The Oxidation of Mandelic Acid

and

Related Compounds

by

Bacterium NCIB 8250

by

Samuel I. T. Kennedy.

Thesis presented for the degree of

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SUMMARY.

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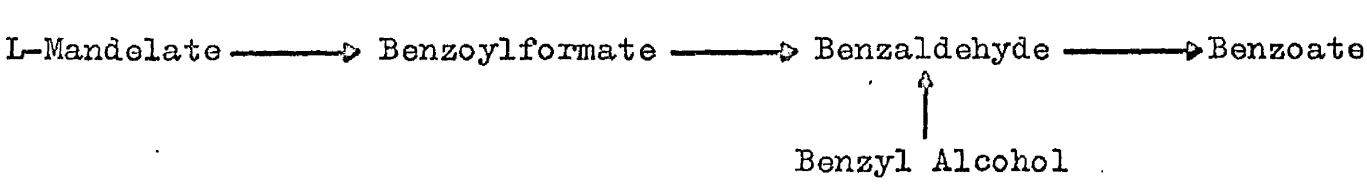
This thesis describes an investigation into the mechanism whereby the bacterium NCIB 8250 (which was formerly known as 'Vibrio 01') carries out the oxidation of mandelate and a number of related compounds.

Bacterium NCIB 8250 was found to utilise L-mandelate, benzoylformate, benzyl alcohol, benzaldehyde or benzoate as sole source of carbon and energy for growth. 2-Hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy derivatives of these compounds can also be utilised. The pathways of oxidation of all these substances were determined, in the first instance, by the technique of simultaneous adaptation. Washed cell suspensions, prepared from bacteria which had been grown on each aromatic compound in turn, were challenged in the Warburg apparatus with a large number of possible intermediates and analogues. The resulting patterns of oxygen utilisation indicated that the side chain of each L-mandelate or benzyl alcohol is oxidised to give the corresponding benzoate.



The enzymes converting the L-mandelates or benzyl alcohols to the appropriate benzoates appear to be non-specific: indeed a variety of

compounds which do not support growth, such as the 3-hydroxy substituted compounds, are also metabolised to the corresponding benzoates but no further. The benzoic acids are then metabolised by a series of specific enzymes which are generally found only when cells have been grown in the presence of the corresponding substrate. Benzoate and 2-hydroxybenzoate are oxidised to catechol while 4-hydroxybenzoate and 4-hydroxy-3-methoxybenzoate are converted to 3,4-dihydroxybenzoate. Catechol and 3,4-dihydroxybenzoate then undergo ring fission.

In order to substantiate the hypothesis that the enzymes converting the L-mandelates or benzyl alcohols to benzoates are non-specific these enzymes were examined in cell-free systems. Cell suspensions were subjected to ultrasonic disruption and, after centrifugation, the supernatant solutions were examined. Spectrophotometric assays were developed for L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase. A suitably sensitive assay for benzoylformate decarboxylase was not obtained. It was found possible to characterise the enzymes by a number of parameters such as Km, Vmax and relative velocity with a range of substrates. Cell-free extracts were prepared after growth on a number of compounds and in all cases the kinetic properties of the enzymes were the same regardless of the growth substrate. Evidence obtained from experiments in which mixtures of substrates were added simultaneously ruled out the possibility of non-specific induction of several substrate-specific enzymes.

The bacterium NCIB 8250 can therefore convert a large number of

aromatic compounds to five key intermediates by means of just four enzymes. The thesis concludes with a discussion of the advantages to the bacterial cell of this type of specificity both in economy of cellular constituents and ecological status.

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Contents

INTRODUCTION.

General Aspects of the Dissimilation of Aromatic Compounds by Microorganisms.

The utilisation of aromatic compounds by a variety of microorganisms is now well known. Numerous workers have described the isolation of bacteria and fungi which are capable of metabolising phenolic substances and, in many cases, using them as sole sources of carbon and energy for growth (Ribbons, 1965). The primary ecological importance of these microorganisms lies in their ability to effect the release of the organic carbon 'locked up' in the aromatic compounds formed so ubiquitously by the plant world (e.g. Brown, 1966). Attention has also recently been focused on the effects of aromatic pesticides on the balance of the biological composition of soil and other natural environments. This has, in turn, emphasised the ecological importance of the chemical transformations of these compounds by microorganisms (e.g. Carson, 1963).

In general the metabolism of aromatic compounds by microorganisms consists of a series of manipulations aimed at producing one of a limited number of hydroxylated compounds. These key intermediates then undergo ring cleavage with subsequent degradation of the aliphatic products to give intermediates of the central metabolic routes of the cell (Ribbons, 1965). A variety of benzenoid compounds is known which can give rise to aliphatic derivatives by

oxygenative cleavage; these include catechol, 3,4-dihydroxybenzoate, 2,3-dihydroxyphenylpropionate, quinol, and 2,3-dihydroxybenzoate. The methods of ring cleavage of the benzenoid nucleus can be divided into two distinct classes (Ribbons, 1965):

1. The oxidative cleavage of the carbon-carbon bond between two adjacent hydroxylated carbon atoms.

2. The oxidative cleavage of the carbon-carbon bond between a hydroxylated carbon atom and a non-hydroxylated carbon atom.

These two methods of benzenoid ring cleavage produce a muconic acid and a muconic acid semialdehyde respectively. The muconic acid is metabolised to β-oxoadipate which is cleaved to succinate and acetyl-CoA. The muconic acid semialdehyde is converted to pyruvate and a carbonyl derivative whose structure depends on the substituents on the original benzenoid nucleus. In some cases different organisms can cleave the same compound by different methods. Thus catechol is cleaved by Pseudomonas putida to give cis-cis muconic acid (Ornston and Stanier, 1964) and by a pseudomonad examined by Dagley and Stopher (1959) to give α-hydroxymuconic semialdehyde.

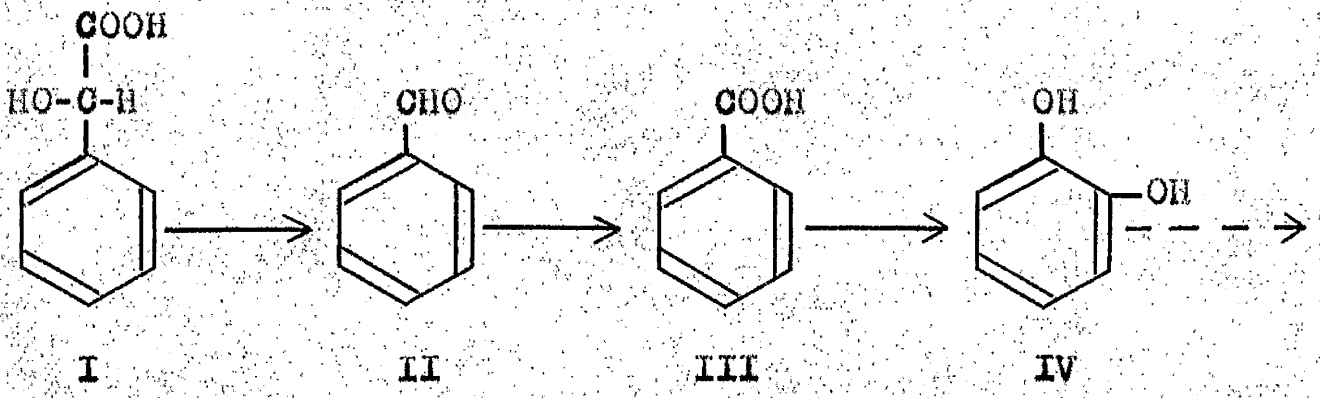
The dissimilation of more complex aromatic compounds is carried out in a step-wise fashion; each ring undergoing hydroxylation and rupture by one or other of the methods described above. Thus phenanthrene is hydroxylated to 3,4-dihydroxyphenanthrene and the hydroxylated ring cleaved (Evans, Fernley and Griffiths, 1965).

The Metabolism of Mandelic Acid and Related Compounds.

Oxidation of mandelate and related compounds to the level of benzoate.

Mandelic acid (I), which is found in nature mainly as mandelonitrile-β-gentiobioside (amygdalin), can be utilised as sole source of carbon and energy by a few bacteria (Stanier, Palleroni and Doudoroff, 1966; Baumann and Doudoroff, In Press).

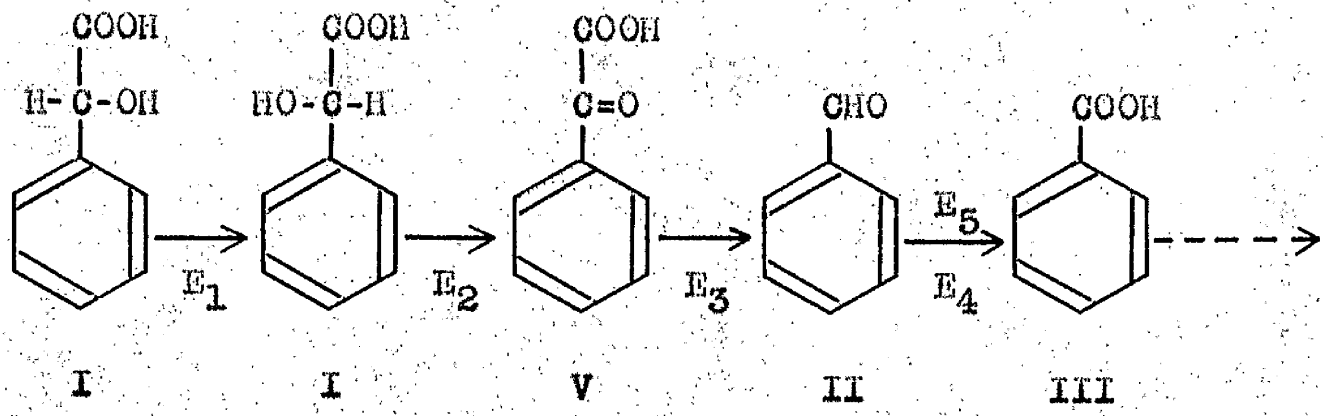
In his classical paper on simultaneous adaptation, which is discussed in a later section (p. 9), Stanier (1947) suggested that in P. putida (then known as P. fluorescens A.3.12 (Stanier et al, 1966)), D,L-mandelate was oxidised via benzaldehyde (II) to benzoate (III):



I II III IV

Stanier and Gunsalus (Gunsalus, Stanier and Gunsalus, 1953; Gunsalus, Gunsalus and Stanier, 1953; Stanier, Gunsalus and Gunsalus, 1953) succeeded in separating four enzymes which effected the conversion

of D,L-mandelate to benzoate in P. putida. The reactions catalysed by these enzymes are:



E_1 - Mandelate racemase (E.C. No. 5.1.2.2)

E_2 - L-Mandelate dehydrogenase (No E.C. number)

E_3 - Benzoylformate (V) decarboxylase (E.C. No. 4.1.1.7) which required T.P.P. as co-factor.

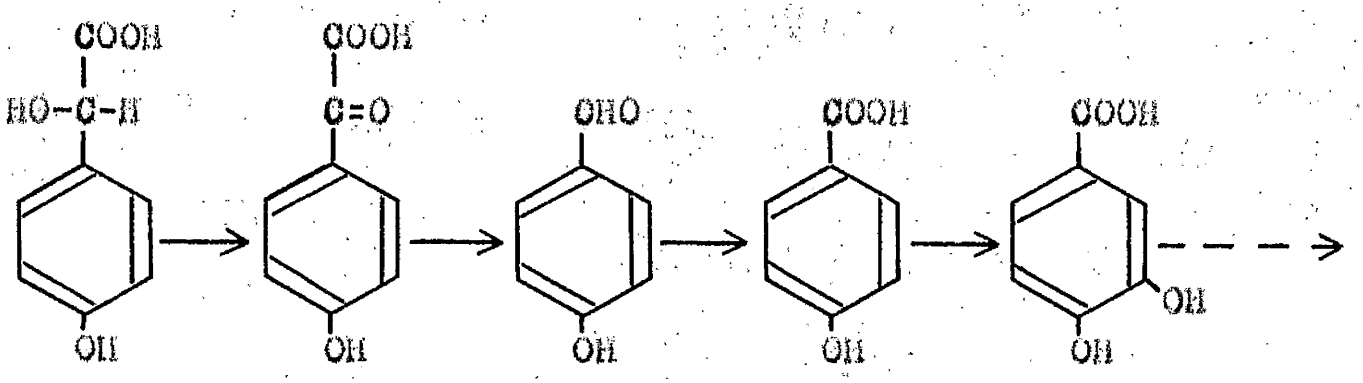
E_4 and E_5 - Benzaldehyde dehydrogenases (E.C. No. 1.2.1.6, and E.C. No. 1.2.1.7) which required NAD^+ and $NADP^+$ as co-factors.

The properties of the mandelate racemase have been described in some detail by Weil-Malherbe (1966).

Benzyl alcohol was considered to be oxidised to benzaldehyde (Stanier, 1950) but the enzyme responsible for this step was apparently never examined.

Gunter (1953), using an analagous approach to that employed by Stanier, elucidated the pathway of dissimilation of 4-hydroxy-

D,L-mandelate (VI) in the same strain of Pseudomonas. She formulated the following degradative scheme:



Here instead of reaching catechol, 4-hydroxy-D,L-mandelate (VI) is oxidised to 3,4-dihydroxybenzoate (VIII). The series of reactions from 4-hydroxy-D,L-mandelate to 4-hydroxybenzoate (VII) are seen to be exactly analagous to that converting D,L-mandelate to benzoate and indeed it was suggested that one series of enzymes might mediate the conversion of both sets of substrates (Stanier, Gunsalus and Gunsalus, 1953). This suggestion was partially substantiated by Stevenson and Mandelstam (1965) using cell-free extracts of Stanier's original strain of P. putida but their results were far from complete.

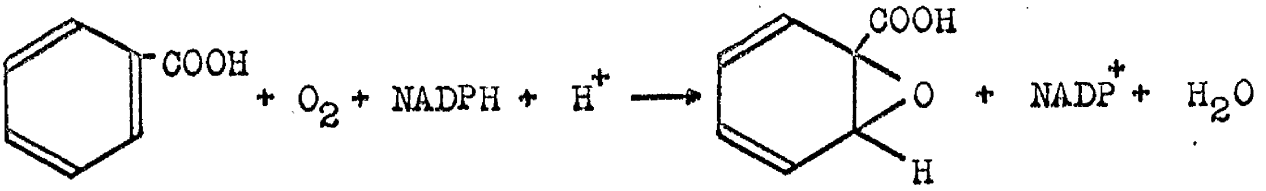
Mandelstam and Jacoby (1965) studied the regulatory mechanisms governing the synthesis of the inducible enzymes which convert D,L-mandelate to benzoate in P. putida. The enzymes which effect this oxidation have been suggested to be co-ordinately induced by D,L-mandelate or benzoylformate (Hegeman, 1966a,b,c; Stevenson and Mandelstam, 1965). The induction of these enzymes is repressed,

not only by the immediate end-product benzoate, but also by more distal end-products such as catechol, succinate or acetate (Stevenson and Mandelstam, 1965). Mandelstam (Mandelstam and Jacoby, 1965) coined the phrase 'multi-sensitive end-product repression' to describe this type of control and showed that this mechanism operates in the induction of all the enzymes which convert D,L-mandelate, through benzoate, to succinate and acetyl-CoA.

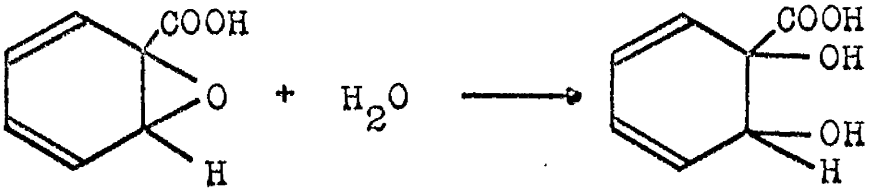
Oxidation of benzoate to catechol.

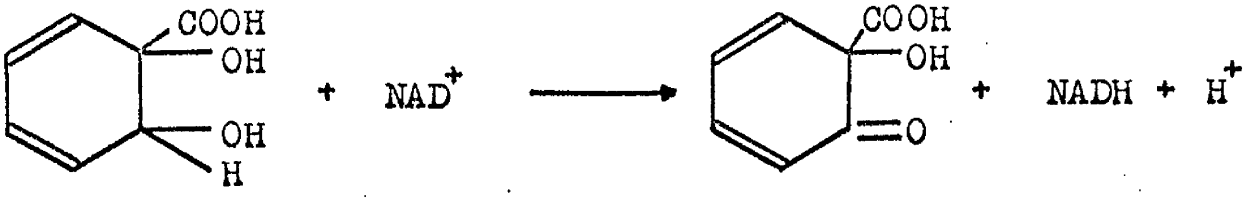
Stanier (1950) postulated that benzoate is oxidised to catechol (IV) in P. putida. Evans (1947) had observed previously that cells of Vibrio 01 grown on benzoate showed an accumulation of small amounts of catechol in the medium. Early attempts to determine the intermediates between benzoate and catechol failed, largely due to the lability of the enzyme systems involved. Sleeper (1951) demonstrated that the ring carbon of benzoate bearing the carboxyl group undergoes hydroxylation in the conversion of benzoate to catechol. Voets (1958) studied the conversion of benzoate to catechol by strains of Azotobacter. By employing the technique of simultaneous adaptation he established that some strains metabolise benzoate directly to catechol, while other strains oxidise benzoate to 2-hydroxybenzoate and thence to catechol. This latter result has not been obtained with any other organism. It was not until 1962 that Ichihara, Adachi, Hosokawo and Takeda (1962) succeeded in

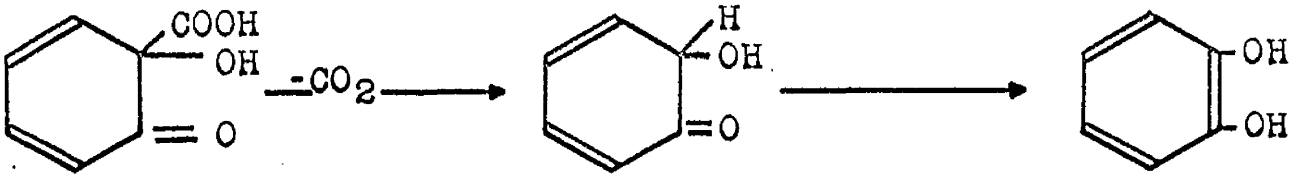
Scheme 1.



Benzoic Acid





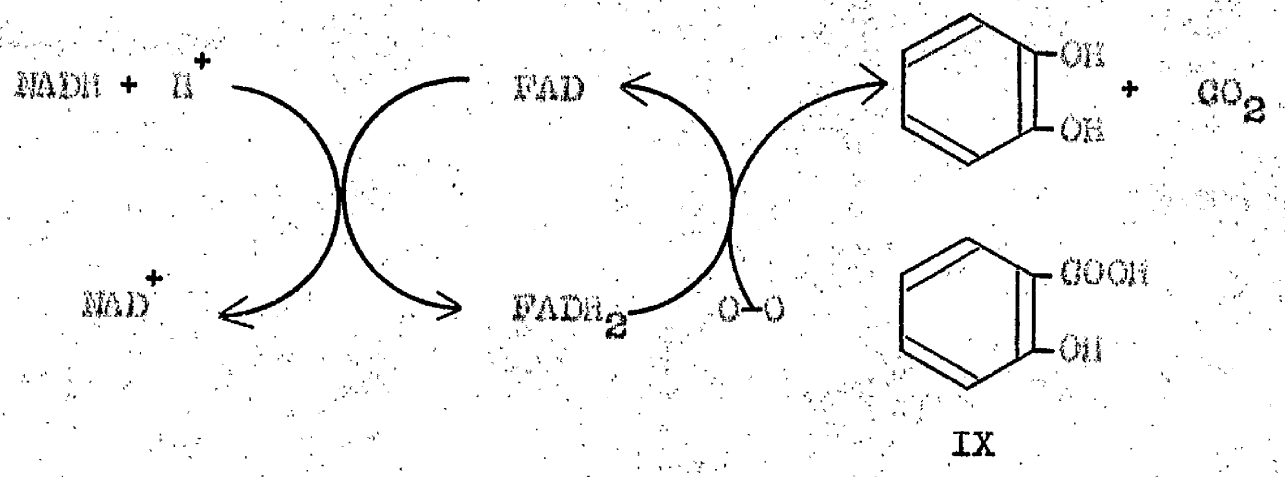


Catechol

preparing cell-free extracts of a pseudomonad which could carry out the conversion of benzoate to catechol and Taniuchi, Hatanaka, Kuno, Hayaishi, Nakajima and Kurihara (1964) suggested that benzoate is oxidised to catechol as shown in Scheme 1. Further investigation is required to substantiate this reaction sequence especially since both oxygen atoms of catechol have been shown to be derived from molecular oxygen (Takeda, Mori, Itada, Taniuchi, Kojima and Hayaishi, In Press).

Oxidation of hydroxy-benzoates and 4-hydroxy-3-methoxy-substituted compounds.

Walker and Evans (1952) showed that 2-hydroxybenzoate (IX) is converted to catechol by a strain of soil Pseudomonas. The enzyme carrying out this oxidative decarboxylation has been extensively purified (Katagiri, Maeno, Yamamoto, Hayaishi, Kitao and Oae, 1965) and shown to contain 1 mole of FAD per mole of protein and to have a requirement for Fe^{++}. The mechanism of action of the enzyme may be represented:



IX

(After Hayaishi, 1966)

The metabolism of 3-hydroxybenzoate has received little attention compared with the oxidative decarboxylation of 2-hydroxybenzoate to catechol. Yano and Arima (1958) extracted two hydroxylases from a soil bacterium, one of which converts 3-hydroxybenzoate to 3,4-dihydroxybenzoate, the other converts 3-hydroxybenzoate to 2,5-dihydroxybenzoate. Both hydroxylases require NADPH as co-factor. The metabolic fate of these two dihydroxybenzoates differ; 3,4-dihydroxybenzoate being cleaved to a muconic acid and thence to succinate and acetyl-CoA whilst 2,5-dihydroxybenzoate is probably metabolised through maleylpyruvate to fumarate and pyruvate.

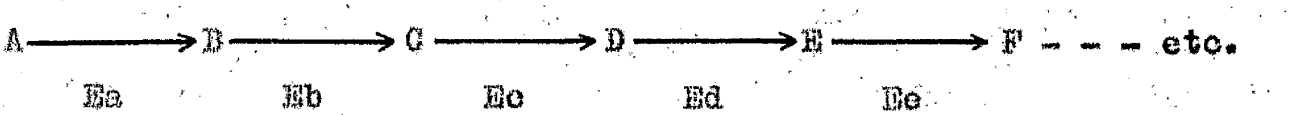
In all organisms examined 4-hydroxybenzoate is hydroxylated to 3,4-dihydroxybenzoate which is then cleaved (Ribbons, 1965).

The metabolism of methoxylated benzoates has been studied particularly with reference to the degradation of plant lignins by soil fungi. Henderson (1956) demonstrated that 4-hydroxy-3-methoxybenzaldehyde is converted to 4-hydroxy-3-methoxybenzoate. Although the demethoxylation of 3-methoxybenzoate to 3-hydroxybenzoate (Henderson, 1957) demonstrated that fungi were capable of further metabolising methoxylated derivatives, no products of ring cleavage were isolated.

Theory and Limitations of the Technique of Simultaneous Adaptation.

In 1937 Karstrom (1937) designated as adaptive those enzymes which are produced by the cell in response to the presence of a specific homologous substrate in the culture medium. He differentiated these "adaptive enzymes" from the "constitutive enzymes" which are always formed by the cells of a given species irrespective of the composition of the medium. Ten years later Stanier (1947) found that the aromatic ring fission enzymes elaborated by P. putida were adaptive in nature. The enzymes were present in the bacterial cells only when growth occurred in the presence of the aromatic substrate. With this information, he devised the technique of simultaneous adaptation for determining the intermediates in an adaptively controlled metabolic pathway.

Consider the pathway:

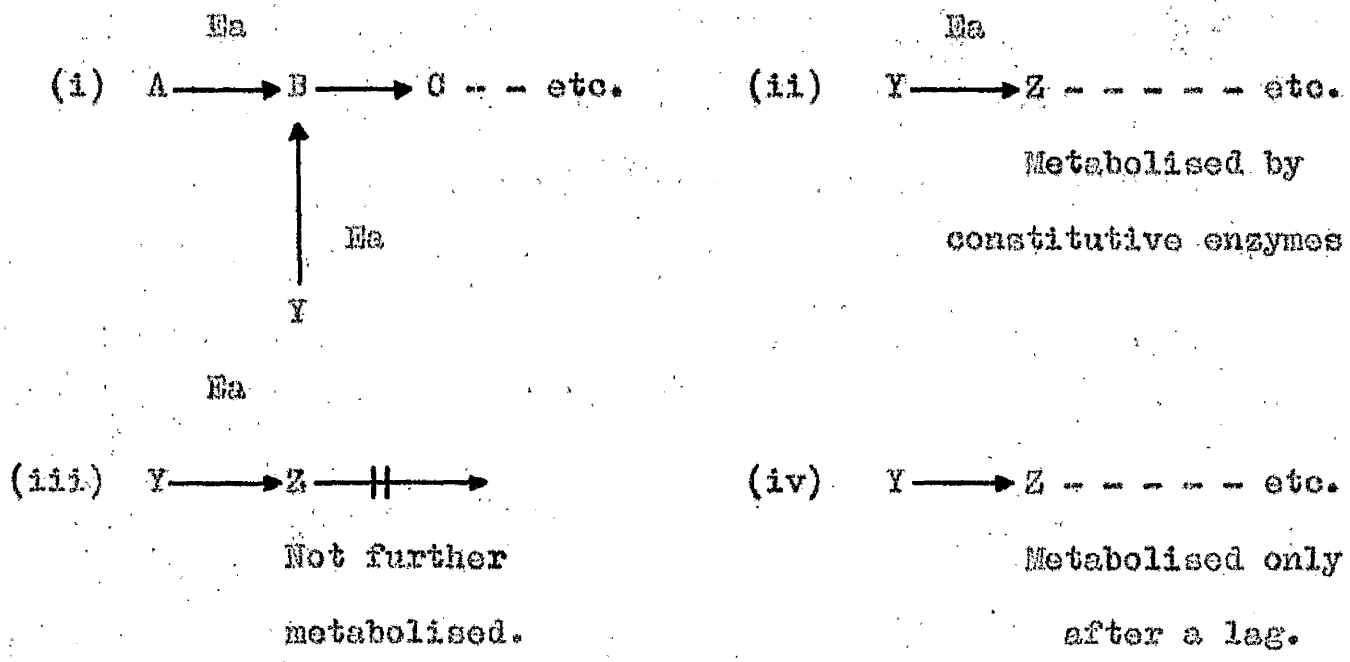


where B, C - - - - are intermediates in the metabolism of A, and Ea, Eb, etc. are the enzymes responsible for converting A to B, B to C etc. By adapting the cells to grow on A and then testing for simultaneous adaptation towards a variety of chemically related compounds, the nature of the intermediates may be determined. Washed cell suspensions grown on the substrate whose metabolism is under investigation (A) are incubated separately with various postulated intermediates, "challenge" substrates, (B,C,D - - -). The presence of a lag in their utilisation is taken to mean that the induced enzymes were not present in the cells grown on the "growth" substrate. In other words if growth on A fails to adapt the cells to X then X cannot be a member of the reaction chain.

The technique is governed by a number of qualifications:

1. That cells be freely permeable to the challenge substrate; if not, a false negative result will be obtained.

2. That a rigid specificity exists for each enzyme with respect to its substrate, i.e. Ea is specific for its substrate A and cannot act even on related compounds. If Ea does not display absolute specificity towards A but can also act on Y say, then we may have:-



The result of (i) and (ii) will be that Y is completely and immediately metabolised - a false positive result; (iii) will result in Y being immediately metabolised by the organism, the reaction stopping at Z. In situation (iv) Y will be immediately converted to Z which can be further metabolised only after an adaptive period during which time new enzymes are being synthesised. Depending on the experimental technique conditions (iii) and (iv) will give a false positive result.

3. A well characterised feature of the adaptive synthesis of enzymes is that in certain cases the product of the enzyme reaction induces that enzyme; i.e. growth on B would induce the formation of Ea. This phenomenon has been demonstrated in E. coli by Jacob and Cohn (1951). In general growth on C will simultaneously adapt the cells to D, E etc. but not to A or B. The probability that growth on C will afford simultaneous adaptation to precursors decreases with

the number of intervening steps, i.e. simultaneous adaptation to B is more likely than to A. This type of limitation to the technique of simultaneous adaptation has become more obvious with the recognition that in many cases several enzymes are controlled by the same regulon (Maas and McFall, 1964). Thus even in the classic example of simultaneous adaptation - the mandelate pathway - there is physiological evidence that groups of enzymes are regulated by a number of operons (Hegeman, 1966a,b,c). Accordingly this pathway is still sequentially induced, but the number of steps is less than was originally thought. (Stanier, Hegeman and Ornston, 1963; Mandelstam and Jacoby, 1965).

4. Growth of the organism on A may induce, not only Ea, but also another enzyme En which can metabolise Y as described in 2 and false positive results be obtained.

5. The detection of a free intermediate in a reaction sequence may be impossible owing to the binding of some member of the sequence to an enzyme which does not release its product till a later stage in the pathway. Under these circumstances, the addition of a free intermediate to a cell suspension in which that intermediate exists only in the bound form will give a false negative result.

The elucidation of any pathway must depend on the use of more than one technique. Studies on simultaneous adaptation must be reinforced with experiments on the isolation and identification of intermediates - perhaps facilitated by tracers and inhibitors - and

the purification and examination of individual enzymes.

Taxonomy and Metabolism of Bacterium NCIB 8250.

Vibrio 01 was isolated by Happold and Key in 1932. It was stated to be a "gram negative vibrio" with an oxidase system, without action on any of the common sugars and not liquefying gelatin. Presumably the organism was motile in view of the designation vibrio and the fact that it was stated to resemble V. tyrosinatica (Happold and Key, 1932) and V. cuneatus (Evans, 1947; Kilby, 1951). There seems little doubt, therefore, that Vibrio 01 was a member of the Pseudomonas-Vibrio group, although a precise classification cannot be arrived at from the information available. Early experiments on the metabolism of aromatic compounds were done with this organism (Evans and Happold, 1939; Evans, 1947; Evans, Parr and Evans, 1949) but the original strain appears to have been lost. Most subsequent work done with Vibrio 01 has, in fact, been done with the bacterium NCIB 8250 which is clearly a different organism. Bacterium NCIB 8250 is neither like the original Vibrio 01 nor characteristic of the genus Vibrio since inter alia, it is a non-motile, oxidase-negative coccobacillus (Fewson, 1967b). Evans (1947) found that Vibrio 01 grew on 3-hydroxybenzoate but not on 2-hydroxybenzoate; bacterium NCIB 8250 shows the inverse pattern (Fewson, 1967a). It is also noteworthy that Dagley, Fewster and Happold (1952) found that "Vibrio 01 originally isolated by Happold and Key" grew on

phenylalanine as sole carbon source, whereas later it was reported (Chapman and Dagley, 1960; 1962; Fewson, 1967a) that bacterium NCIB 8250 showed little or no growth with phenylalanine as carbon source. Bacterium NCIB 8250 does, however, resemble the original strain of Vibrio 01 in its failure to grow on the common sugars and its general ability to metabolise aromatic compounds. Previous report describing experiments carried out with Vibrio 01 should be treated with care unless it is clearly established whether the original Vibrio 01 or the bacterium NCIB 8250 was used.

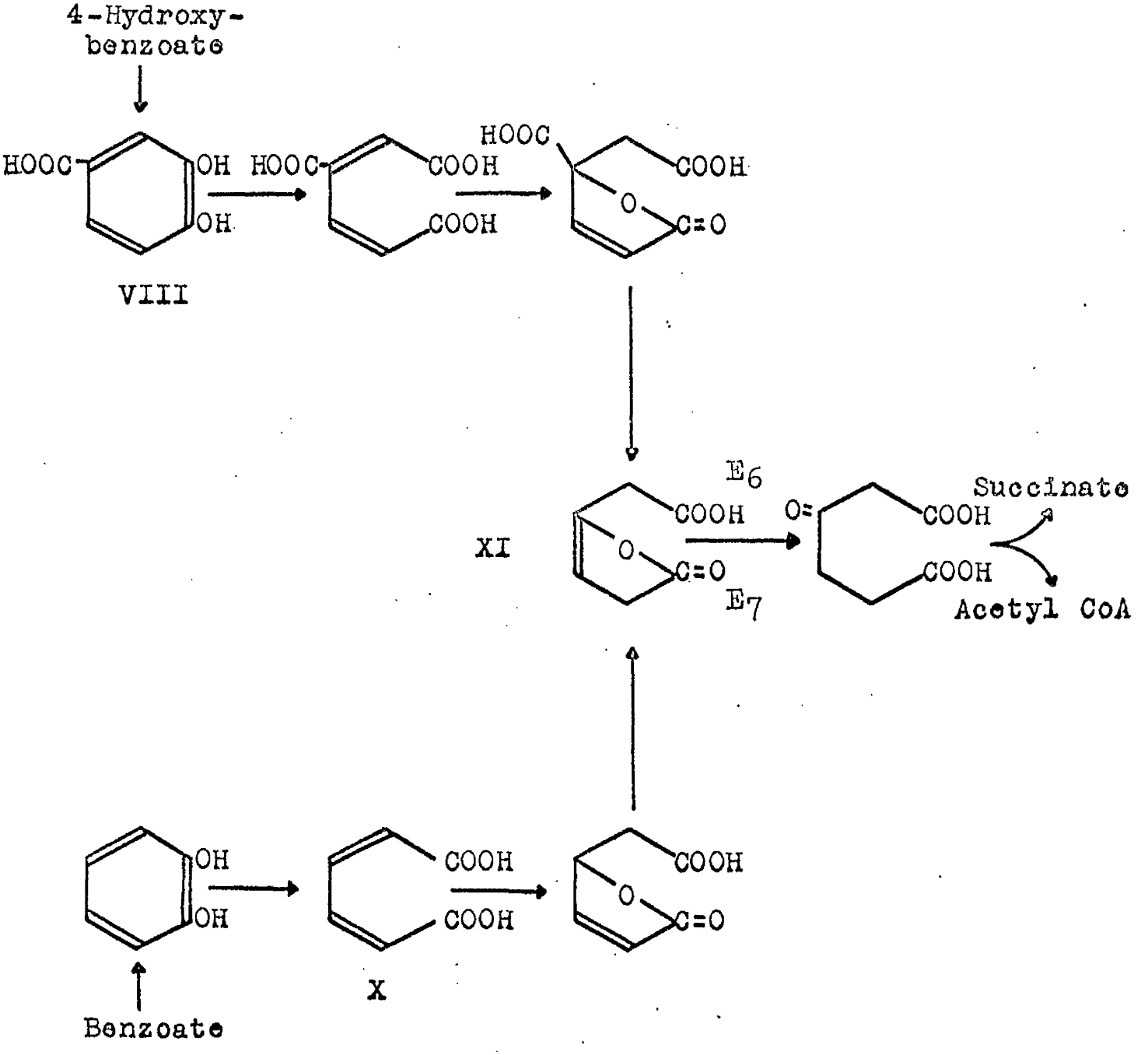
Davis and Park (1962) placed bacterium NCIB 8250 in the genus Comomonas because one of the authors observed "a few motile organisms" and "occasional organisms bearing one or two flagellae". Motility has never been observed in bacterium NCIB 8250 (Fewson, 1967b). These authors (Davis and Park, 1962) also gave conflicting results for the oxidase activity of bacterium NCIB 8250 but it is of interest that the report of motility was made on a culture which was stated to be Kovacs oxidase positive and to show no growth on pyruvate or lactate: compounds which support good growth of bacterium NCIB 8250 (Fewson, 1967a). Bacterium NCIB 8250 is now listed by the National Collection of Industrial Bacteria (1964) as an Achromobacter species but cannot be retained in this genus if the proposals of Brisou and Prévot (1954) and Steel and Cowan (1964) are followed. Sebald and Véron (1963) identified bacterium NCIB 8250 as Moraxella lwoffi (Acinetobacter lwoffii, Steel and Cowan, 1964) apparently very largely on the basis of the GC content of the DNA. Base composition,

although a useful tool in classification cannot be the sole criterion of taxonomy since in view of the small range of GC content possible it is inevitable that quite different organisms will be found to have similar gross DNA analysis (De Ley, 1964). It seems likely, however, that Sebald and Véron (1963) were substantially correct since the characteristics of bacterium NCIB 8250 are typical of those of the Acinetobacter-Moraxella group of bacteria (Steel and Cowan, 1964; Henderson, 1965; Fewson, 1967b). Stanier, Palleroni and Doudoroff (Private Communication) have come to similar conclusions. Work will have to be done with a large number of strains before the question of nomenclature can be settled since it has been shown (Fewson, Unpublished Results) that bacterium NCIB 8250, Acinetobacter lwoffii (NCTC 5866), Acinetobacter anitratus (NCTC 7844) and several other closely related strains differ in their ability to grow on a number of aliphatic and aromatic compounds. Throughout this thesis, therefore, the organism will be referred to as bacterium NCIB 8250.

During the past few years bacterium NCIB 8250 has been employe to delineate not only the pathways of metabolism of a number of aromatic compounds, but also the regulatory mechanisms governing the synthesis of the enzymes involved. Cain (1961), Cain, Ribbons and Evans (1961) and Ornston (1966) have studied the metabolism of catechol and 3,4-dihydroxybenzoate: the degradative pathways of these two compounds by bacterium NCIB 8250 are outlined in Scheme 2.

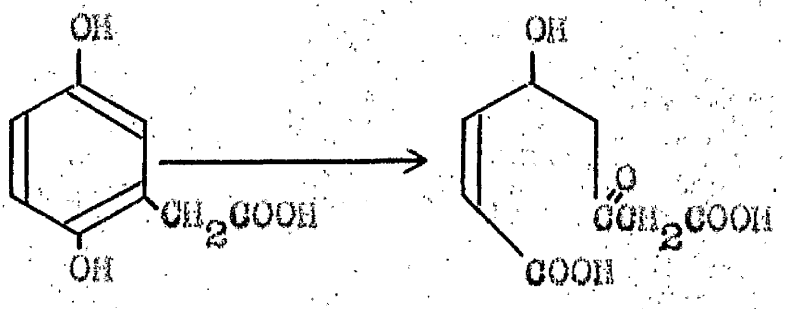
Ornston (1966) has examined the regulatory mechanisms governin the synthesis of the enzymes involved in the catabolism of both

Scheme 2.

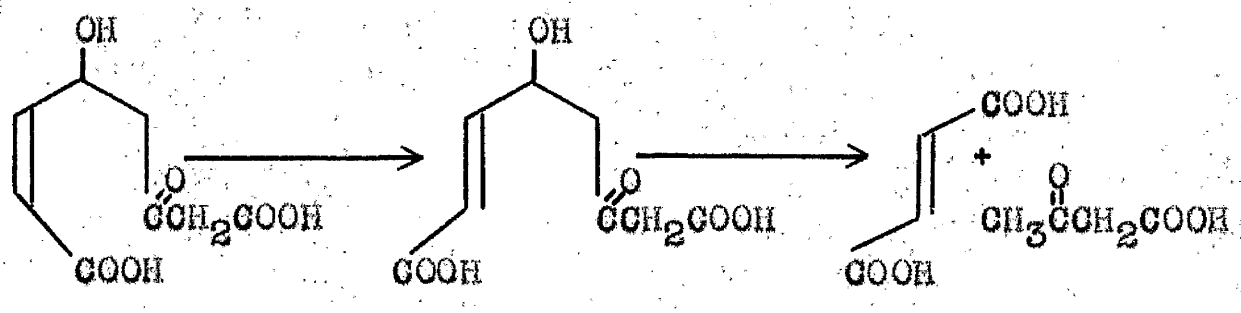

4-Hydroxy-
benzoate
HOOC
OH
OH
VIII
HOOC
COOH
COOH
HOOC
COOH
O
C=O
XI
E6
COOH
O
C=O
E7
O=
COOH
COOH
Succinate
Acetyl CoA
OH
OH
Benzoate
COOH
COOH
X
COOH
O
C=O

catechol and 3,4-dihydroxybenzoate. He concluded that cis-cis muconate (X) is the inducer in bacterium NCIB 8250 for all the enzymes involved in the conversion of catechol to β-oxoadipate enol-lactone (XI). He further found that 3,4-dihydroxybenzoate (VIII) is the inducer for all the enzymes converting 3,4-dihydroxybenzoate to β-oxoadipate enol-lactone. Cis-cis muconate does not induce any of the enzymes involved in the catabolism of 3,4-dihydroxybenzoate, nor does 3,4-dihydroxybenzoate induce any of the enzymes involved in the catabolism of catechol, the induction processes being independent of one another. Bacterium NCIB 8250 synthesises two isofunctional β-oxoadipate enol-lactone hydrolysases E_6 and E_7 depending on the inducer. The first induced by 3,4-dihydroxybenzoate, the second probably by benzoate. Hence the control mechanisms in bacterium NCIB 8250 permits strict economy of induced enzyme synthesis, but require two structural genes governing the synthesis of isofunctional enzymes.

Chapman and Dagley (1962) studied the metabolism of 2,5-dihydroxyphenylacetate (XII) by bacterium NCIB 8250. They suggested that 2,5-dihydroxyphenylacetate is oxidised through the sequence:



XII



This pathway is identical with that described by Ravdin and Crandall (1951) and Knox and Edwards (1955) for the oxidation of 2,5-dihydroxyphenylacetate in rat liver. It is interesting that Chapman and Dagley (1962) studied the oxidation of 2,5-dihydroxyphenylacetate by cells which had been grown on phenylacetate and indeed the authors suggest that the oxidation of phenylacetate by bacterium NCIB 8250 involves the intermediate formation of 2,5-dihydroxyphenylacetate.

The central role of the Krebs cycle in the metabolism of bacterium NCIB 8250 has been indicated by previous workers (Dagley and Rodgers, 1953; Dagley and Patel, 1955; Callely, Dagley and Hodgson, 1958). It may also be presumed that the organism possesses a glyoxylate cycle since it grows on acetate (Fewson, 1967a), and possesses isocitratase and malate synthetase (Callely et al, 1958). Another possible cyclic system of bacterium NCIB 8250 is the butane-2,3-diol cycle (Juni and Heym, 1956) for the dissimilation of

acetoin, diacetyl and butane-2,3-diol (Fewson, 1967a).

Whittaker (In Press) has examined the electron transport system of bacterium NCIB 8250 and concluded that cytochromes a_1, a_2 and b_1 are predominant together with ubiquinone Q-9.

The Present Experiments on the Oxidation of Mandelic Acid and Related Compounds by Bacterium NCIB 8250.

Fewson (1967a) showed that a large number of aromatic compounds can act as sole sources of carbon and energy for the growth of bacterium NCIB 8250. The elucidation, not only of the pathways of catabolism of these compounds, but also of the regulatory mechanisms governing the synthesis of the catabolic enzymes, should prove of considerable interest, particularly since bacterium NCIB 8250 is a non-saccharolytic organism: this property might even facilitate experiments on control mechanisms. It was also of interest to study the comparative biochemistry of mandelate catabolism since bacterium NCIB 8250 belongs to a quite different group of organisms from *P. putida* (Baumann and Doudoroff, In Press) which has hitherto been the only organism in which mandelate metabolism has been studied. Lastly it was felt that the potential of the technique of simultaneous adaptation has not been fully realised and that the technique could be exploited to give a great deal of information about the specificity of enzyme action and induction. This thesis, therefore, describes the elucidation of the pathways of oxidation of mandelate and related

compounds by bacterium NCIB 8250 and an examination of the specificity of the enzymes involved.

MATERIALS AND METHODS.

Organism.

Bacterium NCIB 8250 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland. Stock cultures were maintained in Oxoid cooked meat medium stored at 4°. Subcultures were made into Oxoid nutrient broth at intervals of 2 months and these were also kept at 4°. Inocula were produced by a further subculture into nutrient broth and incubated for 17 - 24 hr. at 30° immediately before use.

Growth of Bacterium NCIB 8250.

Basal medium.

The basal medium used in all these experiments was prepared by dissolving 6 g. KH_2PO_4 + 4 g. $(NH_4)_2SO_4$ in 1l. glass distilled water and adjusting to pH 7.0 with NaOH.

Testing compounds for their ability to serve as carbon and energy sources for growth.

Each carbon source was dissolved in glass distilled water to a concentration of 10mM and the pH adjusted to 7.0 with NaOH or HCl.

Appropriate solutions were dispensed into 250 ml. Erlenmeyer flasks as follows:

Basal medium (ml.)	Carbon source (ml.)	Water (ml.)	Final concentration of carbon source (mM)
25	25.0	0	5.0
25	2.5	22.5	0.5
25	0.5	24.5	0.1
25	0	25.0	0

The flasks were plugged with cotton wool and sterilised by autoclaving at 109°. 4-Hydroxy-D,L-mandelate, 4-hydroxy-3-methoxy-D,L-mandelate, 3,4-dihydroxy-D,L-mandelate, 3,4-dihydroxybenzaldehyde, catechol, 4-hydroxy-3-methoxybenzaldehyde and 4-hydroxy-3-methoxybenzyl alcohol, however, because they are probably heat-labile, were sterilised by filtration through Millipore filters (GSWP 047 00 0.22μ) prior to their addition to suitably diluted sterile basal media. To each flask was added 1.0 ml. sterile 2% (w/v) $MgSO_4.7H_2O$ and, as inoculum, 0.1 ml. of a 24 hr. nutrient broth culture. Growth was followed for 5 days at 30° on a rotary shaker (L. H. Engineering Co., Bells Hill, Stoke Poges, Bucks.; Mk V.) moving at about 180 oscillations/min. Visual estimation of the growth responses to different substrates was made by comparison with standard flasks containing 0.1, 0.3, 1.0 and 3.0 mg. wet wt. bacteria/ml. but in all cases tested the final result could be clearly stated as 'growth' or 'no growth'.

Determination of the growth rates of bacterium NCIB 8250 on the isomeric forms of mandelate.

An aliquot (16 ml.) of a 24 hr. nutrient broth culture of bacterium NCIB 8250 was inoculated into 800 ml. of half-strength basal medium containing 16 ml. of 2% (w/v) $MgSO_4.7H_2O$ and the appropriate mandelate at a final concentration of 1 or 2mM. The medium was contained in 1l. flasks fitted with side arms to facilitate sampling. Cultures were grown under conditions of vigorous aeration in an apparatus similar to that described by Schlegel, Kaltwasser and Gottschalk (1961). Magnet drive assemblies, constructed by Mr. N. Harvey in this Department, were arranged to accommodate the 1l. flasks in a water bath equipped with a Circotherm IIa (Shandon Scientific Company Limited, London N.W.10.) constant temperature unit set at 30°. Aeration was effected by means of 45mm. magnetic stirring bars encased in polypropylene. The vortex produced by the stirring extended to the bottom of the flask and was there distributed by the stirring bar so that bubbles were introduced into the body of the medium (Schlegel et al, 1961). Growth was followed by taking 4 ml. samples at 10 to 20 min. intervals and measuring the extinction at 500mμ in a Spectronic 20 colorimeter. Growth rates were determined by plotting $\log_{10}(E_{500})$ against time: the slopes of the resulting straight lines gave a measure of the mean generation times (Dawes, 1962).

Growth of bacterium NCIB 8250 for whole-cell studies and determination of growth rates.

For each of these experiments 2,100 ml. of half-strength basal medium containing the appropriate carbon source was prepared in a 3l. flat-bottomed flask containing a 45mm. polypropylene coated magnetic stirring bar. 100 ml. of this medium was withdrawn into a 250 ml. Erlenmeyer flask. The 3l. flask, now containing 2l. of medium, and the 250 ml. Erlenmeyer flask were plugged with cotton wool and sterilised by autoclaving at 109°. Heat-labile compounds (p. 21) were sterilised by filtration through Millipore filters (GSWP 047 00 0.22μ) prior to their addition to suitably diluted sterile basal media. In every case the carbon source was present at a final concentration of 1.0mM, except 2-hydroxybenzaldehyde which was present at 0.2mM and D,L-mandelate, 4-hydroxy-3-methoxy-D,L-mandelate, 3,4-dihydroxy-D,L-mandelate and 4-hydroxy-D,L-mandelate which were present at 2.0mM.

The 100 ml. quantity of medium was used to prepare the inoculum. To it were added 2 ml. 2% (w/v) $MgSO_4 \cdot 7H_2O$ and, as inoculum, 0.2 ml. of a 24 hr. nutrient broth culture of bacterium NCIB 8250. The flasks were placed in a 30° hot room on a rotary shaker (L. H. Engineering Co., Bells Hill, Stoke Poges, Bucks.; Mk V.) moving at about 180 oscillations/min. The bacteria were grown to early stationary phase (as determined from the previous growth experiments).

To each of the 2l. quantities of medium, equilibrated to 30°, was added 16 ml. 2% (w/v) $MgSO_4 \cdot 7H_2O$. The 100 ml. amounts of adapted

cells were used as inocula for their respective 2l. of media; inoculation being made by the aseptic addition of the adapted cultures from the Erlenmeyer flasks. The 3l. flasks were placed at 30° on magnetic stirrers constructed by Mr. N. Harvey (Illustration 1). Aliquots (4 ml.) were taken at approximately 30 min. intervals after the first hour. The extinctions were measured at 500mμ in a Spectronic 20 colorimeter and the mean generation times determined as previously described.

Preliminary experiments showed that E_{500}=0.16 was reached at the beginning of the last generation of growth. When the E_{500} of a culture reached 0.16, therefore, the flask was removed from the magnetic stirring device and immediately surrounded by ice. The cells were harvested by batch centrifugation at 12,000g for 30 min. at 4° (M.S.E. "Highspeed 18" Refrigerator Centrifuge). The supernatant solution was decanted and the pellet washed by resuspension in ice-cold distilled water. The cells were recentrifuged at 12,000g for 30 min. at 4°, the supernatant solution decanted, the cells weighed and resuspended in ice-cold distilled water to a concentration of 10 mg. wet wt./ml.. Subsequent experiments in the Warburg apparatus were always started within 4 hr..

Growth of bacterium NCIB 8250 for preparation of cell-free extracts.

(i) For determination of conditions of extraction and assay.

For each of these experiments 2l. of half-strength basal medium, containing either 5mM-D,L-mandelate or 2mM-benzyl alcohol was prepared

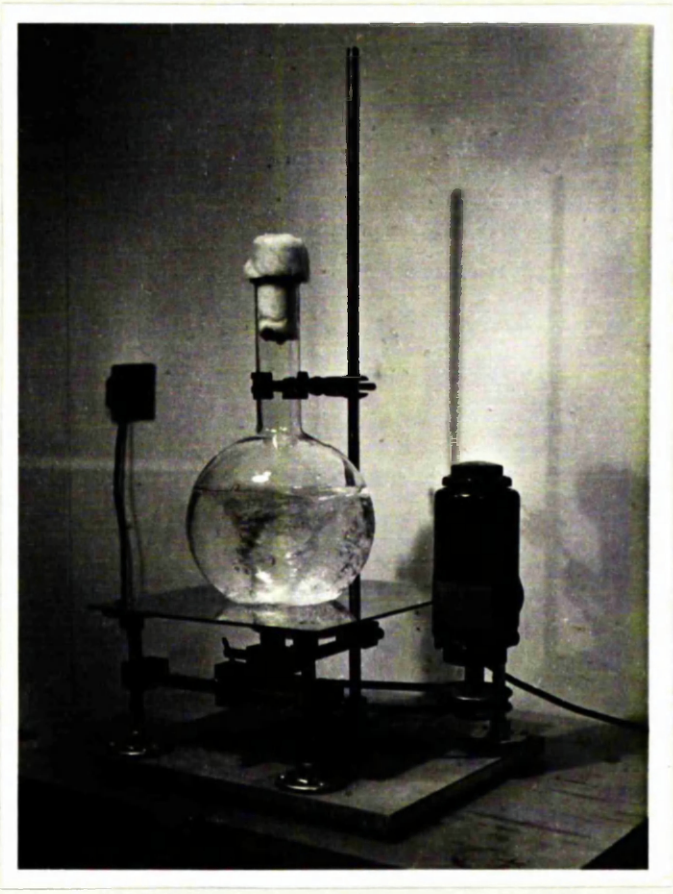


Illustration 1.

in a 3l. round-bottomed flask containing a 45mm. polypropylene coated magnetic stirring bar. The flasks were plugged with cotton wool and the medium sterilised by autoclaving at 109°. To each flask was added 80 ml. sterile 2% (w/v) $MgSO_4.7H_2O$ and, as inoculum, 80 ml. of a 24 hr nutrient broth culture of bacterium NCIB 8250. The flasks were placed at 30° on magnetic stirring assemblies. The bacteria were grown for approximately 16 hr. and the flasks removed from the drive assemblies and immediately surrounded by ice. The cells were harvested by batch centrifugation at 12,000g for 30 min. at 4° (M.S.E. "Highspeed 18" Refrigerator Centrifuge). The supernatant solutions were decanted and the cells washed by resuspension in chilled 0.066M-Na_2HPO_4-NaH_2PO_4 buffer, pH 8.2. The cells were recentrifuged at 12,000g for 30 min. at 4°, the supernatant solutions decanted and the cell pellets weighed. The cells were resuspended for breakage as specified in individual experiments.

(ii) <u>For the determination of the effect of pH on the time-course for the benzaldehyde dehydrogenase.</u>

For this experiment bacterium NCIB 8250 was grown in 2l. quantities of half-strength basal medium containing 5mM-D,L-mandelate. The cells were harvested by batch centrifugation at 12,000g for 30 min at 4°. Three approximately equal portions of the harvested cells were washed in 0.08M-sodium pyrophosphate buffer pH 7.0, 8.5 and 9.5 respectively. These portions were centrifuged as before, the supernatant solutions decanted, and the three washed pellets weighed

and resuspended in their appropriate buffer to 50 mg. wet wt./ml..

(iii) For determining the substrate specificities of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase.

For each of these experiments 2l. of half-strength basal medium containing the appropriate carbon source was prepared in a 3l. flat-bottomed flask containing a 45mm. polypropylene coated magnetic stirring bar. ~~Each~~ D,L-mandelate/or substituted derivative was present at a final concentration of 5mM and ~~each~~ benzyl alcohol/or substituted derivative 4-hydroxy-3-methoxybenzaldehyde was present at 2mM. The exception was 2-hydroxybenzyl alcohol which was present at 1mM. The flasks were plugged with cotton wool and sterilised by autoclaving at 109°. Heat-labile compounds (p. 21) were sterilised by filtration through Millipore filters (GSWP 047 00 0.22μ) prior to their addition to suitably diluted sterile basal medium. The flasks were inoculated and the bacteria grown as described above. The cells were harvested by batch centrifugation at 12,000g for 30 min. at 4° and the supernatant solutions decanted. Two approximately equal portions of the harvested cells were washed in 0.08M-sodium pyrophosphate buffer pH 8.5 or 9.5. These portions were centrifuged as before, the supernatant solutions decanted, and the two washed pellets weighed and resuspended in their appropriate buffer to 50 mg. wet wt./ml..

Measurement of Oxygen Uptake by Washed Cell Suspensions.

The ability of washed cell suspensions of bacterium NCIB 8250 to oxidise a number of challenge substrates was determined manometrically in the Warburg apparatus (B. Braun, Apparatebau, Melsungen, W. Germany; Models V85 and VL85).

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

Main compartment:	0.1 ml. 0.3M-$(NH_4)_2SO_4$.
	0.1 ml. 0.03M-$MgSO_4.7H_2O$.
	0.1 ml. 0.5M-KH_2PO_4-K_2HPO_4 buffer pH 7.0.
	0.2 ml. distilled water.
	0.8 ml. cell suspension (10 mg. wet wt./ml.).
Side arm:	0.5 ml. 7.2mM-"challenge substrate" dissolved in 0.1M-KH_2PO_4-K_2HPO_4 buffer and the pH adjusted to 7.0 with 0.1N-NaOH or 0.1N-HCl.
Centre well:	0.2 ml. 20% KOH and pleated filter paper (Whatman No. 52).
Atmosphere:	Air
Temperature:	30°

The final concentrations of NH_4^+, $SO_4^=$, Mg^{++} and $PO_4^{\equiv}$ in the Warburg flasks were almost identical to those in the medium in which the cells had been grown. The order of addition was: (1) "challenge substrate" into side-arm, (2) $(NH_4)_2SO_4$-$MgSO_4$-phosphate-water (usually added as 0.5 ml. of a mixture prepared from sterilised components), (3) cell suspension, (4) KOH into centre well. The contents of the Warburg vessels were equilibrated for 10 min. in 30° water baths, the manometers were sealed, and the air-tightness of the joints checked five min. later. The reaction was started by tipping the contents of the side-arm into the main compartment. Readings were taken every 10 min. for the first hr., every 20 min. for the second hr. and thereafter every 30 min. up to 6 hr..

Cell Disruption.

Ultrasonic disruption.

A suitable volume (20 - 40 ml.) of a washed cell suspension containing 50 mg. wet wt. cells/ml. was placed in a glass cone-shaped vessel equipped with 4 cooling vanes (Illustration 2) and surrounded by an ice-water slurry. Ultrasonic disruption was effected by means of the 13mm. probe of the Dawe 'Soniprobe' (Dawe Instruments Limited, Acton, London W.3; Type 1130A). The instrument was always used at setting 8 which gave a current of 4 - 5 amps. The total time of actual disruption was up to 20 min. but the current was switched off

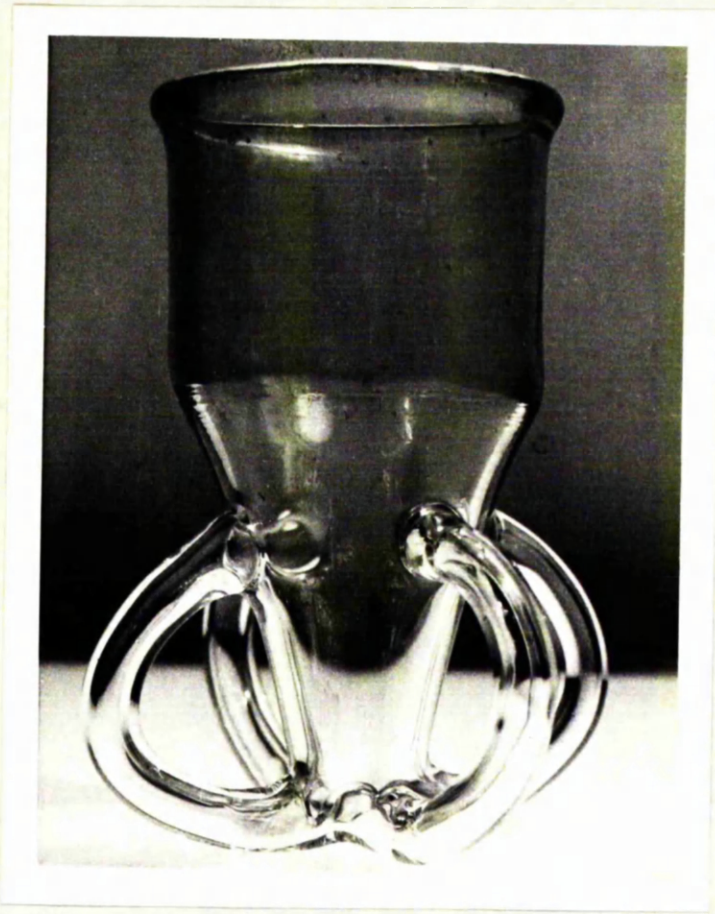


Illustration 2.

(Actual Size)

during every alternate minute to aid cooling. The temperature of the extract never rose above 10°. The extract was centrifuged at 20,000g for 40 min. at 4° (M.S.E. Refrigerated High Speed Angle 13" Centrifuge) to remove whole cells and debris. The supernatant solution, which was light yellow in colour, was decanted and stored at -10°. The enzymatic activity of this cell-free extract was always determined within 48 hr..

Grinding with alumina.

A washed cell pellet (approximately 3.0 g.) was mixed with 2.5 times its weight of alumina (Bacteriological Grade Alumina A-305, Alcoa Chemicals, Aluminium Company of America.) contained in a porcelain mortar surrounded by ice. The cells were broken by vigorous grinding with a glass pestle for 20 min.. To the resulting paste was added chilled 0.066M-Na_2HPO_4-NaH_2PO_4 buffer pH 8.2, to give a concentration of 50 mg. cell materi[illegible] HPO_4-NaH_2PO_4 buffer pH 8.2, to gi[illegible] debris were removed by centrifugation at 20,000g for 20 min. at 4°. The cell-free extract was stored at -10° and assayed within 24 hr..

Hughes Press treatment.

A thick slurry of washed cells containing approximately 1 g. wet wt. cells/3 ml. 0.066M-NaH_2PO_4-Na_2HPO_4 buffer pH 8.2 was frozen to -20° in the cell-block (Shandon Scientific Company Limited, London N.W.1

of a Hughes Press. The cells were disrupted in the conventional manner (Hughes, 1951) with the aid of a fly press. The frozen cell crush was removed and thawed in sufficient ice-cold 0.066M-Na_2HPO_4-NaH_2PO_4 buffer, pH 8.2, to give a final concentration of 50 mg. cell material/ml.. Whole cells, cell debris and a light yellow flocculent precipitate were removed by centrifugation at 20,000g for 40 min. at 4°. The cell-free extract was stored at -10° and assayed within 24 hr..

Spectrophotometric Assays.

Reactions were conducted in 1cm. path-length silica cuvettes containing a total liquid volume of 3.0 ml.. Measurements of extinction were made by means of a Unicam SP. 800 Ultraviolet Spectrophotometer (Unicam Instruments Ltd., York Street, Cambridge.) which was connected to a Servoscribe Chart Recorder (Kelvin Electronics Company, Wembley Park Drive, Wembley, Middlesex.). All reactions were carried out at room temperature (21±1°).

L-Mandelate dehydrogenase.

L-Mandelate dehydrogenase was assayed by adapting the method employed by Hegeman (1966a). The assay was dependent on the reduction of an electron-acceptor dye, 2,6-dichlorophenol-indophenol, to the leuco form concomitant with the oxidation of L-mandelate to benzoylformate. The reaction was measured at 600mμ, near the maximal

extinction of the oxidised form of the dye. Neither L-mandelate nor any of the substituted D,L-mandelates absorb at this wavelength. The standard reaction mixture contained:

0.5 ml. 0.1M-sodium pyrophosphate buffer pH 7.0

0.1 ml. 2mM-2,6-dichlorophenol-indophenol

0.1 ml. enzyme

0.1 ml. 30mM-D,L-mandelate

2.2 ml. distilled water

The reference cuvette contained 3.0 ml. distilled water. The reaction was initiated by the addition of the D,L-mandelate.

The extinction coefficient of the dye at 600mμ and pH 7.0 is 20.6×10^6 cm.^{2}/mole, and therefore a decrease in extinction of 6.87 units corresponded to the oxidation of 1 μmole of L-mandelate to benzoylformate.

Benzyl alcohol dehydrogenase.

Benzyl alcohol dehydrogenase was assayed by following the benzyl alcohol-dependent reduction of NAD^+ at 340mμ. The standard reaction mixture contained:

0.5 ml. 0.1M-sodium pyrophosphate buffer pH 8.5

0.1 ml. 0.015M-NAD^+ in 0.05M-sodium pyrophosphate buffer pH 8.5

0.1 ml. enzyme

0.1 ml. 3mM-benzyl alcohol

2.2 ml. distilled water

The reference cuvette contained 3.0 ml. distilled water. The reaction was initiated by the addition of the benzyl alcohol. With a value of 6.22×10^6 $cm.^2/mole$ for the extinction coefficient of NADH at 340mμ, an increase in extinction of 2.07 units corresponded to the oxidation of 1 μmole of benzyl alcohol to benzaldehyde. Corrections to the initial rate due to the activity of the benzaldehyde dehydrogenase were not made.

Benzaldehyde dehydrogenase.

(i) At 340mμ.

Benzaldehyde dehydrogenase was assayed by following the benzaldehyde-dependent reduction of NAD^+ at 340mμ. The standard reaction mixture contained:

0.5 ml. 0.1M-sodium pyrophosphate buffer pH 9.5

0.1 ml. 0.015M-NAD^+ in 0.05M-sodium pyrophosphate buffer pH 9.5

0.1 ml. enzyme

0.1 ml. 3mM-benzaldehyde

2.2 ml. distilled water

The reference cuvette contained 3.0 ml. distilled water. The reaction was initiated by the addition of the benzaldehyde. With a value of 6.22×10^6 $cm.^2$/mole for the molar extinction coefficient of NADH at 340mμ an increase in extinction of 2.07 units corresponded to the oxidation of 1 μmole of benzaldehyde to benzoate.

(ii) At 282mμ.

Benzaldehyde dehydrogenase was also assayed by exploiting the fact that benzaldehyde has an extinction coefficient of approximately 1.5×10^6 $cm.^2$/mole at 282mμ and pH 9.5, while the product, benzoate, has negligible extinction at this wavelength. The standard reaction mixture contained:

0.5 ml. 0.1M-sodium pyrophosphate buffer pH 9.5

0.1 ml. 0.015M-NAD^+ in 0.05M-sodium pyrophosphate buffer pH 9.5

0.3 ml. enzyme

0.3 ml. 3mM-benzaldehyde

1.8 ml. distilled water

The reference cuvette contained 0.3 ml. enzyme + 2.7 ml. distilled water. The reaction was initiated by the addition of the benzaldehyde.

At 282mμ and pH 9.5 both NAD^+ and NADH absorb:

$$\frac{E_{282}\ NAD^+}{E_{282}\ NADH} = 0.87$$

By measuring the change in extinction at 340mμ due to the formation of NADH, it was possible to calculate the change in extinction at 282mμ due to NADH formation and therefore to correct the 282mμ measurements to give just the rate of disappearance of benzaldehyde.

NADH oxidase.

NADH oxidase was assayed by following the oxidation of NADH at 340mμ. The standard reaction mixture contained:

0.5 ml. 0.1M-sodium pyrophosphate buffer pH 7.0

0.1 ml. 5mM-NADH in 0.05M-sodium pyrophosphate buffer pH 7.0

0.1 ml. enzyme

2.3 ml. distilled water

The reference cuvette contained 3.0 ml. distilled water. The reaction was initiated by the addition of NADH. With a value of 6.22×10^6 cm.^{2}/mole for the extinction coefficient of NADH at 340mμ, a decrease in extinction of 2.07 units corresponded to the oxidation of 1 μmole NADH to NAD^+.

Corrections to the initial rates of oxidation of the benzyl alcohols and benzaldehydes.

The 2-hydroxy, 3-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy-substituted benzaldehydes absorb light at 340mμ. The extinction coefficients of these compounds were determined at pH 9.5 and found to be:

2-Hydroxybenzaldehyde	1.4×10^6 cm.^{2}/mole
3-Hydroxybenzaldehyde	1.6×10^6 cm.^{2}/mole
4-Hydroxybenzaldehyde	21.0×10^6 cm.^{2}/mole
3,4-Dihydroxybenzaldehyde	20.0×10^6 cm.^{2}/mole
4-Hydroxy-3-methoxybenzaldehyde	22.5×10^6 cm.^{2}/mole

None of the benzyl alcohols or benzoates showed any absorption at 340mμ. The measured changes in extinction at 340mμ had therefore to be corrected in order to give a true estimate of the rate of formation of NADH. Corrections factors were calculated in the following way.

Consider the reduction of 1 μmole NAD^+ by 1 μmole 2-hydroxybenzaldehyde in a 3 ml. cuvette.

The increase in E_{340} due to NAD^+ reduction = 2.07

The decrease in E_{340} due to 2-hydroxybenzaldehyde disappearance = 0.47

Therefore total increase in E_{340} = 2.07 - 0.47

= 1.60

Therefore correction factor converting observed change in E_{340} to change in E_{340} due to NADH formation

$$= \frac{2.07}{1.60}$$

= 1.29

The following correction factors were calculated in the same way:

Enzyme	Substrate	Correction factor
Benzyl alcohol dehydrogenase	2-Hydroxybenzyl alcohol	0.82
	3-Hydroxybenzyl alcohol	0.79
	4-Hydroxybenzyl alcohol	0.23
	4-Hydroxy-3-methoxybenzyl alcohol	0.22

Benzaldehyde dehydrogenase	2-Hydroxybenzaldehyde	1.29
	3-Hydroxybenzaldehyde	1.35
	4-Hydroxybenzaldehyde	- 0.42
	3,4-Dihydroxybenzaldehyde	- 0.46
	4-Hydroxy-3-methoxybenzaldehyde	- 0.38

Analytical Methods.

Nitrogen.

Aliquots containing approximately 0.5 mg. wet wt. of cells were digested with 0.25 ml. 1% (v/v) selenium dioxide dissolved in 50% (v/v) H_2SO_4 in a sand bath set at 180°. Ammonia determinations were made on the resulting digests by the Nessler method (Paul, 1958). AnalaR $(NH_4)_2SO_4$ was used as a standard.

Protein.

Total protein in the centrifuged cell-free extracts was estimated by measuring the E_{280} of suitably diluted samples in the Unicam SP. 800 Ultraviolet Spectrophotometer (Unicam Instruments Ltd., York Street, Cambridge.). Measurements 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 means of the Folin-Ciocalteau reagent (Lowry, Rosebrough, Farr and Randall, 1951) using bovine plasma albumin (Armour Pharmaceutical Company Ltd., Eastbourne, England.) as standard. Fewson found that with unfractioned extracts there was no advantage in measuring E_{260} as well as E_{280}.

pH values.

The pH values of solutions having a volume of 25 ml. or more were determined by means of an E.I.L. direct reading pH meter (Electronic Instruments Limited, Richmond, Surrey, England; Model 23A). The pH values of solutions having a volume of less than 25 ml. were determined with a Pye 'Dynacap' direct reading pH meter (W. G. Pye and Co. Ltd., Granta Works, Cambridge, England.) fitted with a microsampling electrode system (Electronic Instruments Limited, Richmond, Surrey, England; Type SMS 23).

Melting point.

Melting points (corrected) were determined using a Gallenkamp melting point apparatus (A. Gallenkamp and Co. Ltd., Sun Street, London; Type MF-370).

Optical rotation.

Optical rotation was measured in a Bellingham and Stanley polarimeter (Bellingham and Stanley Limited, London N.15; Model A). The light source was the D line (5893Å) of sodium and the solution under investigation was contained in a 4dm. glass tube.

Preparation of Buffers.

The buffers which were employed were:

0.5M-KH_2PO_4-K_2HPO_4

0.066M-Na_2HPO_4-NaH_2PO_4

0.08M and 0.1M-sodium pyrophosphate

The 0.5M-KH_2PO_4-K_2HPO_4 and the 0.066M-$Na_2HPO_4$$NaH_2PO_4$ buffers were prepared by dissolving the appropriate quantity of each of the four components in glass distilled water. The two components of each buffer were then mixed to give the required pH. The 0.08M and the 0.1M-sodium pyrophosphate buffers were prepared by dissolving the appropriate quantity of tetra-sodium pyrophosphate in glass distilled water and adjusting the pH to the required value with 1N-NaOH or 1N-HCl. All buffers were stored at 4°.

Preparation of Glassware.

All glassware used for the growth of bacterium NCIB 8250 was washed for 15 min. in boiling 10% nitric acid and thoroughly rinsed in tap-water and distilled water.

Other glassware was cleaned with Haemosol (Meinecke and Co. Inc., Baltimore, U.S.A.).

Materials.

D,L-Mandelic acid, benzyl alcohol, benzaldehyde (AnalaR), 2-hydroxybenzyl alcohol (Saligenin), 2-hydroxybenzaldehyde (Salicylaldehyde), 2-hydroxybenzoic acid (Salicylic acid; AnalaR), 3-hydroxybenzaldehyde, 3-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (Protocatechuic acid), 4-hydroxy-3-methoxy-D,L-mandelate and 4-hydroxy-3-methoxybenzaldehyde (Vanillin) were obtained from British Drug Houses Ltd., Poole, Dorset, England. L(+)-Mandelic acid, D(-)-mandelic acid, NAD^+, $NADP^+$ and NADH were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.. 4-Hydroxy-D,L-mandelic acid (Pure), benzoic acid (Zone Refined) and 4-hydroxy-3-methoxybenzoic acid (Vanillic acid) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. 3-Hydroxy-D,L-mandelic acid, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol and 4-hydroxy-3-methoxybenzyl alcohol (Vanillyl alcohol) were all from K & K Laboratories, Plainview, New York, U.S.A.. 3,4-Dihydroxy-D,L-

mandelic acid and Cleland's reagent were supplied by Calbiochem, Los Angeles, California, U.S.A., benzoylformic acid by Aldrich Chemical Company Inc., Milwaukee, Wisconsin, U.S.A. and 3,4-dihydroxybenzaldehyde by Hopkin and Williams Ltd., Chadwell Heath, Essex, England. Chloramphenicol was obtained from Parke, Davis and Company Limited, Hounslow, England.

D,L-mandelic acid, benzoylformic acid, 2-hydroxybenzyl alcohol, 3-hydroxybenzoic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 3,4-dihydroxybenzoic acid, 4-hydroxy-3-methoxybenzaldehyde and 4-hydroxy-3-methoxybenzoic acid were recrystallised from hot water (80°) until the melting points were within a two-degree range which included the literature value.

Benzyl alcohol, 2-hydroxybenzaldehyde and benzaldehyde were redistilled before use: the fractions which boiled over a two-degree range which included the literature value were collected. The redistilled benzaldehyde was stored under nitrogen.

The optical rotation of the D- and L-isomers of mandelic acid agreed with the values given in the literature (Mislow, 1951).

All other chemicals were the best grade which could be obtained commercially.

RESULTS.

Growth Experiments.

The ability of a number of mandelic acids and related compounds to serve as sole sources of carbon and energy for the growth of bacterium NCIB 8250 is shown in Table 1. 3,4-Dihydroxybenzyl alcohol, 2-hydroxy-D,L-mandelate and the substituted benzoylformates were not tested since these compounds could not be obtained commercially. Attempts to synthesise 2-hydroxy-D,L-mandelic acid from the corresponding nitrile by a method analagous to that used for mandelic acid (Vogel, 1948) produced a dark brown insoluble gum: this has been confirmed by Dr. R. Howe of Imperial Chemical Industries Ltd. who has, however, subsequently prepared this compound by a new method (Private Communication). Preliminary experiments showed that in most cases 1mM was a suitable concentration at which to follow growth. 2-Hydroxy-benzaldehyde, however, was toxic at this concentration and was tested at 0.2mM. The mandelates, being racemic mixtures, were provided at 2mM.

Growth was obtained with D,L-mandelate, benzoylformate, benzyl alcohol, benzaldehyde and benzoate and also with 2-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy derivatives of these compounds. There was no growth with the 3-hydroxy-substituted compounds. In general the mean generation time increased in the order benzoate, benzaldehyde, benzoylformate, D,L-mandelate and also increased with the

Table 1. The ability of a number of aromatic compounds to act as sole source of carbon and energy for the growth of bacterium NCIB 8250.

Bacterium NCIB 8250 was inoculated into basal salts media containing the various growth compounds at a final concentration of 1mM. Growth and mean generation times were determined as described in Methods.

* - Tested at a final concentration of 2mM.

† - Tested at a final concentration of 0.2mM.

\+ - Growth.

\- - No growth.

N.T.- Not tested.

( ) - Mean generation time in minutes.

Growth substrate	Substituents on growth subs			
	Non-substituted	2-Hydroxy	3-Hydroxy	4-Hydroxy
D,L-Mandelate	+ (185*)	N.T.	-	+ (215*
Benzoylformate	+ (115)	N.T.	N.T.	N.T.
Benzyl alcohol	+ (60)	+ (88)	-	+ (88)
Benzaldehyde	+ (73)	+ (127†)	-	+ (70)
Benzoate	+ (50)	+ (70)	-	+ (62)

number and size of ring substituents. Growth on the benzaldehydes was generally slower than on the corresponding benzyl alcohols e.g. a mean generation time of 194 minutes on 4-hydroxy-3-methoxy benzaldehyde compared with 90 minutes on 4-hydroxy-3-methoxybenzyl alcohol. Growth on 3,4-dihydroxy-D,L-mandelate and 4-hydroxy-3-methoxy-D,L-mandelate was extremely slow and accurate estimates of the growth rates were not made. Catechol, acetate and succinate also served as sole sources of carbon and energy giving mean generation times of 50, 45 and 45 minutes respectively.

The ability of the isomeric forms of mandelate to act as sole sources of carbon and energy for the growth of bacterium NCIB 8250 is shown in Table 2. The bacterium grew on the L-isomer of mandelate and on the racemic D,L-mixture with essentially the same mean generation times. The bacterium did not, however, grow on the D-form of mandelate. This result was confirmed repeatedly, even when the inoculum had been grown on D,L-mandelate. The stationary phase population after growth on the L-form was twice (0.216 compared to 0.108) that recorded after growth on an equimolar amount of the D,L-form.

Table 2. The ability of the isomeric forms of mandelate to act as sole source of carbon and energy for the growth of bacterium NCIB 8250.

Bacterium NCIB 8250 was inoculated into basal salts media containing the appropriate mandelates as sole sources of carbon and energy for growth. The mean generation times were determined as described in Methods.

Growth substrate	Concentration (mM)	Mean generation time (min.)	Stationary phase $E_{500\ m\mu}$
D,L-Mandelate	1	140	0.108
D,L-Mandelate	2	136	Not obtained
L-Mandelate	1	153	0.216
L-Mandelate	2	138	Not obtained
D-Mandelate	1	No growth	-
D-Mandelate	2	No growth	-

Experiments with Washed Cell Preparations.

Outline of the method of approach.

The technique of simultaneous adaptation was employed to investigate the pathways of metabolism of mandelate and related compounds. Bacterium NCIB 8250 was grown on each aromatic compound in turn and harvested in the late exponential phase of growth. The cells were washed and their ability to oxidise a large number of "challenge" substrates was determined manometrically as described in Methods. A preliminary experiment was performed to determine the effect of the amount of cells present in the Warburg flask on the rate of oxidation of challenge substrates. The resulting graph was linear up to 8.0 mg. wet wt. whereafter the rate of oxidation began to fall off. In all subsequent experiments, therefore, a wet wt. of 8.0 mg. of cells (containing approximately 160 µg. of nitrogen) was employed. The basal salts medium in which the washed cells were exposed to the challenge substrates was essentially identical to that in which the cells had been grown. All challenge substrates were present at a final concentration of 2mM.

Oxidation of the isomeric forms of mandelate.

The ability of washed cells of bacterium NCIB 8250 which had been grown on D,L-mandelate to oxidise the isomeric forms of mandelate is shown in Fig. 1. The results reflect the observations made in the growth experiment (Table 2). The L- and D,L-forms of mandelate were oxidised, with the L-form taking up 400 µl. of oxygen: approximately twice that of the D,L-form (195 µl.). These oxygen uptakes correspond to 62% and 31% of that required for complete oxidation to CO_2 and H_2O. The D-isomer of mandelate was not oxidised.

Patterns of oxygen utilisation.

During the course of this series of experiments a number of distinct patterns of oxygen utilisation was observed and these are shown in Fig. 2.

Pattern A - Immediate complete utilisation. The initial rate of oxidation of challenge substrates which gave this type of oxygen utilisation pattern was between 40 and 260 µmoles O_2/hr./mg.N. In general the total oxygen uptake was 50 - 80% of that required for the complete oxidation of the challenge substrate to CO_2 and H_2O.

Pattern B - Immediate limited utilisation. The initial rate of oxidation of challenge substrates which gave this type of pattern was between 5 and 120 µmoles O_2/hr./mg.N. In this case, unlike those challenge substrates which gave pattern A, the total oxygen uptake

Fig.1. Oxidation of the isomeric forms of mandelate by washed suspensions of bacterium NCIB 8250.

Cells were grown on 2mM-D,L-mandelate as sole source of carbon, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells.

o- D,L-Mandelate.

Δ- L-Mandelate.

□- D-Mandelate.

o- Control with no added substrate.

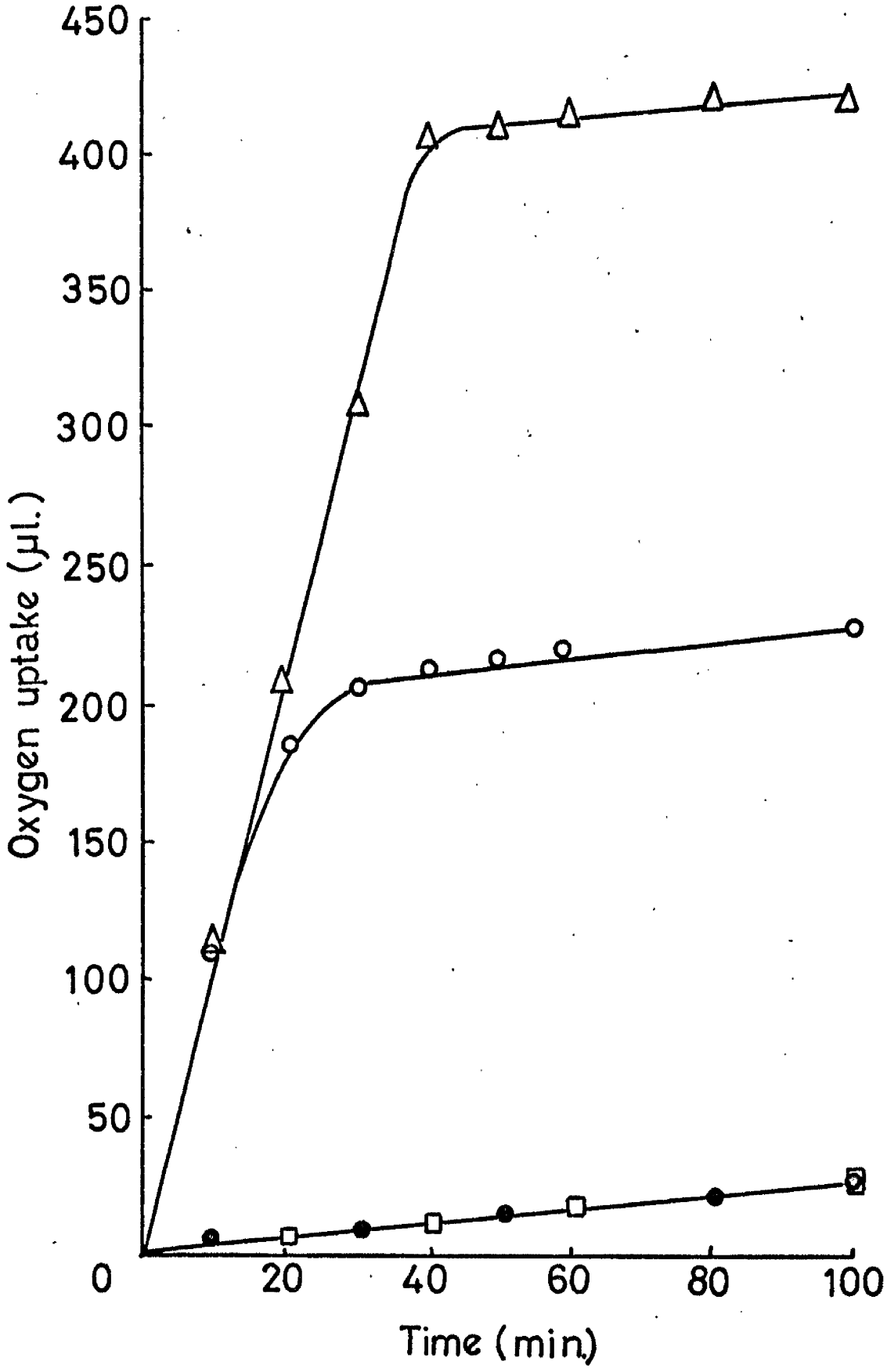

450
400
350
300
250
200
150
100
50
0
20
40
60
80
100
Oxygen uptake (µl.)
Time (min.)

Fig.2. Patterns of oxygen utilisation obtained with bacterium NCIB 8250.

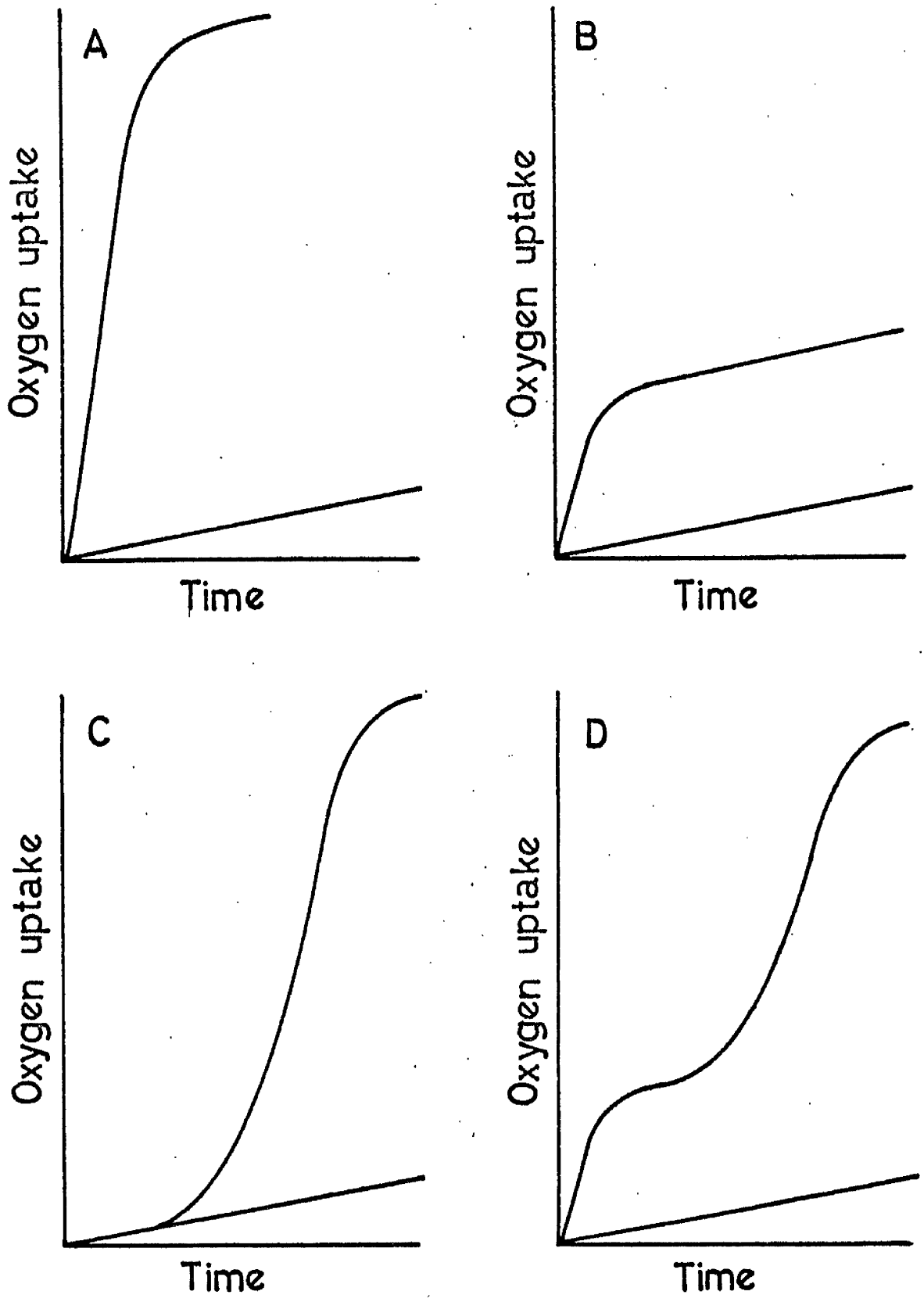
A- Immediate complete utilisation.

B- Immediate limited utilisation.

C- Lag then complete utilisation.

D- Immediate limited utilisation-lag-further utilisation.

In each figure the lower line represents utilisation with no added substrate or no utilisation with added substrate.


A
Oxygen uptake
Time
B
Oxygen uptake
Time
C
Oxygen uptake
Time
D
Oxygen uptake
Time

was only 7 - 15% of that required for the complete oxidation of the challenge substrate to CO_2 and H_2O. This very low percentage oxidation was equivalent to an oxygen uptake of between 1 µatom O_2/µmole of substrate and 1 µmole O_2/µmole of substrate.

Pattern C - Lag then complete utilisation. The duration of the lag varied within wide limits, from only a few minutes to more than 5 hours. The lag was followed by a period of increasing oxygen uptake with both a maximum rate of oxygen uptake and a final stoichiometry of oxygen uptake similar to that observed with challenge substrates which gave pattern A.

Pattern D - Immediate limited utilisation-lag-further utilisation The first phase of this diphasic pattern was similar to pattern B, with an initial rate of oxidation between 5 and 120 µmoles O_2/hr./mg. N. and a plateau in oxygen uptake occurring after 1 µatom or 1 µmole O_2/µmole of substrate had been utilised. The second phase, which is similar to pattern C, showed a lag, again of varying length, a period of increasing oxygen uptake and, like both patterns A and C, a final oxygen uptake of 50 - 80% of that required for complete oxidation of the challenge substrate to CO_2 and H_2O.

The final oxygen uptake of the control with no added substrate was less than 10% of that with challenge substrates which gave complete utilisation of oxygen. A number of challenge substrates failed to raise the oxygen uptake above the endogenous level even after 6 - 24 hours incubation (Pattern E).

Patterns of oxygen utilisation with cells grown on 4-hydroxy-D,L-mandelate.

The patterns of oxygen uptake outlined above can be illustrated by reference to the results obtained with cells grown on 4-hydroxy-D,L-mandelate (Figs. 3, 4a, 4b and 5).

A number of challenge substrates which gave an immediate and complete utilisation of oxygen are shown in Fig. 3. These were 4-hydroxy-D,L-mandelate (the growth substrate), 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoate and 3,4-dihydroxybenzoate. The final oxygen uptake obtained with these challenge substrates was generally between 60 and 75% of that required for complete oxidation; the exception was 4-hydroxy-D,L-mandelate where the oxygen uptake was only 35% of that required for complete oxidation.

The patterns of oxidation obtained with the non-substituted compounds, catechol and β-oxoadipate are shown in Figs. 4a and 4b. D,L-mandelate, benzoylformate, benzyl alcohol in Fig. 4a and benzaldehyde in Fig. 4b all gave a diphasic oxygen uptake pattern (pattern D). The intermediate lag in the oxidation of these substrates occurred after an uptake of 1 µatom O_2/µmole D,L-mandelate, 1 µatom O_2/µmole benzoylformate, 1 µmole O_2/µmole benzyl alcohol and 1 µatom O_2/µmole benzaldehyde. Benzoate and catechol were oxidised only after a lag, whilst β-oxoadipate was oxidised immediately. The final oxygen uptakes were between 60 and 75% of that required for complete oxidation, except in the case of D,L-mandelate where the oxygen uptake was only 33% of that required for

Fig.3. Oxidation of the 4-hydroxy-substituted compounds and 3,4-dihydroxybenzoate by washed suspensions of bacterium NCIB 8250.

Cells were grown on 2mM-4-hydroxy-D,L-mandelate as sole carbon source, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells.

○ - 4-Hydroxy-D,L-mandelate.

△ - 4-Hydroxybenzyl alcohol.

□ - 4-Hydroxybenzaldehyde.

⊙ - 4-Hydroxybenzoate.

△ - 3,4-Dihydroxybenzoate.

⊡ - Control with no added substrate.

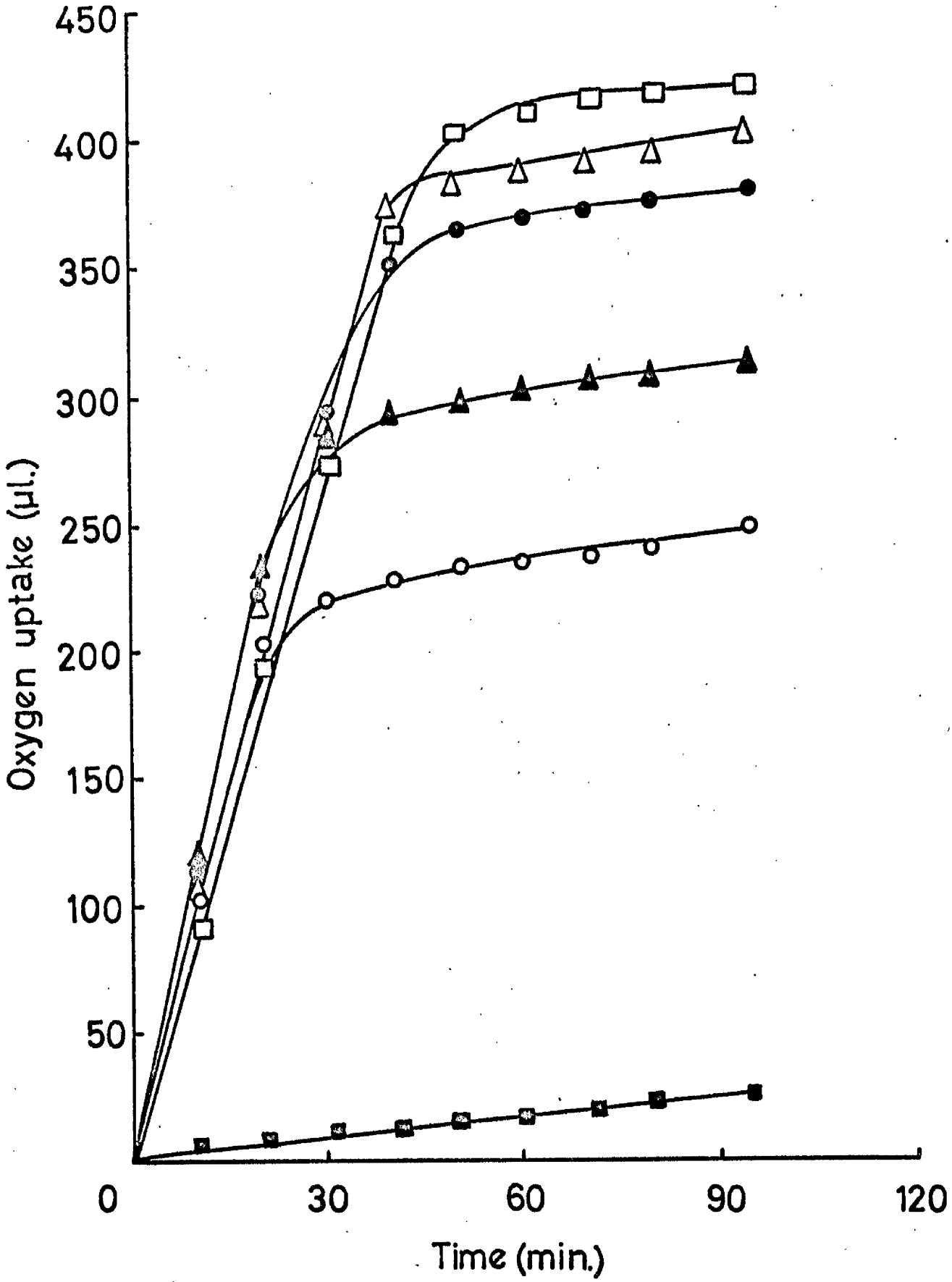

450
400
350
300
250
200
150
100
50
0
30
60
90
120
Oxygen uptake (µl.)
Time (min.)

Fig.4a. Oxidation of some non-substituted compounds by washed suspensions of bacterium NCIB 8250.

Cells were grown on 2mM-4-hydroxy-D,L-mandelate as sole carbon source, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells.

○- D,L-Mandelate.

△- Benzoylformate.

□- Benzyl alcohol.

●- Control with no added substrate.

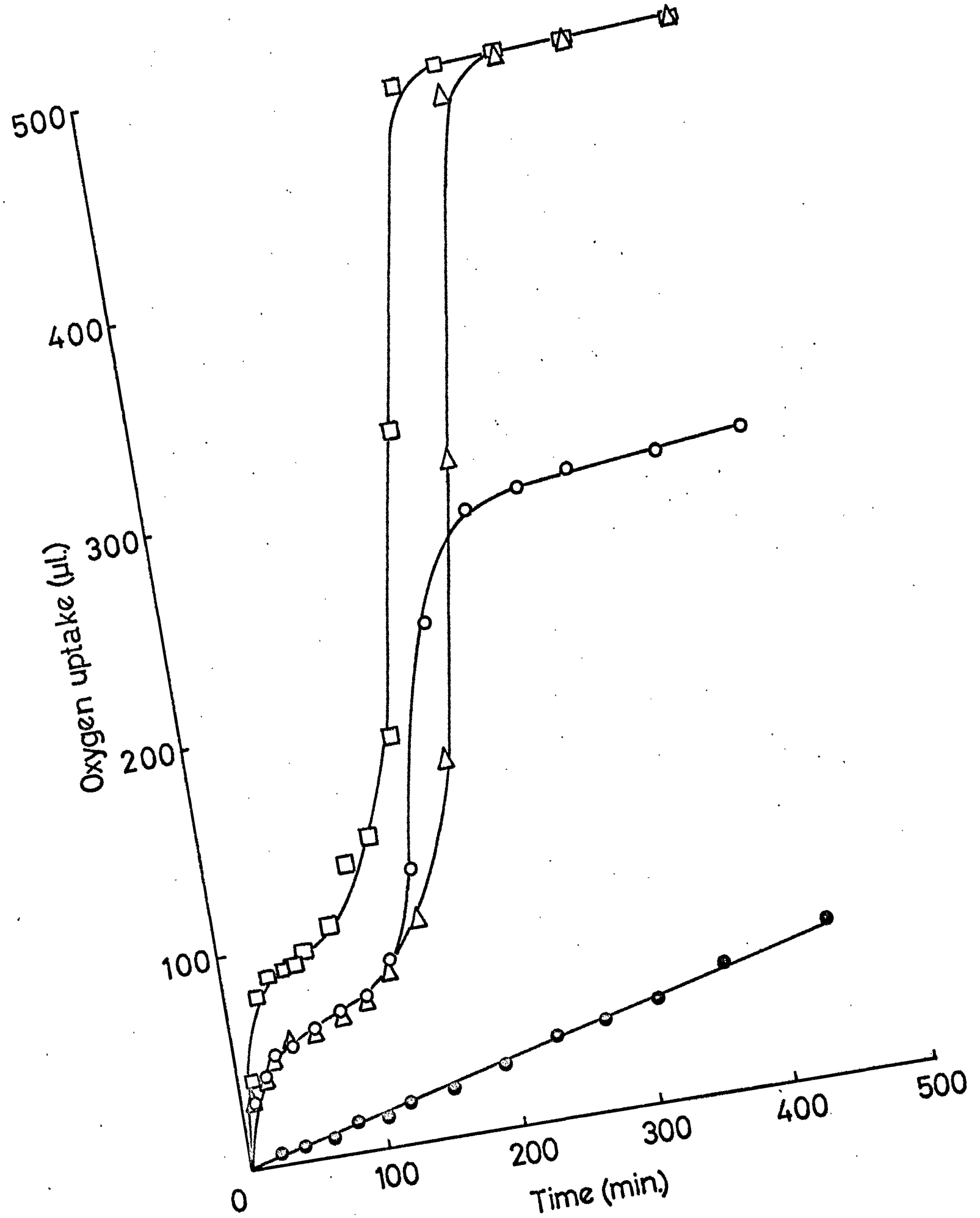

500
400
300
200
100
0
Oxygen uptake (µl)
100
200
300
400
500
Time (min.)

Fig.4b. Oxidation of some non-substituted compounds, catechol and β-oxoadipate by washed suspensions of bacterium NCIB 8250.

Cells were grown on 2mM-4-hydroxy-D,L-mandelate as sole source of carbon, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells.

o- Benzaldehyde.

Δ- Benzoate.

□- Catechol.

⊙- β-Oxoadipate.

▲- Control with no added substrate.

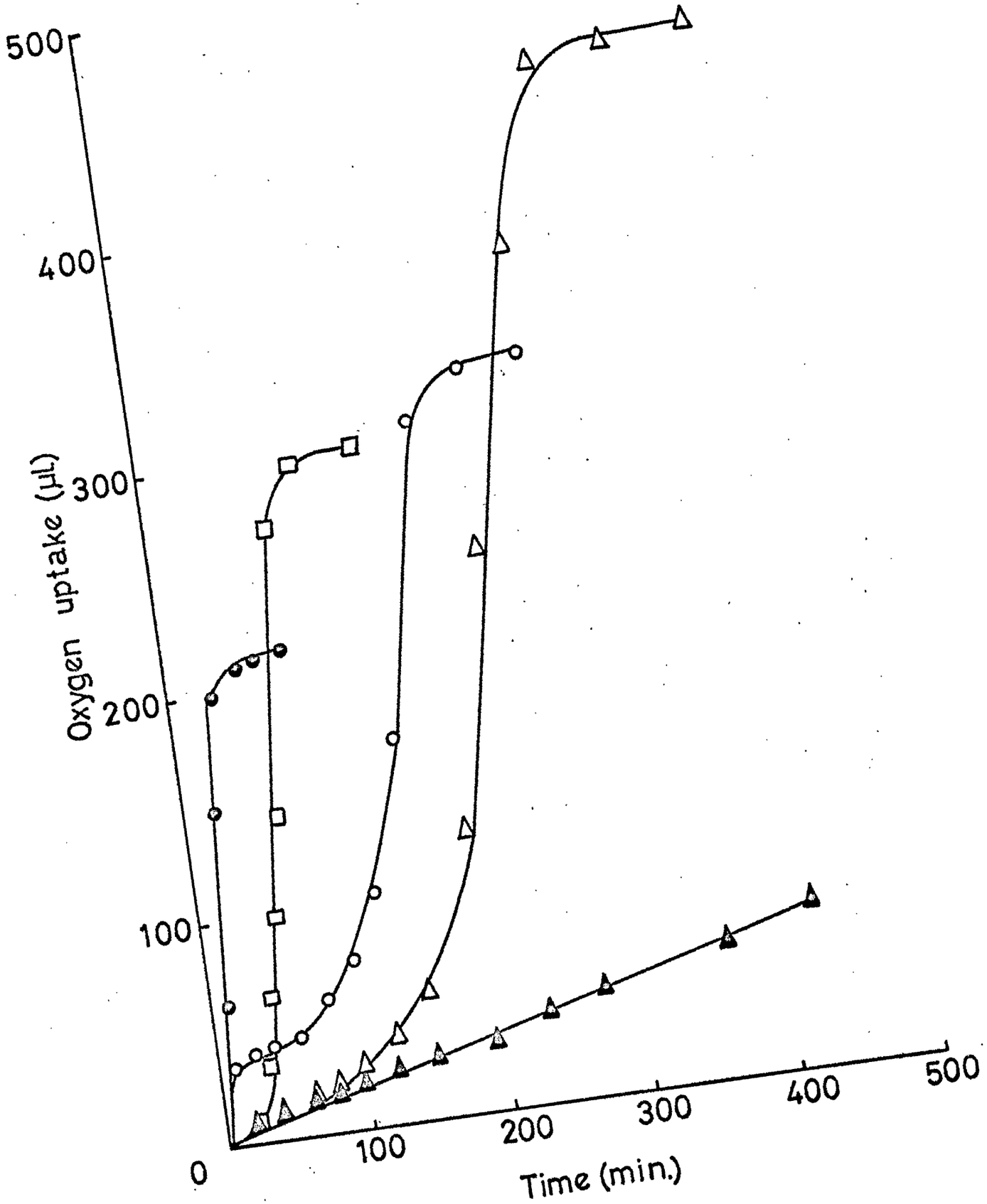

500
400
300
200
100
0
Oxygen uptake (µl)
100
200
300
400
500
Time (min.)

Fig.5. Oxidation of the 3-hydroxy-substituted compounds by washed suspensions of bacterium NCIB 8250.

Cells were grown on 2mM-4-hydroxy-D,L-mandelate as sole carbon source, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells.

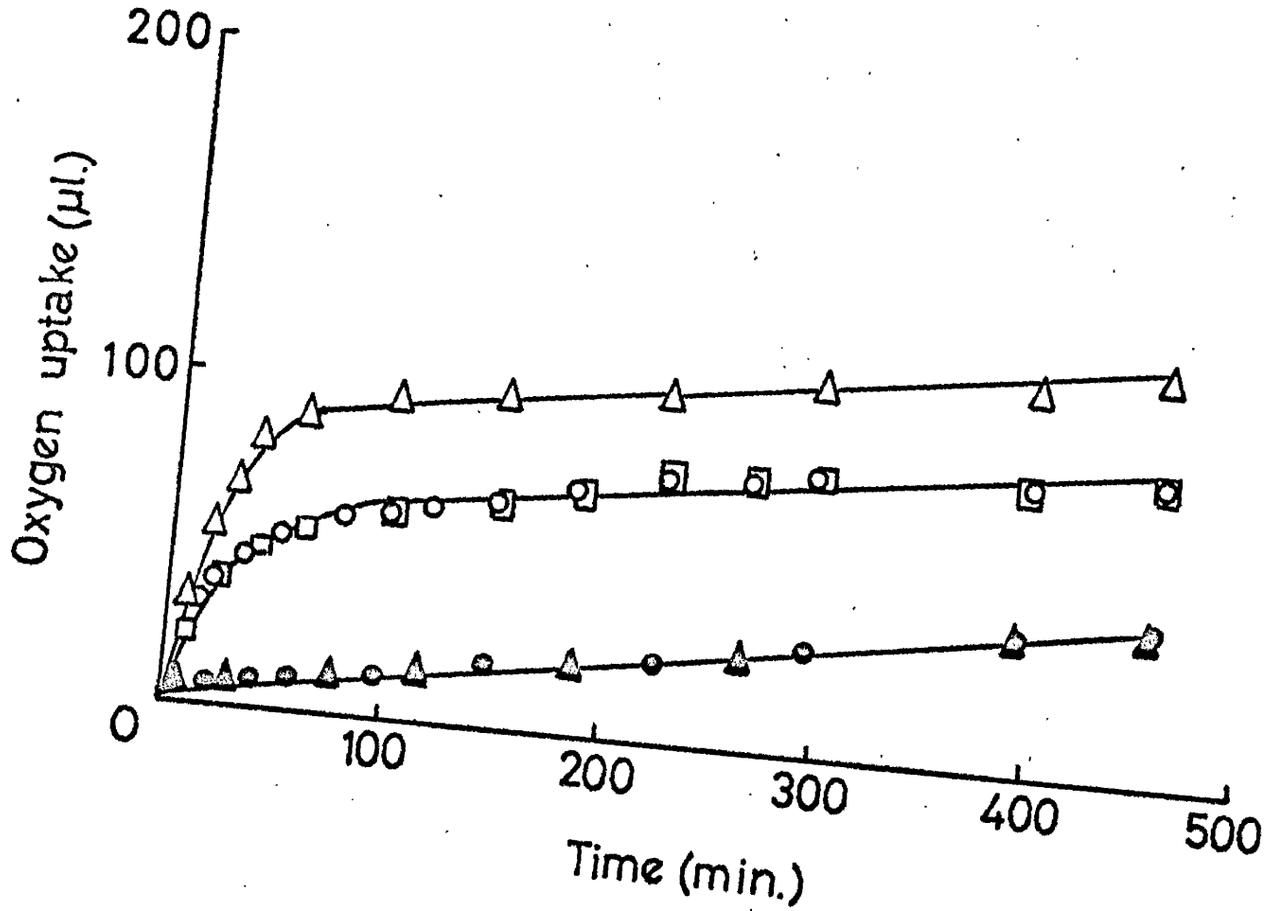
○- 3-Hydroxy-D,L-mandelate.

△- 3-Hydroxybenzyl alcohol.

□- 3-Hydroxybenzaldehyde.

●- 3-Hydroxybenzoate.

▲- Control with no added substrate.


200
100
0
Oxygen uptake (µl.)
100
200
300
400
500
Time (min.)

complete oxidation.

The patterns of oxidation of the 3-hydroxy-substituted compounds are shown in Fig. 5. 3-Hydroxy-D,L-mandelate, 3-hydroxybenzyl alcohol and 3-hydroxybenzaldehyde were all immediately oxidised but to a very low final level. These levels correspond to an oxygen uptake of 1 µatom O_2/µmole of 3-hydroxy-D,L-mandelate, 1 µmole O_2/µmole of 3-hydroxybenzyl alcohol and 1 µatom O_2/µmole of 3-hydroxybenzaldehyde. No oxygen uptake greater than the control was observed with 3-hydroxybenzoate even after prolonged incubation.

Patterns of oxygen utilisation obtained with bacterium NCIB 8250 after growth on all the possible substrates.

The patterns of oxygen utilisation obtained with a wide range of challenge substrates when incubated with washed suspensions of bacterium NCIB 8250 which had been grown on each of the possible substrates have not been presented as figures but are, for convenience in comparison, summarised in Table 3.

In every case incubation of the washed cells with that challenge substrate which had been employed as sole source of carbon and energy for growth gave an immediate and complete utilisation; furthermore all cell suspensions gave an immediate and complete oxygen uptake when incubated with succinate or β-oxoadipate.

Table 3. Patterns of oxygen utilisation obtained with a number of challenge substrates when incubated with washed suspensions of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on each of a number of aromatic compounds in turn, harvested, washed and the ability of the cells to oxidise the various challenge substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells.

M. - D,L-Mandelate.

BF. - Benzoylformate.

B.A. - Benzyl alcohol.

BZ. - Benzaldehyde.

B. - Benzoate.

A- Immediate complete utilisation.

B- Immediate limited utilisation.

C- Lag then complete utilisation.

D- Immediate limited utilisation-lag-further utilisation.

E- No utilisation above the control.

N.T.- Not tested.

Challenge Substrate / Growth Substrate	Non-substituted					2-Hydroxy-			4-Hydroxy-				3,4-Dihydroxy-			4-Hydroxy-3-methoxy-			
	M.	BF.	B.A.	BZ.	B.	B.A.	BZ.	B.	M.	B.A.	BZ.	B.	M.	BZ.	B.	M.	B.A.	BZ.	B.
D,L-Mandelate	A	A	C	C	C	C	C	C	D	C	C	C	N.T.	C	C	D	C	C	C
Benzoylformate	A	A	C	C	C	C	C	C	D	C	C	C	N.T.	C	C	N.T.	C	C	C
Benzyl alcohol	A	A	A	A	C	A	A	C	D	D	D	C	D	D	C	D	D	D	C
Benzaldehyde	A	A	A	A	C	A	A	C	D	D	D	C	N.T.	D	C	D	D	D	C
Benzoate	A	A	A	A	A	A	A	A	C	C	C	C	C	C	C	C	C	C	C
2-Hydroxybenzyl alcohol	D	D	D	D	C	A	A	C	D	D	D	C	N.T.	D	C	N.T.	D	D	C
2-Hydroxybenzaldehyde	D	D	D	D	C	A	A	C	D	D	D	C	N.T.	D	C	N.T.	D	D	C
2-Hydroxybenzoate	C	C	C	C	C	A	A	A	C	C	C	C	N.T.	C	C	N.T.	C	C	C
3-Hydroxy-D,L-mandelate	B	B	E	E	E	E	E	E	B	E	E	E	N.T.	E	E	B	E	E	E
3-Hydroxybenzyl alcohol	B	B	B	B	E	B	B	E	B	B	B	E	N.T.	B	E	N.T.	B	B	E
3-Hydroxybenzaldehyde	B	B	B	B	E	B	B	E	B	B	B	E	N.T.	B	E	N.T.	B	B	E
3-Hydroxybenzoate	E	E	E	E	E	E	E	E	E	E	E	E	N.T.	E	E	N.T.	E	E	E
4-Hydroxy-D,L-mandelate	D	D	C	C	C	C	C	C	A	C	C	C	N.T.	C	C	D	C	C	C
4-Hydroxybenzyl alcohol	D	D	D	D	C	D	D	C	A	A	A	C	N.T.	D	C	N.T.	D	D	C
4-Hydroxybenzaldehyde	D	D	D	D	C	D	D	C	A	A	A	C	N.T.	D	C	N.T.	D	D	C
4-Hydroxybenzoate	C	C	C	C	C	C	C	C	A	A	A	A	N.T.	C	C	N.T.	C	C	C
3,4-Dihydroxy-D,L-mandelate	D	D	C	N.T.	C	C	N.T.	N.T.	A	N.T.	N.T.	N.T.	A	C	C	A	C	C	C
3,4-Dihydroxybenzaldehyde	D	D	D	D	C	D	D	C	A	A	A	C	A	A	C	A	A	A	C
3,4-Dihydroxybenzoate	C	C	C	C	C	C	C	C	A	A	A	A	A	A	A	A	A	A	A
4-Hydroxy-3-methoxy-D,L-mandelate	D	D	C	C	C	C	C	C	D	C	C	C	N.T.	C	C	A	C	C	C
4-Hydroxy-3-methoxybenzyl alcohol	D	D	D	D	C	D	D	C	D	D	D	C	D	D	C	A	A	A	C
4-Hydroxy-3-methoxybenzaldehyde	D	D	D	D	C	D	D	C	D	D	D	C	D	D	C	A	A	A	C
4-Hydroxy-3-methoxybenzoate	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	A	A	A	A

D	C	C	C	N.T.	C	C	D	C	C	C
D	C	C	C	N.T.	C	C	N.T.	C	C	C
D	D	D	C	D	D	C	D	D	D	C
D	D	D	C	N.T.	D	C	D	D	D	C
C	C	C	C	C	C	C	C	C	C	C
D	D	D	C	N.T.	D	C	N.T.	D	D	C
D	D	D	C	N.T.	D	C	N.T.	D	D	C
C	C	C	C	N.T.	C	C	N.T.	C	C	C
B	E	E	E	N.T.	E	E	B	E	E	E
B	B	B	E	N.T.	B	E	N.T.	B	B	E
B	B	B	E	N.T.	B	E	N.T.	B	B	E
E	E	E	E	N.T.	E	E	N.T.	E	E	E
A	C	C	C	N.T.	C	C	D	C	C	C
A	A	A	C	N.T.	D	C	N.T.	D	D	C
A	A	A	C	N.T.	D	C	N.T.	D	D	C
A	A	A	A	N.T.	C	C	N.T.	C	C	C
A	N.T.	N.T.	N.T.	A	C	C	A	C	C	C
A	A	A	C	A	A	C	A	A	A	C
A	A	A	A	A	A	A	A	A	A	A
D	C	C	C	N.T.	C	C	A	C	C	C
D	D	D	C	D	D	C	A	A	A	C
D	D	D	C	D	D	C	A	A	A	C
C	C	C	C	C	C	C	A	A	A	A

(i) Patterns of oxygen uptake obtained with cells grown on the non-substituted compounds.

Cells grown on D,L-mandelate or benzoylformate gave an immediate complete utilisation of oxygen (pattern A) when incubated with any non-substituted compound. These cells gave an immediate limited utilisation-lag-further utilisation (pattern D) when incubated with any substituted D,L-mandelate, benzyl alcohol or benzaldehyde except the 3-hydroxy-substituted derivatives of these compounds which gave an immediate limited utilisation (pattern B). They gave a lag followed by complete utilisation (pattern C) when incubated with any substituted benzoate except 3-hydroxybenzoate which gave no oxygen uptake above the control (pattern E).

Cells grown on benzyl alcohol or benzaldehyde gave an immediate complete utilisation of oxygen when incubated with benzyl alcohol, benzaldehyde or benzoate. These cells gave an immediate limited utilisation-lag-further utilisation when incubated with any substituted benzyl alcohol or benzaldehyde except the 3-hydroxy-substituted derivatives of these compounds which gave an immediate limited utilisation. Both types of cells showed a lag then complete utilisation of oxygen when incubated with benzoylformate, any D,L-mandelate or substituted benzoate: the exceptions were 3-hydroxy-D,L-mandelate and 3-hydroxybenzoate which gave no oxygen uptake above the control.

Cells grown on benzoate gave an immediate complete oxygen uptake only when incubated with benzoate as challenge substrate: all other challenge substrates gave a lag then complete utilisation, except the

3-hydroxy-substituted compounds which gave no oxygen uptake above the control.

Cells grown on any non-substituted compound gave an immediate and complete oxygen uptake when incubated with catechol.

(ii) Patterns of oxygen uptake obtained with cells grown on the 2-hydroxy-substituted compounds.

Cells grown on 2-hydroxybenzyl alcohol or 2-hydroxybenzaldehyde gave an immediate complete utilisation of oxygen when incubated not only with 2-hydroxybenzyl alcohol, 2-hydroxybenzaldehyde or 2-hydroxybenzoate but also with benzyl alcohol, benzaldehyde or benzoate. These cells gave an immediate limited utilisation-lag-further utilisation when incubated with any other substituted benzyl alcohol or benzaldehyde except the 3-hydroxy-substituted derivatives of these compounds which gave an immediate limited utilisation; and a lag then complete utilisation when incubated with benzoylformate, any D,L-mandelate or substituted benzoate. Again, however, 3-hydroxy-D,L-mandelate and 3-hydroxybenzoate gave no oxygen uptake above the control level.

Cells grown on 2-hydroxybenzoate gave an immediate complete oxygen uptake only with 2-hydroxybenzoate or benzoate as challenge substrate; all other challenge substrates gave a lag then complete utilisation, except the 3-hydroxy-substituted compounds which gave no oxygen uptake above the control.

Cells grown on any 2-hydroxy-substituted compound gave an immediate and complete utilisation of oxygen when incubated with catechol.

(iii) Patterns of oxygen uptake obtained with cells grown on the 4-hydroxy-substituted compounds.

Cells grown on 4-hydroxy-D,L-mandelate, as partially described in detail above (Figs. 3 - 5), gave an immediate complete utilisation of oxygen when incubated with any 4-hydroxy-substituted compound or any 3,4-dihydroxy-substituted compound. These cells gave an immediate limited utilisation-lag-further utilisation when incubated with D,L-mandelate, benzoylformate, benzyl alcohol or benzaldehyde or the 2-hydroxy and 4-hydroxy-3-methoxy-substituted derivatives of these compounds which were available. These cells gave an immediate limited utilisation when incubated with the 3-hydroxy-substituted D,L-mandelate, benzyl alcohol or benzaldehyde and a lag then complete utilisation when incubated with the non-substituted, the 2-hydroxy or the 4-hydroxy-3-methoxy-substituted benzoates and no oxygen uptake above the control with 3-hydroxybenzoate.

Cells grown on 4-hydroxybenzyl alcohol or 4-hydroxybenzaldehyde gave an immediate complete utilisation when incubated with 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoate, 3,4-dihydroxybenzaldehyde or 3,4-dihydroxybenzoate. These cells gave an immediate limited utilisation-lag-further utilisation when incubated with benzyl alcohol or benzaldehyde or their 2-hydroxy and 4-hydroxy-3-methoxy substituted derivatives. These cells gave an immediate limited utilisation when incubated with 3-hydroxybenzyl alcohol or 3-hydroxybenzaldehyde and a lag then complete utilisation when incubated with benzoylformate, the non-substituted, 2-hydroxy or 4-hydroxy-3-methoxy-

substituted D,L-mandelates or benzoates and no utilisation above the control with 3-hydroxy-D,L-mandelate or 3-hydroxybenzoate.

Cells grown on 4-hydroxybenzoate gave an immediate oxygen uptake with 4-hydroxybenzoate or 3,4-dihydroxybenzoate and a lag then complete utilisation when incubated with any other substrate except the 3-hydroxy-substituted compounds which gave no oxygen uptake above the control.

Cells grown on any 4-hydroxy-substituted compound oxidised catechol to completion only after a lag.

(iv) Patterns of oxygen uptake obtained with the 3,4-dihydroxy-substituted compounds.

Cells grown on 3,4-dihydroxy-D,L-mandelate were incubated with only a very few challenge substrates. This was because of the scarcity of the compound and the small amount of cells which could therefore be grown. These cells gave an immediate complete utilisation of oxygen when incubated with any 3,4-dihydroxy-substituted compound. They gave an immediate limited utilisation-lag-utilisation with benzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol and 4-hydroxy-3-methoxybenzaldehyde; and a lag then complete utilisation when incubated with benzoate or 4-hydroxy-3-methoxybenzoate.

Cells grown on 3,4-dihydroxybenzaldehyde gave an immediate complete utilisation of oxygen when incubated with 3,4-dihydroxybenzaldehyde or 3,4-dihydroxybenzoate. These cells gave an immediate limited utilisation-lag-further utilisation when incubated with any benzyl alcohol or benzaldehyde other than 3,4-dihydroxybenzaldehyde, except

the 3-hydroxy-substituted derivatives of these compounds which gave an immediate limited utilisation; and a lag then complete utilisation when incubated with benzoylformate, any D,L-mandelate or benzoate. The exceptions were 3-hydroxy-D,L-mandelate and 3-hydroxybenzoate which gave no oxygen utilisation above the control.

Cells grown on 3,4-dihydroxybenzoate gave an immediate complete oxidation only with 3,4-dihydroxybenzoate as challenge substrate; all other challenge substrates gave a lag then complete utilisation except the 3-hydroxy-substituted compounds which were not oxidised above the control.

Cells grown on any 3,4-dihydroxy-substituted compound oxidised catechol to completion only after a lag.

(v) Patterns of oxygen uptake obtained with cells grown on the 4-hydroxy-3-methoxy-substituted compounds.

Cells grown on 4-hydroxy-3-methoxy-D,L-mandelate gave an immediate complete utilisation when incubated with any 4-hydroxy-3-methoxy-substituted compound or any 3,4-dihydroxy-substituted compound. These cells gave an immediate limited utilisation-lag-further utilisation when incubated with D,L-mandelate, 4-hydroxy-D,L-mandelate, benzyl alcohol or benzaldehyde, and a lag then complete utilisation when incubated with benzoate.

Cells grown on 4-hydroxy-3-methoxybenzyl alcohol or 4-hydroxy-3-methoxybenzaldehyde gave an immediate complete utilisation of oxygen when incubated with 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxy-3-

methoxybenzaldehyde, 4-hydroxy-3-methoxybenzoate, 3,4-dihydroxybenzaldehyde or 3,4-dihydroxybenzoate. They gave an immediate limited utilisation-lag-further utilisation when incubated with benzyl alcohol or benzaldehyde or their 2-hydroxy and 4-hydroxy-substituted derivatives. These cells gave an immediate limited utilisation when incubated with 3-hydroxybenzyl alcohol or 3-hydroxybenzaldehyde and a lag then complete utilisation when incubated with benzoylformate, the non-substituted or 2-hydroxy-substituted D,L-mandelates or benzoates and no utilisation above the control with 3-hydroxy-D,L-mandelate or 3-hydroxybenzoate.

Cells grown on 4-hydroxy-3-methoxybenzoate gave an immediate oxygen uptake only with 4-hydroxy-3-methoxybenzoate or 3,4-dihydroxybenzoate and a lag then complete utilisation when incubated with any other challenge substrate except the 3-hydroxy-substituted compounds which gave no oxygen uptake above the control.

Cells grown on any 4-hydroxy-3-methoxy-substituted compound oxidised catechol to completion only after a lag.

(vi) Patterns of oxygen uptake obtained with cells grown on catechol.

Cells grown on catechol oxidised the non-substituted, 2-hydroxy, 4-hydroxy, 3,4-dihydroxy or 4-hydroxy-3-methoxy-substituted compounds only after a lag. When incubated with any 3-hydroxy-substituted compound no oxygen uptake above the control was recorded.

(vii) Patterns of oxygen uptake obtained with cells grown on succinate or nutrient broth.

Cells grown on succinate or nutrient broth oxidised the non-substituted, 2-hydroxy, 4-hydroxy, 3,4-dihydroxy or 4-hydroxy-3-methoxy-substituted compounds after a lag. These cells also oxidised β-oxoadipate only after a lag and showed no oxygen uptake above the control when incubated with any 3-hydroxy-substituted compound.

Stoichiometry of the oxygen uptakes obtained with the 3-hydroxy-substituted compounds.

The patterns of oxygen uptake obtained when the 3-hydroxy-substituted compounds were incubated with each of a large number of washed cell suspensions are shown in Table 3. The 3-hydroxy-substituted compounds gave only two patterns of oxygen utilisation irrespective of the substrate on which bacterium NCIB 8250 had been grown. These two patterns were immediate limited utilisation, pattern B, or no utilisation above the control, pattern E. Different levels were obtained for pattern B depending on the 3-hydroxy-substituted compound which was oxidised. The stoichiometries of the oxygen uptake with the various 3-hydroxy-substituted compounds are as follows. In all cases corrections were made for the endogenous respiration.

Challenge substrate (3.6 µmoles)	Final oxygen uptake (µmoles Mean ± S.E.M.)	Number of experiments
3-Hydroxy-D,L-mandelate	1.77 ± 0.14	4
3-Hydroxybenzyl alcohol	3.50 ± 0.06	12
3-Hydroxybenzaldehyde	1.85 ± 0.08	12
3-Hydroxybenzoate	0	12

Thus the oxidation of 3-hydroxy-D,L-mandelate stopped after the uptake of 1 µatom O_2/µmole of substrate, the oxidation of 3-hydroxybenzyl alcohol after the uptake of 1 µmole O_2/µmole of substrate, and the oxidation of 3-hydroxybenzaldehyde stopped after the uptake of 1 µatom O_2/µmole of substrate.

Stoichiometry of the oxygen uptake with compounds which were completely oxidised.

Most compounds which were oxidised according to patterns A, C or D gave an oxygen uptake corresponding to 60 - 80% of that required

for complete oxidation to CO_2 and H_2O. Probably the balance of 40 - 20% represents oxidative assimilation. The oxidation of any mandelate gave only about half this oxygen uptake: presumably this represents the oxidation of the L-isomer and the inactivity of the D-isomer (e.g. Fig. 1).

In the case of the situations where pattern D was obtained, the stoichiometries of the intermediate oxygen utilisation generally corresponded to 1 µmole O_2/µmole benzyl alcohol or 1 µatom O_2/µmole D,L-mandelate or benzaldehyde. This would correspond to oxidation to the benzoate level. In some cases, where the intermediate plateau was ill-defined, the stoichiometry was difficult to calculate.

The ability of bacterium NCIB 8250 to oxidise a large number of compounds which do not support growth.

The results of the growth experiments (Table 1) demonstrated that bacterium NCIB 8250 could not utilise the 3-hydroxy-substituted compounds as sole sources of carbon and energy for growth. Experiments on the oxidation of these compounds, however, showed that the 3-hydroxy-substituted D,L-mandelate, benzyl alcohol and benzaldehyde were oxidised by washed cell suspensions with a limited utilisation of oxygen (pattern B). These oxygen uptakes would correspond to the oxidation of these compounds to 3-hydroxybenzoate

which Table 3 shows was never oxidised by bacterium NCIB 8250. In an attempt to determine if any other compounds which do not support growth (Fewson, 1967a) undergo partial oxidation, washed cell suspensions of bacterium NCIB 8250 were incubated with almost 100 compounds and the resultant uptake of oxygen recorded.

Washed cell suspensions of bacterium NCIB 8250 which had been grown on 2mM-D,L-mandelate were incubated with each of the compounds in turn. The compounds may be divided into three categories depending on the patterns of oxygen utilisation which they produced:

(i) Compounds which gave an immediate complete oxygen utilisation.

4-Fluoro-D,L-mandelate
4-Fluorobenzyl alcohol
2-Fluorobenzaldehyde
4-Fluorobenzaldehyde
2-Fluorobenzoate
4-Fluorobenzoate

(ii) Compounds which gave an immediate limited oxygen utilisation.

4-Bromo-D,L-mandelate
4-Chloro-D,L-mandelate
3-Hydroxy-4-methoxy-D,L-mandelate
3,4-Dimethoxybenzyl alcohol
3-Chlorobenzyl alcohol
3-Methoxybenzyl alcohol
4-Methoxybenzyl alcohol
3-Chlorobenzaldehyde

4-Chlorobenzaldehyde
3,4-Dichlorobenzaldehyde
2,4-Dihydroxybenzaldehyde
2,5-Dihydroxybenzaldehyde
3-Fluorobenzaldehyde
2-Hydroxy-3-methoxybenzaldehyde
3-Hydroxy-4-methoxybenzaldehyde
5-Hydroxy-3-methoxybenzaldehyde
3-Methoxybenzaldehyde
4-Methoxybenzaldehyde
3-Nitrobenzaldehyde
4-Nitrobenzaldehyde
3-Fluorobenzoate
Cinnamaldehyde
Cinnamyl alcohol

(iii) Compounds which gave no oxygen utilisation above the control.

2-Carboxybenzaldehyde
2,6-Dichlorobenzaldehyde
2,4-Dimethoxybenzaldehyde
2-Methoxybenzaldehyde
2-Nitrobenzaldehyde
2,3,4,5,6-Pentafluorobenzyl alcohol
2,3,4,5,6-Pentafluorobenzaldehyde
3-Aminobenzoate
4-Aminobenzoate
2-Bromobenzoate
3-Bromobenzoate
4-Bromobenzoate
2-Chlorobenzoate
3-Chlorobenzoate
4-Chlorobenzoate
2,4-Dichlorobenzoate
2,6-Dichlorobenzoate
2,3-Dihydroxybenzoate
2,4-Dihydroxybenzoate
2,5-Dihydroxybenzoate
2,6-Dihydroxybenzoate
3,5-Dihydroxybenzoate
3,4-Dimethoxybenzoate
3,5-Dinitrobenzoate
2-Hydroxy-4-aminobenzoate
2-Hydroxy-3-nitrobenzoate
2-Hydroxy-5-nitrobenzoate
4-Hydroxy-3-aminobenzoate
2-Iodobenzoate
3-Iodobenzoate
4-Iodobenzoate
2-Methoxybenzoate
3-Methoxybenzoate
4-Methoxybenzoate
2-Methylbenzoate
3-Methylbenzoate
4-Methylbenzoate
2-Mercaptobenzoate

2-Nitrobenzoate
3-Nitrobenzoate
4-Nitrobenzoate
3,4,5-Trihydroxybenzoate
Atrolactate
Atropate
p-Benzoquinone
Cinnamate
2-Coumarate
3-Coumarate
4-Coumarate
Cyclohexane carboxylate
Cyclohexanol
Homocatechol
2-Hydroxycyclohexanone
3-Nitrophthalate
4-Nitrophthalate
2-Phenylethanol
3-Phenylpropionate
Phenylsuccinate
Phthalate
iso-Phthalate
Resorcinol
Styrene glycol
Terephthalate

Cells grown on D,L-mandelate, therefore, gave an immediate and complete utilisation of oxygen when incubated with any D,L-mandelate, benzyl alcohol, benzaldehyde or benzoate having a fluorine atom on the 2 or 4 position of the aromatic ring (pattern A). In general any other substituted D,L-mandelate, benzyl alcohol or benzaldehyde gave an immediate limited utilisation of oxygen (pattern B). The final levels of oxygen uptake obtained with these compounds were approximately 1 µatom O_2/µmole substituted D,L-mandelate or benzaldehyde and 1 µmole O_2/µmole substituted benzyl alcohol. As previously observed with the 3-hydroxy-substituted compounds these uptakes would correspond to oxidation to the level of benzoate, and like 3-hydroxybenzoate (Table 3) the corresponding benzoates of these compounds were not utilised above the control. A number of compounds, including 2,3,4,5,6-pentafluorobenzyl alcohol and

benzaldehydes with large substituents (e.g. nitro, methoxy, or chloro) on the 2 position of the aromatic ring gave no utilisation above the control. Of all the substituted benzoates tested in this series of experiments only the monofluoro benzoates gave an oxygen uptake above the control with the 2 or 4-fluorobenzoate giving an immediate and possibly complete oxygen utilisation. A number of miscellaneous compounds having similar structures to the D,L-mandelates, benzyl alcohols, benzaldehydes and benzoates were also tested but none of these compounds, including such close analogues as atrolactic acid, gave an oxygen uptake above the endogenous.

Cells grown on 2-hydroxybenzyl alcohol gave similar results to those obtained with cells grown on D,L-mandelate. None of the substituted mandelates however, including 4-fluoro-D,L-mandelate gave an oxygen uptake above the control. 2-Fluorobenzyl alcohol and 2-fluorobenzaldehyde, moreover, gave an immediate limited utilisation whilst 2-fluorobenzoate gave only a very small oxygen uptake above the endogenous. This result contrasts with the immediate complete utilisation of oxygen obtained with the 2-fluoro-substituted compounds when incubated with cells grown on D,L-mandelate.

Cells grown on either 3,4-dihydroxybenzaldehyde or 4-hydroxy-3-methoxybenzyl alcohol gave essentially identical results both to each other and to those obtained with cells grown on 2-hydroxybenzyl alcohol. The notable exceptions were that cells grown on either 3,4-dihydroxybenzaldehyde or 4-hydroxy-3-methoxybenzyl alcohol gave an immediate limited utilisation with both the 2 and 4 fluorodinated

benzyl alcohols and benzaldehydes, while the fluorodinated benzoates gave no oxygen uptake above the control.

Initial rates of oxygen utilisation obtained with washed cell suspensions of bacterium NCIB 8250.

The initial rates of oxygen utilisation obtained in the experiments described above (Table 3) are shown in Table 4. Initial rates of oxygen utilisation, in μmoles O_2/hr./mg. N, were determined from the total oxygen uptake recorded 10 minutes after 'tipping', and hence do not take into account the very short lags displayed by a number of challenge substrates nor the fact that certain challenge substrates which gave an immediate limited utilisation, pattern B, had reached their final level before 10 minutes. Thus, although most challenge substrates which gave a lag then complete utilisation (pattern C) gave an initial rate of 0, certain exceptions were observed e.g. the initial rate of oxygen utilisation of 27 μmoles O_2/hr./mg. N observed with 3,4-dihydroxybenzoate when incubated with cells which had been grown on benzoate. Certain challenge substrates, notably benzaldehyde, on account of their high volatility, distilled over into the main compartment of the Warburg vessel prior to 'tipping' and, where these challenge substrates gave a lag then complete utilisation, a high initial rate was recorded. This high initial rate was presumably due to the somewhat longer time the cells had been exposed to benzaldehyde with the concomitant reduction in

Table 4. Initial rates of oxygen utilisation obtained with a number of challenge substrates when incubated with washed suspensions of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on each of a number of aromatic compounds in turn, harvested, washed and the ability of the cells to oxidise the various challenge substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells. Initial rates are expressed in µmoles oxygen utilised/hour/mg. nitrogen.

M. - D,L-Mandelate.

BF. - Benzoylformate.

B.A. - Benzyl alcohol.

BZ. - Benzaldehyde.

B. - Benzoate.

N.T. - Not tested.

Growth / Challenge / Substrate	Non-substituted					2-Hydroxy-			4-Hydroxy-				3,4-Dihydroxy-			4-Hydroxy-3-methoxy-			
	M.	BF.	B.A.	BZ.	B.	B.A.	BZ.	B.	M.	B.A.	BZ.	B.	M.	BZ.	B.	M.	B.A.	BZ.	B.
D,L-Mandelate	175	120	0	0	0	0	0	0	48	0	0	0	N.T.	0	0	38	0	0	0
Benzoylformate	172	148	0	0	0	0	0	0	48	0	0	0	N.T.	0	0	N.T.	0	0	0
Benzyl alcohol	43	112	186	198	5	194	210	24	82	128	128	0	49	101	0	75	117	115	0
Benzaldehyde	163	150	177	212	30	145	175	108	40	34	43	0	N.T.	34	0	38	48	43	0
Benzoate	182	134	140	188	198	145	118	130	0	0	0	0	0	0	0	0	0	0	0
2-Hydroxybenzyl alcohol	12	29	70	55	0	152	116	5	60	128	128	0	N.T.	107	0	N.T.	73	95	8
2-Hydroxybenzaldehyde	6	11	23	31	0	124	81	0	17	34	43	0	N.T.	34	0	N.T.	39	33	8
2-Hydroxybenzoate	10	10	0	6	6	124	80	166	0	0	0	0	N.T.	0	0	N.T.	0	0	0
3-Hydroxy-D,L-mandelate	10	8	0	0	0	0	0	0	35	0	0	0	N.T.	0	0	31	0	0	0
3-Hydroxybenzyl alcohol	9	18	37	52	0	62	97	0	70	128	128	0	N.T.	101	0	N.T.	91	79	0
3-Hydroxybenzaldehyde	12	8	12	18	0	21	38	0	17	43	56	0	N.T.	56	0	N.T.	57	21	0
3-Hydroxybenzoate	0	0	0	0	0	0	0	0	0	0	0	0	N.T.	0	0	N.T.	0	0	0
4-Hydroxy-D,L-mandelate	19	13	0	0	0	0	0	0	187	0	0	0	N.T.	0	0	12	0	0	0
4-Hydroxybenzyl alcohol	19	20	25	26	0	39	33	0	187	185	210	0	N.T.	60	0	N.T.	125	30	0
4-Hydroxybenzaldehyde	3	5	7	9	0	17	12	0	152	185	210	0	N.T.	19	0	N.T.	108	13	0
4-Hydroxybenzoate	3	5	7	5	0	9	3	13	187	185	210	180	N.T.	0	0	N.T.	0	13	16
3,4-Dihydroxy-D,L-mandelate	25	28	0	N.T.	0	0	N.T.	N.T.	93	N.T.	N.T.	N.T.	34	N.T.	N.T.	46	N.T.	N.T.	N.T.
3,4-Dihydroxybenzaldehyde	7	3	9	18	0	91	14	0	126	147	220	0	34	160	0	130	116	185	0
3,4-Dihydroxybenzoate	7	3	9	18	27	11	7	5	203	147	255	196	99	160	174	194	194	54	172
4-Hydroxy-3-methoxy-D,L-mandelate	15	3	0	0	0	0	0	0	14	0	0	0	N.T.	0	0	46	0	0	0
4-Hydroxy-3-methoxybenzyl alcohol	9	11	18	15	0	19	28	0	31	62	100	0	13	71	0	130	116	120	0
4-Hydroxy-3-methoxybenzaldehyde	4	2	19	9	0	15	16	0	14	23	25	0	7	27	0	114	100	120	0
4-Hydroxy-3-methoxybenzoate	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	96	100	148	185

48	0	0	0	N.T.	0	0	38	0	0	0
48	0	0	0	N.T.	0	0	N.T.	0	0	0
82	128	128	0	49	101	0	75	117	115	0
40	34	43	0	N.T.	34	0	38	48	43	0
0	0	0	0	0	0	0	0	0	0	0
60	128	128	0	N.T.	107	0	N.T.	73	95	8
17	34	43	0	N.T.	34	0	N.T.	39	33	8
0	0	0	0	N.T.	0	0	N.T.	0	0	0
35	0	0	0	N.T.	0	0	31	0	0	0
70	128	128	0	N.T.	101	0	N.T.	91	79	0
17	43	56	0	N.T.	56	0	N.T.	57	21	0
0	0	0	0	N.T.	0	0	N.T.	0	0	0
187	0	0	0	N.T.	0	0	12	0	0	0
187	185	210	0	N.T.	60	0	N.T.	125	30	0
152	185	210	0	N.T.	19	0	N.T.	108	13	0
187	185	210	180	N.T.	0	0	N.T.	0	13	16
93	N.T.	N.T.	N.T.	34	N.T.	N.T.	46	N.T.	N.T.	N.T.
126	147	220	0	34	160	0	130	116	185	0
203	147	255	196	99	160	174	194	194	54	172
14	0	0	0	N.T.	0	0	46	0	0	0
31	62	100	0	13	71	0	130	116	120	0
14	23	25	0	7	27	0	114	100	120	0
0	0	0	0	0	0	0	96	100	148	185

the apparent length of the lag.

Those challenge substrates which gave an immediate and complete utilisation of oxygen, pattern A, also gave the highest initial rate of oxidation; between 43 and 255 umoles O_2 utilised/hr./mg. N.

Those challenge substrates which gave an immediate limited utilisation of oxygen, pattern B, or an immediate limited utilisation of oxygen-lag-further utilisation, pattern D, gave initial rates between 6 and 117 umoles O_2/hr./mg. N.

The effect of chloramphenicol on the oxidation of certain substrates.

In order to check whether the lags in oxygen utilisation observed in pattern C and D were due, as seemed likely, to enzyme induction, the effect of chloramphenicol on these patterns was determined. Bacterium NCIB 8250 was therefore grown to late exponential phase on 2mM-D,L-mandelate as sole source of carbon and energy. The cells were harvested, washed and resuspended and their ability to oxidise a number of compounds in the presence or absence of chloramphenicol was determined manometrically as described in Methods.

The effect of chloramphenicol on the oxidation of D,L-mandelate (Fig. 6) was to very slightly increase the total oxygen uptake (from 32% to 33% of that required for total oxidation), and to very slightly decrease the initial rate of oxygen utilisation (from 195 to 183 umoles O_2/hr./mg. N). There was plainly no effect of chloramphenicol on the pattern of oxygen utilisation in this case. There was, however,

Fig.6. Effect of chloramphenicol on the oxidation of D,L-mandelate and 4-hydroxy-D,L-mandelate by washed suspensions of bacterium NCIB 8250.

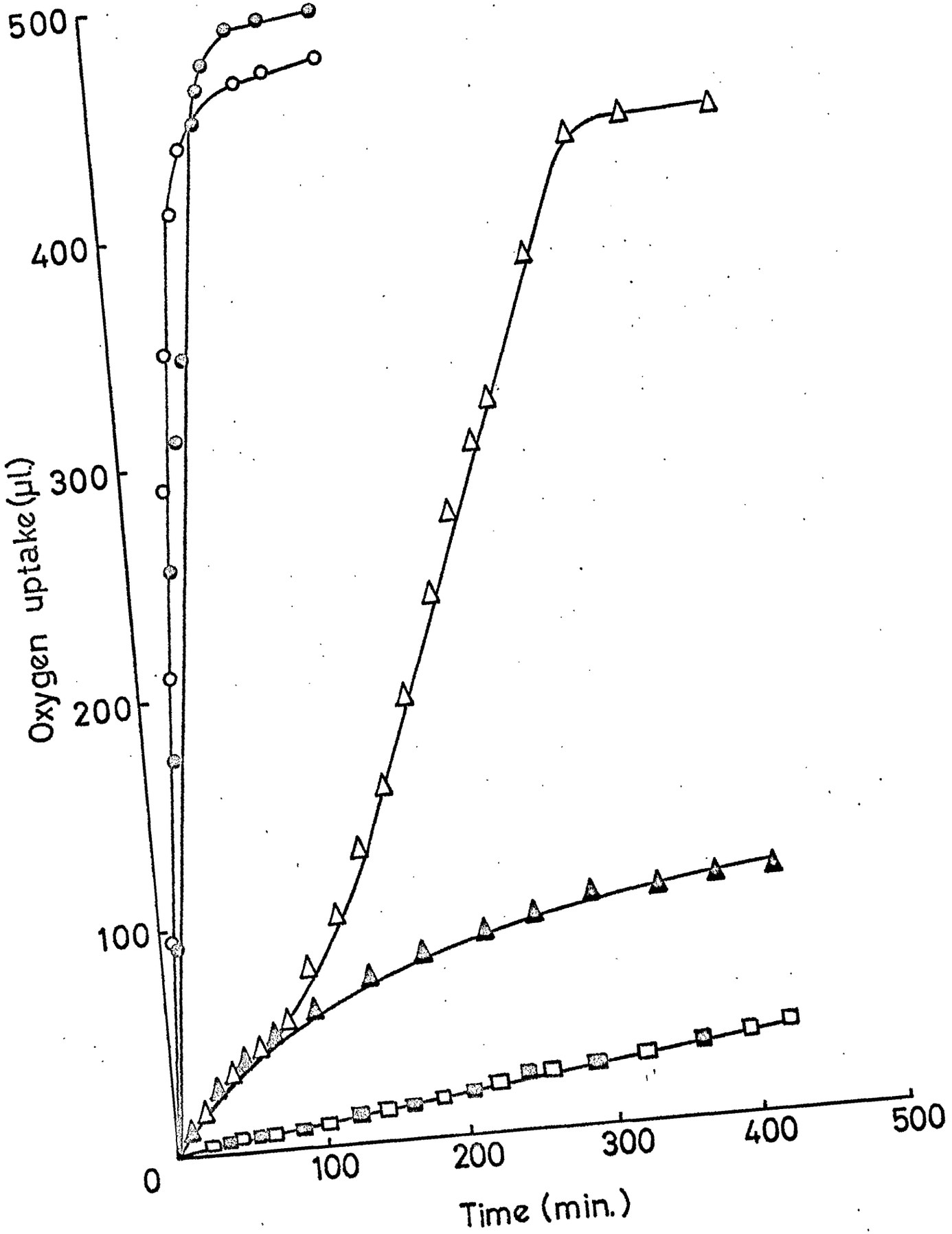
Cells were grown on 2mM-D,L-mandelate as sole carbon source, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells and, where stated, chloramphenicol at a final concentration of 50μM.

o- D,L-Mandelate.
Δ- 4-Hydroxy-D,L-mandelate.
□- Control with no added substrate.

Without chloramphenicol.

o- D,L-Mandelate.
Δ- 4-Hydroxy-D,L-mandelate.
□- Control with no added substrate.

With chloramphenicol.


500
400
300
200
100
0
Oxygen uptake (µl)
100
200
300
400
500
Time (min.)

a dramatic effect of chloramphenicol on the oxidation of 4-hydroxy-D,L-mandelate. Chloramphenicol changed the oxygen uptake pattern from immediate limited utilisation-lag-further utilisation (pattern D) to an immediate limited utilisation (pattern B) corresponding to approximately 1 μatom O_2/umole substrate. There was no effect on the initial rates of oxygen utilisation.

The effect of chloramphenicol on the oxidation of benzoate and 4-hydroxybenzoate by the same cells is shown in Fig. 7. There was essentially no effect of chloramphenicol on the pattern of oxygen utilisation in the presence of benzoate: there was a very slight increase in the total oxygen uptake (65% to 67% of that required for total oxidation), and a very slight decrease in the initial rate of oxidation (from 187 to 177 μmoles O_2/hr./mg. N). Chloramphenicol, however, completely blocked the oxidation of 4-hydroxybenzoate by cells grown on D,L-mandelate. In other words it changed the oxygen uptake pattern from 'lag then complete utilisation' (pattern C) to 'no utilisation above the control' (pattern E).

Fig.7. Effect of chloramphenicol on the oxidation of benzoate and 4-hydroxybenzoate by washed suspensions of bacterium NCIB 8250.

Cells were grown on 2mM-D,L-mandelate as sole carbon source, harvested, washed and their ability to oxidise the various substrates estimated manometrically as described in Methods. Each vessel contained 8.0 mg. wet wt. of cells and, where stated, chloramphenicol at a final concentration of 50µM.

Without chloramphenicol.

- o- Benzoate.
- Δ- 4-Hydroxybenzoate.
- □- Control with no added substrate.

With chloramphenicol.

- o- Benzoate.
- Δ- 4-Hydroxybenzoate.
- ▣- Control with no added substrate.

Application by Samuel I.T. Kennedy

A copy of the thesis has been aent to the Additicnal Examiner who is:

Mr. O.T.G. Jones,
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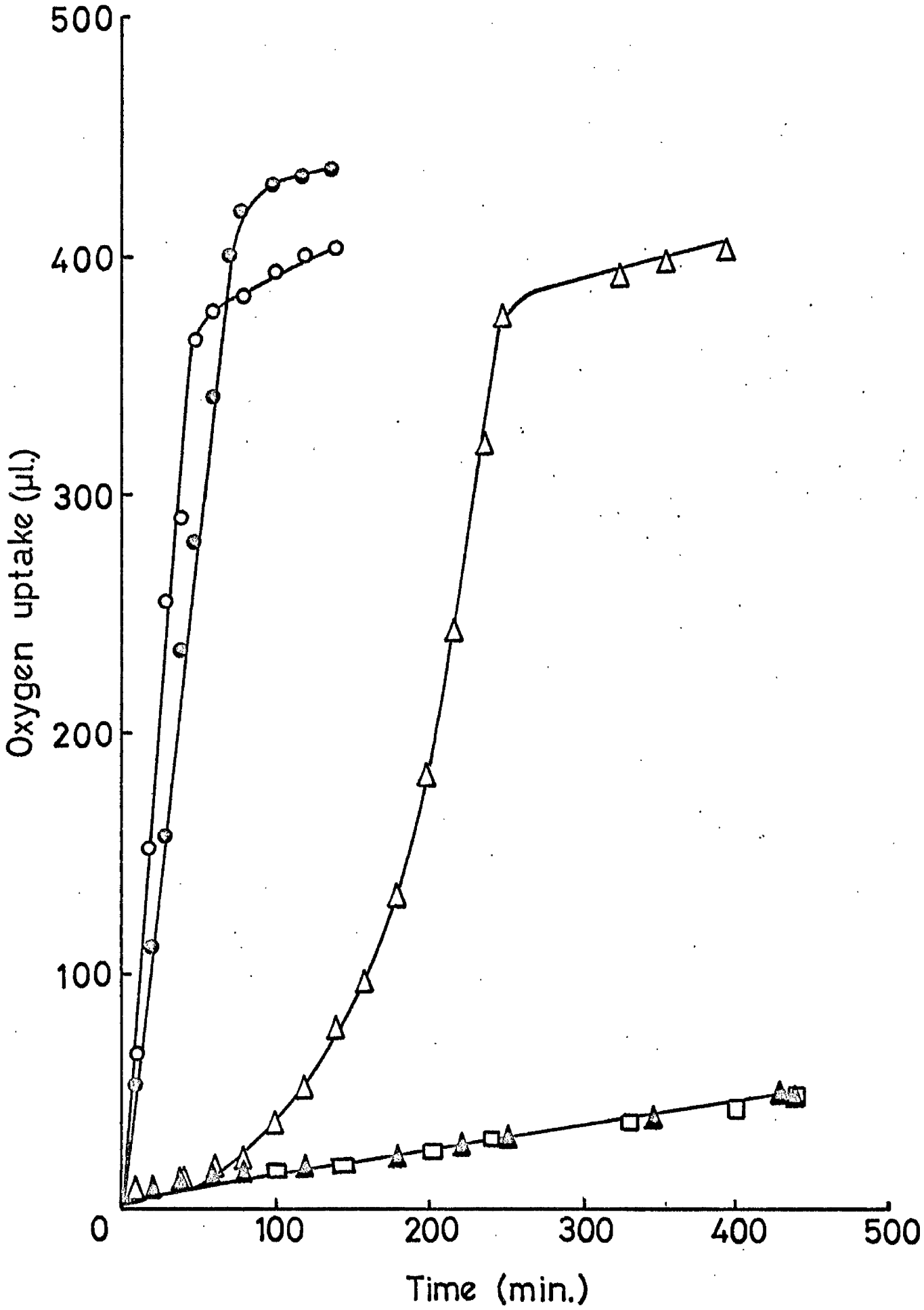
The Special Committee is:


500
400
300
200
100
0
100
200
300
400
500
Oxygen uptake (µl.)
Time (min.)

Experiments using Cell-Free Extracts.

Enzymes studied and outline of methods employed.

In order to examine further the specificities of the enzymes involved in the oxidation of mandelate and related compounds to the level of benzoate, attempts were made to examine these enzymes in cell-free systems. The enzymes of interest were:

L-Mandelate dehydrogenase

Benzoylformate decarboxylase

Benzyl alcohol dehydrogenase

Benzaldehyde dehydrogenase

The enzymes were assayed by adapting the methods employed by Hegeman (1966a) in his study of the corresponding enzymes from P. putida. L-Mandelate dehydrogenase was assayed by following the rate of reduction of 2,6-dichlorophenol-indophenol as estimated by the decrease in extinction at 600mμ. Benzyl alcohol dehydrogenase (which was not measured by Hegeman (1966a)) and benzaldehyde dehydrogenase were measured by following the rate of NAD^+ reduction at 340mμ. Attempts to measure the benzoylformate decarboxylase spectrophotometrically (Hegeman, 1966a) failed because of the low extinction coefficient of benzoylformate and the consequent insensitivity of the assay. There was no sustained effort to measure

this enzyme since no substituted benzoylformates were available to test the specificity of the enzyme. Some experiments were also made with NADH oxidase which was followed by determining the decrease in extinction at 340mμ due to the oxidation of NADH. Full details of the assays are given in Methods.

Preparation of cell-free extracts.

The effectiveness of three methods of cell breakage is shown in Table 5. More protein, 112 mg./g. wet wt., was released by ultrasonic disruption than by either of the other methods of breakage. The specific activity of the benzyl alcohol dehydrogenase was essentially the same irrespective of the method of breakage - 130 mμmoles of benzyl alcohol oxidised/min./mg. protein. The specific activities of the L-mandelate dehydrogenase and the benzaldehyde dehydrogenase were lower when the cells were broken by grinding with alumina than by either of the other two methods. Since breakage of the cells by ultrasonic disruption gave the highest specific activity for the three enzymes this method of breakage, which was also the most convenient and easily controlled method, was always subsequently employed.

The effect of the time of ultrasonic disruption on the activity of four enzymes from bacterium NCIB 8250 is shown in Fig. 8. The activity of the L-mandelate dehydrogenase increased almost linearly from zero time, as did the activity of the NADH oxidase. Subsequent

Table 5. Comparison of the specific activities of three enzymes extracted by various methods from bacterium NCIB 8250.

For each of the three experiments bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole source of carbon and energy, harvested and the cells washed in 0.066M-NaH_2PO_4-Na_2HPO_4 buffer pH 8.2. The cells were disrupted as described in the text and, after centrifugation, the activities of the enzymes in the resulting supernatant solutions were determined as described in Methods. Specific activities are expressed in mμmoles substrate oxidised/min./mg. protein.

Enzyme	Hughes Press	Grinding with alumina	Ultrasonic disruption
	Protein released 46mg./g.wet wt.	Protein released 61mg./g.wet wt.	Protein released 112mg./g.wet wt.
	Specific activity	Specific activity	Specific activity
L-Mandelate dehydrogenase	134	57	140
Benzyl alcohol dehydrogenase	147	115	135
Benzaldehyde dehydrogenase	52	29	60

Fig.8. Enzyme activity released by ultrasonic disruption of bacterium NCIB 8250.

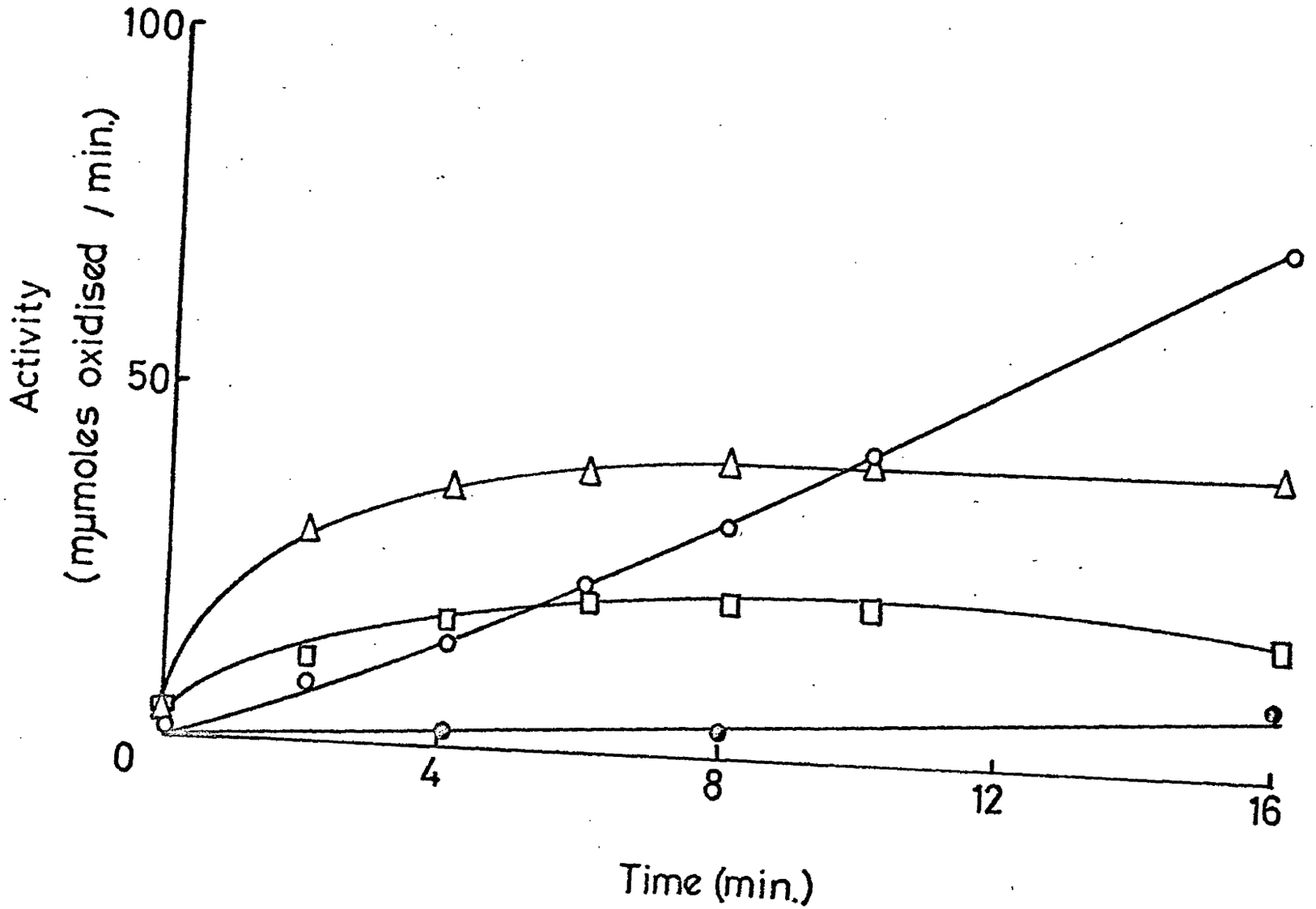
Cells were grown on 5mM-D,L-mandelate as sole carbon source, harvested, washed in 0.066M-Na_2HPO_4-NaH_2PO_4 buffer pH 8.2 and resuspended in the same buffer to 50 mg. wet wt./ml.. The suspension was disrupted with the Dawe Soniprobe as described in Methods. 5ml. samples were taken at appropriate time intervals and centrifuged at 4,500g for 40 min. at 4°. The enzyme activities of the resulting supernatant solutions were determined as described in Methods.

O- L-Mandelate dehydrogenase.

Δ- Benzyl alcohol dehydrogenase.

□- Benzaldehyde dehydrogenase.

⊙- NADH Oxidase.


100
50
0
Activity
(mμmoles oxidised / min.)
4
8
12
16
Time (min.)

experiments, not presented here, showed that this linear release continued for at least 20 minutes. Both the benzyl alcohol dehydrogenase and the benzaldehyde dehydrogenase reached maximum activity within 6 to 8 minutes. In the case of the benzaldehyde dehydrogenase activity began to fall after about 8 minutes whilst that of the benzyl alcohol dehydrogenase remained constant for up to 16 minutes. During this experiment, as in most subsequent experiments, the benzyl alcohol dehydrogenase activity was about twice that of the benzaldehyde dehydrogenase. For all subsequent work a disruption time of 8 minutes was employed as this resulted in the release of ample activity of each of the enzymes.

Conditions of assay of three enzymes from bacterium NCIB 8250.

(i) Nature of the cofactor requirements.

A number of experiments was performed on the cofactor specificity of the benzaldehyde dehydrogenase from bacterium NCIB 8250, since it had been demonstrated that two benzaldehyde dehydrogenases requiring NAD^+ and $NADP^+$ as cofactors were present in cell-free extracts of P. putida. (Gunsalus et al, 1953). Repeated attempts to find an $NADP^+$ dependent benzaldehyde dehydrogenase failed. The enzyme present in cell-free extracts of bacterium NCIB 8250 required NAD^+ as cofactor.

Neither NAD^+ nor $NADP^+$ acted as cofactor in the oxidation of D,L-mandelate by extracts of bacterium NCIB 8250 which displayed

L-mandelate dehydrogenase activity and the enzyme was assayed by following the reduction of 2,6-dichlorophenol-indophenol. Furthermore extracts of bacterium NCIB 8250 which displayed benzyl alcohol or benzaldehyde dehydrogenase activity could not employ 2,6-dichlorophenol-indophenol as cofactor for these enzymes, which were assayed by using NAD^+ as electron acceptor.

(ii) pH value.

The effect of pH on the activities of L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase are shown in Figs. 9, 10 and 11 respectively. The effect of pH on each of the three enzymes was determined in the presence of 16.7mM-sodium pyrophosphate buffer at the appropriate pH. Pyrophosphate is not a good buffer over the whole pH range tested, but it was decided to use this buffer rather than a range of buffers in order to rule out possible effects of the buffers themselves. In any case the pH values of the actual reaction mixtures were measured. The pH optima for the three enzymes were found to be:

L-Mandelate dehydrogenase	7.0
Benzyl alcohol dehydrogenase	8.7
Benzaldehyde dehydrogenase	9.5

Tris or glycylglycine when tested at the same pH values gave essentially the same rates as those obtained using pyrophosphate

Fig.9. The effect of pH on the activity of L-mandelate dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole source of carbon: the cells were harvested and the enzyme prepared as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer at the appropriate pH, 0.2 µmoles 2,6-dichlorophenol-indophenol, 3.0 µmoles D,L-mandelate, enzyme (142 µg. of protein) and water to 3.0 ml. Buffer, water, 2,6-dichlorophenol-indophenol and enzyme were incubated together at room temperature (21±1°) for five minutes prior to the addition of mandelate to start the reaction. After the reaction had been recorded the pH of the reaction mixture was determined by means of microelectrodes.

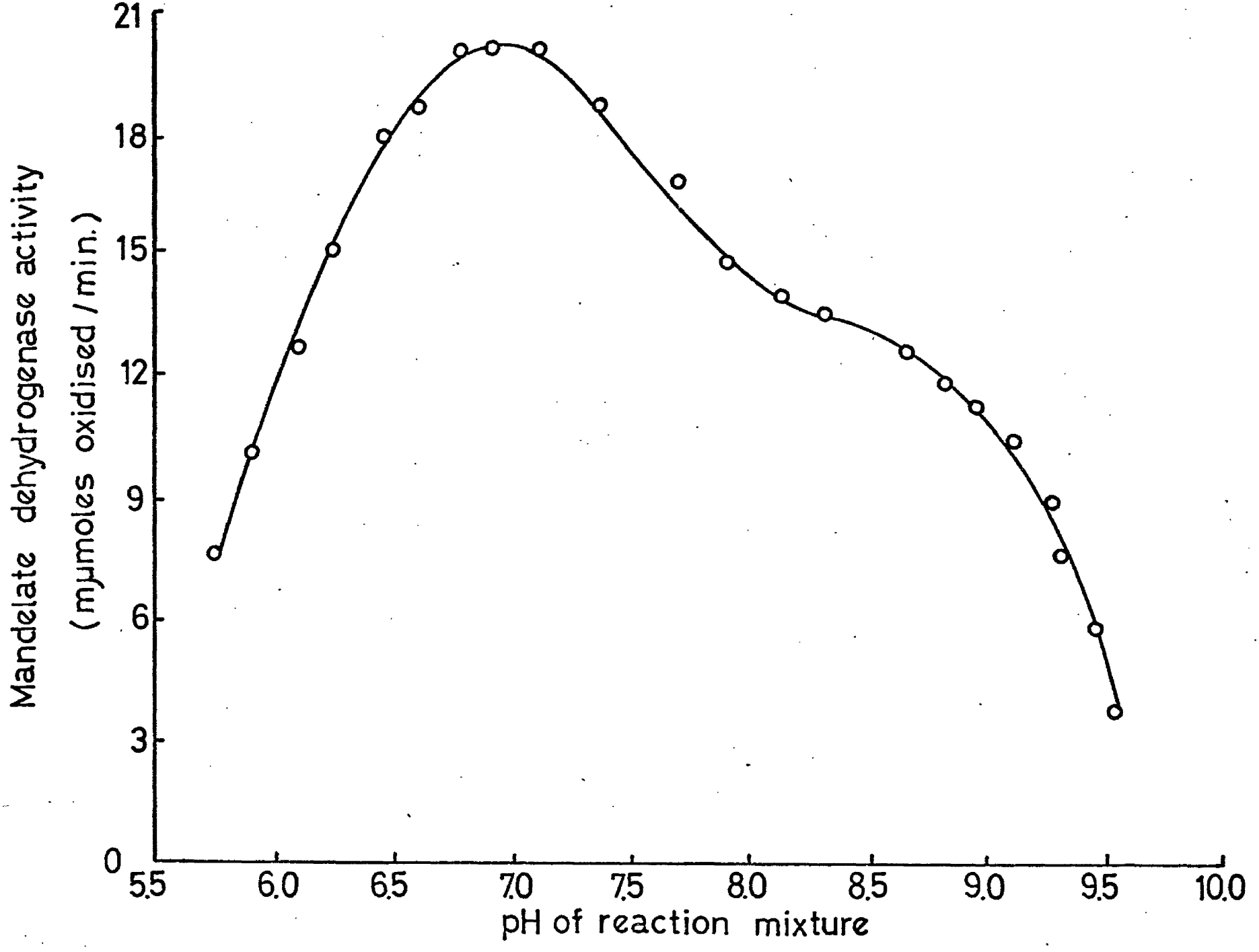

Mandelate dehydrogenase activity
(mμmoles oxidised / min.)
21
18
15
12
9
6
3
0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
pH of reaction mixture

Fig.10. The effect of pH on the activity of benzyl alcohol dehydrogenase.

Bacterium NCIB 8250 was grown on 2mM-benzyl alcohol as sole source of carbon: the cells were harvested and the enzyme prepared as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer at the appropriate pH, 1.5 µmoles of NAD^+, 0.3 µmoles of benzyl alcohol, enzyme (169 µg. of protein) and water to 3.0 ml.. Buffer, water, NAD , and enzyme were incubated together at room temperature ($21\pm1^{\circ}$) for five minutes prior to the addition of benzyl alcohol to start the reaction. After the reaction had been recorded the pH of the reaction mixture was determined by means of microelectrodes.

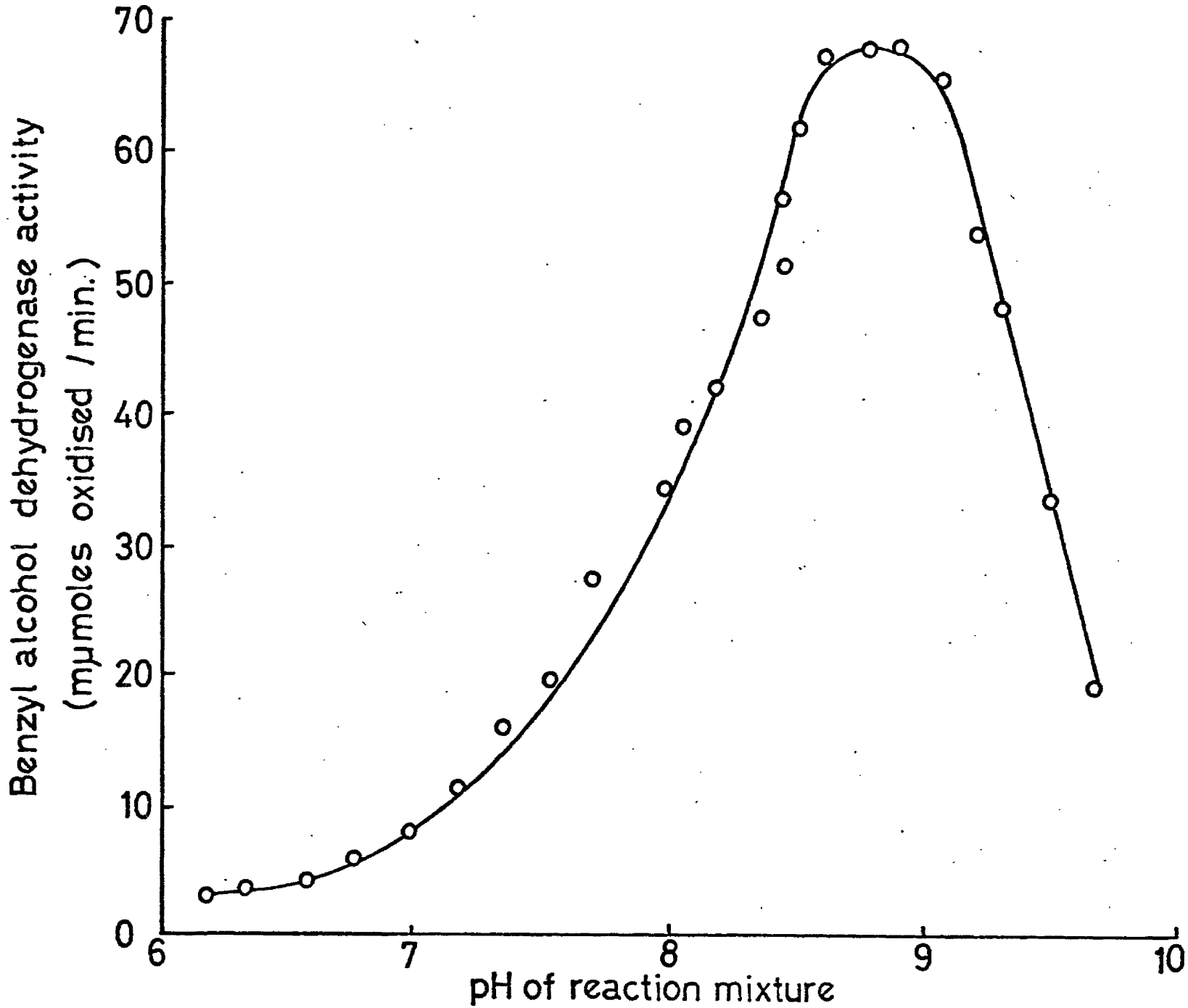
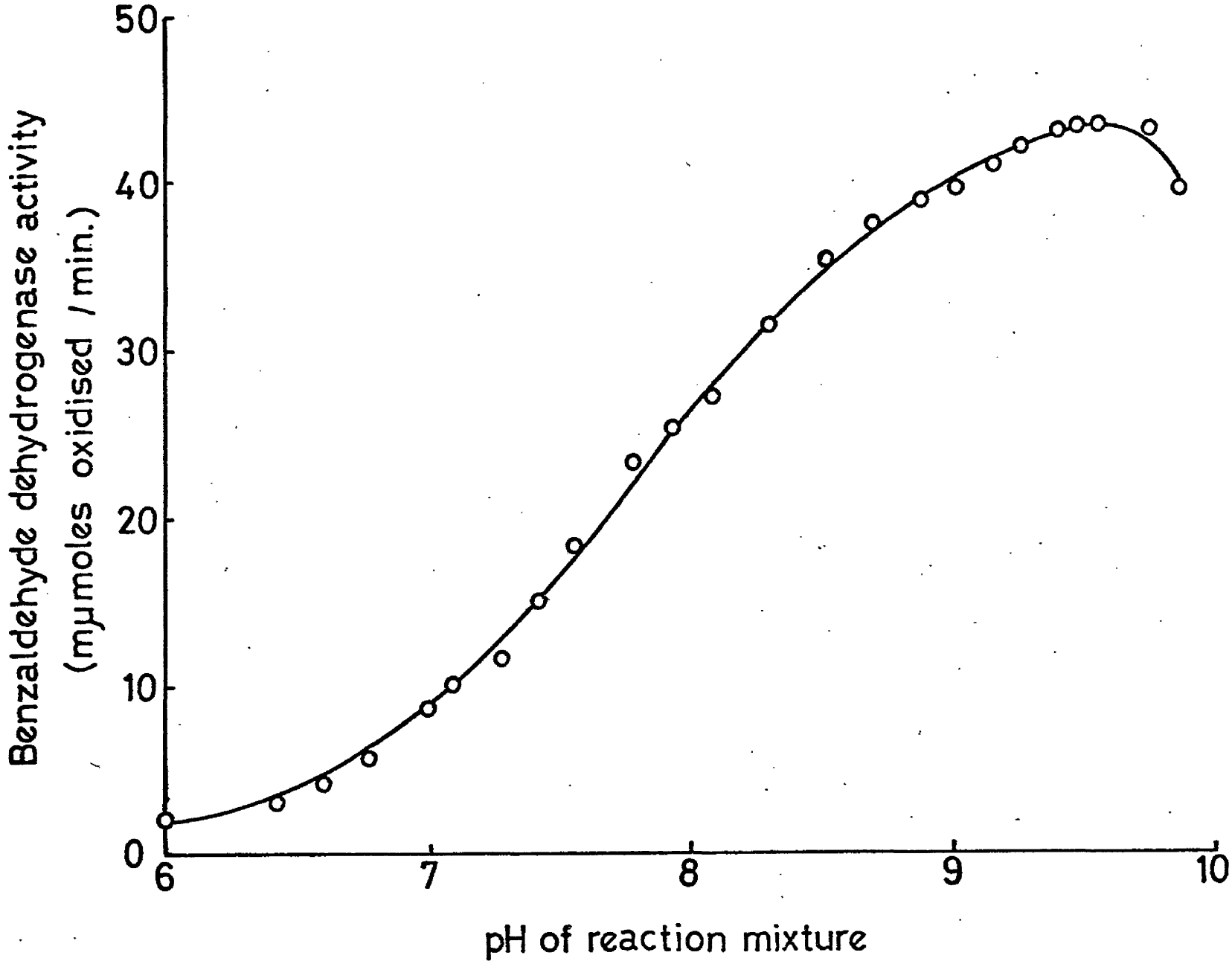

Benzyl alcohol dehydrogenase activity
(mμmoles oxidised /min.)
70
60
50
40
30
20
10
0
6
7
8
9
10
pH of reaction mixture

Fig.11. The effect of pH on the activity of benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 2mM-benzaldehyde as sole source of carbon: the cells were harvested and the enzyme prepared as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer at the appropriate pH, 1.5 µmoles of NAD^+, 0.3 µmoles of benzaldehyde, enzyme (175 µg. of protein) and water to 3.0 ml.. Buffer, water, NAD^+ and enzyme were incubated together at room temperature ($21 \pm 1^{\circ}$) for five minutes prior to the addition of benzaldehyde to start the reaction. After the reaction had been recorded the pH of the reaction mixture was determined by means of microelectrodes.


Benzaldehyde dehydrogenase activity
(mμmoles oxidised / min.)
50
40
30
20
10
0
6
7
8
9
10
pH of reaction mixture

buffer. In all subsequent work the enzymes were assayed at their optimum pH.

(iii) Buffer concentration.

A final concentration of 16.7mM-sodium pyrophosphate buffer was sufficient to maintain the pH of the reaction mixture (± 0.2 pH units) at the appropriate optimum for each of the three enzymes throughout the duration of the reaction.

(iv) Substrate concentration.

A final concentration of 1mM-D,L-mandelate, 0.1mM-benzyl alcohol or 0.1mM-benzaldehyde gave maximum initial rates with the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase respectively.

(v) Cofactor concentration.

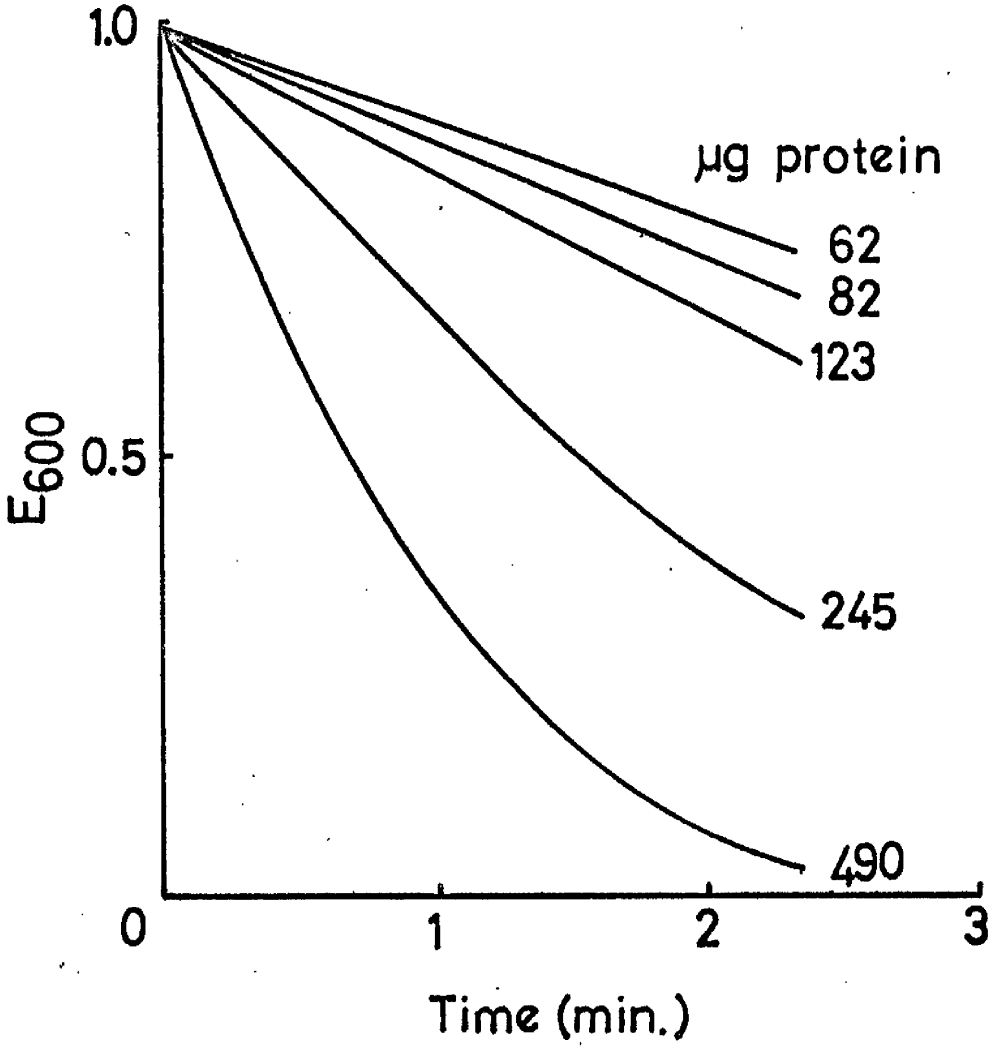
A final concentration of 67μM-2,6-dichlorophenol-indophenol or 0.5mM-NAD^+ were suitable concentrations of the two cofactors for the appropriate enzyme; there was no effect on the initial rate of doubling or halving these concentrations.

(vi) Amount of enzyme.

The effect of the amount of enzyme on the initial rate of oxidation of D,L-mandelate by the L-mandelate dehydrogenase is shown in Fig. 12. A range of enzyme concentrations from 62 μg. protein to

Fig.12. The effect of enzyme concentration on the time-course for L-mandelate dehydrogenase.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole source of carbon: the cells were harvested and the enzyme prepared as described in Methods. Each reaction cuvette contained 50 μmoles sodium pyrophosphate buffer pH 7.0, 0.2 μmoles 2,6-dichlorophenol-indophenol, 3.0 μmoles D,L-mandelate, enzyme (62-490 μg. of protein) and water to 3.0 ml.. The reaction was started by the addition of mandelate.


1.0
0.5
0
E_{600}
µg protein
62
82
123
245
490
0
1
2
3
Time (min.)

490 µg. protein was used. Enzyme concentrations of 62, 82 and 123 µg. of protein gave a linear time-course for the duration of the experiment. Higher enzyme concentrations gave a fall-off in rate after about 1 minute but since the time-courses obtained using the recording spectrophotometer were continuous the initial rates could be measured with reasonable accuracy up to about 500 µg. of protein. The rate of oxidation of D,L-mandelate was proportional to enzyme concentration over the measurable range. Analagous results were obtained for the effect of the amount of enzyme on the initial rates of oxidation with both the benzyl alcohol and benzaldehyde dehydrogenases.

Time-course for the benzaldehyde dehydrogenase.

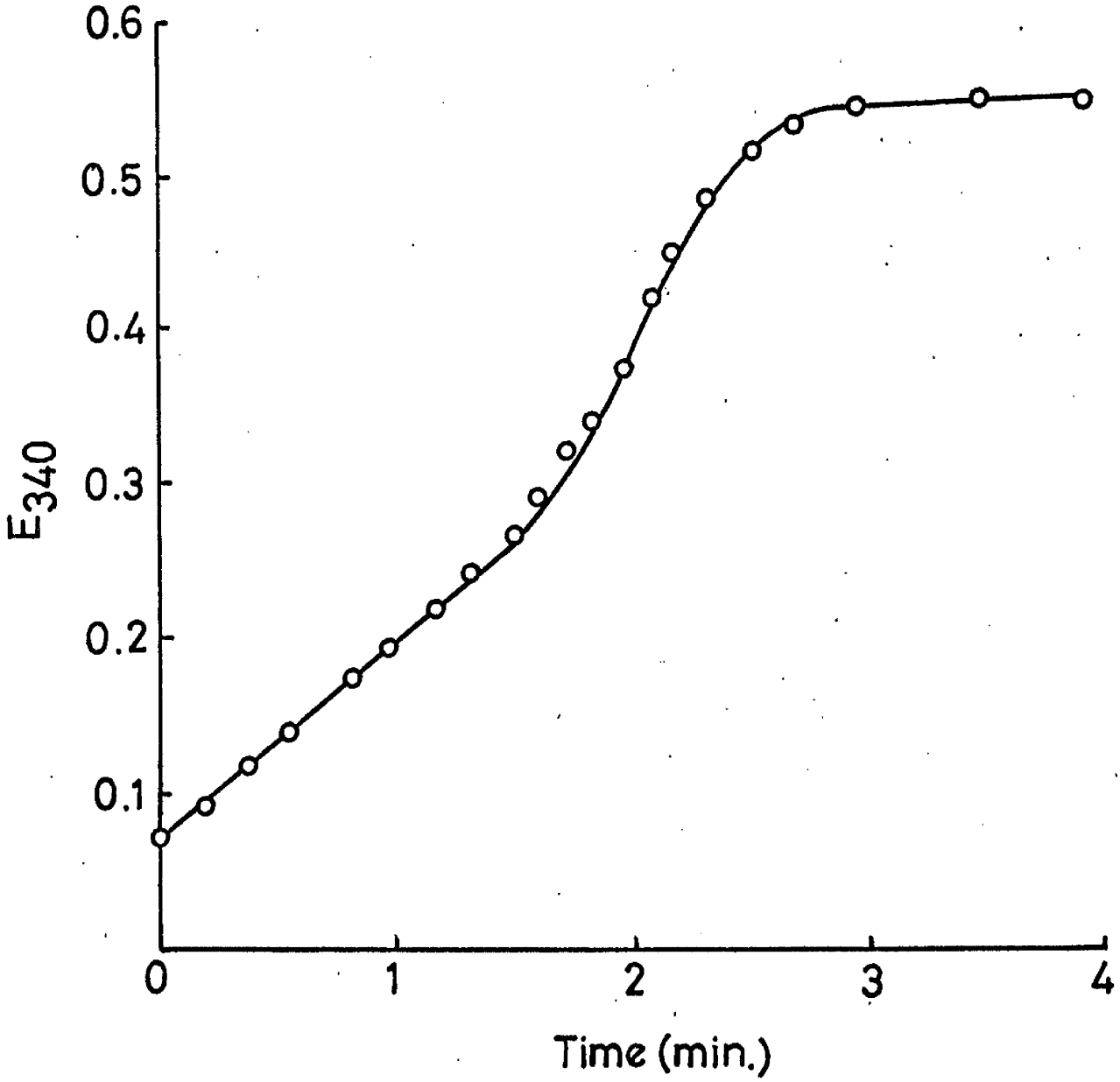
During the early experiments on the oxidation of benzaldehyde by cell-free extracts time-courses of the type shown in Fig. 13 were repeatedly obtained. This time-course consisted of an initial linear portion followed by a sigmoidal section. Before proceeding to study the kinetic properties of this enzyme it was necessary to determine the reason for this type of time-course and the following experiments were carried out.

(i) Concentration of reactants.

The initial linear rate of NAD^+ reduction was proportional to enzyme concentration. Both the length of the initial linear section

Fig.13. Time-course for benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 2mM-benzyl alcohol as sole source of carbon, harvested and the cells washed and disrupted in 0.066M-Na_2HPO_4-NaH_2PO_4 buffer pH 8.2 as described in Methods. The reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 9.5, 0.3 µmoles of benzaldehyde, 1.5 µmoles of NAD^+, enzyme (256 µg. of protein) and water to 3.0 ml.. The reaction was started by the addition of benzaldehyde.


0.6
0.5
0.4
0.3
0.2
0.1
E_{340}
0
1
2
3
4
Time (min.)

and the final E_{340} were directly proportional to the concentration of benzaldehyde added. The final E_{340} obtained was that expected from the stoichiometric oxidation of benzaldehyde to benzoate. There was no effect on the shape of the time-course by doubling or halving the concentration of buffer or NAD^+.

(ii) Effect of ions.

There was no effect on the shape of the time-course when the reaction was carried out in the presence of Mg^{++}, Mn^{++}, or K^+ at a final concentration of 3.3mM.

(iii) Effect of monothiols.

There was no effect on the shape of the time-course when the reaction was carried out in the presence of L-cysteine or reduced glutathione at a final concentration of 2.5mM.

(iv) Effect of a dithiol.

Cleland's reagent (1,4-dithiol-2,3-dihydroxybutane)(Cleland, 1964) at a final concentration of 2.5mM slightly increased the length of the initial linear portion and gave a correspondingly 'flatter' sigmoidal section, i.e. the time-course tended towards linearity.

(v) Effect of KCN.

KCN present at a final concentration of 1mM also changed the time-course in a similar manner to that described for Cleland's reagent. Neither KCN nor Cleland's reagent had any effect on the initial rate of oxidation.

(vi) Effect of preincubation.

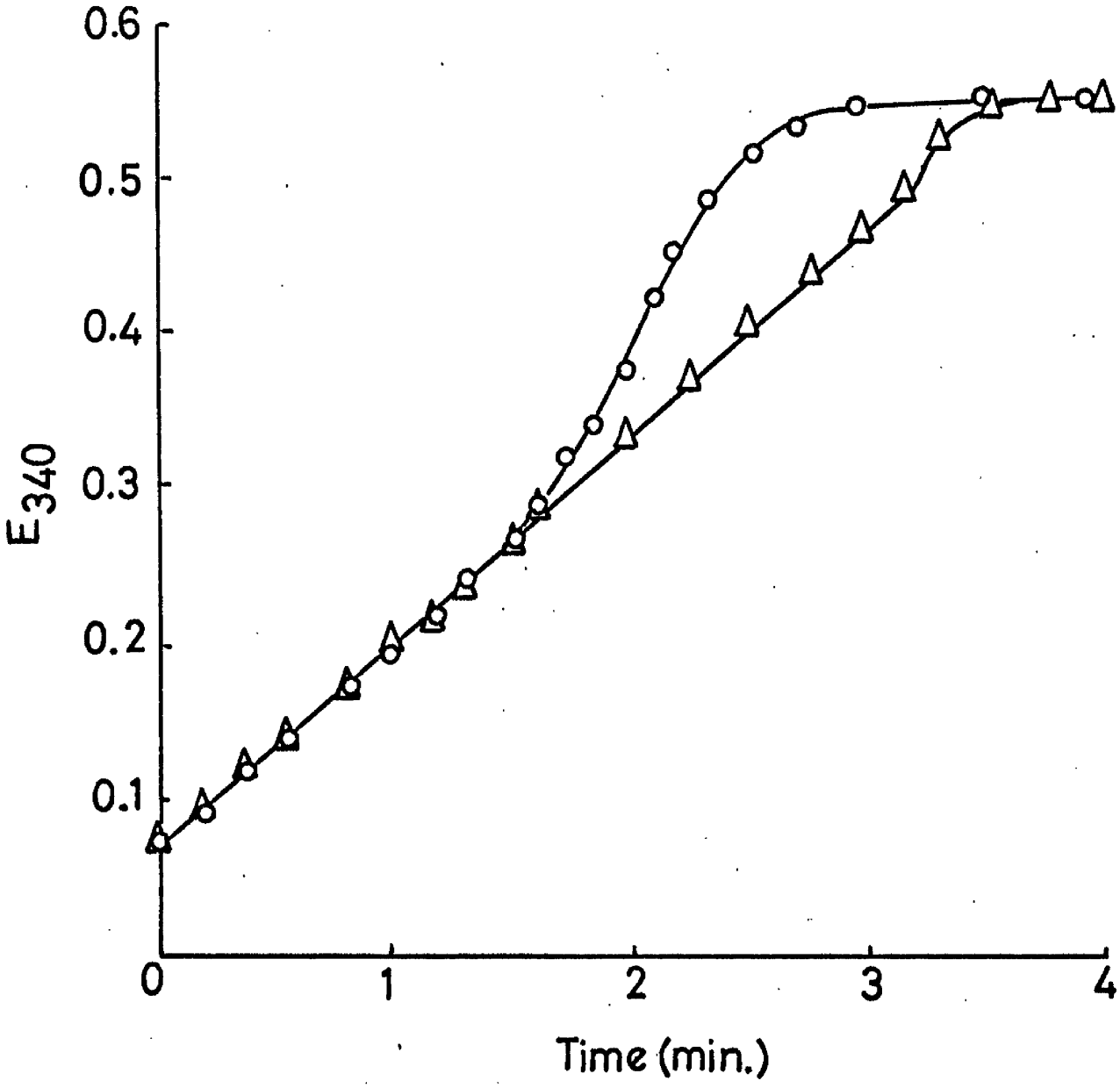
A series of experiments was performed to determine if preincubation of various combinations of the reaction components prior to the initiation of the reaction would affect the shape of the time-course. The following combinations were tried: substrate + enzyme + buffer + water, cofactor + enzyme + buffer + water, substrate + cofactor + water, enzyme + buffer + water and enzyme + water. Preincubation was carried out at room temperature ($21 \pm 1^{\circ}$) for 10 minutes. The effect of preincubation of enzyme + buffer + cofactor + water is shown in Fig. 14. There was a considerable increase in the length of the initial linear section, whilst the 'sigmoidal' section was almost eliminated, there being only a slight positive inflection at the end of the reaction. Identical results were obtained on preincubation of enzyme + buffer, enzyme + buffer + water or enzyme + substrate + water. No effect of preincubation on the time-course was observed with enzyme + water or substrate + cofactor + water. In other words, in order to obtain similar results to those shown in Fig. 14 the reaction components which must be preincubated together were enzyme and buffer. The buffer employed

Fig.14. Effect of preincubation on the time-course for benzaldehyde dehydrogenase.

Bacterium NCIB 8250 was grown on 2mM-benzyl alcohol as sole source of carbon, harvested and the cells washed and disrupted in 0.066M-Na_2HPO_4-NaH_2PO_4 buffer pH 8.2 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 9.5, 0.3 µmoles benzaldehyde, 1.5 µmoles NAD^+, enzyme (256 µg. of protein) and water to 3.0 ml.. The reaction was started by the addition of benzaldehyde.

O- Benzaldehyde added immediately.

Δ- Benzaldehyde added after a 10 minute incubation of the other reaction components at room temperature ($21 \pm 1^{\circ}$).


0.6
0.5
0.4
0.3
0.2
0.1
E_{340}
0
1
2
3
4
Time (min.)

in those studies was sodium pyrophosphate pH 9.5. To see if the increase in linearity observed after preincubation was due to the buffer itself an equimolar amount of Tris also at pH 9.5 was tested. The same results were recorded as with sodium pyrophosphate. If, however, enzyme and sodium pyrophosphate buffer pH 8.5 were incubated together no effect on the time-course was obtained and the typical sigmoidal shape was unchanged. It is noteworthy that the cell-free extract itself was at pH 8.2; the value of the Na_2HPO_4-NaH_2PO_4 buffer in which the harvested cells had been washed and ultrasonically disrupted. There seemed little doubt that the linear time-course obtained on preincubation of the enzyme was due to the effect of incubating the enzyme at pH 9.5

(vii) Effect of pH of both extraction and assay.

The effect of the pH of both ultrasonic disruption and enzyme assay were examined in order to extend the results obtained from the experiments on preincubation. The results obtained, not only with benzaldehyde dehydrogenase, but also with L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and NADH oxidase are shown in Table 6. Maximum rates for each enzyme were obtained when cells were extracted and the resulting supernatant solutions assayed at the pH optimum of each enzyme (c.f. Figs. 9, 10 and 11). There was little effect on the activity of the L-mandelate dehydrogenase by either extraction or assay at the three pH values. There was, however, a dramatic effect of the pH of extraction and assay on the activity of the benzyl

Table 6. The effect of the pH of both extraction and assay on the activities of four enzymes from bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole carbon source. The cells were harvested and three equal portions washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 7.0, 8.5 and 9.5 respectively as described in Methods. The enzyme activities of the four extracts, expressed as a percentage of the maximum activity of each enzyme, were determined at pH 7.0, 8.5 and 9.5 as described in Methods.

Enzyme	Assay pH	pH of extraction 7.0	8.5	9.5
L-Mandelate dehydrogenase	7.0	100	95	97
	8.5	80	90	94
	9.5	75	90	88
Benzyl alcohol dehydrogenase	7.0	6	8	6
	8.5	17	100	8
	9.5	37	75	3
Benzaldehyde dehydrogenase	7.0	23	23	50
	8.5	80	69	92
	9.5	85	92	100
NADH Oxidase	7.0	100	N.T.	N.T.
	8.5	80	20	N.T.
	9.5	60	N.T.	4

alcohol dehydrogenase. Cells extracted at pH 7.0 gave less than 40% maximum activity and cells extracted at pH 9.5 gave less than 10% maximum activity. The effect on the benzaldehyde dehydrogenase was a general decrease in activity both with extraction pH and assay pH from 9.5 to 7.0. The NADH oxidase showed maximum activity after extraction and assay at pH 7.0. Thus extracting the cells at pH 9.5 resulted in very low activity for the benzyl alcohol dehydrogenase and gave optimum activity for the benzaldehyde dehydrogenase. This was the first occasion on which the activities of these two enzymes had been separated.

The effect of the pH of both extraction and assay on the time-course for the benzaldehyde dehydrogenase using the same extracts as had been employed to determine the activities (Table 6) is shown in Table 7. Cells extracted at pH 7.0 or 9.5 gave a linear time-course, whilst cells extracted at pH 8.5 gave a sigmoidal time-course when assayed at either pH 8.5 or 9.5.

The result of this experiment demonstrated that the sigmoidal time-course was obtained only when there was a high benzyl alcohol dehydrogenase activity and, conversely, a linear time-course was obtained when the activity of the benzyl alcohol dehydrogenase was low.

The effect of those conditions which had tended to give a linear time-course for the benzaldehyde dehydrogenase, namely preincubation of the enzyme at pH 9.5 and the addition of KCN or Cleland's reagent were re-examined. In these new experiments the activities of both

Table 7. The effect of the pH of both extraction and assay on the shape of the time-course of the benzaldehyde dehydrogenase from bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole carbon source. The cells were harvested and three equal portions washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 7.0, 8.5 and 9.5 respectively as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 8.5 or 9.5, 0.1 µmoles of benzaldehyde, 1.5 µmoles NAD^+, enzyme prepared as described and water to 3.0 ml.. The reaction was started by the addition of benzaldehyde.

Assay pH	pH of extraction 7.0	8.5	9.5
8.5	Linear	Sigmoidal	Linear
9.5	Linear	Sigmoidal	Linear

the benzyl alcohol and benzaldehyde dehydrogenases were determined. In every case conditions which tended to give a linear time-course for the benzaldehyde dehydrogenase resulted in a concomitant reduction of benzyl alcohol dehydrogenase. None of the conditions which tended to give a linear time-course for the benzaldehyde dehydrogenase gave an increase in the initial rate of oxidation or an alteration in the final E_{340}.

(viii) Time-course for the benzaldehyde dehydrogenase at 340 and 282mμ.

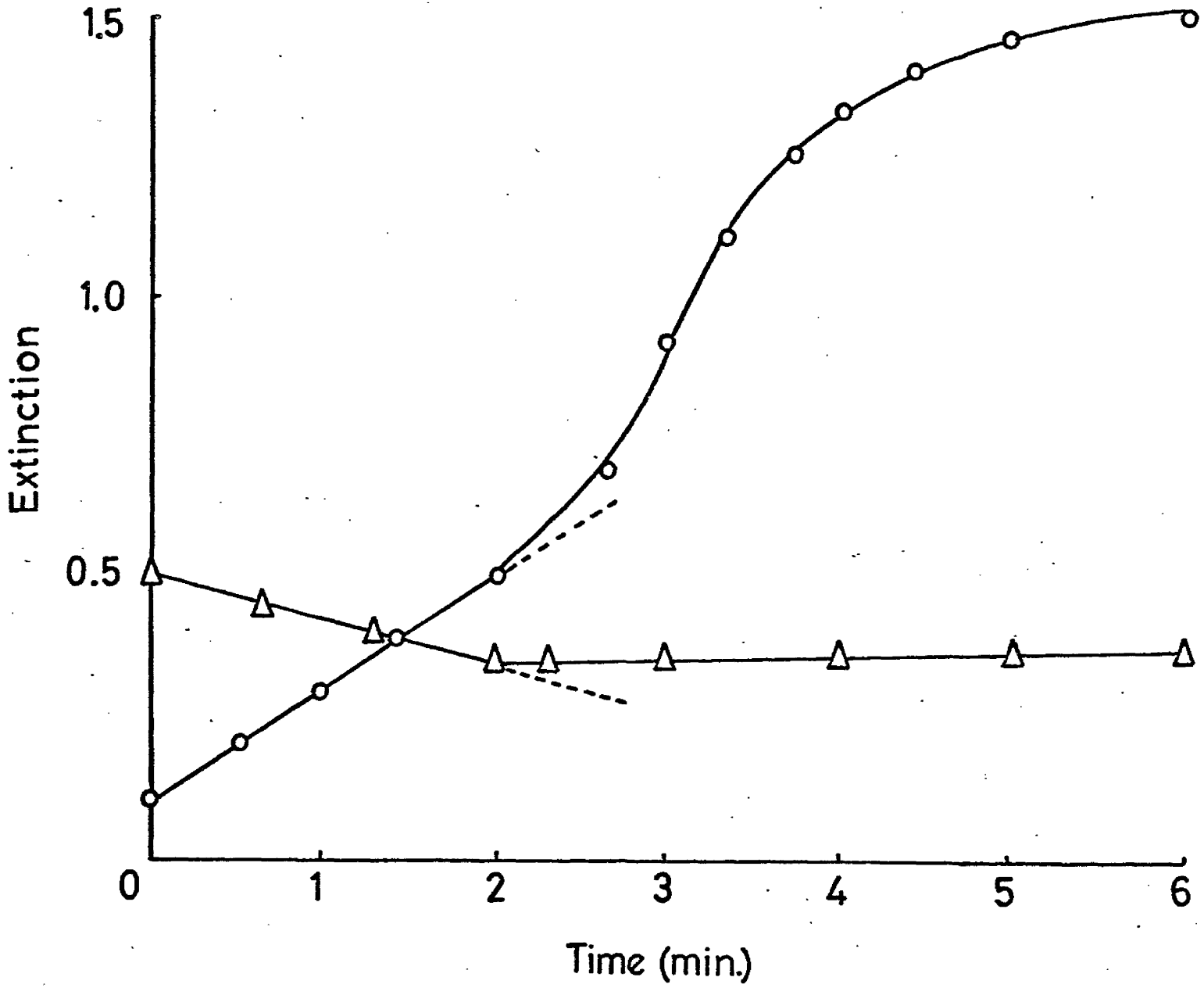
The assay method normally employed for determining the activity of the benzaldehyde dehydrogenase was to follow the production of NADH at 340mμ. In order to follow the reaction by the disappearance of benzaldehyde, experiments were carried out to follow the production of NADH at 340mμ and the disappearance of benzaldehyde simultaneously. Benzaldehyde has an extinction shoulder between 280 and 285mμ where neither benzoate, the presumed product of the reaction, nor benzyl alcohol absorb. The assay was complicated, however, by the higher extinction of NADH than NAD^+ at 282mμ (which was the wavelength subsequently employed). The time-course for the reaction at 340 and 282mμ is shown in Fig. 15. The experiment was performed using cell extracts which had been shown to have a high benzyl alcohol dehydrogenase activity. The time-course at 340mμ was sigmoidal, typical of that obtained in the presence of benzyl alcohol dehydrogenase activity. The time-course at 282mμ showed a linear fall in extinction up to the end of the linear portion of the 340mμ

Fig.15. Time-course for the benzaldehyde dehydrogenase reaction at 340 mμ and 282 mμ.

Bacterium NCIB 8250 was grown on 2mM-benzyl alcohol as sole source of carbon, harvested and the cells washed and disrupted in 0.066M-Na_2HPO_4-NaH_2PO_4 buffer pH 8.2 as described in Methods. Each reaction cuvette contained 50 μmoles sodium pyrophosphate buffer pH 9.5, 1.5 μmoles NAD^+, 0.9 μmoles of benzaldehyde, enzyme (768μg. of protein) and water to 3.0 ml.. The reaction was started by the addition of benzaldehyde. The dotted lines are extrapolations of the linear portions of the graphs.

o- Time-course at 340 mμ.

Δ- Time-course at 282 mμ.


1.5
1.0
0.5
0
Extinction
1
2
3
4
5
6
Time (min.)

time-course. At the point where the 340mμ time-course showed its upward inflection the linear decrease in the 282mμ time-course stopped, presumably due to exhaustion of benzaldehyde, and thereafter showed a slight increase to the end of the reaction. The initial linear rate of NAD^+ reduction therefore corresponded to the period during which all the benzaldehyde disappeared. The higher rate of NAD^+ reduction only started when the benzaldehyde was exhausted. Nevertheless, as stated above, the stoichiometry of the total reaction corresponded to 1 mole NADH produced/mole benzaldehyde oxidised. No attempt was made to investigate the possible transitory appearance of other compounds such as benzyl alcohol.

In all subsequent studies on the activity of the benzaldehyde dehydrogenase the cells were washed and ultrasonically disrupted in 0.1M-sodium pyrophosphate buffer pH 9.5. These preparations, devoid of benzyl alcohol dehydrogenase activity, invariably gave linear time-courses.

Specificity and kinetic constants of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from bacterium NCIB 8250.

(i) Determination of kinetic constants.

In order to obtain additional information about the specificities of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase present in cell-free

extracts of bacterium NCIB 8250, an attempt was made to determine the Km and Vmax for as many as possible of the challenge substrates shown in Table 3. Bacterium NCIB 8250 was grown on each of a number of aromatic compounds in turn. The cells were harvested and the cell-free extracts prepared by ultrasonic disruption as described in Methods. The Km and Vmax of each D,L-mandelate or benzyl alcohol were obtained from Lineweaver-Burk plots (1934). The type of graph obtained is illustrated by Fig. 16, which shows the results obtained with benzyl alcohol, 3-hydroxybenzyl alcohol and 4-hydroxy-3-methoxy-benzyl alcohol as substrates for the benzyl alcohol dehydrogenase. The Km and Vmax for each of these substrates were calculated from the intercepts on the ordinate and absicissa. From Fig. 16:-

Enzyme substrate	K_M (μM)	Vmax (as a percentage of rate with benzyl alcohol)
Benzyl alcohol	12	100
3-Hydroxybenzyl alcohol	50	55
4-Hydroxy-3-methoxybenzyl alcohol	38	32

The reproducibility of this method of determining the Km was investigated. On five separate occasions bacterium NCIB 8250 was

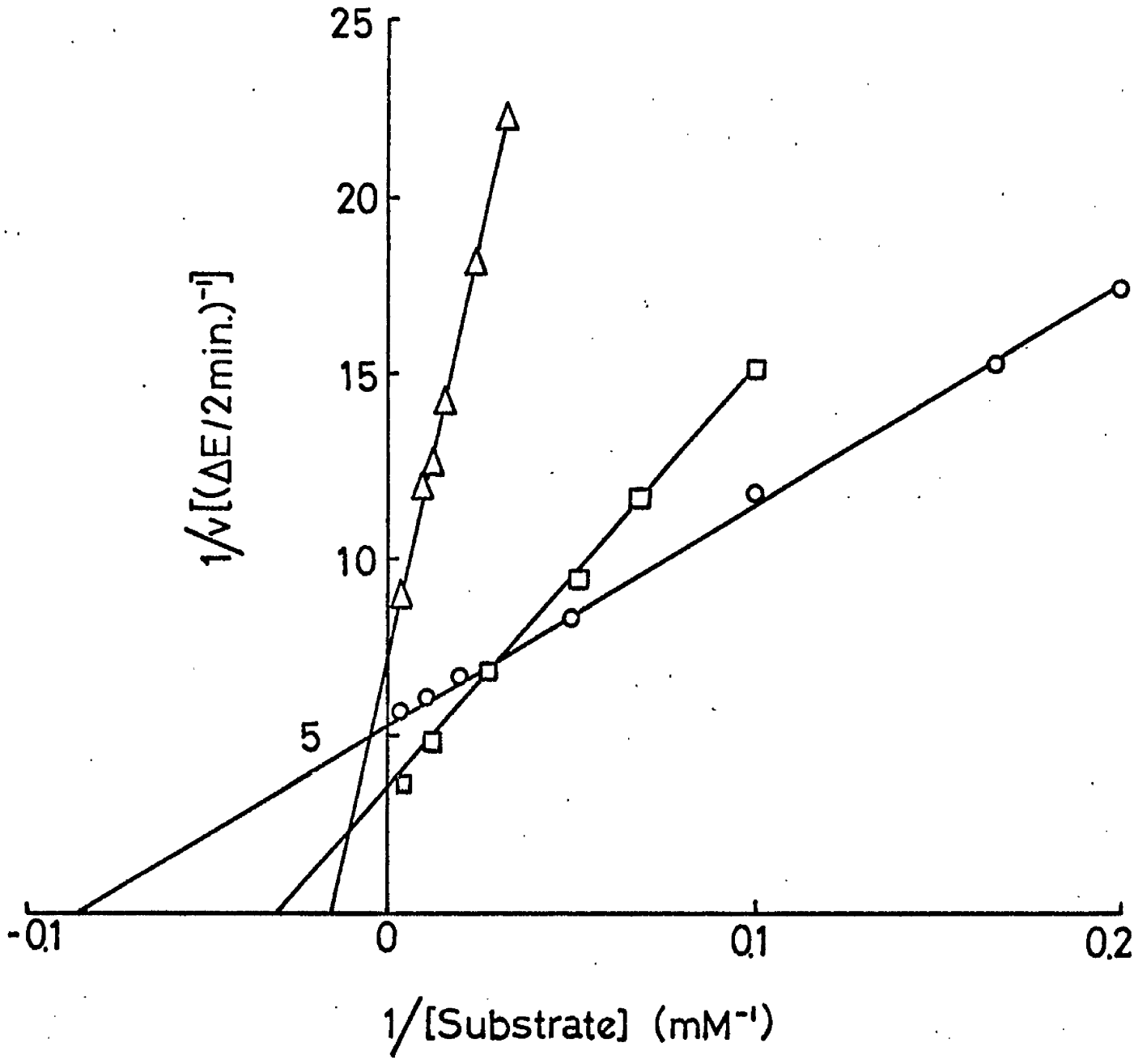
Fig.16. Lineweaver-Burk(1934) plots for benzyl alcohol dehydrogenase using various substrates.

Bacterium NCIB 8250 was grown on 2mM-benzyl alcohol as sole source of carbon, harvested and the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 8.5, 1.5 µmoles NAD^+, substrates at various concentrations, enzyme (125 µg. of protein) and water to 3.0 ml.. The reaction was started by the addition of substrate.

o- Benzyl alcohol.

Δ- 3-Hydroxybenzyl alcohol.

□- 4-Hydroxy-3-methoxybenzyl alcohol.


25
20
15
10
5
1/V[(ΔE/2min.)⁻¹]
-0.1
0
0.1
0.2
1/[Substrate] (mM⁻¹)

grown on D,L-mandelate, harvested and the cells disrupted as described in Methods. The value of the Km with D,L-mandelate as substrate was 235uM ± S.E.M. of 5.2μM.

(ii) L-Mandelate dehydrogenase.

The initial rates of oxidation of the isomeric forms of mandelate by extracts of bacterium NCIB 8250 are shown in Table 8. The results reflect the observations made in the growth experiment (Table 1) and the washed cell experiment (Fig. 1). Saturating concentrations of the L- and D,L-forms were oxidised with essentially the same initial rates. The D-isomer was not oxidised. No decrease was observed in the initial rate of oxidation of the L-isomer by the addition of an equimolar amount of the D-form.

Values for the Km and Vmax for each D,L-mandelate obtained using cell-free extracts of bacterium NCIB 8250 which had been grown on each of a number of D,L-mandelates are shown in Tables 9 and 10. The values of the Km for each D,L-mandelate were essentially the same irrespective of the substrate on which bacterium NCIB 8250 had been grown e.g. the Km for 3-hydroxy-D,L-mandelate was always about 350μM. Preliminary experiments with 3,4-dihydroxy-D,L-mandelate as substrate suggested that the Km was about 1,000μM but owing to the scarcity of this compound no further experiments were performed. The values for Vmax, expressed as a percentage of the rate with D,L-mandelate, were essentially the same for each of the substituted D,L-mandelates irrespective of the growth substrate.

Table 8. The ability of cell-free extracts of bacterium NCIB 8250 to oxidise the isomeric forms of mandelate.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole source of carbon and energy, harvested and the cells washed and disrupted in 0.066M-Na_2HPO_4-NaH_2PO_4 buffer pH 8.2 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 7.0, 0.2 µmoles 2,6-dichlorophenol-indophenol, the mandelate(s) at the appropriate concentration, enzyme (130 µg. of protein) and water to 3.0 ml.. The reaction was started by the addition of the mandelate.

Enzyme substrate	Concentration (mM)	Rate of oxidation (mmoles/min.)
D,L-Mandelate	1	39.3
D-Mandelate	1	0
L-Mandelate	1	40.9
D-Mandelate + L-Mandelate	1 1	39.7

Table 9. Values for the Km of oxidation of various D,L-mandelates by extracts of bacterium NCIB 8250 which had been grown on a number of D,L-mandelates.

Bacterium NCIB 8250 was grown on each D,L-mandelate in turn, as sole source of carbon, at a concentration of 5mM; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 7.0, 0.2 µmoles 2,6-dichlorophenol-indophenol, a D,L-mandelate at an appropriate concentration, enzyme and water to 3.0 ml.. The reaction was started by the addition of the D,L-mandelate. The values for Km(µM) were determined from Lineweaver-Burk (1934) plots as described in the text.

Growth substrate	Enzyme substrate			
	D,L-Mandelate			
	Non-substituted	3-Hydroxy	4-Hydroxy	4-Hydroxy-3-methoxy
D,L-Mandelate	237	366	700	2,100
4-Hydroxy-D,L-mandelate	237	333	620	2,000
4-Hydroxy-3-methoxy-D,L-mandelate	232	340	670	2,100

Table 10. Values for the relative Vmax for various D,L-mandelates obtained using extracts of bacterium NCIB 8250 which had been grown on a number of D,L-mandelates.

Bacterium NCIB 8250 was grown on each D,L-mandelate in turn, as sole source of carbon, at a concentration of 5mM; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 7.0, 0.2 µmoles 2,6-dichlorophenol-indophenol, a D,L-mandelate at an appropriate concentration, enzyme and water to 3.0 ml.. The reaction was started by the addition of the D,L-mandelate. Values for the relative Vmax, expressed as a percentage of the Vmax for D,L-mandelate, were determined from Lineweaver-Burk (1934) plots as described in the text.

Growth substrate	Enzyme substrate			
	D,L-Mandelate			
	Non-substituted	3-Hydroxy	4-Hydroxy	4-Hydroxy-3-methoxy
D,L-Mandelate	100	124	127	51
4-Hydroxy-D,L-mandelate	100	108	116	74
4-Hydroxy-3-methoxy-D,L-mandelate	100	112	120	68

(iii) Benzyl alcohol dehydrogenase.

Values for the Km and Vmax for each benzyl alcohol obtained using cell-free extracts of bacterium NCIB 8250 which had been grown on a number of compounds are shown in Tables 11 and 12. The values of the Km for each benzyl alcohol (c.f. Fig. 16) were essentially the same irrespective of whether the growth substrate was a D,L-mandelate, benzyl alcohol or benzaldehyde e.g. the Km for benzyl alcohol was always about 12μM. The values for the Vmax, expressed as a percentage of the rate with benzyl alcohol, were essentially the same for each of the substituted benzyl alcohols irrespective of the growth substrate.

(iv) Benzaldehyde dehydrogenase.

It was not possible to obtain a value for the Km for benzaldehyde dehydrogenase with benzaldehyde as substrate (the Km was below 6μM) because of the sensitivity limitations imposed on the assay by the extinction coefficient of NADH. For this reason the initial rates of oxidation of each benzaldehyde relative to benzaldehyde itself were determined at one suitable concentration (40μM). Values for the relative velocity for each benzaldehyde obtained using cell-free extracts of bacterium NCIB 8250 which had been grown on a number of compounds are shown in Table 13. The values of the relative velocity, expressed as a percentage of the rate with benzaldehyde, were essentially the same for each of the substituted benzaldehydes irrespective of the growth substrate.

Table 11. Values for the Km of oxidation of various benzyl alcohols by extracts of bacterium NCIB 8250 which had been grown on a number of compounds.

Bacterium NCIB 8250 was grown on each benzyl alcohol or D,L-mandelate in turn, as sole carbon source, at a concentration of 2mM and 5mM respectively; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 8.5, 1.5 µmoles NAD^+, a benzyl alcohol at an appropriate concentration, enzyme and water to 3.0 ml.. The reaction was started by the addition of the benzyl alcohol. The values for Km(µM) were determined from Lineweaver-Burk (1934) plots as described in the text.

Growth substrate	Enzyme substr		
	Benzyl alcoh		
	Non-substituted	2-Hydroxy	3-Hydrox
Benzyl alcohol	12	63	46
2-Hydroxybenzyl alcohol	12	66	50
4-Hydroxybenzyl alcohol	12	65	49
D,L-Mandelate	11	68	46
4-Hydroxy-D,L-mandelate	11	69	54

Table 12. Values for the relative Vmax for various benzyl alcohols obtained using extracts of bacterium NCIB 8250 which had been grown on a number of compounds.

Bacterium NCIB 8250 was grown on each benzyl alcohol or D,L-mandelate in turn, as sole carbon source, at a concentration of 2mM and 5mM respectively; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 8.5, 1.5 µmoles NAD^+, a benzyl alcohol at an appropriate concentration, enzyme and water to 3.0 ml.. The reaction was started by the addition of the benzyl alcohol. Values for the relative Vmax, expressed as a percentage of the Vmax for benzyl alcohol, were determined from Lineweaver-Burk (1934) plots as described in the text.

Growth substrate	Enzyme substra		
	Benzyl alcoh		
	Non-substituted	2-Hydroxy	3-Hydroxy
Benzyl alcohol	100	63	59
2-Hydroxybenzyl alcohol	100	57	55
4-Hydroxybenzyl alcohol	100	57	53
D,L-Mandelate	100	67	66
4-Hydroxy-D,L-mandelate	100	69	61

Table 13. Values for the relative velocity for various benzaldehydes obtained using extracts of bacterium NCIB 8250 which had been grown on a number of compounds.

Bacterium NCIB 8250 was grown on each benzyl alcohol(2mM), 4-hydroxy-3-methoxybenzaldehyde(2mM) or D,L-mandelate(5mM) in turn, as sole source of carbon, harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 9.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 9.5, 1.5 µmoles NAD^+, a benzaldehyde at a concentration of 40µM, enzyme and water to 3.0 ml.. The reaction was started by the addition of the benzaldehyde. Values for the relative velocity, expressed as a percentage of the velocity obtained with benzaldehyde, were determined from Lineweaver-Burk (1934) plots as described in the text.

Growth substrate	Enzyme substrate			
	Benzaldehyde			
	Non-substituted	2-Hydroxy	3-Hydroxy	4-Hyd
Benzyl alcohol	100	9	68	4
2-Hydroxybenzyl alcohol	100	6	65	3
4-Hydroxybenzyl alcohol	100	6	63	3
4-Hydroxy-3-methoxy-benzaldehyde	100	6	69	4
D,L-Mandelate	100	7	72	5

There was slightly more variation in the values of the relative velocities for the various benzaldehydes than of the values of Vmax for either the benzyl alcohols or D,L-mandelates. This was because of the difficulties in obtaining accurate measurements of the initial rate of oxidation of certain benzaldehydes due to the high extinctions of these compounds at 340mμ (see Methods section).

(v) Initial rates of oxidation of two substrates when added either singly or simultaneously to each of three enzymes from bacterium NCIB 8250.

Experiments were performed to determine if bacterium NCIB 8250 could synthesise a number of substrate specific L-mandelate dehydrogenases, benzyl alcohol dehydrogenases and benzaldehyde dehydrogenases in response to growth on any one D,L-mandelate, benzyl alcohol or benzaldehyde. If this were so, then simultaneous addition of two substrates, each at saturating concentration, might be expected to give the additive rate of oxidation obtained with the two substrates when added separately. The cell-free extracts of bacterium NCIB 8250 which had been grown on D,L-mandelate, and employed to determine the substrate specificities presented in the previous section, were employed in this study. The initial rates of oxidation of pairs of D,L-mandelates, benzyl alcohols or benzaldehydes when added either singly or simultaneously to their appropriate enzyme are shown in Tables 14, 15 and 16.

L-Mandelate dehydrogenase. There was little difference between the rates of oxidation of the individual D,L-mandelates (Table 14). This rate was about 20 mμmoles substrate oxidised/min./0.1 mg. protein. The rates obtained with the various combinations of D,L-mandelates when added simultaneously were also about 20 mμmoles substrate oxidised/min./0.1 mg. protein.

Benzyl alcohol dehydrogenase. The initial rates of oxidation of the individual benzyl alcohols (Table 15) varied from 15 to 36 mμmoles substrate oxidised/min./0.1 mg. protein. The rates obtained with the various combinations of benzyl alcohols were in general intermediate between the rates obtained with the two substrates when added separately.

Benzaldehyde dehydrogenase. Due to the limited number of suitable substrates only one combination of benzaldehydes was tested (Table 16). The rate of oxidation of benzaldehyde + 3-hydroxy-benzaldehyde was less than that obtained with benzaldehyde alone.

The initial rates of oxidation of mixtures of two D,L-mandelates, benzyl alcohols or benzaldehydes, therefore, were either identical to, or considerably lower than, the higher of the initial rates obtained with one of the individual substrates. In no case was the rate with a mixture of the substrates even close to the sum of the individual rates.

Table 14. Rates of oxidation of various D,L-mandelates when added either singly or in pairs to extracts of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole carbon source; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 7.0, 0.2 µmoles of 2,6-dichlorophenol-indophenol, the D,L-mandelates at appropriate concentrations, enzyme (230 µg. of protein) and water to 3.0 ml.. The reaction was started by the addition of the D,L-mandelate. Rates are expressed in mµmoles substrate oxidised/min./0.1 mg. protein.

Enzyme substrate	Conc. (mM)	Rate
D,L-Mandelate	1	20.0
	2	19.5
4-Hydroxy-D,L-mandelate	5	21.7
	10	22.0
3-Hydroxy-D,L-mandelate	4	21.2
	8	21.4
D,L-Mandelate + 4-hydroxy-D,L-mandelate	1	22.0
	5	
D,L-Mandelate + 3-hydroxy-D,L-mandelate	1	21.2
	4	
4-Hydroxy-D,L-mandelate + 3-hydroxy-D,L-mandelate	5	19.4
	4	

Table 15. Rates of oxidation of various benzyl alcohols when added either singly or in pairs to extracts of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole carbon source; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 8.5 as described in Methods. Each reaction cuvette contained 50 µmoles sodium pyrophosphate buffer pH 8.5, 1.5 µmoles NAD^+, the benzyl alcohols at appropriate concentrations, enzyme (375 µg. of protein) and water to 3.0 ml.. The reaction was started by the addition of the benzyl alcohol. Rates are expressed in mµmoles substrate oxidised/min./0.1 mg. protein.

Enzyme substrate	Conc. (mM)	Rate
Benzyl alcohol	0.1	20.3
	0.2	21.0
2-Hydroxybenzyl alcohol	1	15.2
	2	15.8
4-Hydroxy-3-methoxybenzyl alcohol	1	34.3
	2	36.7
Benzyl alcohol + 2-hydroxybenzyl alcohol	0.1 1.0	15.8
Benzyl alcohol + 4-hydroxy-3-methoxybenzyl alcohol	0.1 1.0	27.7
2-Hydroxybenzyl alcohol + 4-hydroxy-3-methoxybenzyl alcohol	1.0 1.0	25.0

Table 16. Rates of oxidation of benzaldehyde and 3-hydroxybenzaldehyde when added either singly or together to extracts of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown on 5mM-D,L-mandelate as sole source of carbon; harvested and a portion of the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer pH 9.5 as described in Methods. Each reaction cuvette contained 50 μmoles sodium pyrophosphate buffer pH 9.5, 1.5 μmoles NAD^+, the benzaldehydes at appropriate concentrations, enzyme (1.65 mg. of protein) and water to 3.0 ml.. The reaction was started by addition of the benzaldehyde. Rates are expressed as mμmoles substrate oxidised/min./0.1 mg. protein.

Enzyme substrate	Conc. (mM)	Rate
Benzaldehyde	0.1	26.3
	0.2	27.8
3-Hydroxybenzaldehyde	0.1	12.4
	0.2	12.4
Benzaldehyde + 3-Hydroxybenzaldehyde	0.1 0.1	21.9

Specific activities of three enzymes from bacterium NCIB 8250.

The specific activities of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase examined after the growth of bacterium NCIB 8250 on each of a number of compounds are shown in Table 17. The enzymes were examined using the same cell-free extracts as had been employed to determine the substrate specificities presented in the previous section. The specific activities of the L-mandelate dehydrogenase were higher than those of either the benzyl alcohol or benzaldehyde dehydrogenases. The 4-hydroxy-D,L-mandelate grown cells had a somewhat higher specific activity than cells grown on the other mandelates (347 compared to 237 mμmoles D,L-mandelate oxidised/min./mg. protein). The specific activities of the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were lower when extracted from cells grown on a D,L-mandelate than from cells grown on a benzyl alcohol or benzaldehyde. Cells grown on 4-hydroxybenzyl alcohol had considerably higher specific activity levels of both benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase (2 to 3 times) than cells grown on any other substrate.

Table 17. Specific activities of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase from bacterium NCIB 8250 after growth on a number of compounds.

Bacterium NCIB 8250 was grown on a number of D,L-mandelates (all 5mM), benzyl alcohols (all 2mM) or 4-hydroxy-3-methoxybenzaldehyde (2mM) as sole source of carbon, harvested and the cells washed and disrupted in 0.08M-sodium pyrophosphate buffer as described in Methods. The specific activities, expressed in mμmoles substrate oxidised/min./mg. protein, were determined as described in Methods.

Growth substrate	L-Mandelate dehydrogenase tested with D,L-Mandelate	Benzyl alcohol dehydrogenase tested with benzyl alcohol	Benzaldehyde dehydrogenase tested with benzaldehyde
D,L-Mandelate	237	94.5	60
4-Hydroxy-D,L-mandelate	347	74.5	N.T.
4-Hydroxy-3-methoxy-D,L-mandelate	237	N.T.	N.T.
Benzyl alcohol	-	169	113
2-Hydroxybenzyl alcohol	-	198	123
4-Hydroxybenzyl alcohol	-	440	467
4-Hydroxy-3-methoxy-benzaldehyde	-	N.T.	104

DISCUSSION.

Metabolic Versatility of Bacterium NCIB 8250.

Bacterium NCIB 8250 was found to utilise a number of mandelic acids and related compounds as sole sources of carbon and energy for growth (Table 1). These compounds can be grouped into five families according to the substituents on the aromatic ring. Each family consists of an L-mandelate, benzyl alcohol, benzaldehyde, benzoate and probably, as will be discussed later, a benzoylformate. The five families are the non-substituted, the 2-hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy-substituted compounds. Some of these substances have apparently not previously been reported to support the growth of microorganisms.

Washed cells of bacterium NCIB 8250 which had been grown on succinate or nutrient broth oxidised catechol and 3,4-dihydroxybenzoate only after a lag (p.55). This lag presumably represented the time taken for the washed cells to synthesise the enzymes required to convert catechol and 3,4-dihydroxybenzoate to compounds which can enter the central amphibolic cycles of the cell. Furthermore, washed cells which had been grown on catechol or 3,4-dihydroxybenzoate oxidised the remaining aromatic compounds only after a lag (p.54; Table 3); again presumably due to the time taken to synthesise the appropriate enzymes. Thus in order for bacterium NCIB 8250 to utilise any aromatic compound the bacterial cell must

synthesise the necessary degradative enzymes. The situation in bacterium NCIB 8250 appears to be the same as that in P. putida (Stanier, 1947) in which all the enzymes involved in the catabolism of aromatic compounds are adaptive in nature.

Pathways of Oxidation of Mandelic Acid and Related Compounds.

Metabolism of the isomeric forms of mandelate.

Bacterium NCIB 8250 was found to grow on the L-isomer and racemic D,L-mixture of mandelate but not on the D-isomer (Table 2). The D-isomer, moreover, was not oxidised either by whole-cell suspensions (Fig. 1) or cell-free extracts (Table 8). These results suggest that bacterium NCIB 8250 can metabolise only L-mandelate. Assuming that the L-form comprises 50% of the racemic mixture this suggestion is supported by three observations:

1. The stationary phase population recorded after growth on D,L-mandelate was only half that recorded on an equimolar amount of L-mandelate (Table 2).

2. The final oxygen uptake recorded with D,L-mandelate when incubated with a washed suspension was only half that recorded with an equimolar amount of the L-form (Fig. 1).

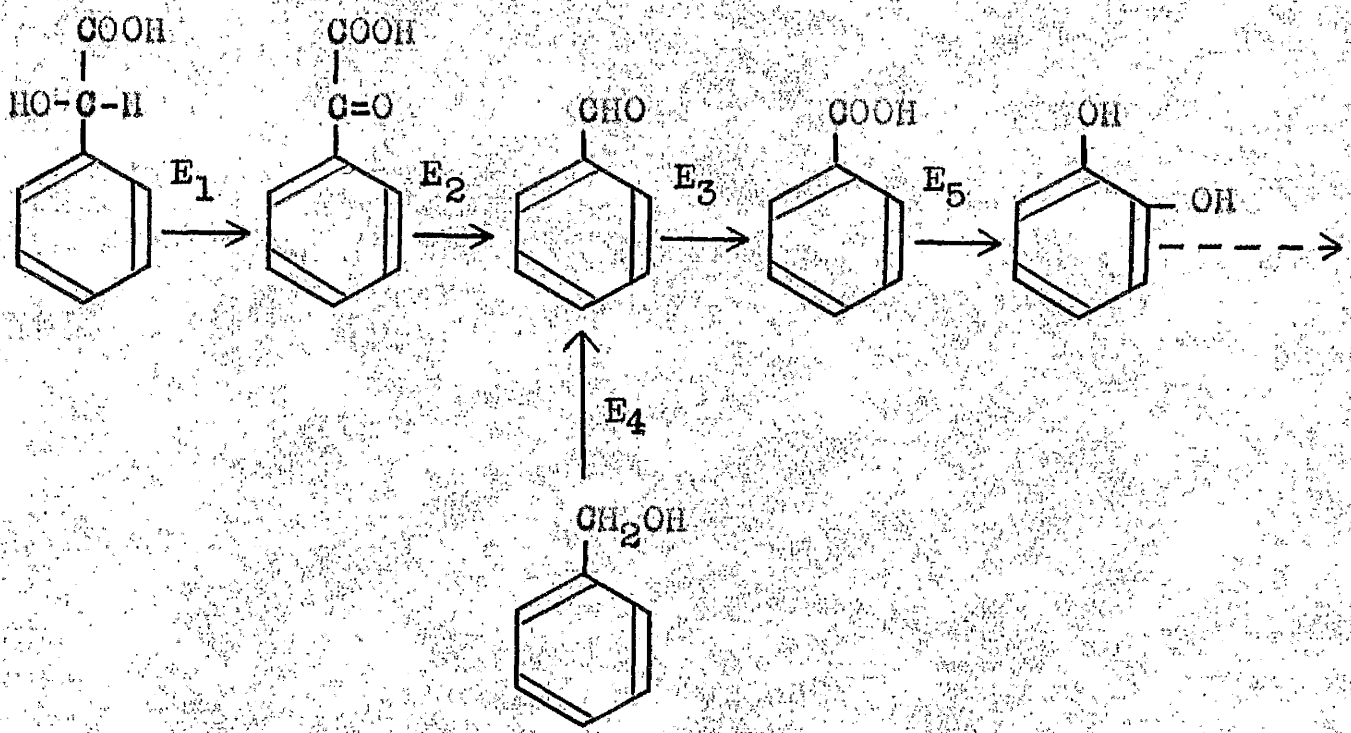
3. The final oxygen uptake recorded with any D,L-mandelate when incubated with washed cell suspensions was only half that recorded with an equimolar amount of a substrate which required the same number of oxygen atoms as the D,L-mandelates for complete oxidation (e.g. the benzyl alcohols).

It should be borne in mind when interpreting the results (e.g. Fig. 4a) that oxygen uptakes of 1 µatom O_2/µmole of D,L-mandelate are equivalent to uptakes of 1 µmole O_2/µmole of L-mandelate.

Stanier et al (1966) in their authoritative study of the pseudomonads found just a few strains which could grow on L-mandelate but not on D-mandelate.

Pathways of oxidation of the non-substituted compounds.

Washed cells of bacterium NCIB 8250 which had been grown on D,L-mandelate immediately and completely oxidised benzoylformate, benzyl alcohol, benzaldehyde, benzoate (Table 3) and catechol. These compounds, therefore, satisfied the criteria set down by the technique of simultaneous adaptation (p. 9). The results were the same as those obtained with P. putida (Stanier, 1947). The pathway of oxidation of L-mandelate is therefore probably:



E_1-L-Mandelate dehydrogenase

E_2-Benzoylformate decarboxylase

E_3-Benzaldehyde dehydrogenase

E_4-Benzyl alcohol dehydrogenase

E_5-Benzoate oxidase

Bacterium NCIB 8250 presumably cannot form the mandelate racemase present in P. putida (Stanier et al, 1953) since, as was suggested earlier, only the L-isomer of D,L-mandelate was oxidised. With this exception the pathway of mandelate oxidation in bacterium NCIB 8250 is identical to the pathway in P. putida.

The conclusion that L-mandelate and benzoylformate precede both benzaldehyde and benzyl alcohol is based on several lines of reasoning

1. A consideration of the chemical structures of L-mandelate and benzoylformate makes the postulated steps probable.

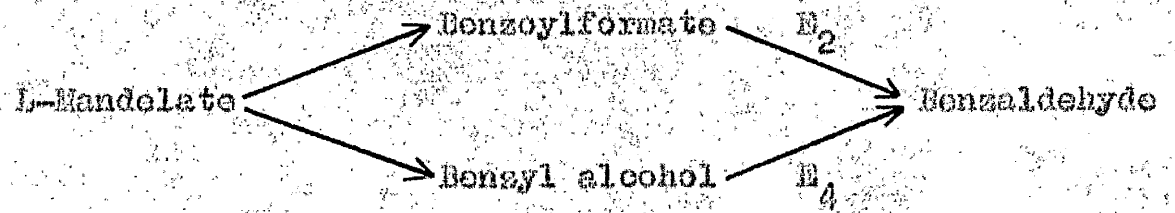
2. The required enzymes could indeed be detected.

3. Cells grown on benzyl alcohol or benzaldehyde oxidised D,L-mandelate and benzoylformate only after a lag.

4. Cell-free extracts from benzyl alcohol grown cells had no L-mandelate dehydrogenase activity.

5. There was a distinct odour of benzaldehyde when cells were growing on D,L-mandelate.

The decision to put benzyl alcohol on a separate arm of the sequence and not to include it in the main reaction chain rests largely on the observation that the initial rate of oxidation of benzyl alcohol by cells which had been grown on D,L-mandelate was much lower (25%) than the rate of oxidation of D,L-mandelate (Table 4). The possibility that L-mandelate is oxidised by two pathways operating simultaneously via benzoylformate and benzyl alcohol, cannot be excluded but has apparently never been considered by Stanier. Thus:

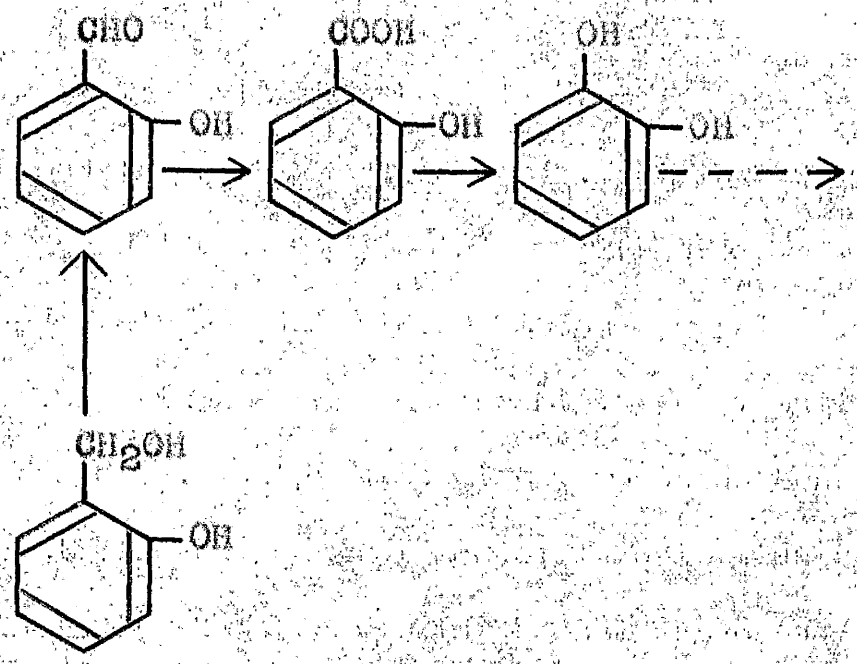


The lower initial rate of oxidation of benzyl alcohol would in this case be merely an indication of the smaller percentage of L-mandelate which was being oxidised through it. The validity of this hypothesis is open to experimentation. If specific inhibition of E_2 or E_4 were to block the oxidation of L-mandelate then it could be concluded that L-mandelate is oxidised exclusively by the 'blocked' pathway. Mutants devoid of L-mandelate dehydrogenase or benzyl alcohol dehydrogenase would also be of interest in this respect.

There seems no reason to doubt that benzyl alcohol is oxidised via benzaldehyde and benzoate as shown on page 86. Various workers (Hegeman, 1966a; Claus and Walker, 1964) using species of Pseudomonas have suggested that benzyl alcohol is oxidised through a pathway not involving benzoate but the evidence has been scanty. In P. putida it seems to be argued (Hegeman, 1966a) that since growth on benzyl alcohol did not induce all the enzymes for mandelate oxidation and since these enzymes, including that converting benzaldehyde to benzoate, are coordinately induced, then benzyl alcohol cannot be oxidised by means of the benzaldehyde dehydrogenase. It would appear, however, as is considered later (p.120), that at least in bacterium NCIB 8250 these enzymes are in fact not all induced coordinately.

Pathways of oxidation of the 2-hydroxy-substituted compounds.

Washed cells of bacterium NCIB 8250 which had been grown on 2-hydroxybenzyl alcohol immediately and completely oxidised 2-hydroxybenzyl alcohol, 2-hydroxybenzaldehyde, 2-hydroxybenzoate (Table 3) and catechol. The pathway of oxidation of these compounds may be represented:

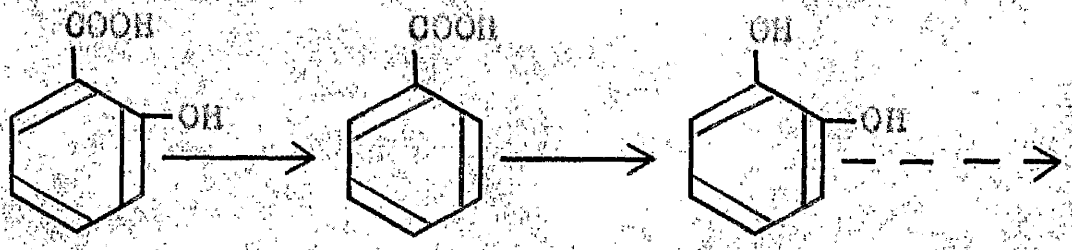


The pathway of oxidation of 2-hydroxybenzyl alcohol to 2-hydroxybenzoate is parallel to the pathway of oxidation of benzyl alcohol to benzoate.

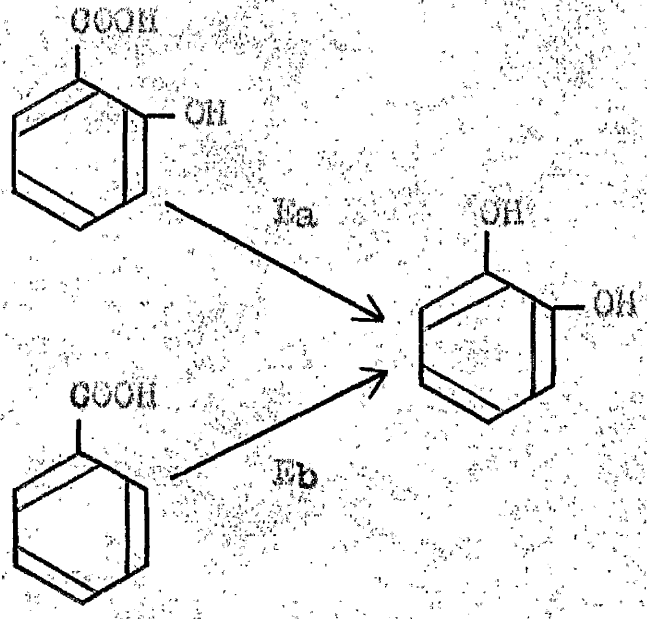
Cells grown on 2-hydroxybenzoate not only immediately oxidised catechol, as would be expected if the above pathway were correct, but also immediately oxidised benzoate. This observation contrasts with the finding that cells grown on benzoate oxidised 2-hydroxybenzoate only after a lag (Table 3). Bearing in mind the limitations

to the technique of simultaneous adaptation (p. 9), these results may be interpreted as follows:

1. That 2-hydroxybenzoate is converted to benzoate and thence to catechol.



2a. That 2-hydroxybenzoate or some intermediate between 2-hydroxybenzoate and catechol acts as an inducer for the benzoate oxidising system:

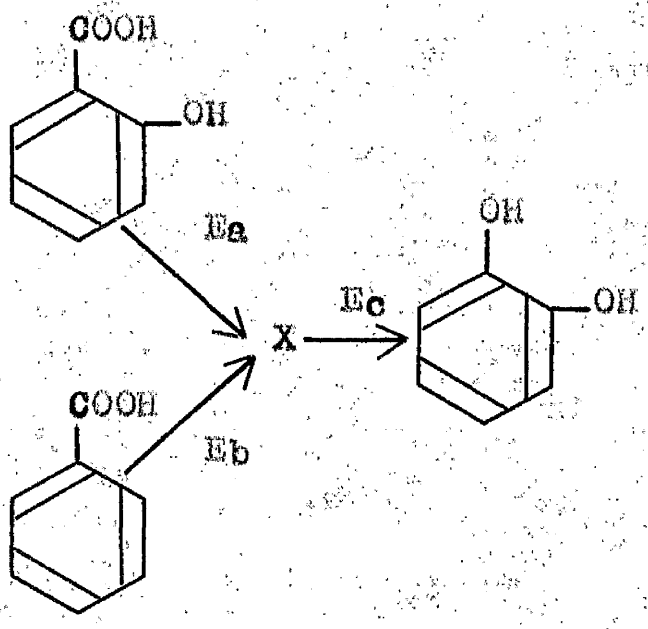


Ea induced specifically by 2-hydroxybenzoate.

Eb induced by benzoate, 2-hydroxybenzoate, or some intermediate between 2-hydroxybenzoate and catechol.

2b. That 2-hydroxybenzoate or some intermediate between 2-hydroxybenzoate and the first intermediate common to the oxidation of

benzoate and 2-hydroxybenzoate (X) acts as inducer for the benzoate oxidising system:



Ea induced by 2-hydroxybenzoate.

Eb induced by benzoate, 2-hydroxybenzoate or some intermediate between 2-hydroxybenzoate and X.

Ec induced by either benzoate or 2-hydroxybenzoate.

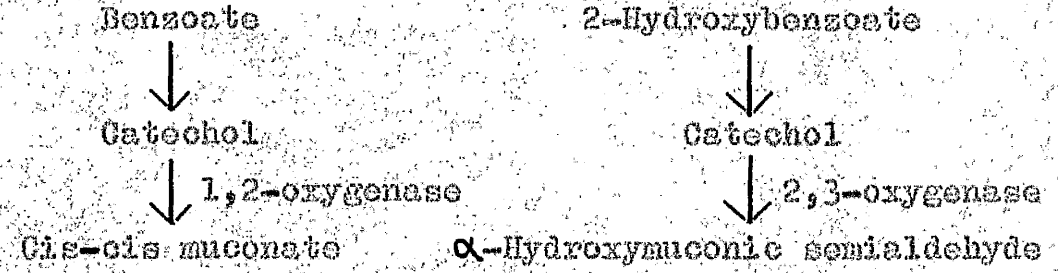
3. That benzoate is converted to X by means of constitutive enzymes; i.e. Eb in the above diagram is constitutive. Benzoate would then induce Ec, and 2-hydroxybenzoate would induce Ea and Ec.

4. That 2-hydroxybenzoate oxidase non-specifically oxidises benzoate.

Both the lability of the enzyme systems and the unknown nature of the intermediates between benzoate or 2-hydroxybenzoate and catechol (p. 6) would make it extremely difficult to establish which, if any, of the above hypotheses is correct. Benzoate has been excluded from the pathway of oxidation of 2-hydroxybenzoate since

the 2-hydroxybenzoate oxidase from a Pseudomonas species produced catechol directly from 2-hydroxybenzoate (Hayaishi, 1966). In a recent report it was suggested that Vibrio O1 (probably bacterium NCIB 8250) could adaptively synthesise either catechol 1,2-oxygenase (which splits catechol to cis-cis muconate; p. 2) or catechol 2,3-oxygenase (which splits catechol to α-hydroxymuconic semialdehyde; p. 2) depending on the nature of the inducer (Griffiths, Rodriques, Davies and Evans, 1964). These authors suggested that catechol 1,2-oxygenase was induced by benzoate, in agreement both with the present study and that of Ornston (1966), whilst catechol 2,3-oxygenase was induced by growth on naphthalene. 2-Hydroxybenzoate is an intermediate in the oxidation of naphthalene by certain soil microbes (Davies and Evans, 1964). It has been established in this thesis that bacterium NCIB 8250 grown on 2-hydroxybenzoate immediately utilised β-oxoadipate (p.48) which is an intermediate occurring exclusively in the pathway of oxidation of cis-cis muconate which in turn is the product of catechol cleavage by the 1,2-oxygenase (Ribbons, 1965). Bacterium NCIB 8250 grown on 2-hydroxybenzoate, therefore, almost certainly contains the 1,2-oxygenase and may also, according to Griffiths et al (1964), contain the 2,3-oxygenase. Very preliminary experiments to detect the 2,3-oxygenase by the spot test of Pankhurst (1965) were not, however, successful. If 2-hydroxybenzoate can indeed induce both catechol oxygenases it might also induce not only the 2-hydroxybenzoate ⟶ catechol system but also the benzoate ⟶ catechol system. Further work is required to

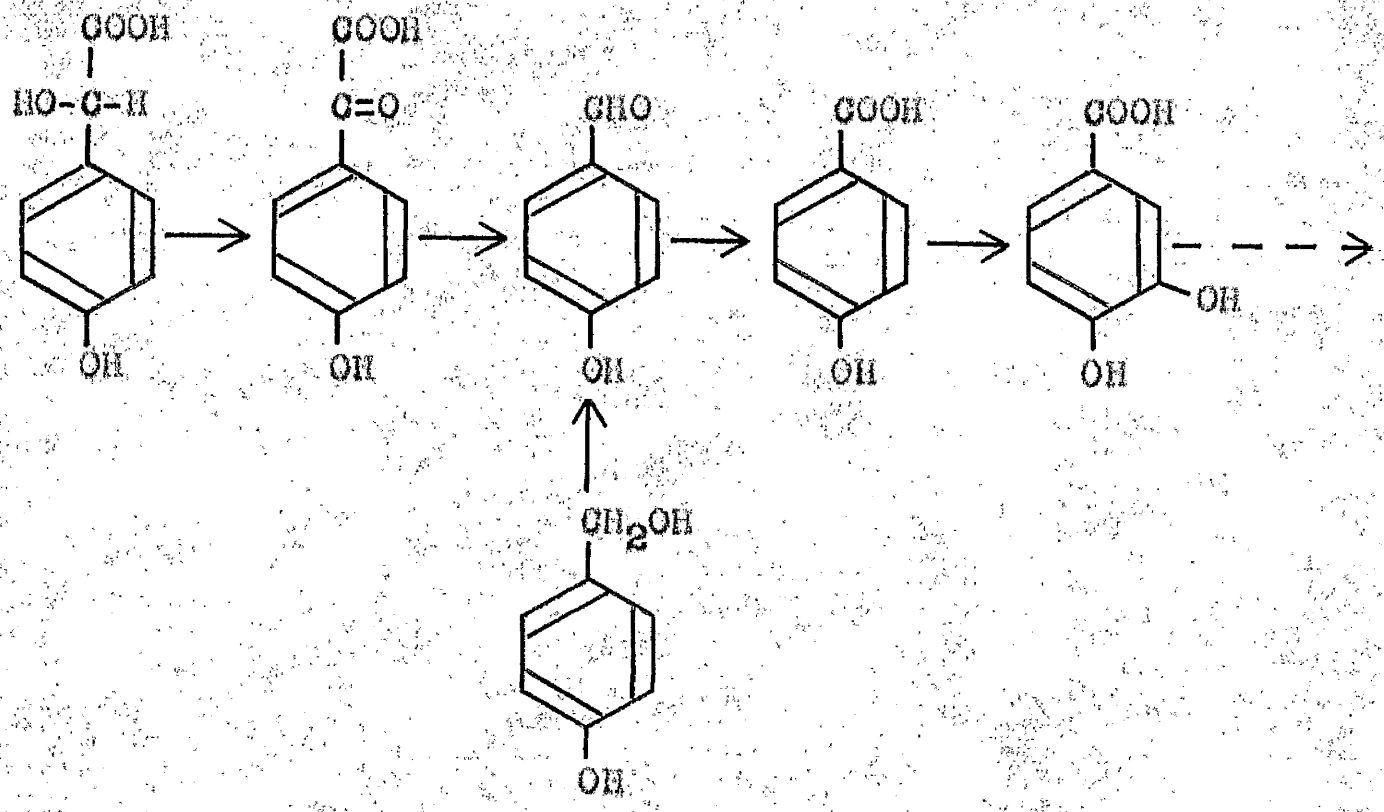
substantiate this hypothesis, but it is intriguing to speculate that if this is indeed so, then catechol derived from benzoate or from 2-hydroxybenzoate would be oxidised in different ways:



In some ways the position in bacterium NCIB 8250 is reminiscent of the inverse situation in some strains of Azotobacter in which growth on benzoate induced enzymes for the metabolism of both benzoate and 2-hydroxybenzoate (Voets, 1958).

Pathway of oxidation of the 4-hydroxy-substituted compounds.

4-Hydroxy-D,L-mandelate, 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, 4-hydroxybenzoate and 3,4-dihydroxybenzoate were all immediately and completely oxidised by washed cells of bacterium NCIB 8250 which had been grown on 4-hydroxy-D,L-mandelate (Fig. 3; Table 3). These data are consistent with the pathway:

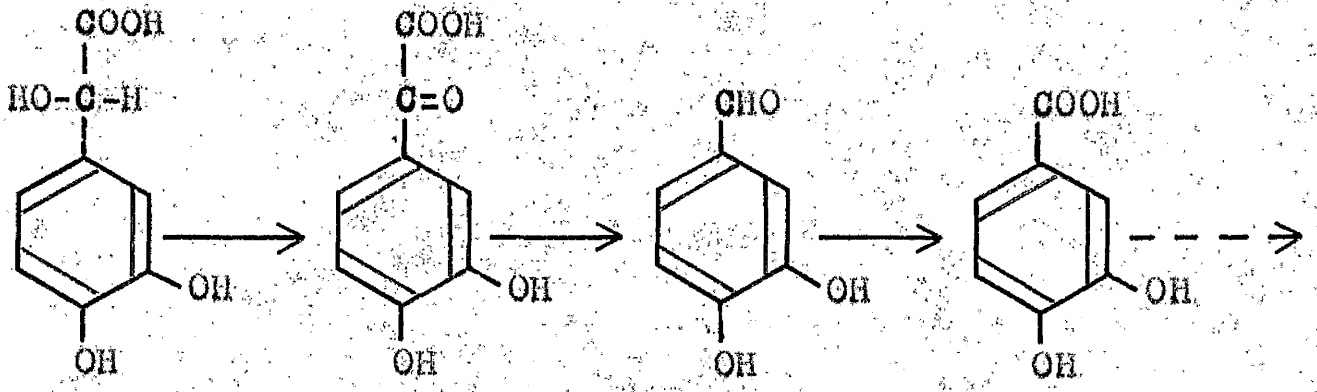


The sequence from 4-hydroxy-L-mandelate to 3,4-dihydroxybenzoate is identical to that described by Gunter (1953) in P. putida. 4-Hydroxybenzoylformate is included by analogy with the pathway of oxidation of the non-substituted compounds. The sequence of reactions from 4-hydroxy-L-mandelate and 4-hydroxybenzyl alcohol is parallel to that converting L-mandelate and benzyl alcohol to benzoate. It is only at the level of the benzoates that the pathways differ. Benzoate is oxidised to catechol whilst 4-hydroxybenzoate is probably converted to 3,4-dihydroxybenzoate. These two compounds are cleaved independently of one another: catechol yielding cis-cis muconate whilst 3,4-dihydroxybenzoate produces β-carboxy,cis-cis muconate (Scheme 2). The significant difference, therefore, in the pathways of

oxidation of L-mandelate and 4-hydroxy-L-mandelate occurs at the benzoate level; the reaction sequence from L-mandelate to benzoate being identical to that converting 4-hydroxy-L-mandelate to 4-hydroxybenzoate.

Pathway of oxidation of the 3,4-dihydroxy-substituted compounds.

Cells grown on 3,4-dihydroxy-D,L-mandelate gave an immediate and complete utilisation of oxygen when incubated with 3,4-dihydroxy-D,L-mandelate, 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoate (Table 3). The pathway of oxidation of 3,4-dihydroxy-L-mandelate is probably:

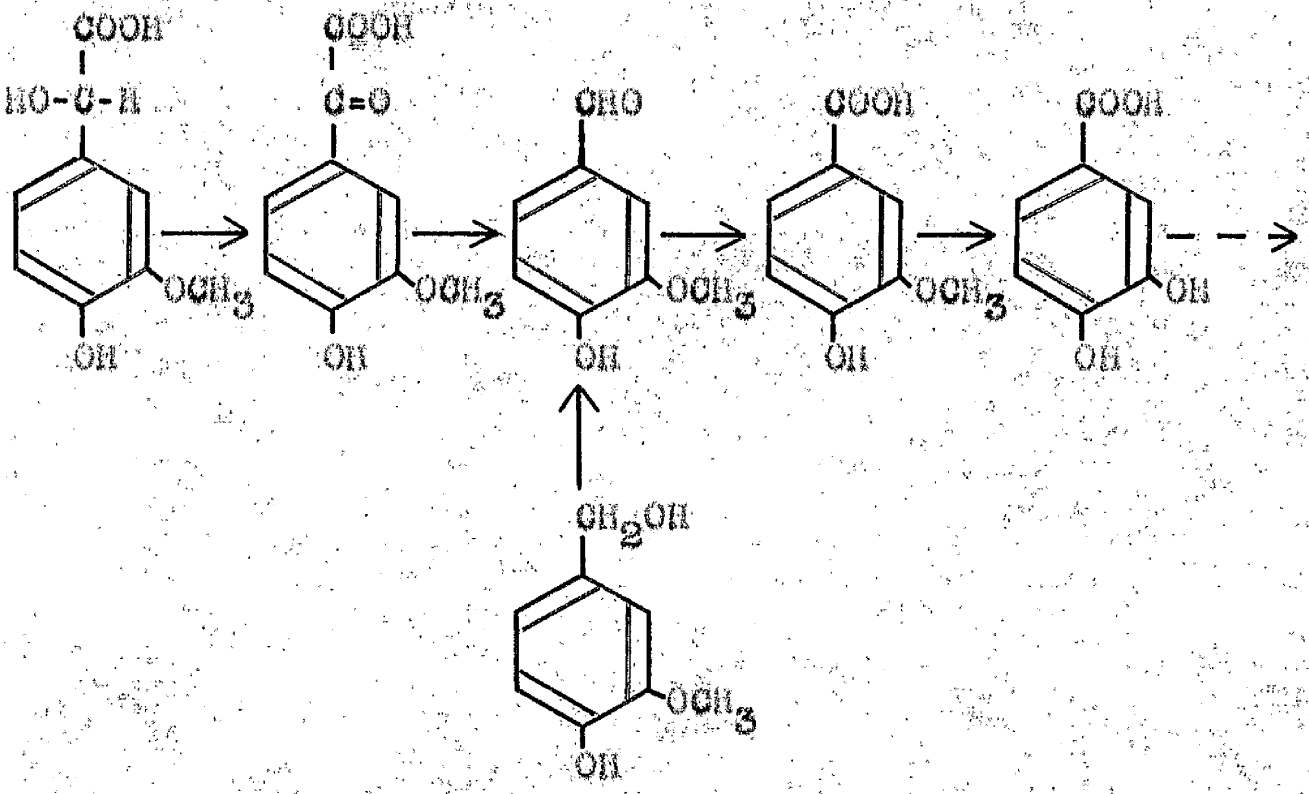


3,4-Dihydroxy-L-mandelate is converted to 3,4-dihydroxybenzoate which is cleaved as previously described. 3,4-Dihydroxybenzoyl-formate is included by analogy with the pathway of oxidation of the non-substituted compounds and again the sequence of reactions from 3,4-dihydroxy-L-mandelate to 3,4-dihydroxybenzoate is parallel to the sequence from L-mandelate to benzoate. 3,4-Dihydroxybenzyl

alcohol is not included in this pathway because this compound could not be obtained.

Pathway of oxidation of the 4-hydroxy-3-methoxy-substituted compounds.

Washed cells of bacterium NCIB 8250 which had been grown on 4-hydroxy-3-methoxy-D,L-mandelate gave an immediate and complete oxygen utilisation when incubated with 4-hydroxy-3-methoxy-D,L-mandelate, 4-hydroxy-3-methoxybenzyl alcohol, 4-hydroxy-3-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzoate and 3,4-dihydroxybenzoate (Table 3). The pathway is probably:



4-Hydroxy-3-methoxy-L-mandelate is converted to 4-hydroxy-3-methoxybenzoate which is demethylated to 3,4-dihydroxybenzoate and this in

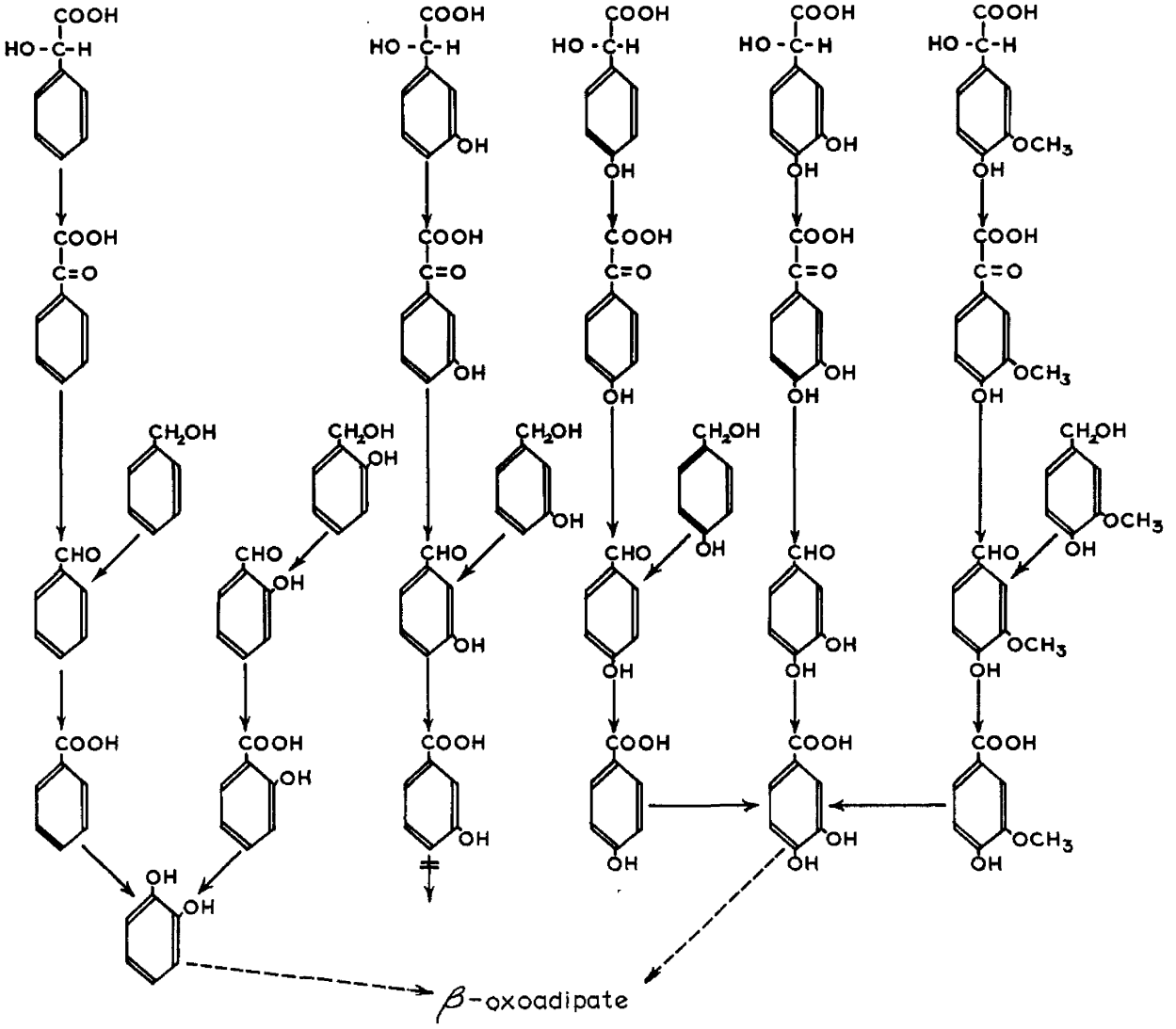
turn undergoes ring cleavage as previously described. 4-Hydroxy-3-methoxybenzoylformate is included by analogy with the pathway of oxidation of L-mandelate and indeed the sequence of reactions from 4-hydroxy-3-methoxy-L-mandelate to 4-hydroxy-3-methoxybenzoate is parallel to the sequence form L-mandelate to benzoate.

Parallelism in the pathways of oxidation of mandelic acid and related compounds.

The reaction sequences described above for each L-mandelate or benzyl alcohol are identical to the level of benzoate. Each L-mandelate is oxidised to the corresponding benzoylformate. Each benzoylformate is decarboxylated to the corresponding benzaldehyde, each benzyl alcohol is oxidised to the corresponding benzaldehyde and each benzaldehyde is oxidised to the corresponding benzoate. All the reactions involve manipulations to the side chain of the aromatic ring and not the aromatic ring or its substituents per se.

It is only at the level of benzoate that the catabolic reactions are centred on the aromatic ring. Thus benzoate and 2-hydroxybenzoate are oxidised to catechol, while 4-hydroxybenzoate and 4-hydroxy-3-methoxybenzoate are converted to 3,4-dihydroxybenzoate. Both catechol and 3,4-dihydroxybenzoate give rise to β-oxoadipate.

Bacterium NCIB 8250, therefore, probably catabolises mandelate and related compounds by the converging metabolic sequence outlined in Scheme 3.



Scheme 3.

Interpretation of the Patterns of Oxygen Utilisation.

Whilst the pathways of oxidations postulated above are consistent with the patterns of oxygen utilisation obtained, several cell preparations displayed a 'double simultaneous adaptation'. Cells grown on 4-hydroxy-D,L-mandelate gave an immediate and complete oxygen uptake when incubated not only with the 4-hydroxy-substituted compounds but also with the 3,4-dihydroxy-substituted compounds (Table 3). Inasmuch as the 3,4-dihydroxy-substituted compounds fulfil the criteria set down by the technique of simultaneous adaptation, it might be postulated that, for example, 4-hydroxy-L-mandelate, instead of being oxidised via the pathway of oxidation of the 4-hydroxy-substituted compounds might be hydroxylated to 3,4-dihydroxy-L-mandelate and metabolised via the pathway of oxidation of the 3,4-dihydroxy-substituted compounds. On the basis of the present study this possibility cannot be excluded, nor indeed can the conversion of 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde to their 3,4-dihydroxy-substituted derivatives. If this interpretation were correct a series of hydroxylases, or a non-specific hydroxylase, would have to be induced by growth on the 4-hydroxy-substituted compounds which would convert each of the 4-hydroxy-substituted compounds to its respective 3,4-dihydroxy-substituted derivative. A similar situation obtains in the oxidation of the 4-hydroxy-3-methoxy-substituted compounds which can also

immediately and completely oxidise the 3,4-dihydroxy-substituted compounds (Table 3). In this case a series of demethylases, or a non-specific demethylase, would be required. In an exactly analagous manner growth on the 2-hydroxy-substituted compounds would require the synthesis of a series of dehydroxylases or a non-specific dehydroxylase (Table 3).

A much simpler explanation of these observations of 'double simultaneous adaptation' is that the enzymes converting the L-mandelates or benzyl alcohols to benzoates are non-specific and only at the benzoate level are specific enzymes required or induced. If this hypothesis is correct, as evidence to be discussed later strongly suggests, then 'double simultaneous adaptation' of one series of compounds for another can be explained by considering, for example, the 'double simultaneous adaptation' of cells grown on 4-hydroxy-D,L-mandelate towards the 4-hydroxy and 3,4-dihydroxy-substituted compounds. Exposure of an unadapted culture of bacterium NCIB 8250 to 4-hydroxy-D,L-mandelate induced the formation of the non-specific enzymes required to convert 4-hydroxy-L-mandelate to 4-hydroxy-benzoate, the specific hydroxylase which converts 4-hydroxybenzoate to 3,4-dihydroxybenzoate and the ring cleavage enzymes required to degrade 3,4-dihydroxybenzoate through β-carboxy,cis-cis muconate to succinate and acetyl-CoA. When these cells were harvested, washed and challenged with 4-hydroxy-D,L-mandelate there was an immediate and complete oxygen utilisation (Fig. 3) signifying that no enzymes had

been lost during the preparation of the cell suspension. When challenged with the 3,4-dihydroxy-substituted compounds (Table 3) these cells showed an immediate and complete oxygen utilisation. This immediate complete utilisation was due to, firstly the conversion of the 3,4-dihydroxy-substituted compounds to 3,4-dihydroxybenzoate by the non-specific enzymes, and secondly the degradation of 3,4-dihydroxybenzoate by enzymes already present in the 4-hydroxy-D,L-mandelate grown cells. These cells, challenged with the non-substituted, the 2-hydroxy or 4-hydroxy-3-methoxy-substituted compounds converted them to benzoate, 2-hydroxybenzoate and 4-hydroxy-3-methoxybenzoate respectively. The enzymes required to degrade these compounds were not present in cells grown on 4-hydroxy-D,L-mandelate and before they could be metabolised the necessary enzymes had to be synthesised. Thus, in general, cells grown on any D,L-mandelate will oxidise all L-mandelates to their respective benzoates but will immediately and completely oxidise only those benzoates whose degradative enzymes have been induced by the growth substrate.

In the light of the pathways postulated above it is possible to speculate on the reasons for the various patterns of oxygen utilisation observed both in the block experiments summarised in Table 3, and on incubation of a number of challenge substrates which do not support growth (p.57).

Immediate complete utilisation (Pattern A).

Challenge substrates which gave this type of oxygen utilisation pattern fulfil the criteria set down by the technique of simultaneous adaptation and the majority of these have already been discussed in the establishment of the pathways of catabolism of the various aromatic compounds.

Immediate limited utilisation (Pattern B).

Cells grown on benzoylformate or any D,L-mandelate gave an immediate limited utilisation of oxygen when incubated with 3-hydroxy-D,L-mandelate, 3-hydroxybenzyl alcohol or 3-hydroxybenzaldehyde (Table 3). The oxygen uptakes recorded with these compounds corresponded to oxidation to 3-hydroxybenzoate (p.55), which was neither oxidised by bacterium NCIB 8250 even after prolonged incubation, nor utilised for growth (Table 1). Thus cells which had been grown, for example, on D,L-mandelate not only oxidised L-mandelate or benzyl alcohol to benzoate as previously described, but also appeared to oxidise 3-hydroxy-L-mandelate and 3-hydroxybenzyl alcohol to 3-hydroxybenzoate. This explanation would be strengthened by the quantitative recovery of 3-hydroxybenzoate. The simplest explanation of this finding is that one set of enzymes mediates the conversion of both sets of substrates. In other words

growth of bacterium NCIB 8250 on D,L-mandelate induces the formation of a series of non-specific enzymes which can convert not only L-mandelate or benzyl alcohol to benzoate but also 3-hydroxy-L-mandelate or 3-hydroxybenzyl alcohol to 3-hydroxybenzoate. Experiments using a range of compounds which, like the 3-hydroxy-substituted compounds, do not give growth supported this contention. Cells grown on D,L-mandelate oxidised almost all of the substituted L-mandelates, benzyl alcohols and benzaldehydes with the uptake of the quantity of oxygen required to produce the respective benzoates (p.60). Corresponding results were obtained with cells grown on 2-hydroxybenzyl alcohol, 3,4-dihydroxybenzaldehyde or 4-hydroxy-3-methoxybenzyl alcohol (p.61). Thus the enzymes which convert L-mandelate, benzyl alcohol or benzaldehyde to benzoate can also convert a host of substituted L-mandelates, benzyl alcohols or benzaldehydes to their respective benzoates.

Lag then complete utilisation (Pattern C).

The most reasonable interpretation of the lag recorded with challenge substrates which gave this type of oxygen uptake pattern is that it represented the time taken for the washed cells to synthesise the enzymes required for the catabolism of the challenge substrate. Evidence to support this suggestion comes from the effect of chloramphenicol on the lag observed in the oxidation of

4-hydroxybenzoate by cells grown on D,L-mandelate (Fig. 7). The presence of chloramphenicol, which inhibits protein synthesis (Collins, 1965), whilst not affecting the oxidation pattern of challenge substrates which were immediately and completely oxidised e.g. benzoate, changed the oxygen uptake pattern observed with 4-hydroxybenzoate from 'lag then complete utilisation' to 'no utilisation above the control level'. Thus chloramphenicol, by its capacity to block protein synthesis, inhibited the synthesis of the enzymes required to oxidatively catabolise 4-hydroxybenzoate and the oxygen uptake with this compound never rose above the endogenous level.

Pardee and Prestidge (1961) studied the initial kinetics of β-galactosidase induction and established that in E.coli there was an induction lag of 3 minutes between the addition of inducer and appearance of enzyme. These experiments were performed using cultures growing on glycerol as source of carbon and energy at the time of addition of inducer. These conditions contrast with those employed in the present study where washed cells were used and where the challenge substrate acted not only as inducer but also as sole source of carbon and energy. The relationship between these functions of the challenge substrate makes any firm conclusions regarding the mechanism or kinetics of induction impossible but probably explains the autocatalytic nature of the curves of oxygen uptake. Thus as enzyme was synthesised so some of the challenge

substrate would be catabolised to give rise to raw materials which in turn would allow more enzyme formation.

Immediate limited utilisation-lag-further utilisation (Pattern D).

This diphasic oxygen uptake pattern can be considered in two parts. Firstly, an immediate limited utilisation analagous to pattern B and secondly, a lag then complete utilisation analagous to pattern C. The stoichiometry of the first phase corresponded to oxidation of the challenge substrate to the benzoate level. Thus cells grown on D,L-mandelate immediately oxidised 4-hydroxy-L-mandelate to 4-hydroxybenzoate. As suggested above, growth on D,L-mandelate appears to induce the formation of a series of non-specific enzymes which not only converts L-mandelate or benzyl alcohol to benzoate but also 3-hydroxy-L-mandelate or 3-hydroxybenzyl alcohol to 3-hydroxybenzoate. These same enzymes might also act on 4-hydroxy-L-mandelate and 4-hydroxybenzyl alcohol converting them to 4-hydroxybenzoate. The intermediate lag, or plateau, in the oxidation of 4-hydroxy-L-mandelate therefore, is presumably a measure of the time taken to synthesise the enzymes required to degrade 4-hydroxybenzoate. This interpretation is supported by the observation that chloramphenicol which has been shown to inhibit the synthesis of the enzymes required by D,L-mandelate grown cells to degrade 4-hydroxybenzoate, changed the oxygen uptake pattern obtained

with 4-hydroxy-D,L-mandelate from 'immediate limited utilisation-lag-further utilisation' to 'immediate limited utilisation' (Fig. 6). Thus chloramphenicol stopped the oxidation of 4-hydroxy-L-mandelate at 4-hydroxybenzoate by inhibiting the synthesis of the enzymes required to degrade 4-hydroxybenzoate. Furthermore, every challenge substrate which gave a diphasic oxygen uptake pattern was also found to support the growth of bacterium NCIB 8250 which suggests that the second phase of the diphasic pattern represented an adaptation towards the 'growth supporting' benzoate. Conversely, all the compounds whose oxidation stopped at the benzoate level do not support growth.

Although the effect of chloramphenicol strongly suggests that the lags observed both in pattern C and pattern D are measures of the time taken to synthesise the necessary degradative enzymes, the possibility that these lags are due to a permeability barrier must also be examined. Consider cells grown on benzyl alcohol. These cells oxidised 4-hydroxybenzoate only after a lag, whilst they gave an immediate limited utilisation-lag-further utilisation when incubated with 4-hydroxybenzyl alcohol or 4-hydroxybenzaldehyde (Table 3). The oxygen uptakes corresponded to oxidation of these compounds to 4-hydroxybenzoate. The enzymes responsible for these oxidations are presumably intracellular. Thus even though 4-hydroxybenzoate was formed within the cell this compound was only further metabolised after a lag. Therefore the inability of bacterium NCIB

8250 to oxidise exogenous 4-hydroxybenzoate immediately was not due to the impermeability of the cell wall to 4-hydroxybenzoate since even when this compound was formed within the cell it was not immediately oxidised. Incidentally the similarity between the specificity results obtained with washed cell suspensions and with cell-free extracts suggest that any permeability barriers, if they do exist, can be no more specific than the enzymes.

No utilisation of oxygen above the control level (Pattern E).

Challenge substrates which were not oxidised above the control level even after prolonged incubation included 3-hydroxybenzoate (Table 3) and a range of substituted benzoates which do not support growth (p.57). These compounds might not be oxidised because of their inability to permeate the cell wall. By an analagous argument to that used above the inability of bacterium NCIB 8250 to oxidise exogenous 3-hydroxybenzoate was not due to a permeability barrier to this compound, since 3-hydroxybenzoate formed within the cell was not oxidised. Analagous conclusions can be reached regarding the oxidation of the majority of the other substituted benzoates which gave no oxygen uptake above the endogenous level.

The Specificity of the Enzymes Oxidising Mandelic Acid and Related Compounds.

The interpretations presented above focussed attention on the ability of whole cells of bacterium NCIB 8250 to oxidise a range of L-mandelates, benzyl alcohols and benzaldehydes to the level of the corresponding benzoates. The activities of the enzymes towards compounds such as the 3-hydroxy derivatives, which do not support growth, extended the whole-cell studies and illustrated the specificity of the enzymes towards compounds having both large and multiple ring substituents. By examining the enzymes in cell-free extracts further information was obtained on the broad specificity spectra of these enzymes. The results of the cell-free studies demonstrated that the kinetic parameters of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase were independent of the substrate on which bacterium NCIB 8250 had been grown. Thus the Km and Vmax for the L-mandelate dehydrogenase (Tables 9 and 10) towards each of the 'growth' mandelates were the same irrespective of the D,L-mandelate on which bacterium NCIB 8250 had been grown. Identical results were obtained for the benzyl alcohol and benzaldehyde dehydrogenase. This is good presumptive evidence that the same enzymes were induced in all cases but final proof must await further studies such as the purification of the enzymes and the preparation of specific antisera. Furthermore

if mutants devoid of activity towards mandelate, for instance, were also devoid of activity towards other mandelates (and vice-versa) this would also be strong evidence that the same enzymes were involved.

It must be considered, however, that growth on any D,L-mandelate might induce the formation, not of one non-specific L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase, but rather of a series of specific enzymes which act on each substrate. This rather unlikely possibility seems to be ruled out by the experiments in which combinations of substrates were presented simultaneously (Tables 14, 15 and 16). The rates of oxidation recorded were never equal to the sums of the individual rates obtained when the substrates were added separately. There is the further possibility that each member of the pairs of substrates inhibited the specific enzyme of its partner. While this possibility cannot be excluded until the enzymes have been extensively purified, the results obtained make this extremely improbable.

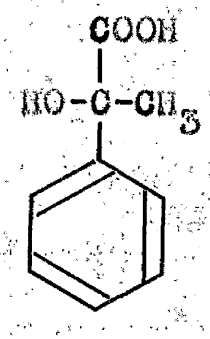
Studies on the kinetic properties of the L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase demonstrated that the concentrations of both the growth substrates employed in the determination of the mean generation times (Table 1), and the challenge substrates present in the Warburg flasks were sufficiently high to saturate the appropriate enzymes. The only exception was 4-hydroxy-3-methoxy-D,L-mandelate

which, as a substrate for the L-mandelate dehydrogenase, has a Km of approximately 2mM (Table 9). It is interesting that this compound gave an extremely slow growth rate. Furthermore the various initial rates of oxygen uptake recorded in the Warburg experiments reflected the values of Vmax obtained with the challenge substrates when assayed in cell-free extracts. Thus an initial rate of 31 μmoles O_2 taken up/hr./mg. N obtained with 4-hydroxy-3-methoxybenzyl alcohol compared with 82 μmoles O_2 taken up/hr./mg. N obtained with benzyl alcohol when these substrates were incubated with 4-hydroxy-D,L-mandelate grown cells (Table 3) reflected the observation that the Vmax for benzyl alcohol is about 2.5 times that for 4-hydroxy-3-methoxybenzyl alcohol (Table 12). This might be the reason for the slow growth obtained with 4-hydroxy-3-methoxybenzyl alcohol compared with benzyl alcohol itself (Table 1).

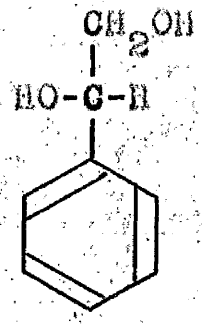
On the assumption that one set of enzymes mediates the conversion of all L-mandelates, benzyl alcohols and benzaldehydes to their respective benzoates, the whole-cell and cell-free studies provide information on the structural requirements which these compounds must meet in order to act as substrates for their respective enzyme.

L-Mandelate dehydrogenase.

Of all the miscellaneous compounds tested (p.60) none of the compounds related to mandelate, but with modified side-chains, was oxidised. The washed cell preparations did not act even on such closely related compounds as atrolactic acid (XIII), where a methyl group replaces the α-hydrogen, or styrene glycol (XIV), where a hydroxymethyl group replaces the carboxyl group:



XIII



XIV

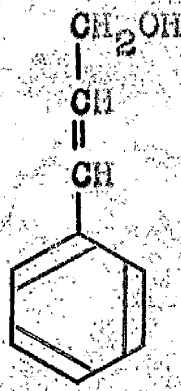
In order for a compound to act as a substrate for the L-mandelate dehydrogenase, that compound must possess the —CH(OH).COOH configuration attached to an aromatic ring. All compounds tested which had this configuration, irrespective of the nature or size of the ring substituents were substrates, but nevertheless were not all equally good substrates. The non-substituted D,L-mandelate was the best substrate as judged by the low Km (Table 9). There was little difference in Vmax between the non-substituted, the 4-hydroxy and 3-hydroxy-substituted compounds (Table 10), whilst 4-hydroxy-3-

methoxy-D,L-mandelate was the poorest substrate giving both the lowest Vmax and highest Km.

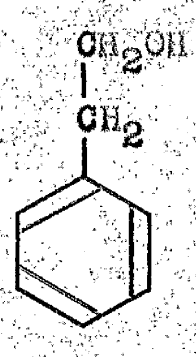
L-Mandelate dehydrogenase was assayed with 2,6-dichlorophenol-indophenol; no experiments were performed to identify the natural cofactor for the enzyme.

Benzyl alcohol dehydrogenase.

The benzyl alcohol dehydrogenase appears to be less stringent in its requirement for substrate structure than the L-mandelate dehydrogenase since cinnamyl alcohol (XV) was oxidised by cells displaying benzyl alcohol dehydrogenase activity. It seems rather surprising that 2-phenylethanol (XVI) showed no activity.



XV



XVI

Of all the benzyl alcohols which supported growth (Table 1) the non-substituted compound was the best substrate as reflected in its having both the lowest Km and highest Vmax. The 4-hydroxy-3-methoxy-

benzyl alcohol was the poorest substrate (Tables 11 and 12).

Benzaldehyde dehydrogenase.

Benzaldehydes with a large group (e.g. nitro, methoxy or carboxy) at the 2 position of the aromatic ring were not oxidised at a detectable rate by cells displaying benzaldehyde dehydrogenase activity. In the conversion of benzaldehyde to benzoate these large substituents would be vicinal to the group undergoing hydration and dehydrogenation and it is not surprising, therefore, that those large substituents interfered with the enzymatic action. Unfortunately corresponding benzyl alcohols were not available to test the ability of the benzyl alcohol dehydrogenase towards compounds with large substituents in the 2 position of the aromatic ring. For reasons already presented (p.79) it was not possible to determine the Km for the benzaldehyde dehydrogenase with benzaldehyde as substrate and the relative velocities were recorded with each of the growth substrates present at 40μM (Table 13). On the basis of relative velocities, benzaldehyde was the best substrate for the benzaldehyde dehydrogenase. Significantly, in the light of the observations made above on the oxidation of substrates with large groups in the 2 position of the ring, 2-hydroxy-benzaldehyde gave a very low relative velocity while the other mono-hydroxylated derivatives gave much higher values.

Several workers (Gunsalus et al, 1953; Stevenson and Mandelstam, 1965; Hegeman, 1966a,b,c) have demonstrated the presence of two benzaldehyde dehydrogenases in extracts in P. putida. The first requires NAD^+ as cofactor, (E.C. No. 1.2.1.6), the second $NADP^+$, (E.C. No. 1.2.1.7). The purpose of the two enzymes has never been explained. Throughout the present work on bacterium NCIB 8250 no $NADP^+$ requiring benzaldehyde dehydrogenase has ever been detected. The enzyme in bacterium NCIB 8250, prepared from cells grown on 5 different compounds, showed an absolute requirement for NAD^+.

Enzymes metabolising the benzoates.

From the evidence presented on page 100 there seems little doubt that the specificity displayed by the enzymes which oxidised the various benzoates is much greater than that displayed by the enzymes converting the mandelates, benzyl alcohols and benzaldehydes to the benzoate level. Only benzoates which supported growth, and in certain cases the fluorodinated benzoates, were oxidised. One of the limitations of the Warburg technique is that by measuring only the oxygen uptake, certain types of reaction (e.g. hydration) would be impossible to detect. Any reaction, however, which produced a 'growth' benzoate would be detected by the further degradation of the benzoate. Bearing these limitations in mind information can be obtained on the specificity of the enzymes metabolising the benzoates.

(i) Benzoate and 2-hydroxybenzoate oxidase.

Cells grown on D,L-mandelate which possessed only the benzoate oxidase immediately oxidised the 2 and 4-fluoro-substituted benzoates (p.58). This presumably reflects the similarities in the van der Waal's radii of the hydrogen and fluorine atoms (Pauling, 1960) since other substitutions, e.g. chloro or bromo gave compounds which were inactive as substrates. Ali, Calloly and Hayes (1962) also showed that bacterium NCIB 8250 grown on benzoate could oxidise the mono-fluorobenzoates and detected free fluoride in the culture medium after the oxidation of 4-fluorobenzoate. Goldman, Milne and Pignataro (1967) have recently reported on the catabolism of 2-fluorobenzoate by a species of Pseudomonas which could grow on this compound. These authors demonstrated the formation of fluorocatechol and fluoromuconate. It is of interest in this context that Calloly and Jones (1965) have suggested that the monofluorobenzoates, compounds which do not support the growth of bacterium NCIB 8250, can, nevertheless, act as inducers of the enzymes required to oxidise benzoate. The evidence which these authors presented, however, was at best suggestive.

Cells grown on 2-hydroxybenzyl alcohol oxidised only 4-fluorobenzoate. This observation is difficult to explain since these cells were fully adapted to benzoate (Table 3) and should therefore presumably have been able to oxidise 2-fluorobenzoate. It would be particularly interesting to test 4-fluoro-2-hydroxybenzoate

and 6-fluoro-2-hydroxybenzoate as these compounds might provide further information on the specificity of the 2-hydroxybenzoate oxidising system.

(ii) 3,4-Dihydroxybenzoate oxygenase.

This enzyme appears to be extremely specific. None of the fluorodinated compounds was oxidised by cells which possessed this enzyme. Furthermore no other combinations of pairs of hydroxyl groups on the aromatic ring permitted oxidation (e.g. 2,3-dihydroxybenzoate, 3,5-dihydroxybenzoate). The trihydroxy derivative (3,4,5-trihydroxybenzoate) was not oxidised above the endogenous level.

(iii) 4-Hydroxybenzoate hydroxylase.

Cells grown on any of the 4-hydroxy-substituted compounds possessed this enzyme. The hydroxylase, like the 3,4-dihydroxybenzoate oxygenase appears to be extremely specific. The enzyme cannot hydroxylate 3-hydroxybenzoate since this compound never gave an oxygen uptake above the control level (Table 3). It is also of interest that there was no detectably activity with 2,4-dihydroxybenzoate which might conceivably have given rise to 2,3,4-trihydroxybenzoate. Hosokawa and Stanier (1966) have recently reported on the hydroxylation of 4-hydroxybenzoate to 3,4-dihydroxybenzoate by an enzyme from P. putida. These authors have crystallised a

flavoprotein containing 1 mole FAD/mole enzyme protein which requires molecular oxygen and NADPH specifically for activity. The enzyme from P. putida, like that from bacterium NCIB 8250, was highly specific for 4-hydroxybenzoate.

(iv) 4-Hydroxy-3-methoxybenzoate O-demethylase.

Cartwright and Smith (1967), in a paper published after the experimental work described in this thesis had been completed, described experiments on the demethylation of 4-hydroxy-3-methoxybenzoate by a Pseudomonas species isolated from coal tar, and suggested that the methyl group was removed sequentially as formaldehyde, formate and carbon dioxide. These authors implicated a reduced nucleotide, probably NADPH, and reduced gluthathione as cofactors in the demethylation. The organism used by Cartwright and Smith (1967) was sequentially adapted to 4-hydroxybenzoate by growth on 4-hydroxy-3-methoxybenzoate so that these workers could not rule out the possibility that 3,4-dihydroxybenzoate arose by demethoxylation followed by hydroxylation. No such ambiguity exists in bacterium NCIB 8250 where cells grown on 4-hydroxy-3-methoxybenzoate oxidised 4-hydroxybenzoate only after a lag.

Additional evidence was obtained in the present work on the specificity of the demethylase in bacterium NCIB 8250. Cells grown on 4-hydroxy-3-methoxybenzyl alcohol gave an immediate limited utilisation when incubated with 3-hydroxy-4-methoxybenzaldehyde. The

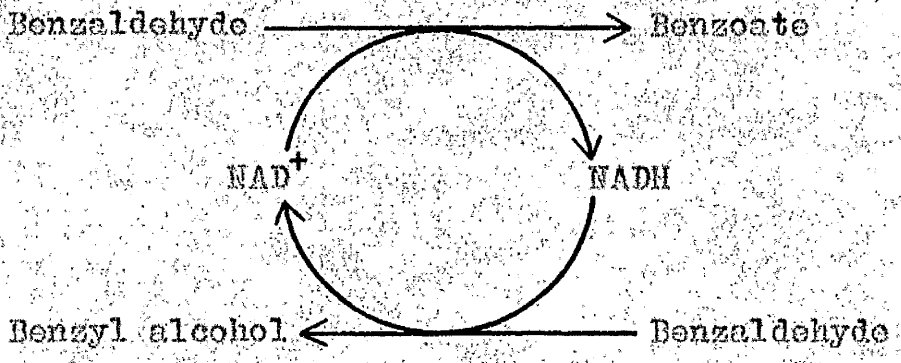
oxygen uptake corresponded to oxidation of this compound to 3-hydroxy-4-methoxybenzoate and no further. If the demethylase had acted upon this compound, 3,4-dihydroxybenzoate would have been formed which would have given a further oxygen uptake. In confirmation of this observation, 3,4-dimethoxybenzoate was not attacked; neither was 4-methoxybenzoate which might have given rise to 4-hydroxybenzoate which in turn would have been oxidised after a lag. The demethylase would seem, therefore, to be specific for the 4-hydroxy-3-methoxy configuration.

A Possible Dismutation of Benzaldehyde.

Throughout the early investigations into the oxidation of benzaldehyde by cell-free extracts, sigmoidal time-courses were invariably obtained (Fig. 13). Experiments to determine the reason for this type of time-course demonstrated that the sigmoidal pattern was uniquely associated with the presence of benzyl alcohol dehydrogenase activity. In other words extracts having benzyl alcohol dehydrogenase activity as well as benzaldehyde dehydrogenase activity invariably gave a sigmoidal time-course, whilst extracts having only benzaldehyde dehydrogenase activity or in which the benzyl alcohol dehydrogenase activity was inhibited gave a linear time-course (Tables 6 and 7; Fig. 14). The simplest and most attractive explanation of these findings is that in the presence of

benzyl alcohol dehydrogenase the reaction proceeded in two stages as follows:

A. Benzaldehyde underwent a dismutation to give benzoate and benzyl alcohol, thus:



Evidence to support this contention comes from the experiments in which benzaldehyde oxidation was followed both directly, by the disappearance of benzaldehyde as recorded at 282mμ, and indirectly, by the production of NADH as recorded at 340mμ (Fig. 15). Since the final E_{340} was equivalent to the amount of benzaldehyde added and since all the benzaldehyde had disappeared when only a fraction of the NADH had been produced, another reaction must have produced a transient product from benzaldehyde. Since a linear time-course was obtained in the absence of benzyl alcohol dehydrogenase it is not unreasonable to assume that the other reaction was the reduction of benzaldehyde to benzyl alcohol.

B. Only when all the benzaldehyde had disappeared did the E_{340} time-course display its upward inflection. This presumably represented the reversal of the benzaldehyde ⟶ benzyl alcohol reaction with the concomitant production of NADH both from the benzyl alcohol ⟶ benzaldehyde and the benzaldehyde ⟶ benzoate reactions.

This explanation would be strengthened by the detection of benzyl alcohol at the end of the linear portion of the E_{340} time-course. It was not the object of the present study, however, to examine the mechanism of benzaldehyde oxidation but rather to establish conditions whereby a linear time-course would be obtained, and for this reason no further experiments were performed. It is interesting that Hegeman (1966a) observed a sigmoidal time-course for the oxidation of L-mandelate by the particulate fraction of P. putida, but it seems hardly likely that a similar sort of reaction could explain his result.

Patterns of Enzyme Induction.

Although the experiments described in this thesis were not performed to investigate the mechanism of induction of the enzymes which oxidise mandelate and related compounds, certain suggestions can be made from the results obtained.

Growth of bacterium NCIB 8250 on benzoylformate induced the formation of the L-mandelate dehydrogenase (Table 3). There are at least three possibilities which would explain this result:

1. That the L-mandelate dehydrogenase and benzoylformate decarboxylase are coordinately induced by either L-mandelate or benzoylformate.

2. That benzoylformate itself acts as a non-specific inducer for the L-mandelate dehydrogenase.

3. That benzoylformate can be reduced either enzymically or non-enzymically to D,L-mandelate and the D,L-mandelate can act as inducer for the L-mandelate dehydrogenase.

The hypothesis of coordinate induction of the L-mandelate dehydrogenase and benzoylformate decarboxylase is of interest because of the conclusion reached by Hegeman (1966a,b,c) that in P. putida the L-mandelate dehydrogenase, benzoylformate decarboxylase and

benzaldehyde dehydrogenase are coordinately induced. It is noteworthy that in bacterium NCIB 8250, cells which had been grown on benzaldehyde could also oxidise benzyl alcohol (Table 3). Similar explanations to those presented above could, of course, explain these findings. Significantly, however, cells grown on benzyl alcohol or benzaldehyde displayed no activity towards L-mandelate or benzoylformate as might be expected if these enzymes were all coordinately induced.

It is interesting to speculate, therefore, that in bacterium NCIB 8250 there are two coordinately linked pairs of enzymes, (1) L-mandelate dehydrogenase and benzoylformate decarboxylase induced either by D,L-mandelate or benzoylformate, (2) benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase induced by either benzyl alcohol or benzaldehyde. The present study therefore, whilst not disproving the sequential nature of the mechanism of induction of the enzymes degrading L-mandelate and benzyl alcohol, suggests that groups of enzymes, rather than individual enzymes, are induced sequentially.

The situation in bacterium NCIB 8250 would at first sight appear to contrast with that in P. putida where the mandelate racemase, mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase have all been considered to be controlled by the same operon (Hegeman, 1966a,b,c; Stevenson and Mandelstam, 1965; Mandelstam and Jacoby, 1965). In fact, however, the studies

with P. putida have been far from complete and rest on physiological rather than genetic evidence. Thus benzaldehyde has apparently never been tested as an inducer, indeed the only experiments performed with this compound were some on repression by Stevenson and Mandelstam (1965) who used concentrations which at least in bacterium NCIB 8250 would be inhibitory. Hegeman (1966a) showed that benzyl alcohol was not an inducer for the mandelate dehydrogenase and benzoylformate decarboxylase but, as mentioned above (p. 88) benzyl alcohol has never been considered to be an integral part of the mandelate pathway by those workers (Hegeman, 1966a).

Metabolic Economy of Bacterium NCIB 8250.

Attention has been drawn in recent years to the ability of microorganisms to elaborate the synthesis of multi-enzyme sequences induced by, and active upon, a range of substrates. Gunsalus, Chapman and Kuo (1965), during a study of the degradation of camphor, examined the specificity of the inducible secondary alcohol dehydrogenase synthesised by a diphtheroid during growth on each of a number of substrates. The same enzyme was active not only on hydroxycamphor but also on a range of hydroxylated bicyclic monoterpenoids. Studies on this and other enzymes which participate in the catabolism of camphor, demonstrated that the same series of enzymes was induced by growth on a number of compounds and that

several of these enzymes displayed activity towards a range of substrates. In an analogous study Dalal, Bhattacharyya and Rangachari (1966) demonstrated that the perillyl alcohol dehydrogenase of a soil pseudomonad was both active on, and induced by, a range of related substrates. Gunter (1953) and Stevenson and Mandelstam (1965) have suggested that a similar situation exists in P. putida for the oxidation of D-mandelate and 4-hydroxy-D-mandelate.

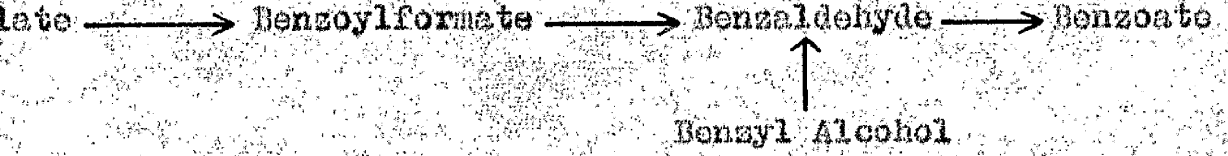
The observations recorded in this thesis have extended these findings both to a quite different organism and to a larger number of compounds. In bacterium NCIB 8250 there is a considerable degree of economy of protein synthesis since just four enzymes mediate the conversion of almost twenty compounds to five benzoates (Scheme 3). These key intermediates are subjected to a series of manipulations resulting in the convergent formation of catechol or 3,4-dihydroxybenzoate which then undergo ring cleavage followed by degradation of the aliphatic products. The possible ecological significance of partial degradations, such as the oxidation of 3-hydroxy-D,L-mandelate to 3-hydroxybenzoate, should not be overlooked. Not only might this type of transformation be capable of providing energy, even if not serving as a carbon source, but the product might be acted upon by other organisms. The situation in bacterium NCIB 8250 is also of interest since the apparent coordinacy of the L-mandelate dehydrogenase and benzoylformate decarboxylase, and the benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase would lead to

economy of genetic material without an undue amount of synthesis of non-functional protein. Thus if each enzyme is induced independently it must have its own regulator, operator and structural gene (Jacob and Monod, 1961), whereas if several enzymes are induced coordinately they require only the one regulator and operator gene. On the other hand if too many enzymes are controlled by one operon, in order for growth to occur on all the enzyme substrates then induction must be non-specific and, furthermore, if the organism is growing on the substrate of the last enzyme of the chain, all the preceding enzymes are wasted. In bacterium NCIB 8250 growth on any member of the mandelate family never leads to the formation of more than two gratuitous enzymes. This type of specificity of enzyme induction and activity may well have a selective advantage for the organism.

SUMMARY.

This thesis describes an investigation into the mechanism whereby the bacterium NCIB 8250 (which was formerly known as 'Vibrio O1') carries out the oxidation of mandelate and a number of related compounds.

Bacterium NCIB 8250 was found to utilise L-mandelate, benzoylformate, benzyl alcohol, benzaldehyde or benzoate as sole source of carbon and energy for growth. 2-Hydroxy, 4-hydroxy, 3,4-dihydroxy and 4-hydroxy-3-methoxy derivatives of these compounds can also be utilised. The pathways of oxidation of all these substances were determined, in the first instance, by the technique of simultaneous adaptation. Washed cell suspensions, prepared from bacteria which had been grown on each aromatic compound in turn, were challenged in the Warburg apparatus with a large number of possible intermediates and analogues. The resulting patterns of oxygen utilisation indicated that the side chain of each L-mandelate or benzyl alcohol is oxidised to give the corresponding benzoate:



The enzymes converting the L-mandelates or benzyl alcohols to the appropriate benzoates appear to be non-specific; indeed a variety of compounds which do not support growth, such as the 3-hydroxy-

substituted compounds, are also metabolised to the corresponding benzoates but no further. The benzoic acids are then metabolised by a series of specific enzymes which are generally found only when cells have been grown in the presence of the corresponding substrate. Benzoate and 2-hydroxybenzoate are oxidised to catechol while 4-hydroxybenzoate and 4-hydroxy-3-methoxybenzoate are converted to 3,4-dihydroxybenzoate. Catechol and 3,4-dihydroxybenzoate then undergo ring fission.

In order to substantiate the hypothesis that the enzymes converting the L-mandelates or benzyl alcohols to benzoates are non-specific these enzymes were examined in cell-free systems. Cell suspensions were subjected to ultrasonic disruption and, after centrifugation, the supernatant solutions were examined. Spectrophotometric assays were developed for L-mandelate dehydrogenase, benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase. A suitably sensitive assay for benzoylformate decarboxylase was not obtained. It was found possible to characterise the enzymes by a number of parameters such as Km, Vmax and relative velocity with a range of substrates. Cell-free extracts were prepared after growth on a number of compounds and in all cases the kinetic properties of the enzymes were the same regardless of the growth substrate. Evidence obtained from experiments in which mixtures of substrates were added simultaneously ruled out the possibility of non-specific induction of several substrate-specific enzymes.

The bacterium NCIB 8250 can therefore convert a large number of aromatic compounds to five key intermediates by means of just four enzymes. The thesis concludes with a discussion of the advantages to the bacterial cell of this type of specificity both in economy of cellular constituents and ecological status.

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