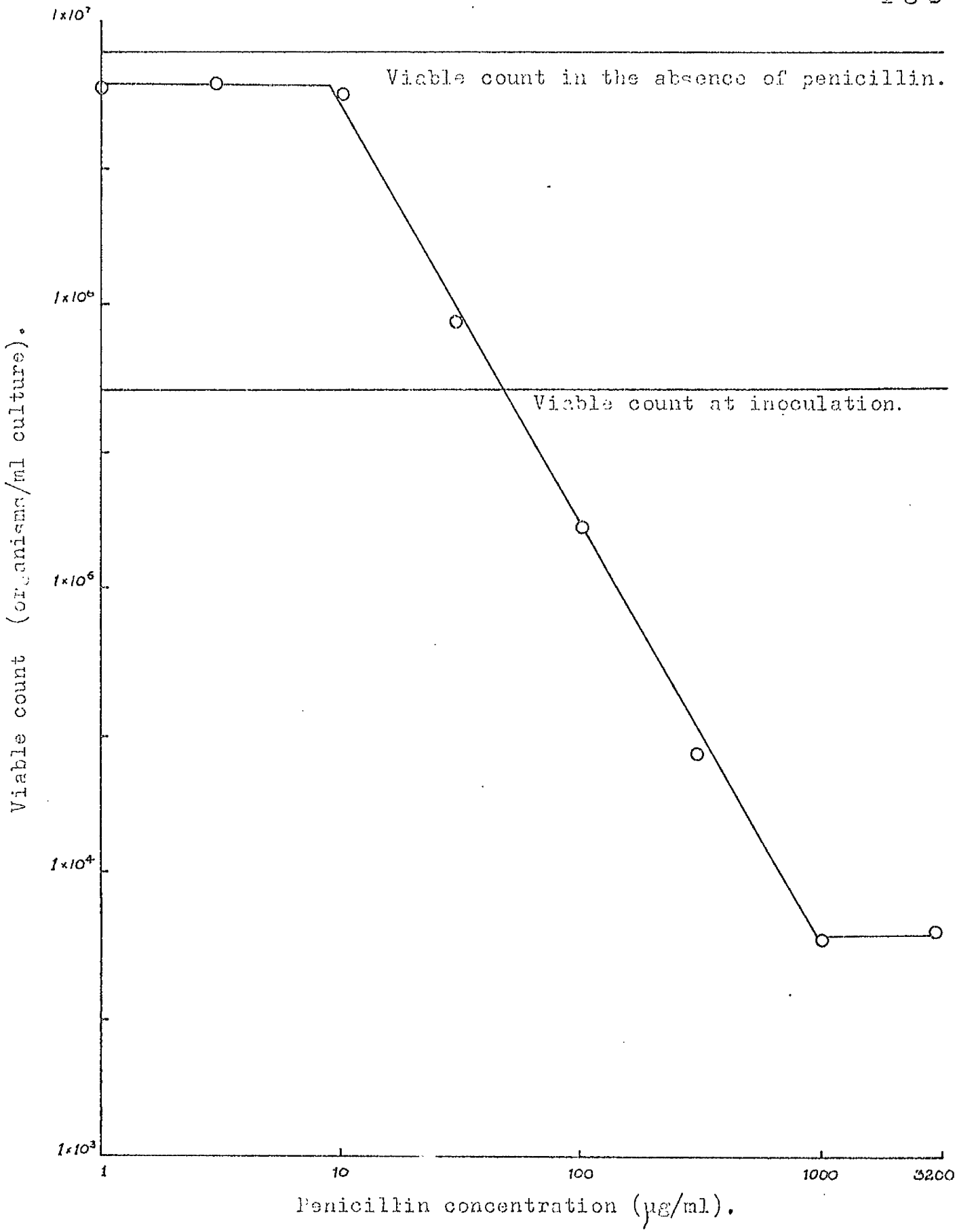

5×10⁹
1×10⁹
1×10⁸
1×10⁷
1×10⁶
Viable count (organisms/ml culture).
50
100
150
175
Time of irradiation (s).

Fig.46. The effect of penicillin V on the growth of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown as described in Methods (p.59) except that the cells were not harvested. A 1% inoculum was added to 50ml 10mM-succinate-salts media containing various concentrations of penicillin V. A viable count was made on a similar culture which contained no penicillin as described in Methods (p.62). The cultures were then grown for 4h at 30° at which time a viable count was made on each culture.


1×10⁷
1×10⁶
1×10⁶
1×10⁴
1×10³
Viable count in the absence of penicillin.
Viable count at inoculation.
Viable count (organisms/ml culture).
1
10
100
1000
3200
Penicillin concentration (µg/ml).

were probably produced as the viable count of cells treated with ultraviolet irradiation was about 25% less on succinate agar plates than on nutrient agar plates.

3.1.1.2. Mutagenesis by incubation with N.T.G.

Since ultraviolet irradiation did not produce any of the desired mutants, it was decided to induce mutagenesis with the more powerful mutagen N.T.G.. Adelberg et al.(1965) have shown that incubation of a culture of E. coli with this compound produced at least one mutation in the genome of every organism of the culture under conditions which gave a 50% kill. For bacterium NCIB 8250, however, it was decided to use conditions which gave a 99.9% kill since Cánovas & Stanier (1967) used these conditions to obtain mutants of A. calcoaceticus. The effect of incubating a suspension of bacterium NCIB 8250 with N.T.G. at a final concentration of 100µg/ml is shown in Table 19. Incubating the cells for 30min at 30° gave approximately a 99.9% kill, and this time interval was therefore used for the isolation of mutants.

In four separate experiments using mutagenesis with N.T.G. followed by the replica plating selection technique, 15 benzyl alcohol mutants, 7 benzoate mutants and 2 mandelate mutants were isolated (Table 20).

It was then decided to attempt the isolation of additional mandelate and benzoate mutants using the limiting nutrient selection technique. Enrichment of the desired mutants by Carboxy Q (a quinolone which has been shown by Fewson

Table 19. The effect of incubating a suspension of bacterium NCIB 8250 with N.T.G. at a final concentration of 100µg/ml.

Bacterium NCIB 8250 was grown as described in Methods (p.59). 9ml of the cell suspension was added to 1ml N.T.G. dissolved in 10mM-sodium citrate buffer pH 6.0 to give a final N.T.G. concentration of 100µg/ml. A viable count was made on the resulting suspension. The suspension was then incubated at 30° in a shaking water bath, 1ml samples were taken at 30min, 45min and 60min, and viable counts were made on them.

Incubation time (min)	No. of colonies	% kill
0	2.0×10^9	0
30	2.5×10^6	99.88
45	8.7×10^5	99.96
60	5.9×10^5	99.97

Table 20. The isolation of blocked mutant strains of bacterium NCIB 8250.

Blocked mutant strains of bacterium NCIB 8250 were isolated using the following procedures which are fully described in Methods (Section 2):

A. Mutagenesis by treatment with ultraviolet irradiation followed by the penicillin enrichment technique.

B. Mutagenesis by treatment with N.T.G. followed by the replica plating selection technique.

C. Mutagenesis by treatment with N.T.G. followed by the limiting nutrient selection technique, with 0.3mM-glutamate (Glu.) or 0.2mM-benzoate (Ben.) as the limiting nutrient.

D. Mutagenesis by treatment with N.T.G. followed by the Carboxy Q enrichment technique, and the limiting nutrient selection technique with 0.3mM-glutamate as the limiting nutrient.

Class of mutant	Mutagenic treatment	No. of organisms treated with mutagen	No. of colonies screened	No. of mutants isolated
Able to grow on benzoate or benzyl alcohol but not on L-mandelate	A	5.5×10^9	9,600	0
	B	8.5×10^9	17,000	2
	C-Glu.	8.7×10^9	656	1
	D-Glu.	8.7×10^9	310	1
	C-Ben.	6.4×10^8	21,000	12
Able to grow on benzoate or L-mandelate but not on benzyl alcohol	A	5.5×10^9	9,600	0
	B	6.3×10^9	9,000	15
Unable to grow on benzoate, L-mandelate, or benzyl alcohol	A	5.5×10^9	9,600	0
	B	6.3×10^9	9,000	7
	C-Glu.	8.7×10^9	656	33
	D-Glu.	8.7×10^9	310	30

(unpublished results) to have a slight bactericidal effect on growing cells of bacterium NCIB 8250) was carried out on one occasion. Other experiments were carried out with no enrichment. In the first two experiments 0.3mM-glutamate + 2mM-benzoate was used as the carbon and energy source in the selection procedure with 0.3mM-glutamate as the limiting nutrient. The mutants obtained in this experiment are shown in Table 20. Although 63 benzoate mutants were isolated, only 2 mandelate mutants were found. Thus, in order to obtain mandelate rather than benzoate mutants, it was decided to grow the mutated culture on 2mM-benzoate, and then spread it on agar plates containing 0.2mM-benzoate (as the limiting nutrient) + 5mM-L-mandelate to eliminate the benzoate mutants. 12 mandelate mutants were obtained in these experiments (Table 20).

3.1.1.3. The isolation of mutants constitutive for L-mandelate dehydrogenase.

Since the required mutants normally form only a very small proportion of the bacterial population under study, it was essential to develop a rapid screening procedure for their isolation. The screening procedure makes use of the fact that L-mandelate dehydrogenase activity can be detected visually by the coupled reduction of the dye, 2,6-dichlorophenol-indophenol. L-Mandelate dehydrogenase was assayed in suspected mutant organisms by destroying the permeability barrier of whole cells with a toluene-ethanol mixture, and then adding the dye and L-mandelate.

The alternate culture technique was used to enrich both spontaneous and N.T.G. induced mutants. 6 independent cultures were set up to enrich spontaneous mutants, and 3 to enrich N.T.G. induced mutants. This enrichment method is based on the presumed selective advantage of constitutive mutants, which do not lag, during the growth lag exhibited by the inducible wild type when transferred from a glutamate- to a L-mandelate-salts medium. After 10-15 cycles alternating between the two carbon and energy sources, the constitutive mutants should have outgrown the wild type. The presumptive enriched culture was spread on nutrient agar plates, and the colonies obtained were tested for a constitutive L-mandelate dehydrogenase after growth on a glutamate-salts medium. Table 21 shows that 42 constitutive mutants were isolated in this way.

3.1.2. The system used to classify the mutants.

The classification system used to designate the mutant strain numbers is summarised as follows:

(1) the letter N signifies mutagenesis by N.T.G.;

(2) the letter C signifies spontaneous mutation;

(3) the second letter, if applicable, refers to the experiment in which the mutant was isolated;

(4) in a one or two figure number, the number represents the chronological order in which the mutant was isolated;

(5) in a four figure number, the first two figures represent the number of the agar plate on which the mutant was situated, and the second two figures the position of the mutant on the

Table 21. The isolation of constitutive mutant strains of bacterium NCIB 8250.

Constitutive mutant strains of bacterium NCIB 8250 were isolated using the following procedures which are fully described in Methods (Section 2):

A. Mutagenesis by treatment with N.T.G. followed by the alternate culture enrichment technique, and selection by means of the L-mandelate dehydrogenase whole cell assay.

B. Spontaneous mutation followed by the alternate culture enrichment technique, and selection by means of the L-mandelate dehydrogenase whole cell assay.

Class of mutant	Mutagenic treatment	No. of organisms treated with mutagen	No. of colonies screened	No. of mutants isolated
Constitutive for L-mandelate dehydrogenase	A	8.7×10^9	420	21
	B	not counted	560	21

plate, in cases where the isolated mutant was further tested before cloning;

and (6) in a five figure number, the first two figures represent the number of the alternate culture experiment, and the last three figures the chronological order in which the mutant was tested for a constitutive L-mandelate dehydrogenase.

3.1.3. The choice of mutants for further study.

All the mutants isolated were characterised as bacterium NCIB 8250 by the tests shown in Table 2. Some of the blocked mutants showed signs of reversion or leakiness, and were not examined further. The mutants used in the rest of the experiments described in Section 3 gave the least indications of leakiness or reversion. Preliminary experiments showed that all the constitutive mutants contained much less L-mandelate dehydrogenase when grown under non-inducing conditions than did the wild type when grown on L-mandelate. Thus these organisms represent meso-constitutive strains according to the phenotypic classification and terminology for enzyme mutants in micro-organisms suggested by Collins, Mandelstam, Pollock, Richmond & Sneath (1965). NE04071, NE02270 and C04211, the three strains which contained the highest levels of L-mandelate dehydrogenase, were used in subsequent experiments.

3.2. Nutritional versatility of the mutant strains.

All the mandelate, benzyl alcohol and constitutive mutants were tested for growth on L-mandelate, benzoylformate,

benzyl alcohol, benzaldehyde and benzoate, and the majority of them on 4-hydroxy-D,L-mandelate, 4-hydroxy-3-methoxybenzaldehyde, 2-hydroxybenzyl alcohol and 4-hydroxybenzyl alcohol. The benzoate mutants were tested for growth on benzyl alcohol, 2-hydroxybenzyl alcohol, benzoate, 2-hydroxybenzoate, catechol and succinate. In order to simplify the presentation of the results, compounds which gave the same growth pattern for the wild type and mutants were omitted from Tables 22 to 25.

In some cases growth is shown as "delayed". In these cases the mutants had failed to grow by the time the wild type was fully grown; but did subsequently grow - sometimes after several days.

3.2.1. The nutritional versatility of the mandelate mutants.

The nutritional versatility of the mandelate mutants is shown in Table 22. These mutants can be divided into two classes: those which grow on benzoylformate, and those which do not. Both classes of mutants, however, fail to grow normally on L-mandelate and 4-hydroxy-D,L-mandelate. Growth of all the organisms on benzyl alcohol, 2-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, benzaldehyde, 4-hydroxy-3-methoxybenzaldehyde or benzoate is similar to that of the wild type.

3.2.2. The nutritional versatility of the constitutive mutants.

The nutritional versatility of the constitutive mutants is shown in Table 23. The constitutive mutants show no or

Table 22. The nutritional versatility of mutant strains of bacterium NCIB 8250 which do not grow normally on L-mandelate, but do on benzyl alcohol or benzoate.

The wild type organism and the mutant strains of bacterium NCIB 8250 were inoculated into basal salts media containing the various growth substrates at two concentrations using a 0.2% unwashed nutrient broth culture as inoculum as described in Methods (p.60). Growth was determined at suitable time intervals after inoculation as described in Methods (p. 60). Since growth was similar for both concentrations of growth substrate, only one result has been recorded in the Table.

-. - no growth.

+. - delayed growth.

++. - normal growth.

Organism	Growth substrate Concn.(mM)					
	L-Mandelate		4-Hydroxy-D,L-mandelate		Benzoylformate	
	1	2	2	4	1	2
Wild type	++		++		++	
MF0113	+		not tested.		-	
MA36 MF2405	-		-		-	
MF1706	-		+		-	
MF1202 MF1408	-		not tested.		++	
MF0410	-		-		++	

Table 23. The nutritional versatility of constitutive mutant strains of bacterium NCIB 8250.

The wild type organism and the mutant strains of bacterium NCIB 8250 were inoculated into basal salts media containing the various growth substrates at two concentrations using a 0.2% unwashed 24h nutrient broth culture as inoculum as described in Methods (p.60). Since growth was similar for both concentrations of growth substrate, only one result has been recorded in the Table.

-. - no growth.

+. - delayed growth.

++. - normal growth.

Organism	Growth substrate Concn.(mM)					
	L-Mandelate		4-Hydroxy-D,L-mandelate		Benzoylformate	
	1	2	2	4	1	2
Wild type	++		++		++	
KE04071 KE02270	+		not tested		+	
C04211	+		-		+	

delayed growth on L-mandelate, 4-hydroxy-D,L-mandelate and benzoylformate. Growth of the strains on benzyl alcohol, 2-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, benzaldehyde, 4-hydroxy-3-methoxybenzaldehyde or benzoate is similar to that of the wild type.

3.2.3. The nutritional versatility of the benzyl alcohol mutants.

The nutritional versatility of the benzyl alcohol mutants is shown in Table 24. One mutant, NB7, is clearly different from the other two as it does not grow on any 4-hydroxy-substituted compound. Strain NB7 also does not grow or gives delayed growth on benzyl alcohol, 2-hydroxybenzyl alcohol, benzaldehyde and 4-hydroxy-3-methoxybenzaldehyde. The other two mutants, N2 and NA37, grow normally on 1mM-benzaldehyde, 4-hydroxy-D,L-mandelate or 4-hydroxybenzoate; but show no or delayed growth on benzyl alcohol, 2-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, 2mM-benzaldehyde and 4-hydroxy-3-methoxybenzaldehyde. Growth of the three mutants on L-mandelate, benzoylformate or benzoate is similar to that of the wild type.

3.2.4. The nutritional versatility of the benzoate mutants.

The nutritional versatility of the benzoate blocked mutants is shown in Table 25. The benzoate mutants grow on 2-hydroxybenzoate or catechol, but not on benzoate. Growth of the strains on 2-hydroxybenzyl alcohol or succinate is similar to that of the wild type. Although it is not shown in Table 25, the mutants did not grow on benzyl alcohol.

Table 24. The nutritional versatility of mutant strains of bacterium NCIB 8250 which do not grow normally on benzyl alcohol, but do on L-mandelate or benzoate.

The wild type organism and the mutant strains of bacterium NCIB 8250 were inoculated into basal salts media containing the various growth substrates at two concentrations using a 0.2% unwashed nutrient broth culture as inoculum as described in Methods (p.60). Growth was determined at suitable time intervals after inoculation as described in Methods (p.60). Where growth was similar for both concentrations of growth substrate, only one result has been recorded in the Table.

-. - no growth.

+. - delayed growth.

++. - normal growth.

Organism	Growth substrate Conc.(mM)							
	4-Hydroxy-D,L-mandelate		Benzaldehyde		4-Hydroxy-3-methoxy-benzaldehyde		Benzyl alcohol	
	2	4	1	2	1	2	1	2
Wild type	++		++		++		++	
M2	++		++	+	-		+	
LA37	++		++	+	-		+	
MB7	-		+		-		+	

Organism	Growth substrate Conc.(mM)							
	2-Hydroxy-benzyl alcohol		4-Hydroxy-benzyl alcohol		4-Hydroxy-benzoate			
	1	2	1	2	1	2		
Wild type	++		++		++			
M2	-		-		++			
LA37	-		+		++			
MB7	-		-		-			

Table 25. The nutritional versatility of mutant strains of bacterium NCIB 8250 which do not grow on L-mandelate, benzyl alcohol, or benzoate.

The wild type organism and the mutant strains of bacterium NCIB 8250 were inoculated into basal salts media containing the various growth substrates at two concentrations using a 0.2% unwashed 24h nutrient broth culture as inoculum as described in Methods (p.60). Growth was determined at suitable time intervals after inoculation as described in Methods (p.60). Since growth was similar for both concentrations of growth substrate, only one result has been recorded in the Table.

-. - no growth.

++. - normal growth.

Organism	Growth substrate Concn.(mM)					
	Benzoate		2-Hydroxy-benzoate		Catechol	
	1	2	1	2	1	2
Wild type	++		++		++	
NA16 NA18 NA21	-		++		++	

3.2.5. Presumed enzyme lesions of the blocked mutants.

Table 26 summarises the nutritional versatility of the blocked mutants from the data given in Tables 22, 24 and 25, and gives their presumed enzyme lesions. Benzaldehyde was not included in the summary as it gave equivocal results with the benzyl alcohol mutants (Table 24).

3.3. Levels of the mandelate pathway enzymes found in non-induced cells of the constitutive mutants.

The levels of the mandelate pathway enzymes in non-induced cells of the constitutive mutants grown on glutamate were determined. The growth rate and enzyme content of the three constitutive mutant strains, NE04071, NE02270 and C04211, were similar, and consequently only the results obtained for strain C04211 are documented in full.

The two growth rates of the mutant organism on 8mM-glutamate are similar to those of the wild type (Fig.47). Samples were taken for enzyme assay during the last generation at the times indicated by the arrows. As the values for the specific activity of the enzymes in the three samples taken from the culture of the mutant organism were similar, and in the case of the wild type between two separate experiments in which three samples were taken each time, the values for the specific activity of a particular enzyme were averaged, and it is this mean value which is the figure quoted in Experiment B, Table 27.

Of the three enzymes of Regulon R1, only L-mandelate

Table 26. Summary of the nutritional versatility of the blocked mutant strains of bacterium NCIB 8250 and their presumed enzyme lesions.

The blocked mutant strains of bacterium NCIB 8250 were inoculated into basal salts media containing the various growth substrates at two concentrations using a 0.2% unwashed 24h nutrient broth culture as inoculum as described in Methods (p.60). Growth was determined at suitable time intervals after inoculation as described in Methods (p.60).

-. - no growth or delayed growth.

+. - normal growth.

n.t. - not tested.

MDH - L-Mandelate dehydrogenase.

BFD - Benzoylformate decarboxylase.

BZD - Labile benzaldehyde dehydrogenase.

POB - One of the enzymes of the 4-hydroxybenzoate pathway.

BO - Benzoate oxidase.

Growth substrate	No. of mutants				
	3	4	2	1	3
L-Mandelate	-	-	+	+	n.t.
4-Hydroxy-D,L-mandelate	-	-	+	-	n.t.
Benzoylformate	+	-	+	+	n.t.
4-Hydroxy-3-methoxybenzaldehyde	+	+	-	-	n.t.
Benzyl alcohol	+	+	-	-	-
2-Hydroxybenzyl alcohol	+	+	-	-	n.t.
4-Hydroxybenzyl alcohol	+	+	-	-	n.t.
Benzoate	+	+	+	+	-
2-Hydroxybenzoate	n.t.	n.t.	n.t.	n.t.	+
4-Hydroxybenzoate	n.t.	n.t.	+	-	n.t.
Catechol	n.t.	n.t.	n.t.	n.t.	+
Presumed lesion	MDH	BFD	BZD	BZD & PCB	BO

Fig. 47. Growth of the wild type organism and the constitutive mutant strain C04211 on 8mM-glutamate-salts medium.

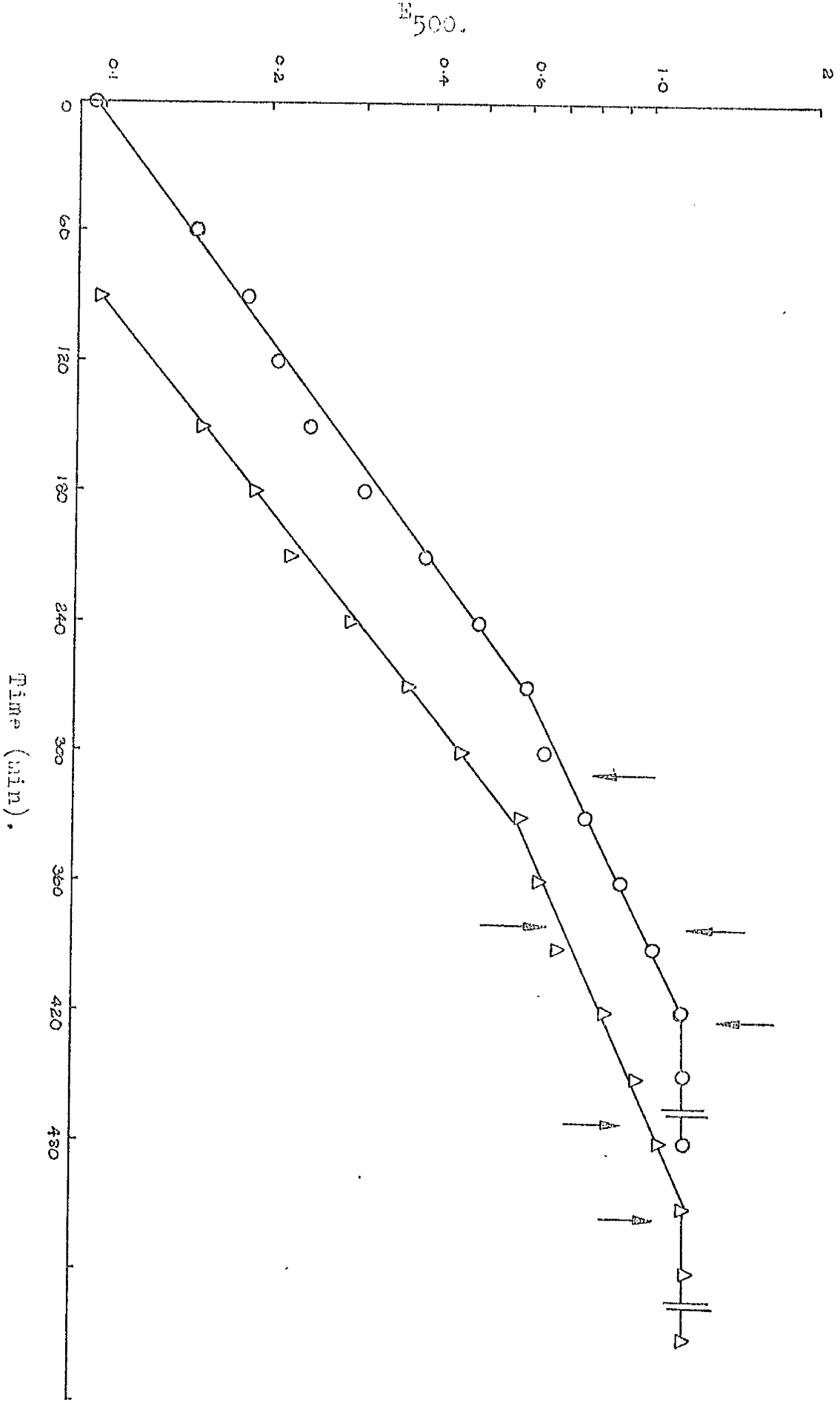
The wild type organism and the constitutive mutant strain C04211 of bacterium NCIB 8250 were inoculated into 8mM-glutamate-salts medium using a 10% unwashed 15h culture grown on 8mM-glutamate-salts medium as inoculum as described in Methods (p.60). Growth and specific growth rate were determined as described in Methods (p.53).

○ - wild type
growth rate - 0.40h^{-1} changing to 0.26h^{-1}.

△ - mutant strain C04211
growth rate - 0.45h^{-1} changing to 0.25h^{-1}.

The time scale of the growth curve of the mutant strain is displaced through 90 min.

↑ - Samples removed for enzyme assay (Table 27.).


E_{500}.
0·1
0·2
0·4
0·6
1·0
2
0
60
120
180
240
300
360
420
480
Time (min).

dehydrogenase and benzoylformate decarboxylase are synthesised constitutively (Table 27). These two enzymes, however, are present in only meso-constitutive amounts since the levels of these enzymes in Experiment A are only about 4% of the levels found in the wild type induced with L-mandelate (Table 28) in a comparable experiment. Although the level of the stable benzaldehyde dehydrogenase is higher in the mutant than in the wild type in Experiment B, it is lower in Experiment A, and taking the average percentage increase over the two experiments as 16%, the enzyme is probably not constitutively synthesised.

3.4. Levels of the mandelate pathway enzymes found in induced cells of various strains of bacterium NCIB 8250.

Since a study of the induction of the mandelate pathway enzymes in the various classes of mutants permits a more refined analysis of the control mechanisms operating in the pathway, the wild type and the blocked and constitutive mutants described in Tables 22 to 24 were induced with L-mandelate, benzoylformate, benzyl alcohol and benzaldehyde.

3.4.1. The wild type.

The levels of the mandelate pathway enzymes in the wild type growing on glutamate supplemented with inducer are shown in Table 28. L-Mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase are induced by L-mandelate or benzoylformate. Low levels of the labile benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase are also induced by L-mandelate or benzoylformate.

Table 27. A comparison of the levels of the mandelate pathway enzymes found in extracts of the wild type organism with the levels found in extracts of the constitutive mutant strain.

Experiment A. The wild type organism and the constitutive mutant strain C04211 of bacterium NCIB 8250 were grown for 2½h on 10mM-glutamate-salts medium as described in Methods (p.61). A 2% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods.

Experiment B. The wild type organism and the constitutive mutant strain C04211 of bacterium NCIB 8250 were grown into the last generation of exponential growth on 8mM-glutamate-salts medium as described in Methods (p.60). A 1% unwashed 15h culture grown on 8mM-glutamate-salts medium was used as inoculum. Samples were then withdrawn from the growth flasks at the times indicated on Fig.47 p.199, harvested, washed, stored, sonicated, and assayed as described in Methods.

Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.
The figures in parenthesis for Experiment A represent the number of experiments on which the specific activity is based.
The figures in parenthesis for Experiment B represent the number of samples on which the specific activity is based.

Enzyme	Experiment A			Experiment B		
	Wild type specific activity	CC4211 specific activity	% of wild type	Wild type specific activity	CC4211 specific activity	% of wild type
L-Mandelate dehydrogenase	1.5 (5)	7.6 (4)	524	0.7 (6)	5.4 (3)	730
Benzoylformate decarboxylase	4.4 (5)	6.9 (4)	157	0.7 (3)	3.7 (3)	500
Stable benzaldehyde dehydrogenase	6.3 (5)	5.4 (4)	86	1.8 (6)	2.6 (3)	144
Labile benzaldehyde dehydrogenase	2.4 (5)	2.3 (4)	96	1.4 (6)	2.0 (3)	144
Benzyl alcohol dehydrogenase	7.4 (5)	7.6 (4)	103	4.2 (6)	5.5 (3)	131
Catechol oxygenase	3.8 (5)	3.9 (4)	103	1.3 (6)	1.6 (3)	119

Table 28. The levels of the mandelate pathway enzymes found in extracts of the wild type organism growing on glutamate-salts medium supplemented with inducer.

Bacterium NCIB 8250 was grown for 2½h on 10mM-glutamate-salts medium supplemented with 5mM-L-mandelate, 5mM-benzoylformate, 5mM-benzyl alcohol, 1mM-benzaldehyde, or no inducer as described in Methods (p.61). A 2% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

* The values for the specific activity of the enzymes when no inducer is present are the average values obtained in Experiment A., Table 27, p.201.

Enzyme	Inducer concentration				
	L-Mandelate 5mM	Benzoylformate 5mM	Benzyl alcohol 5mM	Benzaldehyde 1mM	No* inducer present
L-Mandelate dehydrogenase	178	180	2.6	1.8	1.5
Benzoylformate decarboxylase	198	272	4.3	1.2	4.4
Stable benzaldehyde dehydrogenase	27	34	0	0	6.3
Labile benzaldehyde dehydrogenase	14	14	324	126	2.4
Benzyl alcohol dehydrogenase	18	18	233	143	7.4
Catechol oxygenase	372	208	233	214	3.8
NADH oxidase	122	111	101	111	120

Benzyl alcohol and benzaldehyde induce benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase, but not L-mandelate dehydrogenase, benzoylformate decarboxylase or the stable benzaldehyde dehydrogenase.

3.4.2. Mandelate mutant strains NF1202, NF1408 and NF0410.

Only the results for strain NF0410 will be given in detail as the three strains gave similar patterns of induction. The levels of the mandelate pathway enzymes in strain NF0410 which is presumed to lack L-mandelate dehydrogenase (Table 26) are shown in Table 29. The results confirm this presumption in that no L-mandelate dehydrogenase activity is induced under any experimental condition. Benzoylformate induces amounts of the other enzymes comparable with those of the wild type. L-Mandelate, however, has hardly any inducing power. As expected from the data obtained from the nutritional versatility of this strain, benzyl alcohol and benzaldehyde give normal patterns of induction.

3.4.3. Mandelate mutant strains NF2405, NF0113, NF1706 and NA36.

As the properties of strains NF2405 and NF0113 were similar, only the results obtained for strain NF2405 will be quoted in full. The levels of the mandelate pathway enzymes in strains NF1706, NA36 and NF2405, which are presumed to lack benzoylformate decarboxylase (Table 26), are shown in Table 30. The results confirm this suggestion in that there is very little benzoylformate decarboxylase activity present under any experimental condition.

Table 29. The levels of the mandelate pathway enzymes found in extracts of the mutant strain NF0410 growing on glutamate-salts medium supplemented with inducer.

Mutant strain NF0410 of bacterium NCIB 8250 was grown for 2½h on 10mM-glutamate-salts medium supplemented with 5mM-L-mandelate, 5mM-benzoylformate, 5mM-benzyl alcohol, 1mM-benzaldehyde, or no inducer as described in Methods (p.61). A 2% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Inducer concentration				
	L-mandelate 5mM	benzoylformate 5mM	Benzyl alcohol 5mM	Benzaldehyde 1mM	No inducer present
L-Mandelate dehydrogenase	0	0	0	0	0
Benzoylformate decarboxylase	9.0	224	6.5	0.9	7.5
Stable benzaldehyde dehydrogenase	2.0	28	0	0	7.0
Labile benzaldehyde dehydrogenase	8.0	15	292	155	1.8
Benzyl alcohol dehydrogenase	7.4	23	246	217	6.1
Catechol oxygenase	1.4	177	194	223	1.0
NADH oxidase	132	121	121	119	137

Table 30. The levels of the mandelate pathway enzymes found in extracts of the mutant strains NF1706, NA36, and NF2405 growing on glutamate-salts medium supplemented with inducer.

Mutant strains NF1706, NA36, and NF2405 of bacterium NCIB 8250 were grown for 2½h on 10mM-glutamate-salts medium supplemented with 5mM-L-mandelate, 5mM-benzoylformate, 5mM-benzyl alcohol, 1mM-benzaldehyde, or no inducer as described in Methods (p. 61). A 2% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Mutant strain NF1706					Mutant strain NA36					Mutant strain NF2405				
	Inducer concentration					Inducer concentration					Inducer concentration				
	L-Mandelate 5mM	Benzoylformate 5mM	Benzyl alcohol 5mM	Benzaldehyde 1mM	No inducer present	L-Mandelate 5mM	Benzoylformate 5mM	Benzyl alcohol 5mM	Benzaldehyde 1mM	No inducer present	L-	Benzoylformate 5mM	Benzyl alcohol 5mM	Benzaldehyde 1mM	No inducer present
L-Mandelate dehydrogenase	198	224	2.9	1.4	1.4	165	195	2.2	1.3	2.0		5.4	2.7	3.3	1.9
Benzoylformate decarboxylase	6.0	4.9	0.9	0	1.1	2.5	3.6	1.7	0.3	3.2		1.7	0.9	0.3	1.5
Stable benzaldehyde dehydrogenase	36	37	0	1.4	2.1	40	56	6.4	0	1.7		4.3	5.3	0.9	1.1
Labile benzaldehyde dehydrogenase	157	385	304	102	3.5	21	17	224	182	8.9		239	129	42	2.3
Benzyl alcohol dehydrogenase	235	455	428	103	6.0	22	24	236	177	12		303	191	96	4.6
Catechol oxygenase	37	35	143	221	0.8	15	57	233	283	3.0		65	157	148	1.9
NADH oxidase	69	78	79	56	98	47	82	88	95	87		93	99	62	95

Strains NF1706 and NA36, however, differ from strain NF2405 because they contain normal amounts of L-mandelate dehydrogenase and the stable benzaldehyde dehydrogenase; whereas in strain NF2405 there is only negligible induction of these two enzymes. Another feature of these organisms is that in strains NF1706 and NF2405 L-mandelate and benzoylformate hyperinduce benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase compared with the wild type levels. (These organisms represent inducible-hyper strains according to the phenotypic classification and terminology suggested by Collins et al.(1965)). In comparison with these observations, strain NA36 has levels of the Regulon R_2 enzymes similar to those of the wild type when induced with L-mandelate or benzoylformate. Low levels of catechol oxygenase are induced in the three strains by L-mandelate or benzoylformate.

3.4.4. Benzyl alcohol mutant strains N2, NA37 and NB7.

Only the results for strain NA37 will be documented in full as the three strains gave the same pattern of induction. The levels of the mandelate pathway enzymes in strain NA37, which is presumed to lack the labile benzaldehyde dehydrogenase (Table 26), are shown in Table 31. The results confirm this presumption in that there are only minute amounts of the labile benzaldehyde dehydrogenase present under any experimental condition. This mutant also contains little benzyl alcohol dehydrogenase activity. L-Mandelate and benzoylformate induce normal levels of the Regulon R_1 enzymes. The levels of catechol

Table 31. The levels of the mandelate pathway enzymes found in extracts of the mutant strain NA37 growing on glutamate-salts medium supplemented with inducer.

Mutant strain NA37 of bacterium NCIB 8250 was grown for 2½h on 10mM-glutamate-salts medium supplemented with 5mM-L-mandelate, 5mM-benzoylformate, 5mM-benzyl alcohol, 1mM-benzaldehyde, or no inducer as described in Methods (p.61). A 25% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Inducer concentration				
	L-Mandelate 5mM.	Benzoylformate 5mM	Benzyl alcohol 5mM.	Benzaldehyde 1mM.	No inducer present
L-Mandelate dehydrogenase	221	225	4.8	2.8	2.0
Benzoylformate decarboxylase	176	212	4.0	4.3	2.4
Stable benzaldehyde dehydrogenase	36	41	3.8	5.0	2.2
Labile benzaldehyde dehydrogenase	4.7	2.2	5.6	6.3	1.5
Benzyl alcohol dehydrogenase	7.0	8.6	10	9.7	5.8
Catechol oxygenase	277	231	91	190	0.6
NADH oxidase	140	110	110	127	109

oxygenase induced by L-mandelate or benzoylformate in this organism are similar to the levels found in the wild type. Benzyl alcohol and benzaldehyde, on the other hand, induce lower levels of this enzyme.

3.4.5. The constitutive mutant strains C04211, NE04071 and NE02270.

As the three strains gave similar patterns of induction, only the results obtained for strain C04211 will be given in detail. The levels of the mandelate pathway enzymes in strain C04211 are shown in Table 32. In this organism the meso-constitutive levels of L-mandelate dehydrogenase and benzoylformate decarboxylase found in non-induced cells (Table 27) are not appreciably altered by the presence of L-mandelate or benzoylformate. On the other hand, the stable benzaldehyde dehydrogenase is hyperinduced by L-mandelate or benzoylformate compared with the wild type level. As observed in strains NF1706 and NF2405 (Table 30), benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase are hyperinduced by L-mandelate or benzoylformate in this organism. Benzyl alcohol and benzaldehyde give the normal induction patterns. Appreciable amounts of catechol oxygenase are induced by L-mandelate or benzoylformate.

3.4.6. Levels of the mandelate pathway enzymes induced by thiophenoxyacetate in the wild type, in the constitutive mutant C04211 and in the mandelate mutant NF1706.

L-Mandelate or benzoylformate hyperinduce the Regulon R_2 enzymes in mutant strains NF1706 (Table 30) and C04211 (Table

Table 32. The levels of the mandelate pathway enzymes found in extracts of the constitutive mutant strain C04211 growing on glutamate-salts medium supplemented with inducer.

The constitutive mutant strain C04211 of bacterium NCIB 8250 was grown for 2½h on 10mM-glutamate-salts medium supplemented with 5mM-L-mandelate, 5mM-benzoylformate, 5mM-benzyl alcohol, 1mM-benzaldehyde, or no inducer as described in Methods (p.61). A 2% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Inducer concentration				
	L-mandelate 5mM	benzoyl-formate 5mM	Benzyl alcohol 5mM	Benzal-dehyde 1mM	No inducer present
L-mandelate dehydrogenase	4.7	5.1	3.6	3.3	7.6
Benzoylformate decarboxylase	9.7	9.7	6.2	3.5	6.9
Stable benzaldehyde dehydrogenase	129	129	3.0	0	6.0
Labile benzaldehyde dehydrogenase	129	399	223	100	2.2
Benzyl alcohol dehydrogenase	207	554	227	145	7.6
Catechol oxygenase	157	94	176	131	2.8
NADH oxidase	128	125	129	108	132

32). Since this induction could be brought about by these compounds themselves or alternatively, by a compound formed from their metabolism, it was decided to examine the effect of thiophenoxyacetate, a non-metabolisable inducer of the Regulon R_1 enzymes, on the induction of the Regulon R_2 enzymes in these organisms. Table 33 shows that thiophenoxyacetate induces only the Regulon R_1 enzymes in each of the three organisms; thiophenoxyacetate induces neither the Regulon R_2 enzymes nor catechol oxygenase.

Table 33. The levels of the mandelate pathway enzymes found in extracts of the wild type organism, and the mutant strains C04211, and NF1706 growing on glutamate-salts medium supplemented with 1mM-thiophenoxyacetate.

The wild type organism, the constitutive mutant strain C04211, and the benzoylformate decarboxylaseless mutant strain NF1706 of bacterium NCIB 8250 were grown for 2½h on 10mM-glutamate-salts medium supplemented with 1mM-thiophenoxyacetate as described in Methods (p. 61). A 2% unwashed 12-14h culture grown on 10mM-glutamate-salts medium was used as inoculum. The cells were harvested, washed, stored, sonicated, and assayed as described in Methods. Values for the specific activity of the enzymes are expressed as nmol substrate converted/min/mg protein.

Enzyme	Organism		
	Wild type	CC4211	MF1706
L-Mandelate dehydrogenase	113	6.2	97
Benzoylformate decarboxylase	122	5.8	3.5
Stable Benzaldehyde dehydrogenase	26	33	28
Labile Benzaldehyde dehydrogenase	0	0	0
Benzyl alcohol dehydrogenase	9.8	8.4	6.0
Catechol oxygenase	1.8	2.6	1.8
NADH oxidase	101	130	116

D I S C U S S I O N.

1. DEVELOPMENTAL WORK.

The raison d'être for this thesis was to determine how bacterium NCIB 8250 controls the metabolism of the large number of compounds which it can utilise for growth (e.g. Fig.10), and in particular how this organism regulates the enzymes involved in the aromatic part of the mandelate pathway (Fig.8). At the beginning of this project, the degree to which these enzymes are coordinately controlled was not well understood (Kennedy & Fewson, 1968a,b) so that it was impossible to estimate the activity of a group of enzymes just by measuring the activity of a single enzyme. Indeed the first main object of the experiments on control was to establish the extent of the coordinacy of the mandelate pathway enzymes. Consequently all the enzymes of the pathway had to be measured if worthwhile deductions were to be made from the experiments on regulation. Thus the first priority was to develop convenient assays for as many as possible of the enzymes of the pathway. In addition, NADH oxidase activity was routinely measured as it provided a simple means of gauging the effect of the various inducing conditions on an enzyme not specifically associated with the mandelate pathway.

Rapid, sensitive, accurate and reproducible spectrophotometric assays were obtained for L-mandelate

dehydrogenase, benzyl alcohol dehydrogenase, NADH oxidase, catechol oxygenase (p.128), benzoylformate decarboxylase (p.118), and the labile and stable benzaldehyde dehydrogenase (p.107). Only with benzoate oxidase did the development of a spectrophotometric assay prove impracticable (p.127). This was largely because the cell-free activity obtained with this enzyme was too low to be of use as a starting-point for the development of a spectrophotometric assay. The work on this enzyme was abandoned although P.J. Roach (Fewson et al., 1970) subsequently worked out a method for estimating enzyme activity in whole cells, and this method was used in some experiments.

Having worked out spectrophotometric assays for all but one of the enzymes, a technique was developed for their measurement in a single extract. This was done because of the economy involved (cf. p.48) and the increased flexibility it would allow in experiments on control. The development of this technique is described rather fully in this thesis (Results, Section 1.) since, although the various steps taken followed one another in a logical fashion, it was a process of successive approximation, and the reasons for using the precise conditions would not be understood without a knowledge of the way in which the procedure was developed.

Initially, the chief problem in the accurate measurement of the two benzaldehyde dehydrogenases was to obtain an extract whose pH was high enough to ensure a rate of heat inactivation of the labile enzyme which would reduce its activity to

negligible amounts in 2h at 37° (Table 4, and p.103) when the cells were extracted in the standard extraction buffer (0.04M-Tris-HCl). This situation was made more difficult by the fact that the actual pH of the extract was lower than the pH of the extraction buffer due to the buffering capacity of the cells' cytoplasm (Tables 4 and 5). A similar buffering capacity has been observed with E. coli (Kashket & Wong, 1969). In practice it was found that, using Tris buffer at the highest pH possible (pH 10.3), the actual pH of the extract was never higher than about 9.3. At this pH the rate of heat inactivation of the labile enzyme in extracts prepared in 0.04M-Tris buffer was too low to measure a total benzaldehyde dehydrogenase activity greater than 80 units/mg protein (Table 4). This was because there might have been detectable amounts of the labile enzyme left after 120min denaturation, and thus any residual activity could be due to either the stable or labile enzyme. When this occurred, the cells were extracted in sodium pyrophosphate buffer. The need for a separate extraction of benzaldehyde dehydrogenase in sodium pyrophosphate buffer, if its activity is greater than 80 units/mg protein, is the chief drawback of using Tris-HCl as the extraction buffer. The alternative is to use sodium pyrophosphate as the extraction buffer for all the enzymes, but this lowers the activity of benzoylformate decarboxylase to an unacceptable level (Table 7). In practice, this limitation on the use of Tris-HCl as the extraction buffer only affected the measurement of benzaldehyde dehydrogenase

activity in the blocked and constitutive mutants (Tables 28 to 32) as in the experiments on the kinetics of induction the total benzaldehyde dehydrogenase activity never exceeded 80 units/mg protein.

The other main piece of developmental work was the isolation of the various classes of mutants. Of the two mutagenic treatments tried, the desired types of blocked mutant were isolated using N.T.G. as the mutagen; whereas no suitable mutants were obtained using u.v. irradiation (Table 20). Nevertheless auxotrophic mutants were probably obtained by means of u.v. irradiation since the viable count on agar plates containing succinate as carbon source was less than that on nutrient agar plates (p.181). This lack of specific blocked mutants from u.v. irradiation is in agreement with the results of Whittaker and Jones who independently failed to obtain specific mutants of bacterium NCIB 8250 using this technique, and may be a reflection of the fact that N.T.G. has a more powerful mutagenic effect on bacterium NCIB 8250 than has u.v. irradiation. This situation occurs in other species; e.g. in *E. coli*, Adelberg *et al.*(1965) have obtained a 40% yield of auxotrophic mutants using N.T.G., whereas other workers using u.v. irradiation have only obtained a 1% yield of mutants (Davis, 1950).

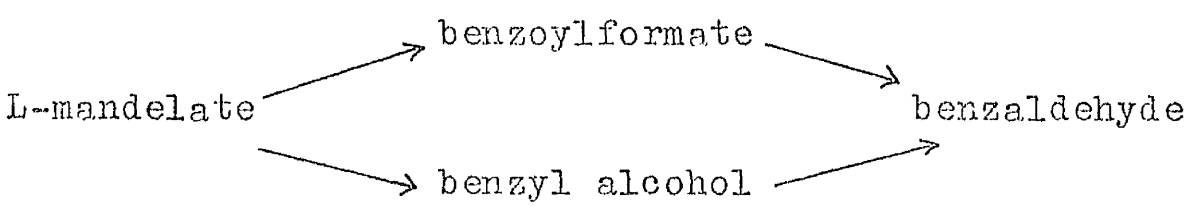
Although only a limited number of mutants was obtained (Table 20), and though many more will be required for any extension of this work, the success of the later experiments

would indicate that incubation with N.T.G. represents a satisfactory mutagenic technique, and that it should not prove too difficult to obtain many more examples of the various mutant classes. Extension of the enrichment techniques with penicillin V, Carboxy Q or other bactericidal agents may improve the efficiency of the procedure.

2. PATHWAYS.

The pathways for the metabolism of mandelate, benzyl alcohol and their substituted derivatives (Fig.4), as outlined by Kennedy (1967) and Kennedy & Fewson (1968a,b), have been further substantiated by the results of this project.

Although Kennedy & Fewson (1968a) postulated that L-mandelate was metabolised solely through benzoylformate largely on account of the fact that benzyl alcohol supported a higher molar growth yield than did L-mandelate, they could not rule out the possibility that L-mandelate could be oxidised by two pathways operating simultaneously via benzoylformate or benzyl alcohol:



Indeed, the existence of the latter pathway would explain why the Regulon R_2 enzymes are induced by growth on L-mandelate (e.g. Table 16). Nevertheless the properties of the various blocked mutants support the idea that L-mandelate is metabolised solely through benzoylformate in this organism.

The benzyl alcohol mutants, which possess little benzyl alcohol dehydrogenase activity (Table 31), show delayed growth on benzyl alcohol, but grow normally on L-mandelate (Table 24). Presumably, if L-mandelate was partially metabolised via benzyl alcohol in these organisms, they would also show slower growth

on L-mandelate. Mandelate mutant strains NF1202, NF1408 and NF0410, which grow on benzyl alcohol but not on L-mandelate (Table 22), lack L-mandelate dehydrogenase activity, but form an active benzyl alcohol dehydrogenase (Table 29). Thus, if L-mandelate was dissimilated through benzyl alcohol in these organisms, the enzymic lesion between L-mandelate and benzoylformate would be by-passed unless the postulated L-mandelate decarboxylase was also lost. Likewise the mandelate mutant strains which contain only low levels of benzoylformate decarboxylase (Table 30) do not grow normally on L-mandelate but grow on benzyl alcohol (Table 22).

Kennedy & Fewson (1968a,b) suggested that bacterium NCIB 8250 oxidised L-mandelate, benzyl alcohol and their substituted analogues to the corresponding benzoates by a series of parallel pathways whose enzymes were non-specific in their activity and induction. Their evidence came from a study of the kinetic properties of these enzymes with a number of substrates and from an examination of the oxidation of these compounds by whole cells. The present work has further substantiated this theory as mutants, which show no or delayed growth on L-mandelate, have a similar growth pattern on 4-hydroxymandelate (Table 22), and mutants, which show slow growth on benzyl alcohol, show no or slow growth on 2- and 4-hydroxybenzyl alcohol and 4-hydroxy-3-methoxybenzaldehyde (Table 24). Final proof of this hypothesis, however, must await the purification of the enzymes and the preparation of antisera.

This non-specificity in the induction and activity of the enzymes which convert L-mandelate and its substituted analogues to the corresponding benzoates may be a feature common to all micro-organisms which can metabolise both mandelate and substituted mandelates. Although restricted to the 4-hydroxy derivatives, this phenomenon has been observed in both fungal and bacterial species. Stevenson & Mandelstam (1965) found that in P. putida cells grown on mandelate or 4-hydroxymandelate possessed both L-mandelate dehydrogenase and 4-hydroxymandelate dehydrogenase activity, and that mutants selected for their inability to grow on mandelate also failed to grow on 4-hydroxymandelate. This type of situation also occurs in Aspergillus niger as extracts of cells grown on mandelate can also oxidise 4-hydroxymandelate as well as mandelate (Jamaluddin et al., 1970). In nature, especially in soil where a particular environment may contain a number of different analogues of the same compound, this non-specificity in enzyme activity and induction has obvious advantages since it leads to an economy of protein synthesis.

Kennedy (1967) could not exclude the possibility that benzoate was an intermediate in the dissimilation of 2-hydroxybenzoate. He observed that cells which could oxidise 2-hydroxybenzoate could also oxidise benzoate but not vice-versa. Benzoate mutant strains NA16, NA18 and NA21 exclude this possibility, however, as they are able to grow on 2-hydroxybenzoate but not on benzoate (Table 25). In addition

to the three strains listed in Table 25, every one of a further 12 mutants, which showed signs of leakiness or reversion in the initial characterisation experiments, failed to grow normally on benzoate, but did on catechol. Thus each of the fifteen mutants tested apparently possessed a defective benzoate oxidase system. This was a rather surprising finding since benzoate oxidase is only one of seven enzymes which catalyse the conversion of benzoate to succinate and acetyl-CoA. There are two possible explanations for this observation.

(1) The benzoate oxidase locus is more susceptible to mutation than the loci for the other enzymes.

(2) Lesions occur in all the enzymes of the catechol ortho cleavage pathway; but catechol and benzoate can be metabolised by different pathways. Griffiths et al.(1964) have already reported the existence of tangential pathways involving catechol as the common intermediate in bacterium NCIB 8250. They found that growth on naphthalene induced the formation of a catechol 2,3-oxygenase, whereas growth on benzoate resulted in the synthesis of a catechol 1,2-oxygenase.

Nevertheless the first explanation is probably the correct one since the observations outlined below suggest that catechol and benzoate are metabolised by the same pathway in this organism:

(1) cells induced with catechol or benzoate synthesise a catechol 1,2-oxygenase (e.g. Fig.42), and can oxidise β-oxoadipate without a lag (Kennedy & Fewson, 1968a);

and (2) all three benzoate mutants which have so far been tested for the ability to decarboxylate benzoate have lacked this capacity (Cook, unpublished results).

3. ENZYMES.

3.1. Benzaldehyde dehydrogenase.

The original heat inactivation experiments of Kennedy & Fewson (1968b) and my results (quoted on p.504 of the same paper and documented in full in this work) on the activation of the heat-stable enzyme by potassium ions suggested that bacterium NCIB 8250 contained two NAD^+-specific benzaldehyde dehydrogenases. This work has now been extended, and has provided the following additional evidence for the existence of two enzymes.

(1) Thiophenoxyacetate which gratuitously induces L-mandelate dehydrogenase but not benzyl alcohol dehydrogenase induces a benzaldehyde dehydrogenase which has a half-life of about 500min at 37°(Fig.14).

(2) Benzyl alcohol which induces benzyl alcohol dehydrogenase but not L-mandelate dehydrogenase induces a benzaldehyde dehydrogenase which has a half-life of about 30min at 37° (Fig.14).

(3) The heat inactivation curves for benzaldehyde dehydrogenase from cells grown on L-mandelate, which induces both L-mandelate dehydrogenase and benzyl alcohol dehydrogenase, and from a mixture of cells induced with benzyl alcohol or thiophenoxyacetate give heat inactivation patterns consistent with the presence of two enzymes (Figs.15 and 16 respectively).

(4) The half-life of the heat-labile enzyme varies with the

extraction buffer and the actual pH of the extract, whereas that of the heat-stable enzyme shows little variation (Tables 4 and 5).

(5) The heat-stable enzyme is activated by monovalent cations ($NH_4^+ > K^+ > Rb^+ > Cs^+ > Na^+$) when extracted in sodium pyrophosphate buffer, whilst the heat-labile enzyme is not activated by any of these ions (Table 3 and Fig.13). The picture is more complicated when the two enzymes are extracted in Tris buffer because the labile enzyme is also activated by monovalent cations, but the percentage activation of the stable enzyme is greater.

(6) The benzyl alcohol mutant strains which show delayed growth on benzyl alcohol but normal growth on L-mandelate (Table 24) have very little labile benzaldehyde dehydrogenase activity, but considerable stable benzaldehyde dehydrogenase activity (Table 31). Conversely, mandelate mutant strains NF2405 and NF0113, which grow on benzyl alcohol but show slow or no growth on L-mandelate (Table 22), possess high labile enzyme activity, but almost negligible amounts of the stable enzyme (Table 30).

Definitive proof for the existence of two enzymes has been provided by Kennedy & Zatman (Zatman, private communication) who have purified the two enzymes.

Both benzaldehyde dehydrogenases appear to require monovalent cations for optimal activity since in the majority of cases the presence of these ions in the reaction mixture

brings the activity of the enzymes extracted in Tris buffer up to the values found in cells extracted in sodium pyrophosphate buffer (Table 3). The two enzymes differ, however, in their response to the various cations (Table 3). As well as the percentage activation of the stable enzyme being generally greater, the individual activations produced by the different cations cover a wider range of values. The requirement of monovalent cations for optimal activity of aldehyde dehydrogenases has been reported for both yeast acetaldehyde dehydrogenase (Sorger & Evans, 1966) and the $NADP^+$-specific benzaldehyde dehydrogenase of *P. putida* (Stachow *et al.*, 1967).

3.2. Benzoate oxidase.

Negligible benzoate oxidase activity was detected in cell-free extracts of bacterium NCIB 8250 using the assay conditions of Ichihara *et al.*(1962) (p.119). This result is in agreement with the results of other workers (Mandelstam & Jacoby, 1965; Kemp & Hegeman, 1968; Hegeman, private communication; Professor J. Mandelstam, Department of Microbiology, University of Oxford, private communication; Dr R.E. Cripps, Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent., private communication) who failed to detect benzoate oxidase activity in cell-free extracts of various *Pseudomonas* spp.. It was found, however, that enzyme activity could be detected in extracts to which FAD had been added (Fig.18), and that this activity did not depend on the

presence of GSH in the assay mixture (Table 11). The enzyme from bacterium NCIB 8250 requires FAD, an NADH generating system and Fe^{++} ions for activity, whereas the enzyme from P. fluorescens Mb-15, M. ureae Et and P. aeruginosa B-23 (the organisms examined by Ichihara et al.(1962)) requires GSH, an NADPH or an NADH generating system and Fe^{++} ions for activity. Nevertheless the two systems are not too dissimilar as only the requirement for GSH and FAD is different. It is possible that these two compounds carry out the same function in the two benzoate oxidase systems as both compounds can participate in oxidation-reduction reactions.

Although attempts were made in the present work to preferentially inhibit or by-pass the endogenous NADH oxidase system, it is possible that this system forms an integral part of the benzoate oxidase system as the "purified" enzyme still has NADH oxidase activity associated with it (Ichihara et al., 1962). Nevertheless it is impossible to decide if this suggestion is valid as these workers gave no details as to the homogeneity of the "purified" enzyme. Indeed they did not even quote a single value for the specific activity of the enzyme or the protein content of the assay, and consequently the value for the specific activity of the enzyme in cell-free extracts of bacterium NCIB 8250 cannot be compared with that of the system described by Ichihara et al.(1962). Neither is it clear why other workers cannot detect benzoate oxidase activity in Pseudomonas spp. using the conditions of Ichihara

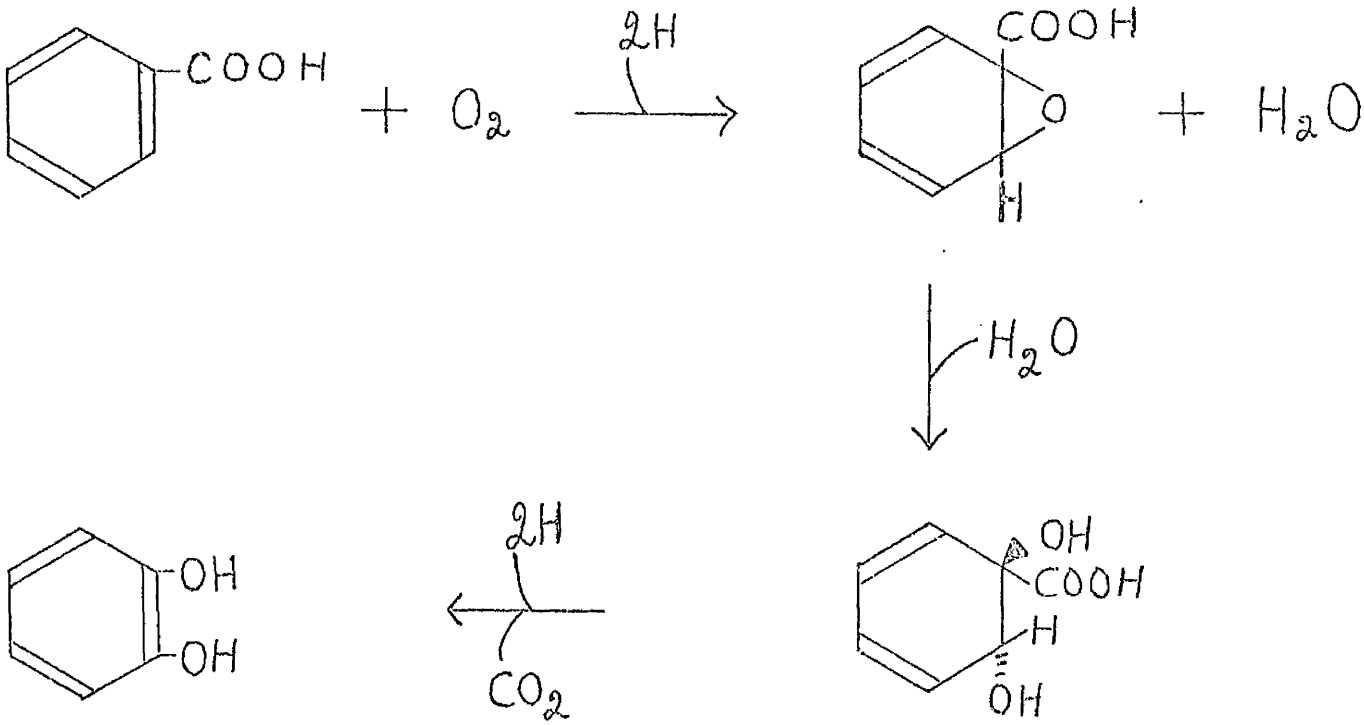
et al.(1962). It would be interesting to test the effect of FAD in these systems. It would also be interesting to discover what steps are necessary to bring the cell-free activity of the enzyme in bacterium NCIB 8250 up to the level expected from experiments with whole cells.

The original mechanism put forward for the conversion of benzoate to catechol involved the intermediate formation of an epoxide (Ichihara et al., 1962; Takeda, Mori, Ueda & Taniuchi, unpublished results in Taniuchi et al., 1964) (Fig. 48). The latter group were able to split the enzyme system into two components, one of which catalysed the formation of an unidentified intermediate in the presence of both NADPH (or NADH) and oxygen, and the other decarboxylated this intermediate to form catechol. This reaction mechanism has had to be discarded in the light of more recent evidence obtained by Takeda et al. (unpublished results in Hayaishi, 1966) who found that both the oxygen atoms incorporated into catechol were derived from molecular oxygen.

The actual reaction mechanism operating may be similar to that suggested for the conversion of anthranilate to catechol (Kobayashi et al., 1964), for the conversion of 2-fluorobenzoate to catechol and 3-fluorocatechol (Milne, Goldman & Holtzman, 1968) and for the conversion of benzene to catechol (Gibson, Koch & Kallio, 1968; Gibson, Cardini, Maseles & Kallio, 1970). All three enzyme systems catalyse the incorporation of one molecule of oxygen into the

Fig.48.

The epoxide mechanism for the conversion of benzoate to catechol.



reaction product, and in each case the formation of a cyclic peroxide intermediate has been postulated.

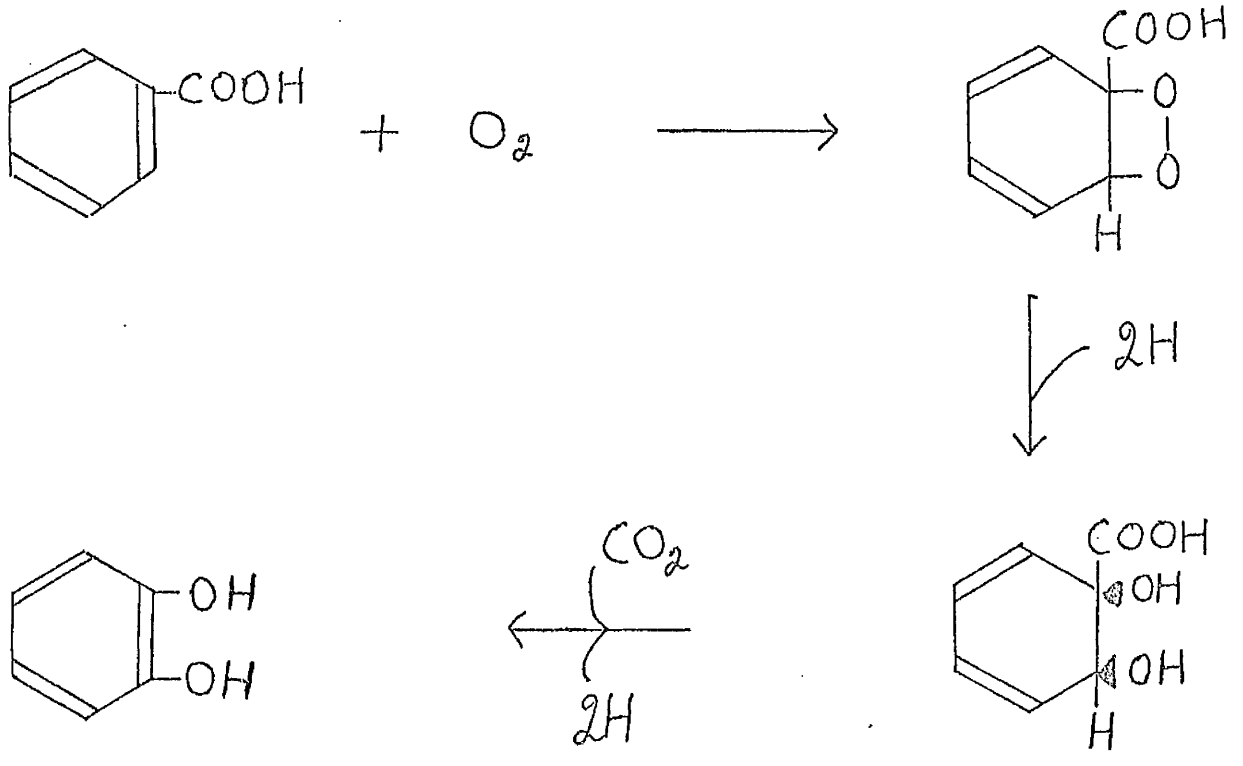
The reaction mechanism of benzoate oxidase from bacterium NCIB 8250 and 2-fluorobenzoate oxidase from the pseudomonad studied by Milne et al.(1968) is probably the same, as cells of bacterium NCIB 8250 grown on benzoate as well as metabolising benzoate can also convert 2-fluorobenzoate to catechol and 3-fluorocatechol (Fewson et al., 1968). Nevertheless 2-fluorobenzoate oxidase has not been studied in cell-free extracts so that its cofactor requirements are not known. On the other hand benzene oxidase has been examined in cell-free systems (Gibson et al., 1968 & 1970). Like benzoate oxidase it requires, in addition to Fe^{++} ions, an NADH generating system for activity which is an uncharacteristic property of dioxygenases. The cofactor complement of the two enzymes, however, is different since benzene oxidase requires cysteine rather than FAD for activity. Nevertheless the two sets of cofactors probably fulfil the same function in the reaction. This role could well be the provision of an electron transport system to supply the reducing power necessary for the formation of a cis-glycol since cis-benzene glycol, whose formation is catalysed by an enzyme system requiring an NADH generating system, Fe^{++} ions and cysteine, has been isolated as an intermediate in the benzene oxidase reaction of P. putida (Gibson et al., 1968 & 1970). The isolation of this compound lends support to the idea that the first intermediate formed is

a cyclic peroxide. The enzyme which catalyses the formation of catechol from cis-benzene glycol requires NAD^+ for activity so that in the overall reaction there is no net oxidation of NADH (Gibson et al., 1968). This poses the question of why an NADH generating system rather than catalytic amounts of NADH is required. The simplest explanation to this problem is that the NADH present in the assay is oxidised by an active NADH oxidase in the enzyme preparation, and thus a continuous supply of NADH is needed. In the benzoate oxidase system, the electron transport chain would consist of the NADH generating system, Fe^{++} ions and FAD, and by analogy with the camphor lactonising system (Conrad, Lieb & Gunsalus, 1965), the electrons would flow sequentially from NADH through FAD and Fe^{++} ions to the terminal electron acceptor. The hypothetical reaction mechanism for benzoate oxidase is shown in Fig.49.

Evidence for this postulated reaction mechanism must await $^{18}O_2$ studies, isolation of intermediates and the purification of the enzyme system. The benzoate oxidase system would consist of at least two enzymes if the reaction mechanism of benzene oxidase and benzoate oxidase is indeed similar. If this suggestion is correct, the observation that all 15 benzoate mutants appear to possess a defective benzoate oxidase system (see p.220) would not be quite so surprising as first thought since the enzyme system concerned would consist of more than one enzyme. These mutants might be convenient tools with which to study the reaction mechanism since they could be used to identify intermediates of the overall reaction.

Fig.49.

The cyclic peroxide mechanism for the conversion of benzoate to catechol.



4. REGULATION OF THE MANDELATE PATHWAY.

4.1. Regulon R_1.

The evidence which suggests that L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase are coordinately synthesised, is outlined below.

(1) The correlation between the P values for the induction of L-mandelate dehydrogenase and those for the induction of the other enzymes shows that only benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase are synthesised coordinately with L-mandelate dehydrogenase (Table 34).

(2) L-Mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase are synthesised before any of the other mandelate pathway enzymes on induction with benzoylformate or L-mandelate (Table 17).

(3) The three enzymes are gratuitously induced by thiophenoxyacetate which does not induce any of the other mandelate pathway enzymes (Table 33).

(4) In the wild type and blocked mutants (Tables 28 to 33), L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase, where synthesised, are induced only by L-mandelate, benzoylformate or thiophenoxyacetate These enzymes are never induced by benzyl alcohol or benzaldehyde, whereas the labile benzaldehyde dehydrogenase, benzyl alcohol dehydrogenase and catechol oxygenase are.

(5) In mandelate mutant strains NF2405 and NF0113 (Table 30),

Table 34.

Correlation between the P values for the induction of L-mandelate dehydrogenase and those for the induction of the other enzymes. The data in this Table are summarised from Fig.42.

Enzyme	Correlation coefficient
Benzoylformate decarboxylase	0.98
Stable benzaldehyde dehydrogenase	0.98
Benzyl alcohol dehydrogenase	-0.17
Labile benzaldehyde dehydrogenase	-0.22
Benzoate oxidase	-0.27
Catechol oxygenase	-0.39

negligible amounts of L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase are induced by L-mandelate or benzoylformate, whereas substantial amounts of the Regulon R_2 and R_4 enzymes are formed.

(6) Both L-mandelate and benzoylformate induce the synthesis of about 190 units/mg protein of L-mandelate dehydrogenase and about 45 units/mg protein of the stable benzaldehyde dehydrogenase in mandelate mutant strains NF1706 and NA36, which possess little benzoylformate decarboxylase activity, whereas the levels of the Regulon R_2 and R_4 enzymes vary from 17-455 units/mg protein and from 15-57 units/mg protein respectively (Table 30).

The evidence presented so far points to the coordinate regulation of L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase, and therefore to these enzymes belonging to a common operon controlled by a single repressor and operator gene. Nevertheless there is reason to believe that the control at the genetic level is more complex. This conclusion is based on the following observations:

(1) the stable benzaldehyde dehydrogenase is not synthesised constitutively in the mutants meso-constitutive for L-mandelate dehydrogenase and benzoylformate decarboxylase (Table 27), and is hyperinduced on induction with L-mandelate or benzoylformate (Table 32) compared with the wild type levels (Table 28), whereas L-mandelate dehydrogenase and benzoylformate

decarboxylase are not induced by these two compounds; and (2) on induction with thiophenoxyacetate, stable benzaldehyde dehydrogenase activity appears approximately 10min after that of L-mandelate dehydrogenase and benzoylformate decarboxylase (Fig.38); whilst on induction with L-mandelate or benzoylformate (Table 17), the stable benzaldehyde dehydrogenase is synthesised before or at the same time as L-mandelate dehydrogenase.

A possible and testable model, compatible with the above findings, can be constructed for the regulation of the Regulon R_1 enzymes. Regulon R_1 may be controlled in a similar manner to the arg system of E. coli (Jacoby & Gorini, 1969) in that unlinked genes possessing their own operator genes are controlled by a common repressor. In the case of Regulon R_1, this would mean that L-mandelate dehydrogenase and benzoylformate decarboxylase would form one subgroup, and that the stable benzaldehyde dehydrogenase would constitute a second subgroup.

Evidence for a common repressor is based on two observations.

(1) The three enzymes are coordinately derepressed by a number of inducers (Fig.42), which suggests that the inducers are combining with the same repressor molecule.

(2) Mandelate mutant strains NF2405 and NF0113 are inducible for none of the Regulon R_1 enzymes (Table 30), which suggests that they may be regulatory mutants, for instance similar to

the i^S mutants of *E. coli* (Jacob & Monod, 1961; Gilbert & Müller-Hill, 1966). On the evidence presented so far, they could also be, for example, polarity mutants (cf. Newton, Beckwith, Zipser & Brenner, 1965).

The mutation which has occurred in strains NE04071, NE02270 and C04211 has had a simultaneous but markedly different effect on the inducibility and constitutive levels of the stable benzaldehyde dehydrogenase on the one hand, and L-mandelate dehydrogenase and benzoylformate decarboxylase on the other hand (Tables 27 and 32). Unless a double mutation has occurred in three independently isolated organisms, which is extremely unlikely, the most reasonable conclusion is that the mutation has occurred in a regulatory gene which governs the synthesis of a repressor molecule which binds to two different sites on the genome. The differences between the inductive properties of these organisms and the wild type (Tables 28 and 32) could then be explained by a differential effect of the altered repressor on the two subgroups of genes.

The existence of two operator genes would also offer the simplest explanation for the observation that the stable benzaldehyde dehydrogenase can be synthesised before, after or at the same time as L-mandelate dehydrogenase (Fig.38 and Table 17) since enzymes which are controlled by different operators need not be synthesised in a definite chronological order when different inducers are used.

Genetic mapping experiments would help to test this

hypothesis, but as yet, there is no means of genetic recombination in bacterium NCIB 8250. A more amenable way of substantiating this hypothesis would be a study of enzyme formation using a larger number of inducers and under different experimental conditions such as induction at various temperatures or in the presence of non-inhibitory levels of chloramphenicol.

Furthermore if this hypothesis is correct, mandelate mutant strains NF2405 and NF0113 would be regulator gene mutants rather than polarity mutants as the Regulon R_1 enzymes would not be situated on a common operon. This being the case, a study of revertants able to grow on L-mandelate or benzoylformate would be of interest as it might be possible to isolate organisms which synthesise L-mandelate dehydrogenase and benzoylformate decarboxylase but not the stable benzaldehyde dehydrogenase. This class of mutant would be able to grow on L-mandelate or benzoylformate as they could still synthesise the labile benzaldehyde dehydrogenase. Some of these revertants might be constitutive for L-mandelate dehydrogenase and benzoylformate decarboxylase as a result of a mutation in the operator or regulator gene for these enzymes. The existence of the former type of mutant, however, depends on the occurrence of a negative rather than a positive type of control, and consequently the failure to isolate this class of revertant would not necessarily contradict the model outlined above. This model also depends on the applicability of some

sort of Jacob-Monod system to the Regulon R_1 system. It might be, of course, that Regulon R_1 is controlled in a completely different way.

An interesting feature about the constitutive mutants is their enrichment in a procedure which involves alternate growth on L-mandelate and glutamate despite the fact that their growth rate on L-mandelate is much slower than that of the wild type (Table 23). The mutants must be growing on either L-mandelate or one of its metabolic products. Benzaldehyde is a likely candidate for this compound as there is a smell of benzaldehyde in the medium when the wild type is growing on L-mandelate although the amount present has not been measured. Benzaldehyde accumulates in the medium possibly because the benzaldehyde dehydrogenase activity of the wild type is less than its benzoylformate decarboxylase activity (e.g. Table 28) although it is risky to assume that enzyme activity as measured in vitro is similar to its activity in vivo. This benzaldehyde may well support the growth of the mutants since these organisms possess a high benzaldehyde dehydrogenase activity when grown in the presence of L-mandelate (Table 32). The mutant strain can never become the predominant organism in the culture under these growth conditions as its growth depends on the metabolism of L-mandelate by the wild type. The phenomenon of one organism producing conditions suitable for the growth of another is well known; e.g. the streptococcus-lactobacillus flora produce lactate from carbohydrates in the rumen and this

lactate is in turn fermented to acetate and propionate by bacteria such as Veillonella alcalescens and Selenomonas ruminantium (Hobson, 1969).

In this project, the original intention was to isolate magno-constitutive mutants by means of the alternate culture technique; but unfortunately only meso-constitutive strains were obtained. A better method for obtaining magno-constitutive mutants is to grow the culture on a carbon or nitrogen source which is a substrate but not an inducer of the enzyme in question. Unluckily, however, there is no compound of this type known for the Regulon R_1 system.

All four mandelate mutants which are unable to grow on benzoylformate (Table 22) possess low levels of benzoylformate decarboxylase activity (Table 30). The ability of these organisms to decarboxylate benzoylformate to some extent is also suggested by the fact that L-mandelate and benzoylformate induce low levels of catechol oxygenase, whose induction requires the metabolism of these compounds to catechol. Lack of growth on benzoylformate, although the organisms possess some benzoylformate decarboxylase activity, may be indicative of the fact that the energy derived from benzoylformate is less than that required for the maintenance of the organism. It is not known whether the ability of these organisms to decarboxylate benzoylformate is due to residual benzoylformate decarboxylase activity or to the non-specific action of other enzymes. Likewise, the basal level of this enzyme in non-

induced cells could be due to the non-specific action of other enzymes. On the other hand, the loss of the basal activity of L-mandelate dehydrogenase in mutant strains NF1202, NF1408 and NF0410 indicates that this activity is indeed due to L-mandelate dehydrogenase.

It was impossible to tell from the experiments on the kinetics of induction whether L-mandelate, benzoylformate or a metabolic product formed from their metabolism induces the Regulon R_1 enzymes. A study of the blocked mutants, however, gives an answer to this question. In mandelate mutant strains NF1202, NF1408 and NF0410 (Table 29), which lack a functional L-mandelate dehydrogenase, L-mandelate does not induce any of the enzymes of the mandelate pathway. On the other hand, benzoylformate gives an inductive pattern comparable with that of the wild type (Table 29) except, of course, for the synthesis of an active L-mandelate dehydrogenase. These results indicate that benzoylformate and not L-mandelate is the inducer of the Regulon R_1 enzymes. That benzoylformate is the sole inducer of the Regulon R_1 enzymes is reinforced by the observation that in mandelate mutant strains NF1706 and NA36 (Table 30), which possess little benzoylformate decarboxylase activity, benzoylformate induces the formation of wild type amounts of L-mandelate dehydrogenase and the stable benzaldehyde dehydrogenase.

Regulon R_1 is thus induced by the product of the first enzyme of the regulatory group. Product-induction of

regulatory units is a relatively frequent occurrence in micro-organisms; e.g. protocatechuate induces shikimate dehydrogenase in A. calcoaceticus (Cánovas, Wheelis & Stanier, 1968), and cis,cis-muconate induces catechol oxygenase in P. putida (Ornston, 1966c).

4.2. Regulon R_2.

There are several pieces of evidence which suggest that the labile benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase are synthesised coordinately.

(1) The correlation between the P values for the induction of benzyl alcohol dehydrogenase and those for the induction of the other enzymes shows that only the labile benzaldehyde dehydrogenase is synthesised coordinately with benzyl alcohol dehydrogenase (Table 35).

(2) Only the time-course of induction of these two enzymes flattens off after 20 to 35min induction with L-mandelate or benzoylformate (Figs.35 and 39).

(3) Their time of appearance lies between that of the Regulon R_1 and R_4 enzymes (Table 17).

(4) The benzyl alcohol mutant strains (Table 31) are non-inducible for both enzymes.

(5) In mandelate mutant strains NF1706, NF2405 and NF0113 (Table 30) and in the constitutive mutants (Table 32), both enzymes are hyperinduced compared with the wild type levels (Table 28) on induction with L-mandelate or benzoylformate.

Table 35.

Correlation between the P' values for the induction of benzyl alcohol dehydrogenase and those for the induction of the other enzymes. The data in this Table are summarised from Fig.43.

Enzyme	Correlation coefficient
L-Mandelate dehydrogenase	-0.17
Benzoylformate decarboxylase	-0.14
Stable benzaldehyde dehydrogenase	-0.10
Labile benzaldehyde dehydrogenase	0.97
Benzoate oxidase	0.67
Catechol oxygenase	0.55

In the experiments on the kinetics of induction, L-mandelate, benzoylformate, benzyl alcohol and benzaldehyde all induced benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase. Induction by L-mandelate or benzoylformate may be brought about by the two compounds themselves or by the benzaldehyde formed from their metabolism. No clear-cut decision can be made between these two possibilities as the evidence obtained in the present work is ambiguous. L-Mandelate, however, can by excluded as an inducer since it does not invoke the synthesis of the Regulon R_2 enzymes in the mandelate mutant strains which lack L-mandelate dehydrogenase (Table 29). Benzoylformate, on the other hand, gives results which are difficult to interpret.

The flattening-off in the rate of formation of the labile benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase in the experiments on the kinetics of induction (Fig.35) lends support to the idea that it is the benzaldehyde formed from the metabolism of benzoylformate and L-mandelate which induces these enzymes. The initial induction of these enzymes followed by a cessation in their synthesis can be explained by benzoylformate decarboxylase producing benzaldehyde faster than the stable benzaldehyde dehydrogenase can remove it, resulting in an accumulation of benzaldehyde. The benzaldehyde formed would then induce the Regulon R_2 enzymes. Consequently the total benzaldehyde dehydrogenase activity would rapidly rise, and bring about the removal of

the surplus benzaldehyde from the medium; thereby eliminating the inducer, and causing the rate of synthesis to flatten off. The occurrence of a strong smell of benzaldehyde just at the time when the Regulon R_2 enzymes are induced is circumstantial evidence in support of this hypothesis. The lower activity of the Regulon R_2 enzymes found in the wild type (Table 28) and in the mandelate mutant strain NA36 (Table 30) after 2½h induction with L-mandelate or benzoylformate, compared to the peak values obtained in the experiments on the kinetics of induction (Fig.35), could be attributed to enzyme decay which might have occurred in the intervening 2h. Indeed decay of the Regulon R_2 enzymes was found to occur when protein synthesis was completely inhibited by the addition of chloramphenicol (20μM).

Although the results presented so far are consistent with the suggestion that it is the benzaldehyde formed from benzoylformate or L-mandelate which induces the Regulon R_2 enzymes, the induction of these enzymes by benzoylformate or L-mandelate in the constitutive mutants (Table 32) and in the mandelate mutant strains NF0113, NF2405 and NF1706 (Table 30) indicates that at least in these organisms, benzoylformate itself may serve as an inducer. This conclusion is based on the assumption that it offers the simplest way of reconciling the fact that the levels of the Regulon R_2 enzymes are 10-20 fold higher in the mandelate mutant strain NF1706 (Table 30) and in the constitutive mutants (Table 32) than in the wild

type (Table 28), although these mutants possess little benzoylformate decarboxylase activity and a stable benzaldehyde dehydrogenase activity which is never less than that of the wild type. Presumably these mutants do not transiently accumulate a greater amount of benzaldehyde than does the wild type as the ratio of their benzoylformate decarboxylase to stable benzaldehyde dehydrogenase activity is far less than that of the wild type. Furthermore the lower levels of catechol oxygenase induced by benzoylformate or L-mandelate in these mutants compared with the levels induced by benzaldehyde or benzyl alchohol in the same mutants and by benzoylformate or L-mandelate in the wild type suggest that the decarboxylation of benzoylformate is the rate limiting step. Thus it appears that at least in these mutants, the transient accumulation of benzaldehyde, if it occurs at all, is not large enough to account for the higher levels of the Regulon R_2 enzymes induced by L-mandelate or benzoylformate. This leaves benzoylformate as the likely inducer of these enzymes. The possibility that L-mandelate acts as an inducer has been excluded as it does not induce the Regulon R_2 enzymes in mandelate mutant strains which lack L-mandelate dehydrogenase activity (Table 29).

If it is a correct deduction that benzoylformate is an inducer of the Regulon R_2 enzymes in at least some strains, it poses the question of why it does not have this function in all strains of bacterium NCIB 8250 (see Table 30). One possible explanation could be that Regulon R_1 is genetically

linked in some way to Regulon R_2 so that in the wild type the amounts of the Regulon R_2 enzymes induced by benzoylformate are cut to a minimum by some type of repression mechanism which is lost as a result of a mutation in a Regulon R_1 locus. Genetic linkage between the two regulons can be ruled out as thiophenoxyacetate does not induce the Regulon R_2 enzymes in the constitutive mutant strain C04211 and in the mandelate mutant strain NF1706 (Table 33). It is also difficult to envisage how a mutation in the benzoylformate decarboxylase structural gene can simultaneously affect the regulatory genes controlling the Regulon R_2 enzymes; a situation which occurs in mutant strain NF1706 (Table 30) if it is assumed that the loss of only benzoylformate decarboxylase of the Regulon R_1 enzymes in this organism is due to a structural mutation.

The six mutant organisms which are hyperinducible for the Regulon R_2 enzymes (Tables 30 and 32) were all independently derived since they were either isolated in separate experiments or can be differentiated from one another by their Regulon R_1 enzyme complement (Table 30) or by their growth characteristics (Table 22). Consequently it is extremely improbable that these organisms represent double mutants in which the mutations in the Regulon R_1 and R_2 loci arose independently of one another.

Another possible explanation for the induction of the Regulon R_2 enzymes by benzoylformate is that benzoylformate itself can act as an inducer in both the wild type and mutant

organisms, but that an enzyme-bound intermediate between benzoylformate and benzaldehyde acts as a repressor by competitively inhibiting the induction by benzoylformate. Thus the six mutant strains which are hyperinducible for the Regulon R_2 enzymes (Tables 30 and 32) cannot form a sufficient amount of this intermediate to cause repression; whereas mutant strain NA36 which synthesises only low levels of these enzymes on induction with L-mandelate or benzoylformate (Table 30) can. The existence of enzyme-bound intermediates for decarboxylases which catalyse the thiamine pyrophosphate dependent decarboxylation of α-oxoacids (i.e. the group of decarboxylases to which benzoylformate decarboxylase belongs) has been reported (Krampitz, Suzuki & Gruell, 1961). This hypothesis would also offer an alternative explanation for the flattening-off in the rate of induction of the Regulon R_2 enzymes in the experiments on the kinetics of induction (Fig.35). Instead of this effect being caused by the exhaustion of benzaldehyde, it could be due to a build-up of repressor which antagonises the induction by benzoylformate.

Although a possible explanation has been put forward for the hyperinduction of the Regulon R_2 enzymes, it only represents a working hypothesis on which to base future experiments as there is only circumstantial evidence in favour of it at present. Future experiments which may give an insight into the nature of this phenomenon include:

(1) a study of the kinetics of induction of the Regulon R_2 enzymes in a mutant which lacks only the stable benzaldehyde

dehydrogenase of the Regulon R_1 enzymes should provide evidence as to whether it is the accumulation of benzaldehyde formed from the metabolism of benzoylformate which causes the induction of the Regulon R_2 enzymes since the labile benzaldehyde dehydrogenase would be required for the metabolism of benzaldehyde in this organism;

(2) the isolation of further mutants which fail to grow on benzoylformate together with a study of the faulty enzymes should give a fuller picture as to the types of mutant which are hyperinducible for the Regulon R_2 enzymes;

and (3) an analysis of the induction of the Regulon R_2 enzymes by benzoylformate in the presence of a specific inhibitor of benzoylformate decarboxylase should provide evidence as to whether benzoylformate metabolism is required for repression to take place.

The question of whether benzaldehyde and benzyl alcohol can both induce the Regulon R_2 enzymes has been left unanswered since the three benzyl alcohol mutants analysed have all lacked both enzymes (Table 31). A solution to this problem would be given by the isolation of mutants some of which lack benzyl alcohol dehydrogenase, and others which lack the labile benzaldehyde dehydrogenase. The delayed growth of the three benzyl alcohol mutants on benzaldehyde or benzyl alcohol (Table 24) is probably caused by leakiness rather than reversion. This is because:

(1) no revertants were isolated when the organisms were spread on agar plates containing benzyl alcohol as sole source

of carbon;
and (2) the organisms did not grow on 4-hydroxy-3-methoxybenzaldehyde or 2-hydroxybenzyl alcohol which are poorer substrates for benzaldehyde dehydrogenase and benzyl alcohol dehydrogenase respectively (Kennedy & Fewson, 1968b). It is also possible that the mutants grow on aldehydes by making use of the basal amounts of the stable benzaldehyde dehydrogenase present in the cell.

It is interesting that there is very little benzyl alcohol dehydrogenase or labile benzaldehyde dehydrogenase activity in the three benzyl alcohol mutants examined. This may be because:
(1) they are double mutants - this is unlikely as the mutants were isolated in separate experiments;
(2) they are regulatory mutants;
(3) owing to polarity effects, a mutation in one of the two genetic loci has prevented the expression of the other;
and (4) the two enzymes share a common polypeptide whose structure has been altered as a result of the mutation.

It must be stressed, however, that the enzyme content of only three benzyl alcohol mutants has so far been analysed, and it could be a coincidence that there is a simultaneous loss of both enzyme activities. The isolation and examination of further benzyl alcohol mutants could lead to the occurrence of one enzyme activity in the absence of the other.

4.3. Regulon R_3 (benzoate oxidase) and Regulon R_4 (catechol oxygenase.

The correlation between the P values for the induction of benzoate oxidase and those for the induction of L-mandelate dehydrogenase (-0.27), benzyl alcohol dehydrogenase (-0.14) and catechol oxygenase (0.24) indicates that this enzyme is independently regulated (Figs.42 to 44). Likewise catechol oxygenase has been shown to form a separate regulon. The time (Table 17) and time-course (Fig.35) of appearance of these enzymes provide additional evidence for their independent regulation. One puzzling feature is that catechol oxygenase appears to be synthesised before benzoate oxidase. This could reflect a relatively high saturation constant for the induction of benzoate oxidase compared with a relatively low one for the induction of catechol oxygenase.

Regulon R_3 is probably induced by benzoate as the time of appearance of benzoate oxidase on induction with L-mandelate or benzyl alcohol rules out its induction by the substrates of the Regulon R_1 and R_2 enzymes (Table 17), and as product-induction by catechol is negated by the absence of induction with this compound (Fig.44). Conclusions as to the identity of the inducer of Regulon R_4 cannot be made from the results obtained in this project; but Farr & Cain (1968) showed that cis,cis-muconate induced this enzyme in bacterium NCIB 8250.

4.4. Repression studies.

End-product repression of L-mandelate dehydrogenase occurs in bacterium NCIB 8250 (Table 18) as well as in P. putida (Mandelstam & Jacoby, 1965; Stevenson & Mandelstam, 1965). Although Mandelstam and his co-workers never examined the repression produced by a mixture of acetate + succinate, the repression they obtained using succinate alone was comparable to the repression observed in the current experiments using a mixture of acetate + succinate. Benzyl alcohol dehydrogenase is subject to a more pronounced repression than L-mandelate dehydrogenase in bacterium NCIB 8250 (Table 18); but analogous data for P. putida are not available as benzyl alcohol dehydrogenase was not assayed by Mandelstam and his colleagues. Benzyl alcohol dehydrogenase is also repressed by L-mandelate in bacterium NCIB 8250 (Table 18).

Hegeman (1966a) did not observe end-product repression in the same strain of P. putida; but the growth conditions he employed were so dissimilar from those of Mandelstam and his colleagues and those used in the present work that there is not necessarily a contradiction between the two sets of findings. Mandelstam & Jacoby (1965) and Stevenson & Mandelstam (1965) added inducer + repressor to cells growing exponentially on glucose, and then measured enzyme activity in samples taken from the culture during the following hour. In comparison, Hegeman (1966a) inoculated cells grown on succinate or asparagine into a medium containing asparagine or succinate

+ mandelate, and then measured enzyme activity in a sample taken from the culture towards the end of exponential growth. Thus it is possible that Hegeman measured enzyme activity in a sample in which the succinate or asparagine had been exhausted.

Multi-sensitive end-product repression plays an important role in the control of the mandelate pathway since it prevents the synthesis of the earlier enzymes of the pathway when intermediates lower down the pathway are present in the growth medium. This type of repression is rather broad in its specificity. The synthesis of L-mandelate dehydrogenase is repressed in the presence of both aliphatic and aromatic intermediates of the pathway which include benzoate, catechol, succinate and acetate. As yet, the site of repressor action is not known. At least two possibilities exist:

(1) inhibition of induction by preventing the permeation of inducer;

and (2) interference with the transcription or translation mechanisms.

4.5. A summary of the control of the mandelate pathway.

As postulated by Kennedy & Fewson (1968b), the aromatic part of the mandelate pathway can be divided into 4 regulons. Regulon R_1 consists of L-mandelate dehydrogenase, benzoylformate decarboxylase and the stable benzaldehyde dehydrogenase; Regulon R_2 consists of benzyl alcohol dehydrogenase and the labile benzaldehyde dehydrogenase; Regulon R_3 consists of

benzoate oxidase; and Regulon R_4 consists of catechol oxygenase. There is also reason to believe that Regulon R_1 can be divided into two subgroups consisting of L-mandelate dehydrogenase and benzoylformate decarboxylase on the one hand and the stable benzaldehyde dehydrogenase on the other hand. The inducers of Regulons R_1, R_3 and R_4 are benzoylformate, benzoate and cis,cis-muconate respectively, but there is not yet conclusive evidence as to the inducer of Regulon R_2. Fig.50 summarises the present knowledge as to the regulation of the aromatic part of the mandelate pathway in bacterium NCIB 8250.

REGULATION OF THE AROMATIC PART OF THE MANDELATE PATHWAY IN BACTERIUM NCIB 8250

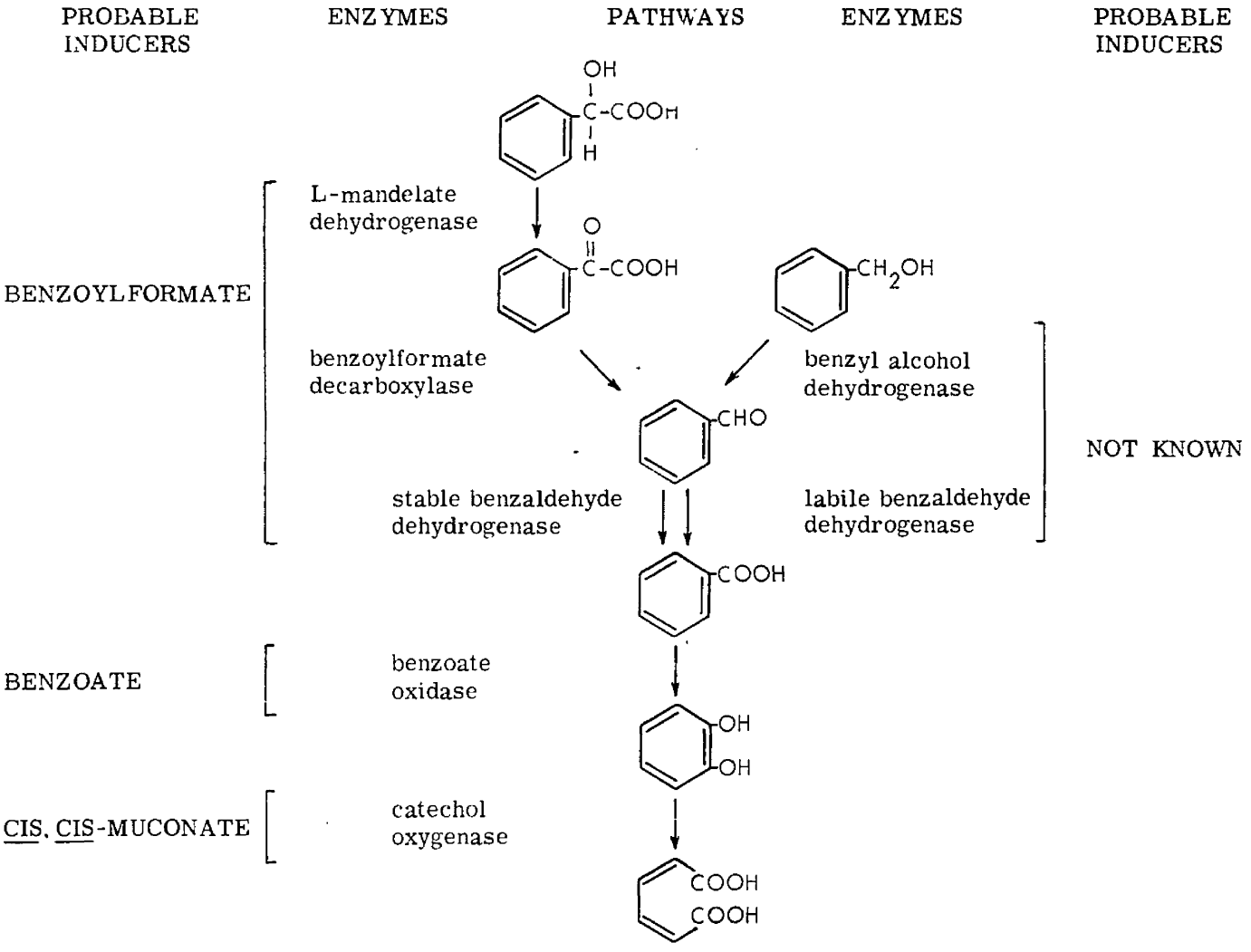


Fig. 50.

5. A COMPARISON OF THE PATHWAYS FOR THE CONVERSION OF MANDELATE AND BENZYL ALCOHOL TO BENZOATE IN DIFFERENT MICRO-ORGANISMS.

The pathways which exist in different micro-organisms for the conversion of mandelate and benzyl alcohol to benzoate are extremely varied from the point of view of their intermediates, enzymology and regulation.

Of the four organisms in which the biochemistry of the pathway for the conversion of mandelate to benzoate has been studied, two, Aspergillus niger (Fig.3) and P. putida (Fig.1), can oxidise D,L-mandelate, whereas the other two, P. aeruginosa (Rosenberg & Hegeman, 1969) and bacterium NCIB 8250 (Fig.2), can oxidise only the L-isomer. Moreover, Aspergillus niger and P. putida have different pathways for the metabolism of D-mandelate to benzoylformate: the former organism converts D-mandelate directly to benzoylformate (Fig.3), whereas in the latter organism, D-mandelate is oxidised to benzoylformate via L-mandelate (Fig.1). Of these four organisms, only in bacterium NCIB 8250 has it been established that benzyl alcohol is converted to benzoate via benzaldehyde (Fig.2) although the preliminary experiments of Stanier (1948) indicated that a similar pathway operates in P. putida (Fig.1).

The fungal and bacterial mandelate dehydrogenases have different properties. The bacterial L-mandelate dehydrogenase is associated with the particulate fraction of the cell, and is not stimulated by nucleotide cofactors (Stanier et al.,

1953; Kennedy & Fewson, 1968b), whereas the fungal enzyme is found in the soluble fraction of the cell, and is stimulated by FAD and FMN. On the other hand, the fungal D-mandelate dehydrogenase is associated with the particulate fraction (Jamaluddin *et al.*, 1970). No comparative work has yet been done with the other enzymes.

Although the chemical point of convergence of the pathways for the metabolism of benzyl alcohol and mandelate is benzaldehyde, the enzymic point of convergence in the two organisms so far studied is benzoate. Bacterium NCIB 8250 contains two benzaldehyde dehydrogenases, one of which, the stable benzaldehyde dehydrogenase is associated with the enzymes that convert mandelate to benzaldehyde (Table 34), and is induced by benzoylformate; and the other, the labile benzaldehyde dehydrogenase, is associated with the enzyme that converts benzyl alcohol to benzaldehyde. There is reason to believe that *P. putida* also synthesises isofunctional benzaldehyde dehydrogenases associated with the benzyl alcohol and L-mandelate pathways (Stevenson & Mandelstam, 1965; Hegeman, 1966a). The benzaldehyde dehydrogenase induced by growth on benzaldehyde or benzyl alcohol has not yet been detected in cell-free extracts of this organism; but its existence can be inferred from a number of observations with whole cells (Stevenson & Mandelstam, 1965; Hegeman, 1966a). It would be interesting to look for a labile benzaldehyde dehydrogenase in *P. putida* using very careful techniques to avoid inactivation. Furthermore in bacterium NCIB 8250

(Kennedy & Fewson, 1968b) and in P. aeruginosa (Rosenberg & Hegeman, 1969), only one benzaldehyde dehydrogenase is associated with the regulon to which benzoylformate decarboxylase belongs; whereas in Aspergillus niger (Jamaluddin et al., 1970) and in P. putida (Hegeman, 1966a), for some unknown reason, two benzaldehyde dehydrogenases are associated with this regulon.

The regulation of the enzymes involved in the conversion of mandelate to benzoate is different in all three bacterial species in which the control of the pathway has been studied. In bacterium NCIB 8250 (Fig.50) and P. putida (Fig.7), the enzymes which convert mandelate to benzoate are coordinately regulated; but the specificity of induction is greater in bacterium NCIB 8250 as only benzoylformate can act as an inducer, whereas in P. putida D- and L-mandelate as well as benzoylformate can act as inducers. In P. aeruginosa L-mandelate dehydrogenase forms an independent regulon induced by L-mandelate, and benzoylformate decarboxylase and benzaldehyde dehydrogenase constitute a second regulon induced by benzoylformate (Rosenberg & Hegeman, 1969).

If, as suggested by Cánovas, Ornston & Stanier (1967), dissimilarities in the regulation of a biochemical pathway reflect the independent evolution of a pathway in different micro-organisms, then the mandelate pathway has had a separate evolution in bacterium NCIB 8250, P. putida and P. aeruginosa. It is surprising that the mandelate pathway has had an

independent evolutionary origin in the two Pseudomonas species as the catechol part of the pathway was postulated to have a common evolutionary origin (Fig.9). These observations could mean that at least in Pseudomonas species, the pathways for aromatic ring cleavage and the subsequent metabolism of the aliphatic products formed had the same evolutionary origin; but that the pathways involved in the formation of dihydroxyphenols from their aromatic precursors arose later in evolutionary time. An extension of this type of work might provide clues as to how catabolic capabilities have been built up in micro-organisms.

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