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PERMEATION AND METABOLISM OF INTERMEDIATES OF THE  
MANDELATE PATHWAY IN BACTERIUM NCIB 8250

By Alasdair M. Cook, B.Sc.

Many bacteria have a very wide metabolic versatility, generally in the form of convergent, inducible, catabolic pathways for the enzymic degradation of organic compounds. The regulation of these pathways is poorly understood. Theories on enzyme regulation are built on the operon model for the control of gene expression and the concept of allosteric control of enzyme activity, while the permeability of the cell membrane has also been considered as a possible locus to mediate enzyme regulation.

The mandelate pathway of an Acinetobacter species, a soil organism known as bacterium NCIB 8250, is one defined unit in a convergent, inducible catabolic sequence. The experimental work described in this thesis is concerned with the control of the enzymes converting L-mandelate and benzyl alcohol to catechol in bacterium NCIB 8250 and specifically with the role of permeation in the control mechanisms.

A sensitive radiochemical assay was developed to measure the utilisation of mandelate, benzyl alcohol or benzoate during growth of bacterium NCIB 8250 in batch culture. An exponential growth curve was observed when either benzoate or benzyl alcohol was the sole source of carbon and energy. Growth in mandelate-salts medium was complex and involved the exhaustion of mandelate before the end of growth, a transient accumulation of benzaldehyde and subsequent growth on excreted material.

Growth in dual substrate media was examined and certain cases of the

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preferential use of one substrate were observed. The utilisation of mandelate was suppressed when benzoate, catechol or succinate was present, but not when benzyl alcohol was present. Suppression of the utilisation of benzyl alcohol in the presence of mandelate occurred after a delay, while benzoate suppressed benzyl alcohol utilisation in cells preinduced to growth on benzoate but not in cells preinduced to growth on benzyl alcohol. No suppression of the utilisation of benzoate was observed.

The use of cell-free enzyme assays in the presence of other intermediates showed that enzyme inhibitions were not the cause of the suppressions of substrate utilisation. In whole cells, the utilisation of mandelate, benzyl alcohol or benzoate was generally unaffected by other intermediates though catechol and succinate did have inhibitory effects (about 30%) of unknown origin.

The utilisation of mandelate in cells possessing the mandelate enzymes was not inhibited by benzoate, so suppression of mandelate utilisation in the presence of benzoate was ascribed to repression of protein synthesis. At least some part of the effects of succinate and catechol in suppressing the utilisation of mandelate was also ascribed to repression of protein synthesis. The enzymes for the utilisation of benzyl alcohol were repressed by L-mandelate, but no repression by benzoate, catechol or succinate was detected. No repression of benzoate oxidase was detected.

A radiochemical assay to measure very low quantities of intracellular aromatic intermediates was developed. A general permeability barrier to carbohydrate was exploited to estimate the intracellular water space and intracellular material was expressed as a concentration in that same water

space.

Varying degrees of permeability barrier to mandelate were displayed by cells without L-mandelate dehydrogenase. Rapid decarboxylation of mandelate was observed in induced wild type cells. An inducible mandelate transport system was therefore proposed. A high radiochemical reagent blank for benzyl alcohol prevented use of the radiochemical assay to study the entry of benzyl alcohol into cells in suspension. However, an inducible benzyl alcohol transport system was proposed on indirect evidence from growth experiments: benzoate suppressed the utilisation of benzyl alcohol in cells preinduced to growth on benzoate without inhibition or repression in cells preinduced to growth on benzyl alcohol. No permeability barrier to benzoate itself was observed, and no substantial inhibition of the decarboxylation of benzoate by whole cells was detected in the presence of a wide range of analogues of benzoate. One of these compounds, p-fluorobenzoate, was decarboxylated by suspensions of cells induced to growth on benzoate. This decarboxylation was totally prevented by the presence of benzoate. p-Fluorobenzoate did not penetrate the cell membrane in the presence of benzoate. Accordingly, a constitutive benzoate transport system was postulated.

It thus appears that although a permeability barrier can prevent enzyme induction by preventing entry of inducer into the cell, repression of enzyme synthesis in the mandelate pathway of bacterium NCIB 8250 is a control at the level of protein synthesis that is not mediated by an inhibition of transport. The possible relevance of the transport systems which have been postulated in this organism and the controls, in terms of multisensitive, end-product repression, are discussed.

Permeation and Metabolism  
of  
Intermediates of the Mandelate Pathway  
in  
Bacterium NCIB 8250

by

ALASDAIR MACLEOD COOK

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

The standard abbreviations found in Biochem. J. (1971). 121, 1. are used throughout this thesis. In addition the following abbreviations are used:

NCIB	National Collection of Industrial Bacteria
Cyclic AMP	Cyclic adenosine-3',5'-monophosphate
$\mu$	Specific growth rate.

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



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S U M M A R Y.

- (1) The thesis starts with a description of the mandelate pathway of the Acinetobacter species known as bacterium NCIB 8250. This pathway is one unit in a convergent, inducible, catabolic sequence whose regulation is ill-understood. Present knowledge of the permeation of bacterial cell membranes, enzyme regulation and inter-relationships between these two facets of metabolism is then summarised.
- (2) The experimental work described in the thesis is concerned with the control of the enzymes converting L-mandelate and benzyl alcohol to catechol in bacterium NCIB 8250, and specifically the role of permeation in the control mechanisms.
- (3) A sensitive radiochemical assay was developed to measure the utilisation of mandelate, benzyl alcohol and benzoate during growth of bacterium NCIB 8250 in batch culture. This was based on the decarboxylation of the  $^{14}\text{C}$ -labelled moiety in the sidechain of [carboxy- $^{14}\text{C}$ ]-mandelate, [carbinol- $^{14}\text{C}$ ] benzyl alcohol or [carboxy- $^{14}\text{C}$ ]-benzoate. A careful choice of inocula for growth experiments was shown to be necessary to facilitate the interpretation of data.

An exponential growth curve was observed when either

benzoate or benzyl alcohol was the sole carbon and energy source. Growth in mandelate-salts medium was complex and involved the exhaustion of mandelate before the end of growth; subsequent growth was on excreted material, including benzaldehyde.

Growth in dual substrate media was examined and certain cases of the preferential utilisation of one substrate were observed:

a) in general, mandelate utilisation was suppressed in the presence of a second substrate. This was observed with inocula both of mandelate-grown cells and of non-induced cells when benzoate, catechol or succinate was present. In contrast, benzyl alcohol did not suppress mandelate utilisation.

b) benzyl alcohol utilisation in cells adapted to benzyl alcohol was suppressed by L-mandelate (and/or phenylglyoxylate) but only some time after the addition of mandelate. The onset of suppression coincided with the initiation of mandelate utilisation. Cells preadapted to benzyl alcohol co-utilised benzoate and benzyl alcohol in dual substrate media, but benzyl alcohol utilisation was totally suppressed when a benzoate-grown inoculum was used.

c) no suppression of benzoate utilisation was detected in the presence of catechol or succinate.

- (4) Cell-free enzyme assays showed that L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase, the two benzaldehyde dehydrogenases and benzyl alcohol dehydrogenase were not inhibited by the DL-mandelate, phenylglyoxylate, benzaldehyde, benzyl alcohol, benzoate or succinate. Whole cell decarboxylation of mandelate was unaffected by benzoate or benzyl alcohol though catechol and succinate caused 30% inhibitions. The disappearance of benzyl alcohol from cell suspensions was not substantially affected by benzoate or mandelate, though inhibitions by catechol and succinate were noted.
- (5) The failure of bacterium NCIB 8250 to assimilate or dissimilate a number of carbohydrates, including notably fructose-1, 6-diphosphate, was interpreted as denoting a complete permeability barrier to these compounds. This permeability barrier was demonstrated directly and the aqueous volume from which glucose was excluded was termed intracellular water and used in subsequent calculations.
- (6) A radiochemical assay to measure very low quantities of intracellular aromatic intermediates was developed. This involved filtration of samples of cells suspended in solutions containing radioactively labelled compounds. Raw data were first corrected for the radioactive "reagent blank" associated with the filter membrane and then for material presumably trapped between cells; the net radioactivity was expressed as a concentration in the intra-

cellular water space.

- (7) Varying degrees of permeability barrier to mandelate were displayed by cells without L-mandelate dehydrogenase. Cells grown on benzoate, for example, showed a very slow permeation of mandelate which was completely inhibited in the presence of benzoate. Rapid mandelate decarboxylation was observed in induced wild type cells but no intracellular mandelate was detected under these conditions. An inducible mandelate transport system was therefore proposed.
- (8) The filtration assay could not be used when benzyl alcohol was the substrate, as the radioactive reagent blank was very high. However, an inducible benzyl alcohol transport system was proposed on indirect evidence from growth experiments which showed that the utilisation of benzyl alcohol was suppressed in benzoate-grown cells in the presence of benzoate, while co-utilisation of these two compounds, with no apparent suppression of utilisation, was observed in cells preinduced to benzyl alcohol.
- (9) No permeability barrier to benzoate itself was observed, and no substantial inhibition of whole cell benzoate decarboxylation was detected in the presence of a wide range of substituted benzoates and analogues of benzoate. One of these compounds, p-fluorobenzoate, was decarboxylated by benzoate-grown cells, but this decarboxylation was totally prevented by the presence of

benzoate. p-Fluorobenzoate did not penetrate the cell membrane in the presence of benzoate. A constitutive benzoate transport system was postulated.

- (10) The rates of substrate disappearance from cell suspension indicated that inhibition of transport by other members of the mandelate pathway was generally absent in induced cells: the utilisation of each substrate was inhibited both by catechol and succinate but the locus of the inhibition has not been established.
- (11) Mandelate utilisation in cells possessing the mandelate enzymes was not inhibited by benzoate, so suppression of mandelate utilisation during growth in the presence of benzoate was ascribed to repression of protein synthesis. At least some part of the effects of succinate and catechol in suppressing mandelate utilisation during growth was ascribed to repression of protein synthesis.
- (12) The enzymes of benzyl alcohol utilisation were repressed by L-mandelate (and/or phenylglyoxylate), but no repression by benzoate, catechol or succinate was detected.
- (13) No repression of benzoate oxidase was detected.
- (14) Although a permeability barrier could prevent enzyme induction by preventing entry of inducer, the repression of enzyme synthesis in the mandelate pathway of bacterium NCIB 8250 is seen to be a control at the level of protein synthesis and not mediated by an inhibition of transport. The thesis concludes with a discussion of the control in



terms of multisensitive end-product repression (Mandelstam & Jacoby, 1965) and the possible relevance of the transport systems which have been postulated in this organism.

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

### 1. MICROBIAL DISSIMILATION OF AROMATIC COMPOUNDS.

#### 1. 1. The importance of aromatic compounds.

A vast range of aromatic compounds is synthesised in the biosphere, ranging from simple compounds like phenylalanine and coumarins through oestrogens and alkaloids to the ill-defined polymers such as melanin, tannin and lignin. The plant world produces the largest quantities and the widest variety of these aromatic compounds, though an increasing number is being produced industrially to be used as pesticides, detergents, plastics, etc.. These latter compounds are frequently halogenated. Completion of the part of the carbon cycle involving aromatic compounds falls to microorganisms (e.g. Alexander, 1961; Dagley, 1967; Evans, 1968; Kearney & Kaufman, 1969). Several bacterial and fungal species are able to metabolise a large variety of benzenoid structures either as sources of organic carbon for growth (e.g. Ribbons, 1965; Dagley, 1967; Evans, 1968; Dutton & Evans, 1969) or by co-metabolism with other substrates (e.g. Fewson, Kennedy & Livingstone, 1968; Jamison, Raymond & Hudson, 1969; Focht & Alexander, 1971).

The variety of aromatic compounds dissimilated by different organisms has aroused interest not only in the catabolic pathways and their enzymology but also in the control of the

expression and operation of these pathways. The control circuits are of particular interest because of the complexity of many of the pathways and because the organisms responsible naturally occur in that myriad complex of organisms and organic and inorganic matter called soil, about which so little is as yet known at other than a descriptive level.

1. 2. The Moraxella-Acinetobacter group of soil bacteria.

Members of the Moraxella-Acinetobacter group of bacteria occur ubiquitously in soil and water (Henderson, 1965; Baumann, Doudoroff & Stanier, 1968) and often represent a large proportion of the bacterial population (Baumann, 1968). They appear as Gram-negative, non-motile rods (ca. 1.5 - 2.5 by 0.9 - 1.5 $\mu$ m) which characteristically occur in pairs. The organisms are catalase-positive, oxidase-negative, obligate aerobes which are generally unable to metabolise carbohydrates but which can utilise a wide variety of organic compounds as sole sources of carbon.

1. 2. 1. Bacterium NCIB 8250.

Bacterium NCIB 8250 has been placed in the Moraxella-Acinetobacter group of bacteria by Sebald & Véron (1963), Véron (1966), Fewson (1967a) and Baumann et al. (1968). Experiments on DNA homologies (Johnson, Anderson & Ordal, 1970) support this classification. Nevertheless, the nomenclature of the Moraxella-Acinetobacter group is still the subject of debate and a large

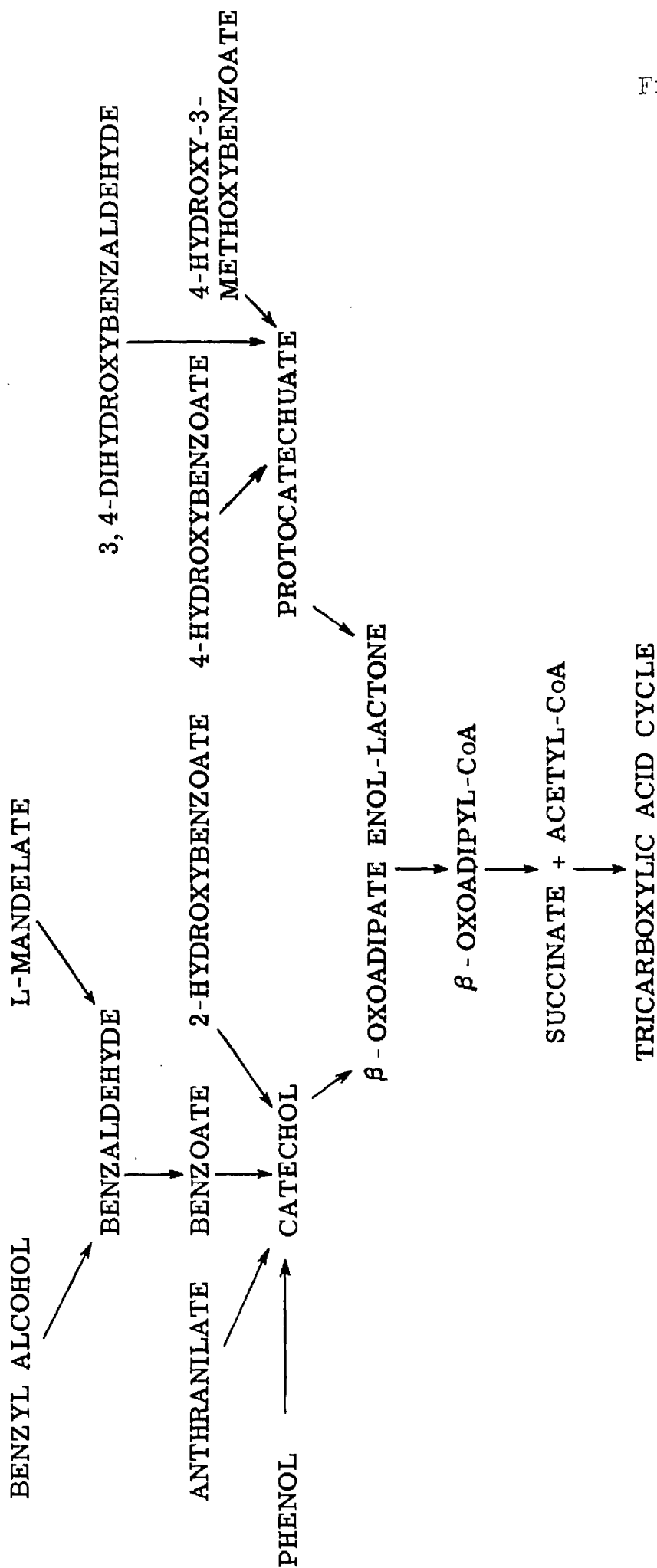
number of generic names (e.g. Acinetobacter, Herellea, Mima and Moraxella) and specific names (e.g. calcoaceticus and lwoffii) are in current use (Baumann et al., 1968). The organism is therefore always referred to as bacterium NCIB 8250 in this laboratory.

There has been some confusion over the identity of this organism. Professor W.C. Evans (Department of Biochemistry and Soil Science, University College of North Wales, Bangor) deposited an organism, which was thought to be *Vibrio* 01 (Happold & Key, 1932), with the National Collection of Industrial Bacteria where it was catalogued as number 8250. This organism is now known not to correspond with the original *Vibrio* 01 (Fewson, 1967a) and work subsequent to 1953, even that nominally done with *Vibrio* 01, has probably been done with bacterium NCIB 8250 (Fewson, 1967a). It is not clear when the original strain was lost.

### 1. 3. Converging catabolic pathways.

The dissimilation of a group of aromatic compounds by bacterium NCIB 8250 illustrates (Fig.1) the general pattern of catabolism in this organism which can grow on well over 100 different carbon sources (Fewson, 1967b). Dissimilation is catalysed by successive, converging pathways of inducible, catabolic enzymes. For instance the degradation of mandelate and benzyl alcohol converges on benzaldehyde and further catabolism converges with that of 2-hydroxybenzoate (Kennedy &

A SUMMARY OF CONVERGING METABOLIC PATHWAYS IN BACTERIUM NCIB 8250



Fewson, 1968ab), anthranilate (Fewson, unpublished data) and phenol (Beveridge & Tall, 1969) at catechol. Similarly, protocatechuate is the point of convergence of the 4-hydroxy-, 3,4-dihydroxy- and 4-hydroxy-3-methoxybenzoate pathways (Kennedy & Fewson, 1968ab). After ring cleavage of the two catechols, the aliphatic manipulations converge on  $\beta$ -oxoadipate enol-lactone, the last intermediate specific to the aromatic compounds. The enol-lactone is converted to  $\beta$ -oxoadipyl-CoA and cleaved to succinate and acetyl-CoA. The latter compounds are dissimilated by the Krebs cycle whose presence is well established in this organism (Dagley & Walker, 1956).

Convergent catabolic pathways are not unusual. Indeed, in aerobic organisms commonly using the Krebs cycle as an amphibolic pathway (Davis, 1961), all degradative pathways will converge on that sequence of reactions. Pseudomonads, for example, also have a wide metabolic versatility (Stanier, Palleroni & Doudoroff, 1966) and groups of converging inducible pathways are well documented. Rosenberg & Hegeman (1969) illustrate the convergence of *p*-hydroxybenzoate, anthranilate and mandelate dissimilation in *Pseudomonas aeruginosa* and Hegeman (1966a) shows the convergent catabolism of *p*-hydroxybenzoate, mandelate and tryptophan in *Pseudomonas putida*. On the other hand, the convergent pathways for carbohydrate catabolism in *Escherichia coli* do not form families of inducible enzymes in the same way. Generally a carbohydrate (e.g. galactose, lactose or glycerol) is

channelled by one inducible enzyme system into a constitutive pathway, glycolysis.

Most studies on the control of metabolic pathways have centred on induction and repression of enzymes concerned with carbohydrate metabolism, especially the lac operon of E. coli (e.g. Jacob & Monod, 1961; de Crombrugghe, Chen, Anderson, Nissley, Gottesman, Pastan & Perlman, 1971) and on the repression and inhibition of the divergent, branched-chain pathways of amino acid biosynthesis in E. coli and Salmonella typhimurium (e.g. Cohen, 1965; Datta, 1969). Relatively little is known of the control systems in operation in convergent, inducible, catabolic pathways. For this reason, work in this laboratory has centred on the examination of one branched catabolic unit within the system illustrated in Fig. 1, the mandelate pathway.

#### 1. 4. The mandelate pathway of bacterium NCIB 8250.

The metabolism of mandelate can be considered in two parts which are: a) the manipulation of the sidechain and ring hydroxylation leading to the formation of catechol, and b) the ring cleavage of catechol and the subsequent metabolism of the aliphatic compounds formed.

This thesis considers only the control of those enzymes involved in the manipulation of aromatic intermediates in the mandelate pathway. The reactions involved in the conversion of

catechol to  $\beta$ -oxoadipyl-CoA have been studied in Acinetobacter species by Cánovas, Ornston & Stanier (1967).

1. 4. 1. Intermediates and enzymes in the mandelate pathway.

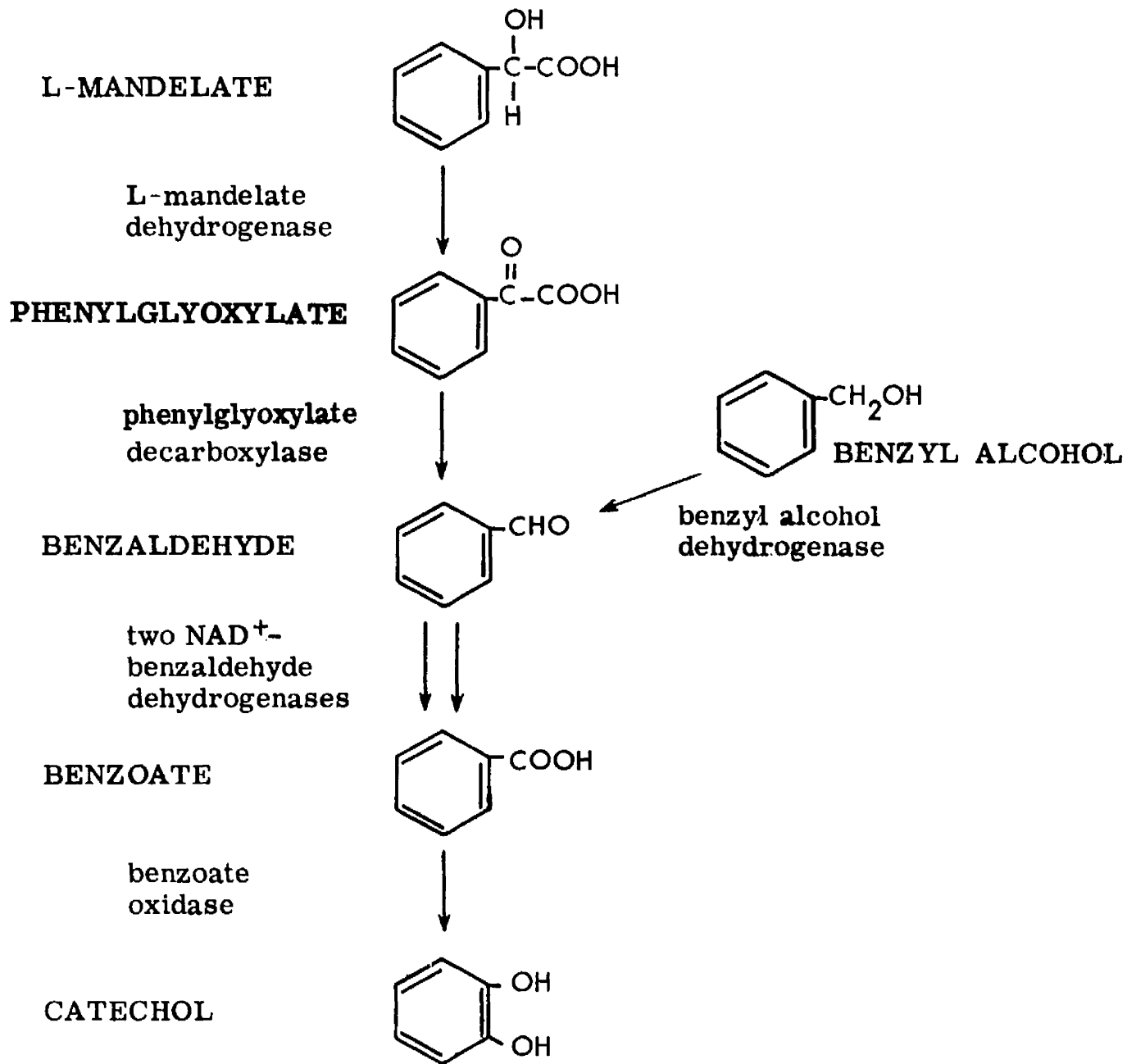
Kennedy & Fewson (1968a) proposed from simultaneous adaptation experiments (Stanier, 1947) that L-mandelate was oxidised to catechol via phenylglyoxylate, benzaldehyde and benzoate (Fig. 2); a convergent pathway degraded benzyl alcohol to catechol via benzaldehyde and benzoate. This formulation of the pathways was supported by the identification of the following enzyme activities in cell-free extracts, L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase, two NAD<sup>+</sup> linked benzaldehyde dehydrogenases (one heat-stable, one heat-labile), benzyl alcohol dehydrogenase and catechol oxygenase (Kennedy & Fewson, 1968b) and benzoate oxidase (Fewson, Livingstone & Roach, 1970). L-mandelate dehydrogenase was shown to be particulate (Kennedy & Fewson, 1968b) while the other enzymes with the exception of benzoate oxidase, are known to be soluble (Kennedy & Fewson, 1968b). Definitive proof of the existence of two benzaldehyde dehydrogenases was provided by Kennedy (1970) when he purified the two enzymes.

The enzymes involved in the oxidation of L-mandelate and benzyl alcohol to benzoate are relatively non-specific as regards substituents on the aromatic ring (Kennedy & Fewson, 1968a).



Fig. 2.

OXIDATION OF MANDELATE TO CATECHOL IN BACTERIUM NCIB 8250



The 2-, 3- and 4- hydroxy- and 4-hydroxy-3-methoxy- derivatives of any one compound are all metabolised by the one enzyme. On the other hand, the catabolism of each hydroxy-substituted benzoate is catalysed by a specific oxidase. Some compounds, for example the 3-hydroxy-derivatives, are metabolised no further than the corresponding benzoate because there is no suitable oxidase.

#### 1. 4. 2. Regulation of the mandelate pathway.

Kennedy & Fewson (1968ab) found that groups of enzymes in the mandelate pathway were induced coordinately (Table 1). A coordinately controlled group of enzymes was considered to be under the genetic control of one regulon. A regulon (Maas & Clark, 1964) is a system of enzyme-forming units which responds to a repressor. The term was propounded by Maas & Clark (1964) when they determined that the genes coding the enzymes of arginine biosynthesis in E. coli, though controlled by a common repressor, were arranged in at least four operons. An operon is a cluster of genes, transcribed as a unit and regulated together (Jacob & Monod, 1961). The existence of an operon (e.g. the lac operon of E. coli, Jacob & Monod, 1961) requires genetic proof and as the genophore (Maas & McFall, 1964) of bacterium NCIB 8250 has not been studied, the less rigorous definition, regulon is employed for coordinately controlled genes in this organism.

Table 1.

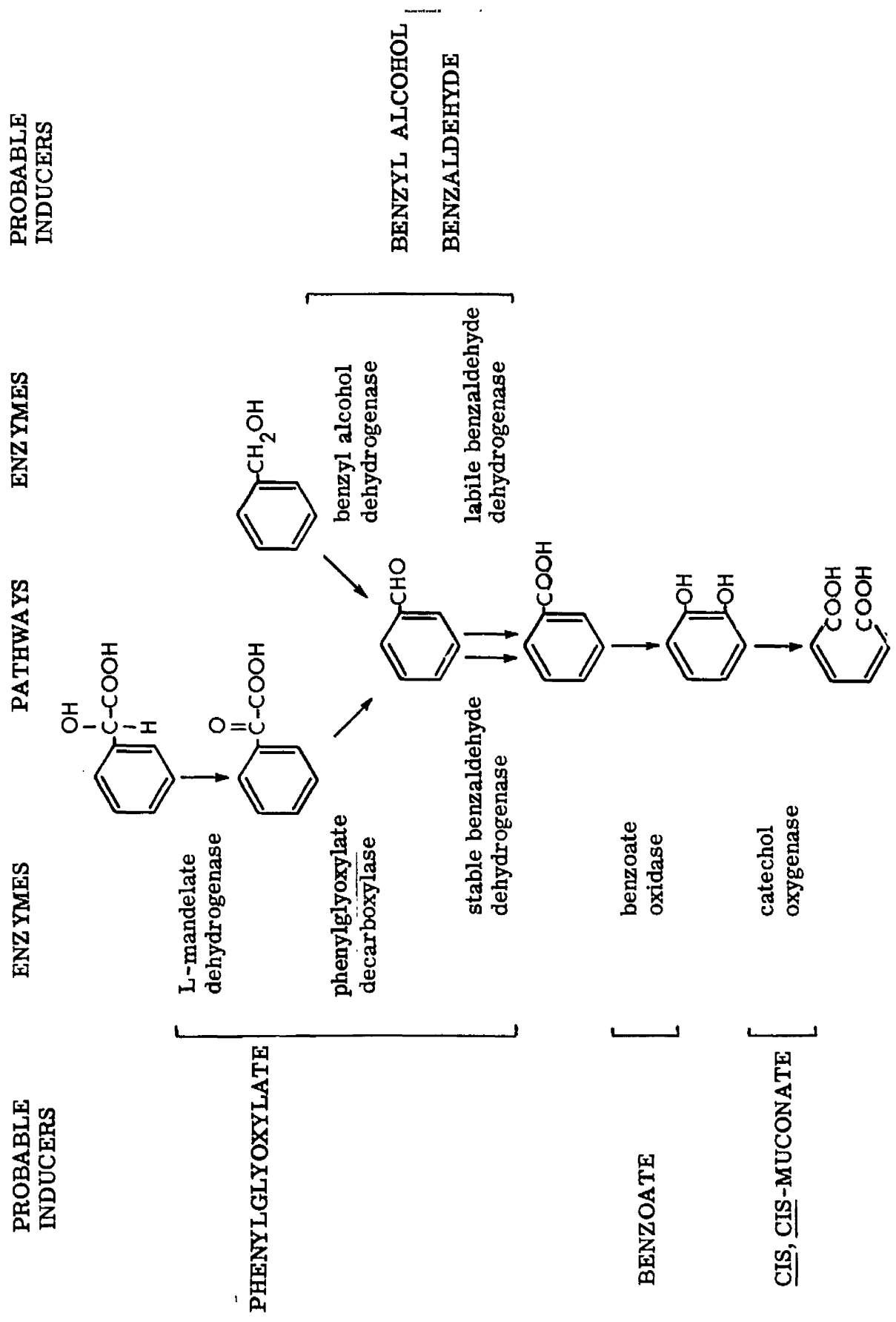
The pattern of the syntheses of the mandelate pathway enzymes observed in bacterium NCIB 8250 by Kennedy & Fewson (1968a, Table 3; 1968b, Tables 1 and 2 and Fig. 1).

Enzyme	Growth Substrate					
	DL-Mandelate	Phenylglyoxylate	Benzyl alcohol	Benzaldehyde	Benzoate	Catechol
L-Mandelate dehydrogenase	+	+	-	-	-	-
Phenylglyoxylate carboxylase	+	+	-	-	-	-
Stable benzaldehyde dehydrogenase	+	+	-	-	-	-
Labile benzaldehyde dehydrogenase	+	+	+	+	-	-
Benzyl alcohol dehydrogenase	+	+	+	+	-	-
Benzoate oxidase	+	+	+	+	+	-
Catechol 1,2-oxygenase	+	+	+	+	+	+

L-Mandelate dehydrogenase, phenylglyoxylate carboxylase and the stable benzaldehyde dehydrogenase were designated regulon  $R_1$  (Fig. 3). Although low levels of benzyl alcohol dehydrogenase and the heat-labile benzaldehyde dehydrogenase were detected during growth on mandelate or phenylglyoxylate (Table 1), Kennedy & Fewson (1968a) rejected the possibility that mandelate was metabolised via benzyl alcohol because benzyl alcohol supports a higher molar growth yield (Bauchop & Elsdon, 1960) than does mandelate. Furthermore, mutant strains lacking benzyl alcohol dehydrogenase are able to grow normally on L-mandelate or phenylglyoxylate (Livingstone & Fewson unpublished data). Benzyl alcohol dehydrogenase and the heat-labile benzaldehyde dehydrogenase were designated regulon  $R_2$ . Benzoate oxidase was assigned to regulon  $R_3$  and catechol oxygenase comprises regulon  $R_4$ .

Livingstone (1970) has confirmed these regulatory groupings by studying the kinetics of induction of the various enzymes in the wild type organism and in blocked mutants. Different inducers for each regulon of the pathway were used during growth in glutamate-salts medium. The data were expressed as "P-values" (Monod, Papenheimer & Cohen-Bazire, 1952), the gradient of the graph of increase in enzyme units/ml culture against increase in bacterial protein/ml culture, and the correlation between the P-values for the induction of one enzyme and the P-values for the induction of the other enzymes

REGULATION OF THE AROMATIC PART OF THE MANDELATE  
PATHWAY IN BACTERIUM NCIB 8250



was used as a test for coordinacy.

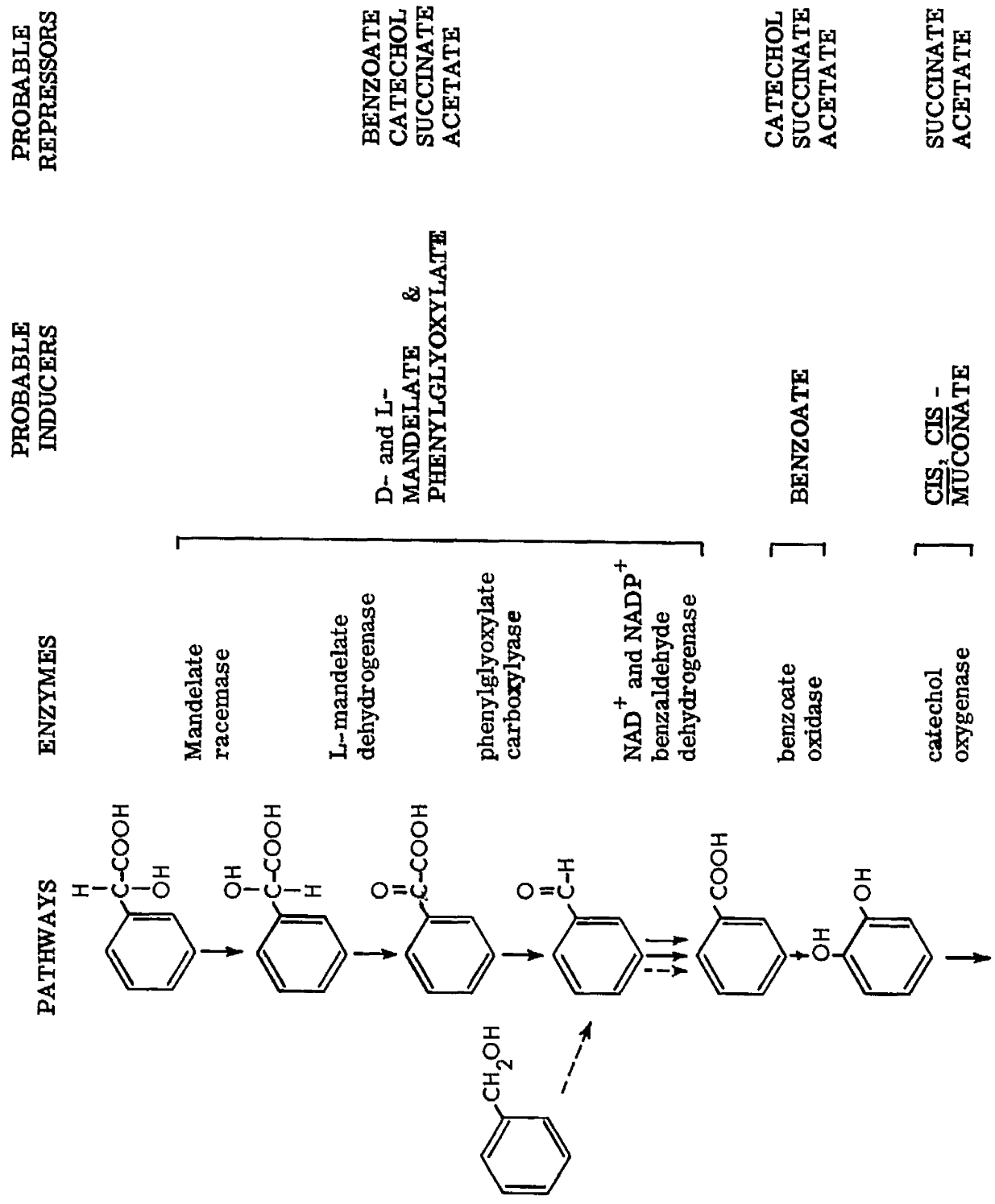
Livingstone (1970) also determined that the probable inducer of regulon  $R_1$  is phenylglyoxylate (Livingston & Fewson, 1971). Both benzyl alcohol and benzaldehyde serve as inducers of regulon  $R_2$  (Livingstone & Fewson, unpublished data). Regulon  $R_3$  is believed to be induced by benzoate (Livingstone, 1970). Farr & Cain (1968) have demonstrated that catechol oxygenase is induced by cis-cis-muconate although the results of Beveridge & Tall (1969) suggest that this is not exclusive.

#### 1. 5. Mandelate pathways in other organisms.

Mandelate can be used as the sole source of carbon and energy by a few bacteria belonging to the genus Pseudomonas (Stanier et al., 1966) or to the genus Acinetobacter (Baumann et al., 1968) while the fungus Aspergillus niger has also been shown to metabolise mandelate (Jamaluddin, Subba Rao & Vaidyanathan, 1970). The fungal mandelate pathway (Jamaluddin et al., 1970) and that of P. aeruginosa (Rosenberg & Hegeman, 1969) have been defined, but by far the most studied mandelate pathway is that in P. putida (ATCC 12633, also known as P. fluorescens A. 3. 12 (Stanier et al., 1966)) (Fig. 4).

The degradation of mandelate to catechol in P. putida was proposed by Stanier (1947, 1948) and Sleeper & Stanier (1950) and verified by Stanier, Gunsalus & Gunsalus (Gunsalus, Stanier & Gunsalus, 1953; Gunsalus, Gunsalus & Stanier, 1953; Stanier,

REGULATION OF THE MANDELATE PATHWAY IN PSEUDOMONAS PUTIDA



Gunsalus & Gunsalus, 1953) and Sleeper (1951). The oxidation of benzyl alcohol via benzaldehyde and benzoate was proposed by Stanier (1948) but the enzymes involved in the production of catechol from benzyl alcohol have not been detected (Hegeman, 1966a). Little further work has been done, presumably because benzyl alcohol supports only very slow growth in P. putida (Fewson, personal communication). However, during growth in benzaldehyde-salts medium, Stevenson & Mandelstam (1965) suspected the presence of a benzaldehyde dehydrogenase which was distinct from those in the mandelate group of enzymes. Stevenson & Mandelstam (1965) were unable to detect this enzyme in cell-free extracts but Fewson (personal communication) has detected a very labile benzaldehyde dehydrogenase activity in cell extracts of P. putida grown in benzaldehyde-salts medium. While this evidence may be interpreted in support of convergent mandelate and benzyl alcohol degradation in P. putida, and benzyl alcohol dehydrogenase activity has been observed in a Pseudomonas species (Katagiri, Takemori, Nakazawa, Suzuki & Akagi, 1967), Claus & Walker (1964) working on an unidentified pseudomonad suggested that benzyl alcohol was catabolised by a different and unidentified pathway.

The induction of the enzymes of mandelate degradation in P. putida (Fig. 4) has been studied by Stanier, Hegeman & Ornston (1965), Hegeman (1966 a,b,c) and Ornston (1966). Hegeman (1966a) offered evidence that under the conditions of his



experiments there was no catabolite repression (see p. 36). Also, a mutant strain of P. putida lacking L-mandelate dehydrogenase but containing the racemase did not concentrate mandelate within the cell and mandelate apparently entered induced and non-induced cells with equal facility (Hegeman, 1966b).

In contrast to the work of Hegeman (1966a), but under different conditions, Mandelstam & Jacoby (1965) and Stevenson & Mandelstam (1965) reported catabolite repression of the enzymes of mandelate dissimilation. The latter authors described the phenomenon as "multisensitive, end-product repression", Fig. 4. The repression was not mediated by an amphibolic intermediate because blocked mutants also displayed repression. The best example of this was in a mutant lacking benzoate oxidase in which the presence of benzoate caused repression of the synthesis of the enzymes of the preceding regulon (Mandelstam & Jacoby, 1965).

Mandelstam & Jacoby (1965) were unable to ascribe any locus to these effects, and suggested that they were either at the level of transport of inducer into the cell or at the level of protein synthesis. In view of the failure to detect a permease for mandelate (reported by Hegeman, 1966b), Mandelstam concluded that repression operated at the level of enzyme formation (Stevenson & Mandelstam, 1965).

Despite the possible contradictions between Hegeman's results and those of Mandelstam, the control situations observed

in the mandelate pathway of P. putida do provide a precedent and a stimulus to examine the control systems operating in the mandelate pathway of bacterium NCIB 8250. Since the regulatory patterns of induction are quite different in the mandelate pathways of the various bacteria so far examined, even in different species of Pseudomonas (Hegeman, 1966 a,b,c; Rosenberg & Hegeman, 1969), it seems not unlikely that other aspects of control may be quite different in different organisms even though the chemical nature of the intermediates of the pathways are identical. The known types of bacterial control system are reviewed in more detail in the next section in order to provide a formal background to the experimental work described in this thesis.

## 2. Permeation and enzyme regulation.

Szent-Györgi (1966) used the quotation "A drug is a substance which, injected into an animal, produces a paper.". This thought applies equally well to bacterial transport systems where cataloguing is well in advance of understanding. It is perhaps naive to expect to understand completely any system embodied in the cell membrane when the membrane itself is so poorly defined. However, specific permeation of the cell membrane is an integral part of cell function and there is preliminary evidence, discussed in the following sections, which suggests that permeation may often be an important locus for the control of cell metabolism.

## 2. 1. Permeability of the cell membrane.

A semi-permeable cell membrane was first postulated because of the behaviour of animal and plant cells under varying osmotic conditions (described in e.g. Loewy & Siekewitz, 1969). Stein (1967) has reviewed the nature and extent of passive diffusion across cell membranes and concluded:

- "a) diffusion within the membrane ... results in a 100- to 1000-fold reduction of transfer rate in comparison with an equivalent thickness (50 Å) of water, however,
- b) to enter the membrane, each hydrogen-bonding acceptor or donor group that the permeant molecule makes with the water molecules of the aqueous phase has to be broken, a step which lowers the transfer rate by a further 6- to 10-fold, and,
- c) ... each bare  $-\text{CH}_2-$  group in the permeant will increase the transfer rate by some twofold."

To exploit this permeability barrier, therefore, a cell requires specific translocation systems in the membrane to permit, for example, the entry of nutrients. The permeability properties of the bacterial cell are a function of the membrane alone; the wall is freely penetrated by most small molecules (e.g. Mandelstam & McQuillen, 1968). To date, three general types of translocation across the bacterial cell membrane have been recognised (e.g. Kaback, 1970); facilitated diffusion, active transport and group translocation. Bulk transport does not exist in bacteria (e.g. Loewy & Siekewitz, 1969).

### 2. 1. 1. Facilitated diffusion.

Facilitated diffusion systems operate on an existing electrochemical gradient of the permeant and lead to the disappearance of the gradient. The permeant has the same chemical identity on both sides of the membrane. These systems require no further input of free energy, except - in the long term view - for that required to maintain the structure of the cell membrane, a criterion which excludes active transport systems (Stein, 1967).

Facilitated diffusion systems per se are very poorly documented in bacteria. Possibly the best known is that for glycerol translocation in E. coli (Sanno, Wilson & Lin, 1968). The evidence is that the intracellular glycerol concentration never exceeds the extracellular concentration in mutant strains deficient in glycerol kinase, and that glycerol enters induced cells of the mutants very much more rapidly than it enters non-induced cells. Intracellular glycerol is converted to L- $\alpha$ -glycerophosphate by an ATP-dependent kinase, thus maintaining the concentration gradient of glycerol across the cell membrane.

Much more extensive studies on facilitated diffusion have been done in eukaryotic cells, notably glucose transport in erythrocytes (e.g. Wilbrandt & Rosenberg, 1961). Substrate specificity, saturation kinetics, competitive inhibition by chemical analogues and non-competitive inhibition by enzyme

poisons have been observed. Perhaps the best experimental confirmation of this facilitated diffusion system was the "counterflow" experiment (Widdas, 1952) first reported by Park, Post, Kalman, Wright, Johnson & Morgan (1956). To an erythrocyte suspension preloaded with xylose, a high concentration of glucose was added. Glucose has a high affinity for the carrier. A transient net movement of xylose out of the cell against a concentration gradient was noted. This behaviour is the result of the inhibition of influx of xylose by the presence of glucose, with little or no effect on efflux.

More recent data have allowed Lieb & Stein (1971) to propose an elegant model for the facilitated diffusion of glucose in the erythrocyte. Nonetheless, the reaction mechanism of this transport system is unknown and this dearth of detail concerning molecular behaviour within the membrane is common to all transport mechanisms.

### 2. 1. 2. Active transport.

In active transport, the permeant is accumulated inside the cell against an electrochemical gradient, and has the same chemical identity on both sides of the membrane.

The accumulation of amino acids against a concentration gradient has been recognised for many years (e.g. Gale, 1947; Cohen & Rickenberg, 1956; Britten & McClure, 1962; Kay & Gronlund, 1971) but as yet little detail has become apparent.

The systems may be oligomeric. Anraku, (1968 a,b,c) studied the release of a leucine binding protein from E. coli under conditions of osmotic shock, and found that the restoration of leucine transport required another protein component also released into the shock fluid. The second protein did not bind leucine. Ames & Lever (1970) have found the histidine transport system of Salmonella typhimurium to be oligomeric and Wilson & Holden (1969) have found different components in the arginine transport system of E. coli.

Several ions are subject to active transport across the bacterial cell membrane (e.g. Lin, 1970). Largely because of genetic evidence both sulphate transport into Salmonella typhimurium and potassium transport into E. coli are thought to be catalysed by oligomeric structures.

The oligomeric nature of these uptake systems leads to the idea that active transport is in fact an extension of the facilitated diffusion system by the inclusion of an energy coupling system (e.g. Cohen & Monod, 1957; Koch, 1964; Winkler & Wilson, 1966; Kaback, 1970).

The most studied active transport system in bacteria is assuredly the lac permease of E. coli. Though the permease gene product, the M-protein, has been isolated (Fox & Kennedy, 1965; Fox, Carter & Kennedy, 1967) it no longer binds its substrate. The lac permease system has been shown to consist of at least two mechanisms, a facilitated diffusion system and an energy coupling

system (Koch, 1964; Winkler & Wilson, 1966; Wong & Wilson, 1970). In the presence of energy poison, a facilitated diffusion system operates as shown by counterflow experiments, while the presence of this energy poisoned entry system increases the rate of entry of lactose by 1,000 - to 10,000 -fold over the rate due to passive diffusion (Winkler & Wilson, 1966). Energy coupling in this system principally alters the affinity of substrate for carrier on the inside of the membrane (Winkler & Wilson, 1966) without changing the number of operative carriers. A mutant strain with a partly defective energy coupling system has been isolated (Wong, Kashket & Wilson, 1970). It is not yet known whether this mutation maps in the lac y-gene, thus there is no evidence to indicate whether the lac permease is a single protein or an oligomeric structure. Whatever the protein complement of the transport system the membrane in which it is mounted must be correctly constituted (e.g. Wilson & Fox, 1971).

ATP was originally suggested as the energy source for galactoside accumulation (Scarborough, Rumley & Kennedy, 1968) but the interpretation of these experiments has been challenged by West (1969). More recently Barnes & Kaback (1970) have suggested that redox energy was utilised directly as a source of energy and West (1970) has shown that the influx of lactose is coupled to a flow of protons. West (1970) interpreted this in terms of the Mitchell hypothesis of chemiosmotic coupling in energy production (e.g. Mitchell, 1966) and this would be consistent with

the less explicit conclusions of Kepes (1971).

In other active transport systems different modes of energy coupling are employed. Okada & Halvorson (1964) found that energy coupling is genetically distinct from the facilitated diffusion of thioethylglucoside in Saccharomyces cerevisiae and gave evidence that energy coupling increased the affinity of the substrate for the carrier on the outside of the membrane. In certain animal cells the immediate source of energy for the active transport of amino acids and sugars appears to be the sodium ion concentration gradient (e.g. Stein, 1967).

Active transport thus appears as a translocation complex that may be oligomeric and depends on membrane structure for proper function. The complex contains a facilitated diffusion component which is coupled directly or indirectly to metabolic energy to concentrate material inside the cell.

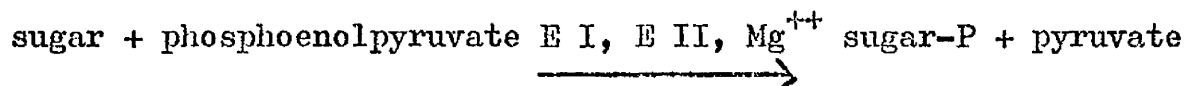
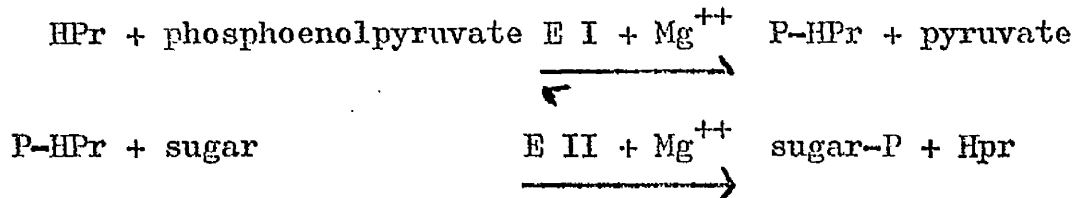
### 2. 1. 3. Group translocation.

Group translocation is a transport system in which the permeant is chemically modified as an integral part of the transport process. The best known example of this and, indeed, the best characterised transport phenomenon in bacteria is the vectorial phosphorylation of sugars in the phosphoenolpyruvate-phosphotransferase system.

Kundig, Ghosh & Roseman (1964) reported the following



system for the phosphorylation of sugars in E. coli:



HPr refers to a constitutively synthesised, cytoplasmic, low molecular weight, heat-stable protein which is phosphorylated on a histidine residue by E I, a constitutive, cytoplasmic enzyme, at the expense of phosphoenolpyruvate. The phosphorylated, heat-stable protein acts as the phosphate donor to the sugar in a reaction catalysed by E II. E II is the generic term for a group of membrane-bound enzymes. Each sugar transported has its own specific E II. Certain E II's are constitutive, for example that for glucose, while others are inducible, for example that for mannose. In addition to the proteins of known function, further components have been found under different conditions in different organisms (reviewed by e.g. Roseman, 1969; Kaback, 1970). Some sugars are subject to dephosphorylation inside the cell (Kundig, Kundig, Anderson & Roseman, 1966; Gachelin, 1970) but the importance of this effect is not known.

The phosphoenolpyruvate-phosphotransferase system has

been found in a wide range of facultative anaerobes, but not in strict aerobes (Romano, Erberhard, Dingle & McDowell, 1970). The range of sugars transported by this system varies in different organisms. Lactose, for example, is transported by vectorial phosphorylation in Staphylococcus aureus (Hengstenberg, Egan & Morse, 1967) while the possibility that galactosides are accumulated by vectorial phosphorylation in E. coli (Kundig et al., 1966) has been eliminated by Pastan & Perlman (1969) and Barnes & Kaback (1970). Hengstenberg et al., (1967) found that lactose, maltose, sucrose, galactose, mannitol, trehalose, fructose, mannose, melizitose and ribose were not utilised in mutant strains of Staphylococcus aureus lacking parts of the vectorial phosphorylation system. Whether all these compounds are transported by this system is open to doubt as similar mutant strains of E. coli are unable to grow on lactose or glycerol under normal conditions (Kundig et al., 1966; Wang & Morse, 1968) though lactose transport is active and glycerol enters the cell by facilitated diffusion.

Kaback (1970) has emphasised that the passive diffusion of free sugars and of sugar phosphates across preparations of cell membranes are, in practice, very similar. The phosphorylation involved in translocation is thus not a trapping mechanism (e.g. Gachelin & Kepes, 1970) but a mechanism of transport. Kaback (1970) points out that vectorial phosphorylation need not be the unique group translocation, and

he visualises vectorial acetylation as a plausible transport system.

Several different types of transport system have been identified. Very little is known of the actual mechanism of transport within the membrane, but some knowledge of the nature and role of the frankly enzymic ancillary systems is being acquired in the more complex transport systems. Each system consists of one or more proteins and that system is essential if the permeant is to enter the cell at a useful rate. In the absence of a suitable transport system the cell membrane varies from being relatively permeable to a permeant like glycerol to being highly impermeable to a permeant like lactose.

The study of transport presents an awkward problem. Classically, biochemistry has involved the stripping of a given system to its individual components, analysing each component and then reconstituting the system with known units. However, to destroy a membrane is to destroy the basis on which transport depends, and the membrane structure would have to be understood in detail to give reconstruction experiments any validity. In addition, facilitated diffusion and active transport have no chemical effect on the permeant, so an analysis of chemical mechanisms in vitro would present problems. The observer is thus forced to analyse a complex unit in operation. Consequently, data must be examined most carefully before conclusions are drawn, and this is well illustrated both in the controversy over

-21-

the nature of the lactose transport system in E. coli (see p. 25) and over the identity of the energy source for the same system (p. 22).

## 2. 2. Enzyme regulation.

Current understanding of the regulation of bacterial enzymes stems directly from the work and inspiration of Jacob & Monod (1961) and Monod, Changeux & Jacob (1963). The basic concept is startlingly simple; small molecules may modulate the activities of proteins. These modulations occur at two organisational levels in the cell, to control enzyme activity and to regulate gene expression. As with all cellular activities, these control circuits represent only one facet of the dynamics of metabolism in which the controlling small molecules are themselves subject to manipulation by the systems they control. For this reason, strict division of allosteric (Monod et al., 1963) controls into "metabolic" and "genetic" subdivisions is arbitrary because they represent different parts of one spectrum.

### 2. 2. 1. Modulation of enzyme activity.

The first allosteric effect to be identified in bacteria was feedback inhibition in isoleucine biosynthesis in E. coli (Umbarger, 1956). Since then feedback inhibition has been

observed in a large number of anabolic pathways (reviews by e.g. Umbarger, 1961; Cohen, 1965; Atkinson, 1969; Datta, 1969). Indeed, several forms of feedback inhibition have been identified depending on the enzymic complement of the pathway. The simplest form is seen in a linear pathway, for example histidine biosynthesis, where the first enzyme in the pathway is inhibited by histidine, the end product (Ames, Martin & Garry, 1961). In divergent, branch-chain, biosynthetic pathways, more complex controls are found to allow economic use of different branches at different rates; the systems have been reviewed (e.g. Datta, 1969) and, in addition to feedback inhibition of the first committed step, these include:

- a) synthesis of isofunctional enzymes at the first reaction in the pathway with each enzyme under the control of a different end product;
- b) concerted feedback inhibition, which requires two or more end products to achieve inhibition, and,
- c) cooperative feedback inhibition in which the inhibition of the common enzyme is greater in the presence of a mixture of end products than in the presence of a single end product.

Within the generally divergent biosynthetic pathways there are elements of convergence, for example methionine biosynthesis involves the methylation of homocysteine by a specific fraction of the tetrahydrofolate pool. In Neurospora crassa these

convergent pathways are synchronised by feedback inhibition controls on both convergent paths and a cross-pathway activation by the specific methyltetrahydrofolate (Selhub, Savin, Sakami & Flavin, 1971).

The activity of amphibolic enzymes is subject to a much more extensive and highly sophisticated series of modulations than observed in anabolic enzymes (e.g. Atkinson, 1969; Sanwal, 1970). This reflects the critical importance of the balance between channelling intermediates to anabolic pathways and to energy production. The complexity probably also arises from the lack of compartmentation within the bacterial cell as compared to the eukaryotic cell, and the wide range of carbon sources utilised by the organisms studied.

Both activations and inhibitions of enzyme activity are known in amphibolic pathways. Sanwal (1970) concludes that amphibolic pathways consist of critical junctions joined by paths which can be examined both individually and as an integrated whole. Feedback inhibition of the first committed step in a path is seen, as well as feedforward activation to indicate the supply of precursor. To complement these modulations, controls indicative of the energy charge of the cell are superimposed. Where paths converge, notably in and around the Krebs cycle, yet another control may be required and NADH serves as such an effector in enteric forms (Sanwal, 1970).

The modulation of activity of catabolic pathway enzymes

is less well documented than that of anabolic and amphibolic pathways. Indeed, during growth on a single carbon source there might be little advantage in reducing the supply of nutrient, as bacteria generally grow at the maximum possible rate in a given condition. Hegeman (1966c), for example, could find no evidence of feedback interactions by pathway intermediates on the mandelate enzymes of P.putida, but Sanwal (1970) quotes several cases in which the activity of the first enzyme of several amino acid degradative pathways was subject to modulation by the energy charge of the cell. It would be interesting to see whether pathways for growth substrates metabolised exclusively via the amphibolic pathways are also subject to modulation by energy charge.

In both anabolic and amphibolic pathways, the first committed step in a reaction sequence is subject to modulation. In a catabolic pathway, the first committed step might be entry into the cell, and certainly a pathway with an active transport system would be most economically controlled at the level of transport while a facilitated diffusion system could be effectively controlled at the level either of transport or of the first metabolic enzyme.

Feedback inhibition of transport has been recorded. Crabeel & Grenson (1970) have demonstrated that in the yeast Saccharomyces cerevisiae histidine uptake is subject to product inhibition by internal histidine. Kaback (1970) has found that

the phosphoenolpyruvate-phosphotransferase system in isolated membranes of E. coli is subject to rigorous control. At least two inhibitory sites in the membrane regulate the transport of glucose and related sugars, one for glucose-1-phosphate and related -1-phosphate esters (feedback inhibition) and one for glucose -6-phosphate and related -6-phosphate esters (product inhibition). The sites are separate, distinct, under independent control and antagonistic to one another. Product or feedback inhibition has been cited in other systems, for example, in sulphate transport in Salmonella typhimurium the approach to equilibrium by overshoot has been attributed to feedback inhibition by 3'-phosphoadenosine-5'-phosphosulphate (Dreyfuss & Pardee, 1966). Control of a facilitated diffusion system at the level of the first catabolic enzyme is known; glycerol uptake into E. coli may be controlled at the level of glycerol kinase which is subject to inhibition by fructose-1, 6-diphosphate (Zwaig & Lin, 1966).

During growth on two carbon sources, interactions at or close to the level of transport have been detected in some cases. On the one hand, McGinnis & Paigen (1969) have concluded that E. coli has a general regulatory mechanism, to be termed catabolite inhibition, which controls the activity of early reactions in carbohydrate catabolism allowing certain substrates to be used preferentially. In contrast, Clarke & Brammar (1964), working on the amidase system of P. aeruginosa, found no



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inhibition of uptake of gratuitous inducer in the presence of a growth substrate (succinate) which does cause repression of amidase synthesis.

McGinnis & Paigen (1969) observed catabolite inhibition in E. coli growing exponentially in medium with radioactive galactose or lactose as the sole carbon source when glucose was added to the culture. A 60% inhibition in the rate of utilisation of the radioactive compound was noted. The inhibition was completely abolished on the removal of glucose. In the cases of galactose and lactose, work with mutant strains showed the effect to be exerted either on transport or on the first catabolic enzyme. Glucose has been shown to inhibit the transport of galactose (Horecker, Thomas & Monod, 1960) and galactosides (e.g. Kessler & Rickenberg, 1963; Winkler & Wilson, 1967) in cell suspensions of E. coli. It is likely that catabolite inhibition is effective at the level of transport in these cases. Catabolite inhibition of the utilisation of maltose, mannose, arabinose, xylose and glycerol was also noted (McGinnis & Paigen, 1969). In the case of glycerol, it is possible that utilisation is subject to control at the level of the first enzyme because glycerol kinase is subject to inhibition by fructose - 1, 6 - diphosphate (Zwaig & Lin, 1966), although the modulator of catabolite inhibition is not yet known. The utilisation of glucose is itself subject to inhibition by glucose - 6 - phosphate and this may be a verification in whole cells of the inhibition of glucose transport

in isolated membrane vesicles (Kaback, 1970).

The enzymes of catabolic pathways seem to be subject to very few modulations, especially when a single carbon source is being utilised. However, when more than one carbon source is available enzyme inhibitions early in the catabolic pathway may operate in certain organisms to allow preferential use of one carbon source.

### 2. 2. 2. Regulation of gene expression.

Studies on the regulation of gene expression in bacteria have centred on the operon model (Jacob & Monod, 1961) for the control of the lac region of E. coli. The model proposed negative control of gene expression at the level of transcription by means of a cytoplasmic repressor. The repressor was coded by one regulatory gene (i) and bound to another regulatory site (o; operator) which was contiguous with the structural genes coding enzymes  $\beta$ -galactosidase,  $\beta$ -galactoside permease and thiogalactoside transacetylase. The presence of inducer removed the repressor from the operator thus allowing transcription and consequently protein synthesis.

This theory was verified by isolation of the repressor (Gilbert & Müller-Hill, 1966), a protein containing no detectable nucleic acid (Riggs & Bourgeois, 1968). The repressor bound specifically to the lac operator and was released by inducer

(Gilbert & Müller-Hill, 1967; Riggs, Suzuki & Bourgeois, 1970).

The repressor (i-gene product) is not the only protein regulating transcription of the lac operon. Another protein, the cyclic AMP receptor protein (de Crombrughe et al., 1971) must be bound to DNA in the p-region, which is contiguous with o but distal to the structural genes (Ippen, Miller, Scaife & Beckwith, 1968), for the correct binding of RNA polymerase. Regulated, cell-free transcription of lac mRNA proceeds only in the presence of cyclic AMP, to cause binding of the receptor protein to DNA, and of inducer, to remove the lac repressor from the operator (de Crombrughe et al., 1971). In the absence of cyclic AMP, no substantial synthesis of lac mRNA occurs.

The cell-free system thus has the properties both of induction, due to the lac repressor-inducer interaction, and of catabolite repression (Magasanik, 1961; to be discussed later) due to the cyclic AMP receptor protein - cyclic AMP interaction. The dominant control is the cyclic AMP concentration because it modulates binding of RNA polymerase to its substrate, but thereafter, transcription of lac mRNA is only possible when inducer is present. In whole cells, given exogenous inducer, the degree of induction of the lac operon will firstly depend on the level of cyclic AMP in the cell and then on the concentration of inducer in the cell. Intracellular inducer concentration in turn depends on the permeability of the cell

membrane and then on the degree of catabolite inhibition (p. 32) if any.

Genetic control of the gal operon in E. coli is exactly analogous to control in the lac operon (Miller, Varmus, Parks, Perlman & Pastan, 1971). The glycerol regulon in E. coli is subject to negative control (Cozzarelli, Freedberg & Lin, 1968) and catabolite repression is relieved by cyclic AMP (de Crombrughe, Perlman, Varmus & Pastan, 1969). It therefore seems likely that this type of genetic control is quite widespread. Indeed, as proposed by Jacob & Monod (1961) the repressible synthesis of enzymes in anabolic pathways is probably subject to a similar basic control system in which a cytoplasmic apo-repressor is activated by a small molecule (the end product of the biosynthetic pathway) to repress transcription. The similarity in the mechanism of the genetic control of inducible and repressible enzymes is emphasised by the regulatory properties of some mutant strains. Jacoby & Gorini (1969) isolated a mutant strain of E. coli in which arginine could induce rather than repress the synthesis of the enzymes of arginine anabolism, while Myers & Sadler (1971) have isolated a mutant strain of E. coli that is repressible rather than inducible for the lac operon. Over the years the Jacob-Monod model has been extended to include regulons (Maas & Clark, 1964; Cozzarelli et al., 1968) and operons under positive control, the best defined of which is the

arabinose system in E. coli (Englesberg, Irr, Power & Lee, 1965; Irr & Englesberg, 1971).

Proof that control of enzyme synthesis occurs at the level of transcription does not exclude translation as a further site of control (e.g. Cline & Bock, 1966). Miller et al., (1971) were unable to rule out translational control in the expression of the gal enzymes. Control at the level of translation has been shown for two enzymes of the arginine pathway (McLellan & Vogel, 1970) by an unknown mechanism. Much more information in defined systems is required to determine whether this is a general effect.

Another source of controversy over recent years has been catabolite repression (Magasanik, 1961). The term initially referred to the repression of the synthesis of inducible enzymes in E. coli, Aerobacter aerogenes and Salmonella typhimurium in the presence of glucose or related compounds like glucose - 6 - phosphate. The enzyme systems affected include those degrading lactose, galactose, glycerol, arabinose, L-tryptophan, and D-serine (de Crombrughe et al., 1969). Similar phenomena are widespread in bacteria (reviewed by e.g. Paigen & Williams, 1970). Schlegel & Trilper (1966) found that molecular hydrogen represses synthesis of enzymes of the Entner-Doudoroff pathway in Hydrogenomonas. In P. aeruginosa the enzymes of glucose catabolism are subject to repression in the presence of citrate (e.g. Hamlin, Ng & Dawes, 1967), and the amidase is

subject to repression in the presence of succinate (e.g. Clarke & Brammar, 1964). Multisensitive end-product repression in the mandelate pathway of P. putida (Mandelstam & Jacoby, 1965) is another example of catabolite repression.

Catabolite repression has been studied in detail in the lac and gal operons of E. coli. Lengeler (1966) and Adhya & Echols (1966) showed that catabolite repression of the gal operon comprised two separate mechanisms. One of these effects involved transport of the inducer into the cell and has been termed catabolite inhibition by McGinnis & Faigen (1969) (this thesis, p. 32). The other effect, the "real" catabolite repression, has been exhaustively studied by Perlman & Pastan and their colleagues (e.g. de Crombrughe et al., 1971). Repression is mediated by the concentration of cyclic AMP in the cell (see p. 34). Glucose and other compounds which cause "catabolite repression" alter the intracellular concentration of cyclic AMP. A complete mechanistic explanation of this effect is not yet available. Glucose can cause E. coli to lose cyclic AMP to the growth medium (Makman & Sutherland, 1965). Adenyl cyclase activity may be modulated both by altered substrate (ATP) and allosteric effector (pyruvate) concentrations (e.g. Tao & Lipmann, 1969) which in turn reflect the carbon source in use.

Definitive proof of the role of cyclic AMP in the regulation of gene expression in the lac and gal operons and

even the evidence of its involvement in repression of the synthesis of other inducible enzymes in E. coli and similar organisms (de Crombrughe et al., 1969) does not mean that cyclic AMP is the effector of catabolite repression throughout the bacterial world. Sanwal (1970) gave this warning " the 'unity of biochemistry' concept which has been so useful in the study of other biochemical phenomena has paid little dividend in the field of controls. Unity of principles underlying controls certainly exists but the manifestations are necessarily as divergent as the diversity of the organisms." In view of the wide variety of catabolite repressions in different organisms (p.56 and Paigen & Williams, 1970) it seems unlikely that the cyclic AMP mediated system will be the only one in existence. It is difficult to envisage how, for example, multisensitive end-product repression (p.16 and Mandelstam & Jacoby, 1965) could be mediated by cyclic AMP. Each repressor would require a binding site on adenylyl cyclase, and if each repressor lowered the concentration of cyclic AMP how would that repressor induce the enzymes for its own catabolism?

Thimann (1963) prefaced his book "The Life of Bacteria" with a quotation from Leeuwenhoek's draughtsman "Dear God, what marvels there are in so small a creature." Some of these marvels are now better understood. Permeation of the cell membrane, however complex, is found to be an integral part of the metabolism of the cell. As such it can, but need not, be the

site of action of control mechanisms (see p. 31). A large net of intracellular control sequences is known but even these do not seem adequate to cover the known situations. This in itself is a sufficient stimulus for further research.

### 3. Scope of this thesis.

This project was initiated with the aim of examining the permeability of the cell membrane of bacterium NCIB 8250 to aromatic intermediates of the mandelate pathway. It was intended that the properties of these systems should then be examined, as well as their role in the regulation of metabolism.

The filtration technique was chosen to follow the uptake of material into cells in suspension. However, although this is a well-established technique for other systems (Britten, Roberts & French, 1955), considerable problems were encountered in developing rapid, sensitive, accurate and reproducible methods under the required range of conditions with bacterium NCIB 8250. As the work progressed, it became obvious that the transport systems for aromatic compounds were very difficult to detect by direct assay and probably did not lead to the concentration of large amounts of substrate in cell pools. The approach to the problem was, therefore, broadened.

Livingstone (1970) had just verified and extended the work on the regulation of the mandelate pathway initiated by Kennedy & Fewson (1968 ab). Multisensitive end-product



repression had been reported in the mandelate pathway of P. putida (Mandelstam & Jacoby, 1965). It was, therefore, decided to examine the mandelate pathway of bacterium NCIB 8250 for catabolite repression and other control circuits in the hope that physiological behaviour during growth would provide evidence for transport systems which could then be tested directly.

Growth in dual substrate media was studied, where diauxie and the patterns of substrate utilisation were the tests for catabolite repression and other possible interactions (Monod, 1942; Hamilton & Dawes, 1959; Paigen & Williams, 1970). Conditions for growth experiments had to be adapted from those used in the laboratory (Fewson, 1967b) as they were not really suited for routine use in the new type of experiments. Radiochemical assays to study the utilisation of aromatic substrates were developed to provide a more complete account of the growth patterns recorded.

On the basis of the growth characteristics of the organism, and with complementary evidence from enzyme assays and the uptake of material into cells in suspension, a number of cases of preferential use of substrates have been described and three permeation systems have been proposed, but their properties have not been examined.

## M E T H O D S .

### 1. BACTERIOLOGICAL TECHNIQUES.

#### 1. 1. Organisms.

Bacterium NCIB 8250 was obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, where it is maintained as an Achromobacter sp.. A mutant strain of bacterium NCIB 8250, deficient in L-mandelate dehydrogenase (strain N.F.1408 isolated by Livingstone, 1970), was also used in some experiments.

Escherichia coli ML 308 was obtained as stock number 15224 from the American Type Culture Collection, Rockville, Maryland, U.S.A. The mutant strain of E. coli ML 308, deficient in the lactose permease, was isolated in this laboratory by Dr. A.G. Robertson.

#### 1. 2. Storage of organisms.

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

#### 1. 3. Growth media.

All glassware used for growth of organisms was washed

by boiling in either 10% (v/v) nitric acid or 1% (w/v) Haemosol (Meinecke & Co. Inc., Baltimore, Maryland, U.S.A.) followed by thorough rinsing in tap water and then in glass-distilled water. Glassware containing defined medium was washed with nitric acid before use.

1. 3. 1. Media for the growth of bacterium NCIB 8250.

The "basal medium" used throughout this work consisted of 2g  $\text{KH}_2\text{PO}_4$  + 1g  $(\text{NH}_4)_2\text{SO}_4$  in 1l glass-distilled water adjusted to pH 7.0 with 5M - NaOH (Fewson, 1967b). "Salts medium" consisted of basal medium + 20ml/1 sterile 2% (w/v)  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  added aseptically after sterilisation of the basal medium.

In the preparation of growth media, if the carbon source could be autoclaved, the basal medium + carbon source was adjusted to pH 7.0 after addition of carbon source. Heat labile carbon sources, and those added during growth of a culture on another carbon source, were dissolved in water and the pH adjusted to 7.0 before Millipore filtration.

250ml and 500ml Erlenmeyer flasks were plugged with Dispo Plugs (T.1385; American Hospital Supply Corp., Edison, New Jersey, U.S.A.). 1l Sidearm flasks had a silicone rubber bung (Esco Rubber, Ltd., London E.C.3.) in the main neck and a Morton Culture Tube Closure with Fingers (T 1390-20; Scientific Products, American Hospital Supply Corp., Evanston, Illinois, U.S.A.) on the sidearm. 2l and 10l flasks were plugged with non-absorbent cotton wool. All plugs were covered with aluminium foil prior

to autoclaving.

### 1. 3. 2. Media for the growth of *Escherichia coli*.

The salts medium used for the estimation of cell water/dry wt. in *E. coli* ML 308 was that of Winkler & Wilson (1966). Studies on the uptake of thiomethylgalactoside by *E. coli* used the salts medium described by Holms & Bennett (1971).

### 1. 3. 3. Sterilisation.

All media, with the exception of the compounds listed below, were sterilised by steam at 109°C for periods of time which varied with the volume of the liquid. The efficacy of sterilisation was originally determined by Fewson (unpublished results) and was always checked by the colour change of Browne's tubes (Albert Browne, Ltd., Leicester). There was a 3% loss of volume from flasks on autoclaving. The following compounds, because of their probable heat lability, were sterilised by filtration through Millipore filters (GSPW 04700) in Sterifil units (XX110 4700: Millipore Corp., Bedford, Mass, U.S.A.), stored in sterile containers and added aseptically to sterile salts media: DL-mandelate, phenylglyoxylate, benzyl alcohol and catechol.

Radiochemicals used in growth experiments were dissolved without added carrier in distilled water and sterilised by filtration in Nalgene disposable, sterile filter units (120; Nalge Sybron Corp., Rochester, New York, U.S.A.).

### 1. 3. 4. Storage of media.

All media with a volume of 100ml or less were stored at 4°C until required. Media for growth and substrate analysis were prepared within a fortnight of use and stored at room temperature. Otherwise, media were stored in the hotroom at 30°C till required.

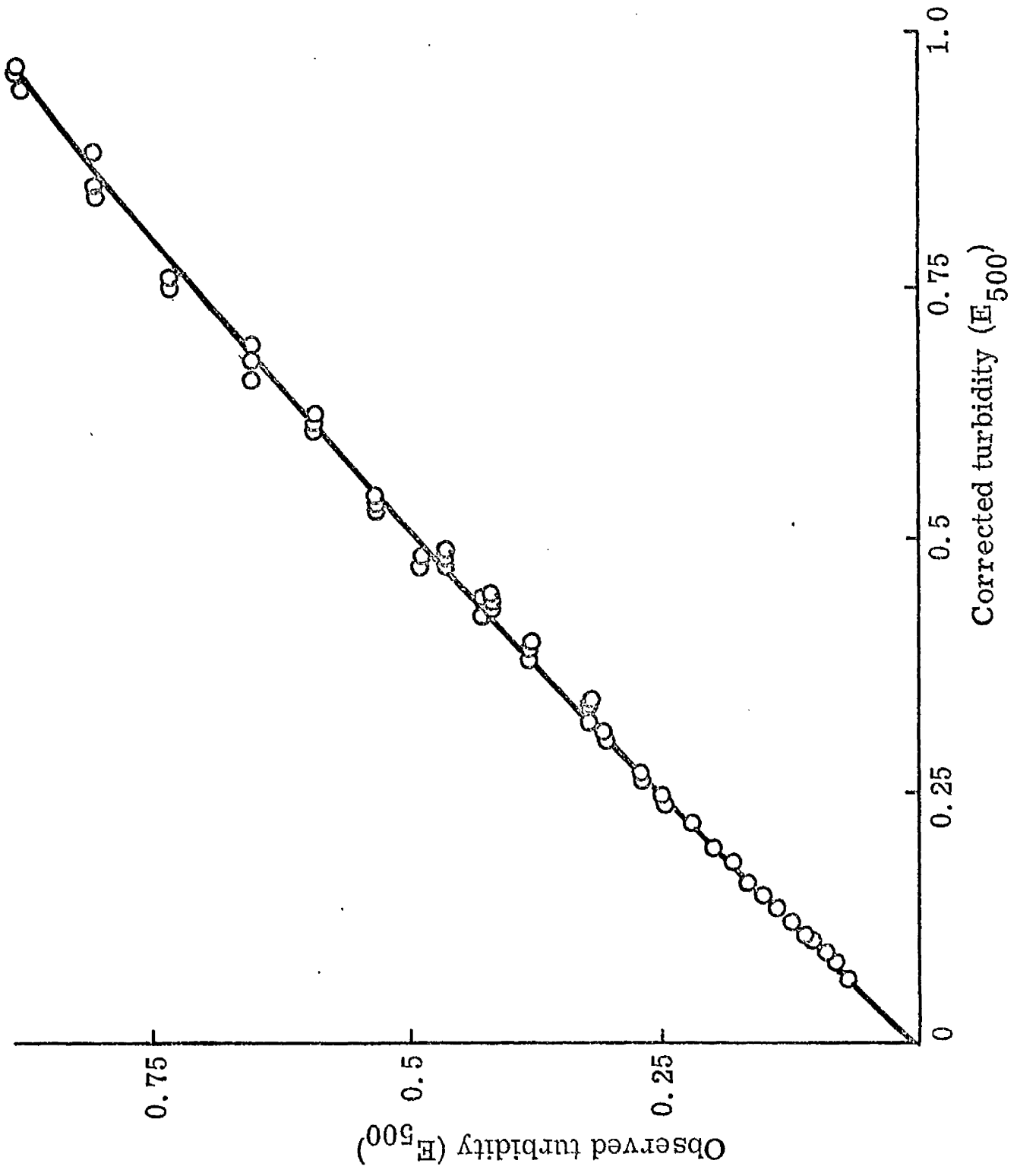
### 1. 4. Measurement of growth.

Growth was measured turbidimetrically at 500nm in a Unicam SP 800 Ultraviolet Spectrophotometer (Pye Unicam Instruments Ltd., Cambridge) connected to a Servoscribe Chart Recorder (Kelvin Electronics Co., Wembley, Middlesex). 1cm Light path glass cuvettes were used, up to four at a time in the automatic sample changer on the Unicam SP 800. No reference cuvettes were used as the samples were read against air. The average of the first four values recorded for a given sample was corrected by subtraction of the E<sub>500</sub> of the uninoculated medium in the same cuvette and sample position. This net observed turbidity was corrected for non-linearity in the response of the Unicam SP 800 by reference to a standard curve (Fig. 5). The turbidimetric response of the spectrophotometer was checked periodically, and after servicing, using suspensions of polystyrene latex in solutions of Triton X-100 (Thorpe, Horsfall & Stone, 1967).

In growth experiments, one cuvette was used for each culture. The cuvette was not rinsed between samples; when the sample was discarded the inverted cuvette was tamped down on Kleenex tissues (Kimberley-Clark Ltd., Kent) to remove liquid and then stored inverted on paper tissues ready for the next sample.

Fig. 5. Turbidity correction curve.

Bacterium NCIB 8250 was grown for 15h from a 1% ( $v/v$ ) inoculum in 0.6l 5mM-benzyl alcohol-salts medium (Methods, p. 46). A 100ml volume of this culture was harvested at 12,000g for 20min at 4°C (M.S.E. "13" Refrigerated Centrifuge) resuspended to 100ml in basal medium and kept at 27°C. This 100ml suspension was progressively diluted in basal medium. At each dilution a portion of the suspension was diluted  $1/2$ ,  $1/5$  and  $1/10$  in basal medium and the turbidity of that sample and its dilutions were measured. The actual turbidity of each sample was calculated from those dilutions whose turbidities lay on the linear section of the curve.



### 1. 5. Growth of bacterium NCIB 8250.

The methods used for growing bacterium NCIB 8250 were modifications of those used by Fewson (1967b), Kennedy & Fewson (1968a,b) and Livingstone (1970).

Inocula for growth of bacterium NCIB 8250 in defined media were prepared immediately before they were required. 0.1% (v/v) inocula from the nutrient broth stock cultures (Methods, 1. 2.) were added to 100ml amounts of nutrient broth contained in 500ml Erlenmeyer flasks, and the cultures were incubated without shaking for 24h at 30°C.

Subsequent cultures were grown at 30°C. Cultures of small volume (30-50ml) were grown in 250ml Erlenmeyer flasks on a rotary shaker (Mk. V; L.H. Engineering Co., Stoke Poges, Bucks.) at about 180 oscillations/min. Larger cultures were grown in flat-bottomed flasks under conditions of vigorous aeration produced by 45mm polypropylene-coated stirring bars in the apparatus of Harvey, Fewson & Holms (1968). This apparatus consists of magnetic drive assemblies which effect high speed rotation of the magnetic stirring bars.

#### 1. 5. 1. Inocula for experiments on growth and substrate utilisation.

Preliminary experiments were done with 4% (v/v) inocula from a 24h nutrient broth culture used directly and without harvesting, but inocula preinduced to defined media were adopted for later experiments (Results p. 85). 11 Salts medium containing the



appropriate carbon source (DL-mandelate, benzyl alcohol or benzoate as detailed in Legends to the appropriate Figures) and a stirring bar was prepared in a 2l flat-bottomed flask. As inoculum, a 24h nutrient broth culture was used, 0.5ml with benzoate, 1.0ml with benzyl alcohol and 4ml with mandelate as carbon source. The culture was grown at 30°C for about 9h (overnight) on the apparatus described by Harvey et al. (1968) and used for inoculation when it reached the required turbidity (detailed in Legends to the appropriate Figures). In experiments where the inoculum was harvested, 40ml portions of the overnight culture were centrifuged in sterile 50ml polycarbonate bottles (59416, Oak Ridge Type; Measuring and Scientific Equipment (M.S.E.) Ltd., Crawley, Sussex) at 12,000g for 10min at 4°C (M.S.E. "13" Refrigerated Centrifuge). A uniform resuspension was prepared by blowing chilled basal medium from a pipette on to the pellet, with brief agitation on a vortex mixer ("Whirlymix"; Fisons Scientific Apparatus, Ltd., Loughborough). The volume of basal medium used was chosen to give a starting turbidity ( $E_{500}$ ) of about 0.02 with a 1-2% (v/v) inoculum. The chilled suspensions were bulked and used for inoculation within 5min.

1. 5. 2. Experiments to follow growth and substrate utilisation simultaneously.

Experiments were done at 30°C with 300ml batches of medium in 1l sidearm flasks without forced aeration on the apparatus described by Harvey et al. (1968). To the basal medium were added 6ml sterile 2% (w/v)  $MgSO_4 \cdot 7H_2O$ , 4.5ml volumes of sterile

carbon sources at 100mM (200mM in the case of DL-mandelate) and water to keep the final volumes the same in each flask.

At this point a sample was taken from each flask to provide pre-inoculation blanks for turbidity readings (Methods, 1. 4) and background values for liquid scintillation counting. A 4.5ml sample was taken by 5ml plastic pipette (7529; Falcon Plastics, Oxnard, California, U.S.A.) into a 6 x  $\frac{3}{4}$  in test tube. Immediately a 1.0ml portion of the sample was taken by 1ml plastic pipette (7503; Falcon Plastics) on to 1.0ml 0.2M-HCl in a Polytube (15x41mm; Metal Box Co. Ltd., Plastics Group, Portslade, Sussex) and mixed by rolling between the hands. When all the flasks had been sampled, the balance of each sample was used as a blank for the turbidity readings (Methods, 1.4).

5ml Sterile, "carrier-free", radiochemically labelled ( $3\mu\text{Ci/ml}$ ) carbon source (Methods, 1. 3. 3.) were then added to each growth flask.

Flasks were inoculated at 30s intervals to permit processing of samples. One minute was allowed for temperature equilibration and mixing after inoculation, then a 4.5ml sample was taken and manipulated as described above for the blanks. Cultures were sampled at intervals throughout growth. Plastic pipettes were re-used but discarded when moisture adhered to them.

Samples, acidified to remove  $^{14}\text{CO}_2$ , were left in open Polytubes for 90min with occasional mixing and the tubes then capped. 250 $\mu\text{l}$  Portions of these samples were added to scintillation

fluid in a scintillation vial at some time in the next 48h. This treatment was necessary as illustrated for a sample taken on to acid from a culture of bacterium NCIB 8250 grown to stationary state in 1.5mM- [carboxy-<sup>14</sup>C] benzoate-salts medium. Portions of this sample were counted by liquid scintillation spectrometry immediately after acidification and at intervals after the sample had been mixed open to the atmosphere (above). Between 2h and 48h after sampling, the residual radioactivity remained constant and most of this radioactivity was retained on a filter membrane (0.22µm pore diameter). It was assumed that this treatment removed effectively all the <sup>14</sup>CO<sub>2</sub> present.

Time after sampling h	% Original radioactivity	
	Acidified sample	Filter membrane
0	10	not tested
2	2	not tested
24	2	1.5
48	2	not tested

The radioactivity that remained in stationary state cultures after acidification, was assumed to have accumulated during growth and to have the same specific rate of increase

as growth. Benzoate utilisation at time, t, was thus calculated as:

$$U = \frac{(R_0 - R_t) \cdot C}{R_0 - R_{ss}}$$

where U = benzoate utilisation, nmol/ml culture;  $R_0$  = radioactivity at zero time, pCi/ml culture;  $R_{ss}$  = radioactivity in stationary state, pCi/ml culture;  $R_t$  = radioactivity at time, t, pCi/ml culture; C = concentration of radioactively labelled carbon source at zero time, nmol/ml culture.

1. 5. 3. Growth of cells for cell-free assay of mandelate pathway enzymes.

The conditions of Livingstone (1970) were used. 4l Salts medium with a stirring bar and containing 5mM-L-mandelate or 10mM-glutamate + 1mM-thiophenoxyacetate or 5mM-benzyl alcohol was prepared in a 10l flat bottomed flask. As inoculum, 10ml of a 24h nutrient broth culture was added to the flask. The culture was grown for 10-15h at 30°C on the apparatus described by Harvey et al. (1968). An additional quantity of carbon and energy source equal to the amount present at inoculation and in a volume not exceeding 200ml was then added to the culture. Threequarters of a

-21-

generation time later, the culture was removed from the growth apparatus and the cells harvested at  $6000g$  for 20min at  $4^{\circ}C$  (M.S.E. "Mistral 6L" Refrigerator Centrifuge). The supernatant solution was discarded and the pellet was washed by resuspension in ice-cold, sterile, distilled water. The cells were recentrifuged at  $12,000g$  for 25min at  $4^{\circ}C$  (M.S.E. "13" Refrigerated Centrifuge), the supernatant solution discarded, the cells weighed and stored at  $-60^{\circ}C$ .

#### 1. 5. 4. Growth of cells for cell suspension assays.

Batches of 5l nutrient broth (filtered through  $0.22\mu m$  pore diameter Millipore membranes to remove particulate matter) or salts medium containing the appropriate carbon source were prepared in 10l flat bottomed flasks containing 45mm stirring bars. As inoculum a 24h nutrient broth culture was used, 1.0ml with nutrient broth benzoate-salts and succinate salts, 2.0ml with benzyl alcohol-salts and 5ml with mandelate-salts and phenylglyoxylate-salts media. These cultures were grown for about 10h (overnight) at  $30^{\circ}C$  on the apparatus described by Harvey et al. (1968) and harvested at the required turbidity detailed in Legends to the appropriate Figures. Flasks were placed on ice and harvested by batch centrifugation at  $6000g$  for 20min at  $4^{\circ}C$  (M.S.E. "Mistral 6L" Refrigerator Centrifuge). The supernatant fluid was discarded and the pellet was washed by resuspension in ice-cold  $0.05M-KH_2PO_4$  adjusted to pH 7.0

with 5M-NaOH. The cells were recentrifuged in tared 50ml centrifuge tubes (59407: M.S.E.) at 12,000g for 20min at 4°C (M.S.E. "13" Refrigerated Centrifuge), the supernatant fluid discarded, the cells weighed, stored on ice and used within 6h.

1. 6. Growth of *Escherichia coli*.

The method used to grow *Escherichia coli* in defined media was that of Holms & Bennett (1971). For growth in basal medium containing casamino-acids (Winkler & Wilson, 1966; this thesis, Methods, Section 2.1.2.) the technique of Holms & Bennett was adapted to the use of 5l culture volumes in 10l flasks (e.g. Methods, p. 46).

## 2. EXPERIMENTS WITH CELL SUSPENSIONS.

### 2. 1. Quantitative measurements in cell suspensions.

#### 2. 1. 1. Correlation between bacterial dry weight and turbidity.

3 dram Trident containers (Johnsen & Jorgensen, Ltd., London) were dried to constant weight at 105°C.

11 Batches of salts medium containing 2.5mM- and 3.75mM- benzyl alcohol, both in duplicate, were prepared in 21 flat-bottomed flasks containing 45mm stirring bars. As inoculum, 5ml of a 24h nutrient broth culture was added to each flask. The cultures were grown for 15h at 30°C on the apparatus described by Harvey et al. (1968). Duplicate cultures were bulked and the turbidity of each bulked culture measured.

Duplicate 600ml batches of each bulked culture (with washings from measuring cylinders used) were harvested in 750ml buckets at 6000g for 45min at 4°C (M.S.E. "Mistral 6L" Refrigerator Centrifuge). The supernatant fluid was discarded and the pellet washed with chilled sterile, distilled water. The cells were resuspended in chilled, sterile, distilled water, transferred by Pasteur pipette to 50ml centrifuge tubes with thorough rinsing and the volume made up to about 40ml with sterile water. The cells were recentrifuged at 18,000g for 45min at 4°C (M.S.E. "High Speed 18" Refrigerator Centrifuge), the supernatant fluid discarded and the pellets washed

in chilled, sterile, distilled water. The pellets were resuspended in chilled, sterile, distilled water and transferred to tared dry weight vials and dried to constant weight at 105°C.

Dry weights of 0.1508g/600ml culture (values of 0.1507 and 0.1509g) and 0.0997g/600ml culture (values of 0.990 and 0.1004g) were observed at corrected turbidities ( $E_{500}$ ) of 0.885 and 0.635 respectively. This gave an average value of 273µg dry wt./corrected turbidity unit at 500nm on a Pye Unicam SP800 Ultraviolet Spectrophotometer.

### 2. 1. 2. Estimation of cell water.

The method was developed from that of Winkler & Wilson (1966). All liquids were Millipore filtered (0.22µm pore diameter) and cotton-wool bungs were wrapped with muslin to reduce fluff and dust in cell pellets.

Pellets of cells, grown and harvested as described in Methods (p.51), were resuspended to 30mg wet wt./ml in the appropriate chilled salts medium or buffer (Results, p.80) and stored on ice. The resuspension was divided into two portions. The first portion was used to measure the bacterial dry weight present; 3 x 35ml samples were centrifuged in 50ml centrifuge tubes at 18,000g for 45 min at 4°C (M.S.E. "High Speed 18" Refrigerator Centrifuge). The supernatant fluid was discarded and the pellet washed in chilled, sterile, distilled water. The pellet was resuspended in chilled, sterile, distilled water to about 40ml and recentrifuged in the same tubes at 18,000g for 45 min at 4°C. The dry weight was measured as described in Methods 2. 1. The second portion of the cell re-



suspension was used to estimate intracellular water. 3 x 35ml Samples of cell suspension were pipetted into a 100ml ground-glass stoppered test tube; 3.0ml of radioactively labelled material was added and thoroughly mixed. (Methoxy -  $^3\text{H}$ ) inulin, inulin (carboxylic acid -  $^{14}\text{C}$ ), glucose -  $^{14}\text{C}(\text{U})$ , fructose -  $^{14}\text{C}(\text{U})$  and ribose -  $^{14}\text{C}(\text{U})$  were used. Tritiated solutions were added at 10 $\mu\text{Ci/ml}$  while  $^{14}\text{C}$  - labelled compounds were added at 3 $\mu\text{Ci/ml}$ . Great care was necessary to ensure that the radioactive material was in solution. Solutions of inulin were heated to 50°C for 30 min before use. 3 x 35ml Portions of the radioactively labelled cell suspension were pipetted into 50ml tared centrifuge tubes and centrifuged at 12,000g for 3.0 min at 4°C (M.S.E. "13" Refrigerated Centrifuge). The supernatant fluid was decanted and 4 x 20 $\mu\text{l}$  samples by Eppendorf pipette (Eppendorf Marburg Mikropipet; Netheler & Hinz GmbH, Hamburg, Germany) were counted by liquid scintillation spectrometry. The centrifuge tube was swabbed and weighed. The cell pellet was resuspended to 10ml in a graduated glass test tube and 4 x 200 $\mu\text{l}$  samples by Eppendorf pipette counted by liquid scintillation spectrometry. The graduated test tubes were calibrated gravimetrically from the weight of distilled water at known temperature when filled to the mark. Eppendorf pipettes were also calibrated gravimetrically from the weight of distilled water at a known temperature dispensed by the pipette. The pipettes tested delivered the stated volume within 0.1%.

Cell water was expressed as a function of dry weight and

was calculated from the following equations:

$$V = \frac{T}{S} \dots \dots \dots (1)$$

where V = volume of extracellular fluid in cell pellet,  $\mu$ l;  
T = total radioactivity in cell pellet, pCi; S = supernatant  
radioactivity per unit volume, pCi/ $\mu$ l.

$$IW = \frac{P - D - V^*}{D} \dots \dots \dots (2)$$

where IW = intracellular water,  $\mu$ l/mg dry wt.; P = wet wt. of pellet,  
mg; D = dry wt. of pellet, mg (the observed dry weight after  
correction for the dilution on addition of radiochemical label); and  
V\* = wt. of extracellular fluid in cell pellet, mg (the same numerical  
value as V in equation (1) ).

2. 1. 3. Quantitative relations for arithmetic  
manipulation of data.

In all experiments the quantitative measurement of bacteria  
present was turbidity at 500nm on a Unicam SP800 Ultraviolet Spec-  
trophotometer (Methods, 1. 4). Results were therefore expressed as  
turbidities unless different units were obviously more suitable.

The following relationships were used in calculations:

273 $\mu$ g dry wt./corrected turbidity unit (Methods 2. 1. 1.)

1.65 $\mu$ l cell water/mg dry wt. (Results p.80), this value of  
cell water excludes water in the cell wall. These two relations  
were combined to give

0.451 $\mu$ l cell water/corrected turbidity unit.

However, other relationships are available.

In the experiment to determine dry wt./turbidity (Methods, 2. 1. 1), the bulked cultures were also used to estimate protein/turbidity. Protein was measured by the method of Kennedy & Fewson (1968b).

The relationship of protein to turbidity, 166 $\mu$ g bovine serum albumin equivalent/corrected turbidity unit at 500nm was the mean of 163 and 169 $\mu$ g/corrected turbidity unit.

Two different dry weight samples from the same experiment (Methods, 2. 1. 1.) were subsequently subjected to duplicate elementary analyses (numbers 5758 and 5759; Weiler & Strauss Microanalytical Laboratory, Oxford, England). The results of these analyses were averaged and expressed as percentages of the dry weight: C, 45.8; H, 6.8; N, 13.1; P, 2.1; S, 0.6; Ash, 10.7.

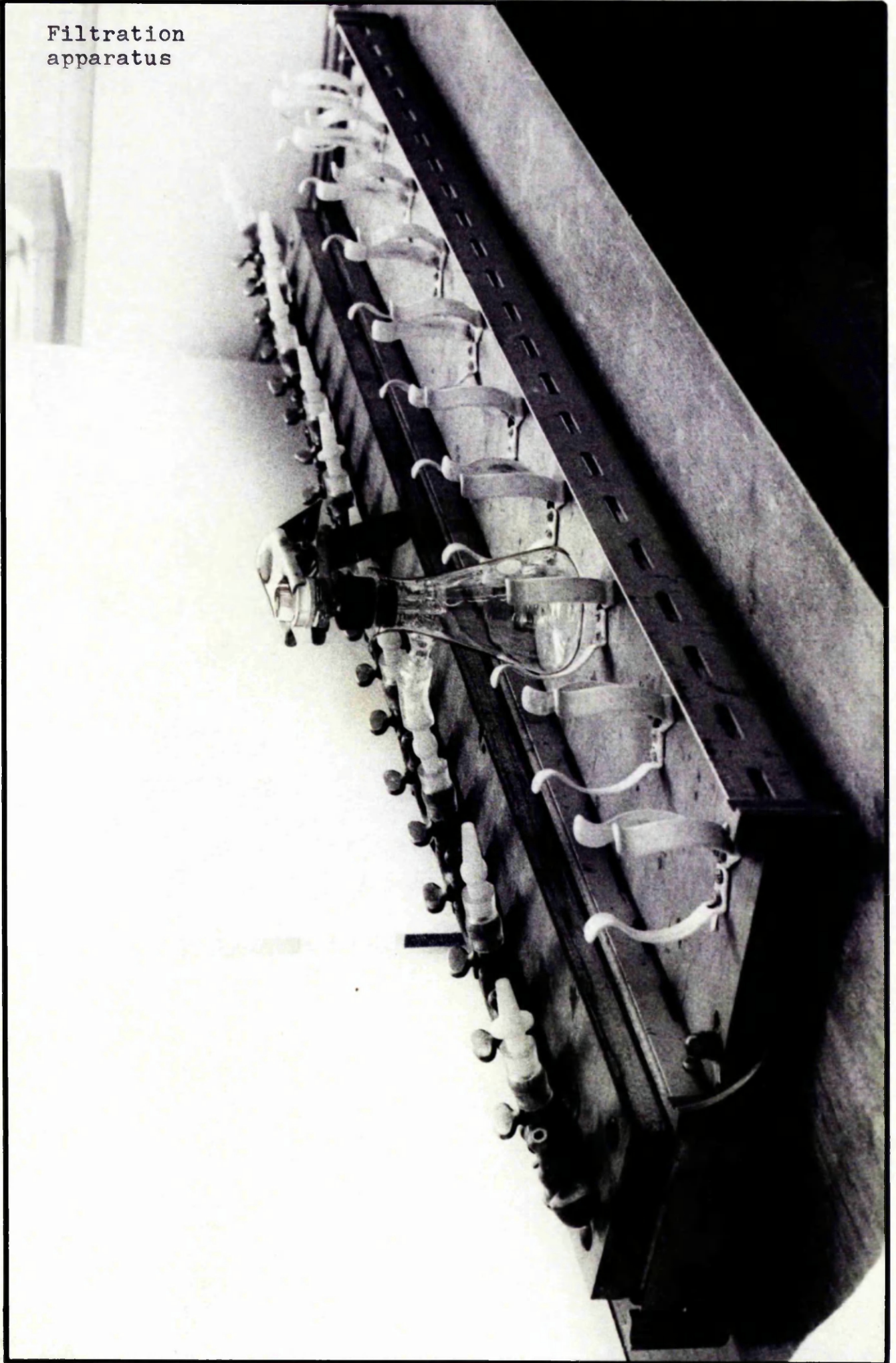
## 2. 2. Construction of filtration apparatus for assays of uptake of radioactivity into cell suspensions.

Samples were harvested by filtration under vacuum on an apparatus (Fig. 6) consisting of units derived from Millipore XX1002500 assemblies.

An angle iron skeleton (Handy Angle, Hayes, Middlesex) was mounted at the corners on rubber feet (Handy Angle). Ten 125ml thick-walled conical flasks with sidearm (XX1002505; Millipore) could be accommodated in line in spring clips (Half clips; A. Gallenkamp & Co. Ltd., Stockton-on-Tees, Durham) on a horizontal  $\frac{3}{4}$ in blockboard base (43 x 6in). A manifold of gas taps, one tap per flask, was mounted horizontally behind the flasks and level with the sidearm. To facilitate rapid changing of flasks, 2 in

Fig. 6.

Filtration  
apparatus



lengths of vacuum tubing (NT19; Portex, Ltd., Hythe, Kent) were attached individually to gas taps and to flask sidearms and corresponding pieces of tubing joined by polypropylene connectors (Portex).

A trap was placed between the manifold and the main vacuum line and a simple vacuum gauge (GJ 330/350; Gallenkamp) inserted in the line. The vacuum was produced by a Speedivac vacuum installation unit (Type 420/50TA; Edwards High Vacuum Ltd., Crawley, Sussex).

The Millipore assembly was basically a sintered glass support for a 25mm diameter filter membrane. This support was connected to the vacuum supply through the bung in the 125ml conical flask. A funnel was clamped over the filter membrane. The Millipore funnel was replaced by a short (0.5in) perspex funnel of the same dimensions as the base of the Millipore component. These perspex funnels were machined by Mr. N.L. Harvey of this Department.

The apparatus was placed parallel to and some 4in back from the edge of the bench. An Eppendorf stand was placed at the right of the assembly. Stocks of numbered and filled scintillation vials were stored on a shelf to the right of the assembly, while vials in use were placed, open, behind the apparatus. To the left, an ice-water slurry contained the Zippettes (2ml size; Jencons Ltd., Hemel Hempstead, Herts.) used to dispense chilled wash fluids. The 4in space in front of the apparatus was lined with paper towels to absorb water dripping from Zippettes.

## 2. 3. Cell suspension assays for substrate uptake, incorporation and utilisation.

### 2. 3. 1. General techniques in cell suspension assays

Assays were done at 30°C in 10ml final volume cell suspensions (called "reaction media") contained in 50ml Erlenmeyer flasks which were shaken at 110 strokes/min in a water bath (I.H. 350/354; Gallenkamp). Complete reaction medium contained 500µmol  $\text{KH}_2\text{PO}_4$  (adjusted to pH 7.0 with NaOH), 50mg wet wt. cells, and the organic compounds indicated in the Legends to the appropriate Figures. Incomplete reaction media of 7.5ml volume were prepared the evening before an experiment and stored overnight at 4°C. This solution contained all the organic compounds, including radioactive label, and 75% of the phosphate buffer. Initiation of the reaction with cells suspended in the phosphate buffer completed the reaction medium. Cell pellets (Methods, p.51) were resuspended in chilled 0.05M- $\text{KH}_2\text{PO}_4$  (adjusted to pH 7.0 with NaOH) and stored on ice. Intracellular water in the stock suspension was estimated from the turbidity of the suspension.

### 2. 3. 2. Manipulative procedures in cell suspension assays.

Incomplete reaction media (7.5ml) and 5ml portions of the stock cell suspension were pre-incubated separately in 50ml Erlenmeyer flasks in the shaking water bath while the filtration apparatus was set up for the assay, i.e. about 5min. Open scintillation vials

containing scintillation fluid were placed, with caps, behind the apparatus. Filter membranes (11307025; 0.22µm pore size, 25mm diameter, "weight constant" type; Sartorius Membranfilter GmbH, Göttingen, Germany.) were mounted eccentrically on the filtration units, fractionally over the edge of the filter base to allow rapid removal. Filters were thoroughly wetted before the funnel was clamped on, and then left under vacuum. Under these conditions filter membranes maintained the applied vacuum (up to 740mm Hg); presumably surface tension in the pores withstood the pressure. Liquid applied to these membranes immediately started to filter and filtration was complete in 1-3s. The use of dry membranes gave a poor vacuum and samples filtered slowly because residual dry areas allowed considerable air flow. When incompletely wetted, filter membranes rapidly dried out.

The incomplete reaction medium was sampled (2 x 20µl by Eppendorf pipette) to estimate the specific radioactivity in that assay. This sample was taken before initiation and corrected for subsequent volume change, because some radioactivity was lost from the reaction medium in the form of  $^{14}\text{CO}_2$  and it was not feasible to take time early in an experiment for these samples. Experiments were initiated by the addition of cells in a large volume (2.5ml), with the shaker already switched on, in order to promote rapid mixing of the complete reaction medium.

At intervals, 100µl samples by Eppendorf pipette were taken from the reaction medium. The shaker on the water bath had to be switched

off for some three seconds to do this, and experience was required to successfully remove samples from the shallow suspension.

The sample was then streaked on a filter membrane, to increase filtration speed and improve subsequent washing. Streaks were kept just away from the funnel which marked the edge of the sintered glass base, where filtration was slow. Filtration was complete within two seconds for most cell types, e.g. those grown in nutrient broth, benzoate -, benzyl alcohol -, phenylglyoxylate - and mandelate-salts media.

Wash-fluid was added after the liquid being filtered had disappeared, because washing before the reaction medium disappeared caused spuriously high results. The wash fluids were dispensed by Zippette. A 1ml wash took about 4s to filter. The clamp and funnel were removed as the last of the liquid disappeared and the filter was lifted, by duck-billed forceps, directly into scintillation fluid in the open scintillation vial. The vial was capped and swirled briefly. The vacuum to the filter was switched off as the membrane was removed, to maintain vacuum in the system. Samples could be taken at 20-25s intervals.

In some experiments, substrate decarboxylation was studied. 100µl samples were taken on to 0.1M-HCl in Polytubes (15 x 41mm; Metal Box) which were then treated as the corresponding samples in growth experiments (Methods, p.48).



### 3. ANALYTICAL METHODS.

#### 3. 1. Liquid scintillation spectrometry.

##### 3. 1. 1. Liquid scintillation fluid.

To 1l toluene (A.R., British Drug Houses Ltd., Poole, Dorset) was added 300ml ethanol (fermented, absolute; Burroughs Wellcome & Co., London) and 5g 2,5-diphenyloxazole (puriss; Koch-Light Laboratories, Colnbrook, Essex). 13ml Portions of this fluid were dispensed by Zippette (30ml size) into glass vials (6001008; Packard Instrument, Downers Grove, Illinois, U.S.A.) with disposable plastic caps (22R3; Metal Box Co. Ltd., Plastics Group, Portslade, Sussex).

Ethanol was present in the scintillation fluid to dissolve water associated with samples studied. 13ml Scintillation fluid dissolved the water associated with a damp filter membrane to give a single liquid phase at counting temperature ( $7^{\circ}\text{C}$ ). The standard volume of fluid had a capacity for 250 $\mu\text{l}$  aqueous solution at about 0.1M and at counting temperature.

Neither chemiluminescence nor phosphorescence was detected with this scintillation fluid under the conditions used. A count of about 20 c.p.m. per vial over machine background (about 30 c.p.m.) was traced to the alcohol in the fluid and was presumably caused by radioactivity in fermented alcohol which is effectively derived

from plant material (Rapkin, 1964).

### 3. 1. 2. Apparatus.

Samples were counted in Philips Liquid Scintillation Analysers (PW 4510/00; Philips Scientific Equipment, Eindhoven, Netherlands) fitted with calculators to convert c.p.m. to absolute units.

Efficiency was calculated by the channels ratio technique (Wang & Willis, 1965). Polar quenching agents such as ethanol and chloroform gave overlapping efficiency curves in toluene-based scintillation fluids and wide-range efficiency curves were prepared with chloroform as the quenching agent. For each curve, ten vials were prepared containing a known quantity of radioactive reference material (or materials) and varying quantities of chloroform. The radioactive reference materials used were toluene -  $^{14}\text{C}$  (batch 7,  $5.10 \times 10^5$  d.p.m./g (1968); Packard Instrument Co. Ltd., Downers Grove, Illinois, U.S.A.) and n-hexadecane-1, 2- $^3\text{H}$  (batch 8, 2.47y-Ci/g on 1st August, 1968; The Radiochemical Centre, Amersham, Bucks). Counting efficiency was expressed as a quadratic function of the observed channels ratio with the aid of programmes ST1002 and ST1003 of the Programma 101 desk computer (British Olivetti Ltd., London W.1.). The constants of these quadratic equations were entered into the calculators in the Liquid Scintillation Analysers.

The efficiency of counting  $^{14}\text{C}$  carbon was 84% and tritium, 20-24%. When double label counting was done there were no counts due to tritium in the  $^{14}\text{C}$  carbon channels.  $^{14}\text{C}$  Carbon was counted at

at about 50% efficiency in its own channel, with a 9-10% spillover into the tritium channel. Tritium was counted at about 20% efficiency.

### 3. 2. Cell extraction and enzyme assays.

#### 3. 2. 1. Cell extraction.

Enzymes were extracted by the method of Kennedy & Fewson (1968b) as modified by Livingstone (1970). This involved ultrasonic disruption with the 13mm probe of the Dawe "Soniprobe" (Type 1130A; Dawe Instruments Ltd., London).

Cells were resuspended to 50mg wet wt./ml in 0.04M-tris-HCl buffer pH 8.5, and a 5ml amount was pipetted into a 1 dram Trident container (Johnsen & Jorgensen Ltd., London). The container was placed in a chilled brass holder (Holms & Bennett, 1971) which was screwed on to the horn of the soniprobe and lowered into an ice-water slurry. The total time of sonication was  $6\frac{1}{2}$ min at a current of 2.5A, but the current was switched off every alternate half minute to aid cooling, thus giving a  $3\frac{1}{2}$ min actual disruption. The extract was centrifuged at 12,000g for 35min at 4°C (M.S.E. "13" Refrigerated Centrifuge) to remove whole cells and debris, and the supernatant fluid was used to measure enzyme activity.

#### 3. 2. 2. Spectrophotometric enzyme assays.

The assays and conditions were those of Livingstone (1970) and were based on those of Kennedy & Fewson (1968b) and Hegeman (1966a).

All assays were done at 27°C in 1cm light-path silica cuvettes containing a total volume of 3.0ml. Measurements of extinction were made with a Unicam SP800 Ultraviolet Spectrophotometer

fitted with the automatic sample changer and connected to a Servoscribe Chart Recorder. No reference cuvettes were used as the samples were read against air.

### 3. 2. 2. 1. L-Mandelate dehydrogenase.

L-Mandelate dehydrogenase was assayed by the reduction of 2, 6-dichlorophenol-indophenol to the leuco form concomitant with the oxidation of L-mandelate to phenylglyoxylate. The reaction was measured at 600nm, near the maximal extinction of the oxidised form of the dye. In addition to enzyme the reaction mixture contained:

200 $\mu$ mol  $\text{KH}_2\text{PO}_4$  -  $\text{K}_2\text{HPO}_4$  buffer pH 7.0

200nmol 2, 6-dichlorophenol-indophenol.

1.5 $\mu$ mol L-mandelate,

The reaction was initiated by the addition of L-mandelate.

### 3. 2. 2. 2. Phenylglyoxylate carboxy-lyase.

Phenylglyoxylate carboxy-lyase was assayed directly as the disappearance of phenylglyoxylate at 334nm and at pH 6.0. Under these conditions benzaldehyde and subsequent products had negligible extinction. In addition to enzyme the reaction mixture contained:

100 $\mu$ mol  $\text{KH}_2\text{PO}_4$  -  $\text{K}_2\text{HPO}_4$  buffer pH 6.0.

100 $\mu$ g thiamine pyrophosphate chloride (dissolved in 0.05 M-phosphate buffer pH 6.0).

2.5 $\mu$ mol phenylglyoxylate.

-10-

The reaction was initiated by the addition of phenylglyoxylate.

### 3. 2. 2. 3. Labile and stable benzaldehyde dehydrogenases.

These enzymes were assayed in extracts which contained only one benzaldehyde dehydrogenase. The assay measured benzaldehyde - dependent reduction of  $\text{NAD}^+$  at 340nm. In addition to enzyme the reaction mixture contained:

200 $\mu\text{mol}$  tris-HCl buffer pH 9.5.

150 $\mu\text{mol}$   $\text{KH}_2\text{PO}_4$ .

1.5 $\mu\text{mol}$   $\text{NAD}^+$ .

300nmol benzaldehyde.

The reaction was initiated by the addition of benzaldehyde.

### 3. 2. 2. 4. Benzyl alcohol dehydrogenase.

The assay measured the benzyl alcohol-dependent reduction of  $\text{NAD}^+$  at 340nm. In addition to enzyme the reaction mixture contained:

200 $\mu\text{mol}$  sodium pyrophosphate buffer pH 9.0.

1.5 $\mu\text{mol}$   $\text{NAD}^+$ .

300nmol benzyl alcohol.

The reaction was initiated by the addition of benzyl alcohol.

### 3. 3. Spectrophotometric assay for benzyl alcohol.

To a chilled 15ml Corex tube (Corning Glass Works U.S.A.)

containing 1.5ml 30% (<sup>w</sup>/<sub>v</sub>) perchloric acid was added 7.5ml solution of unknown benzyl alcohol concentration. After mixing by inversion over Parafilm (Gallenkamp) the sample was stored on ice for 30min before centrifugation at 12,000g for 15min at 4°C (M.S.E. "13" Refrigerated Centrifuge). 8.0ml Decanted supernatant solution was added to a chilled 15ml Corex tube containing 1.5ml 10M-KOH, mixed by inversion over Parafilm and centrifuged at 12,000g for 15min at 4°C (M.S.E. "13" Refrigerated Centrifuge). The supernatant solution was decanted and 8.0ml added to 5.0ml chloroform (May & Baker, Ltd., Dagenham, England) in a 6 x  $\frac{3}{4}$  in test tube and vortex mixed for 30s. After phase separation, the organic layer was removed by pipette on to 100mg anhydrous Na<sub>2</sub>SO<sub>4</sub> in a ground-glass stoppered test tube and vortex mixed. The chloroform sample was then transferred to a stoppered 1cm path-length quartz cuvette and equilibrated to 27°C. The extinction of the sample was measured against a chloroform blank at 258nm, the maximal extinction of benzyl alcohol, on a Unicam SP800 Ultraviolet Spectrophotometer set at 5x scale expansion and connected to a Servoscribe Chart Recorder.

Over the range of benzyl alcohol concentration tested, the spectrophotometric response was linear (Fig. 7). Neither benzoate nor succinate interfered with this assay (Fig. 7).

#### 3. 4. Spectrophotometric assay for benzaldehyde.

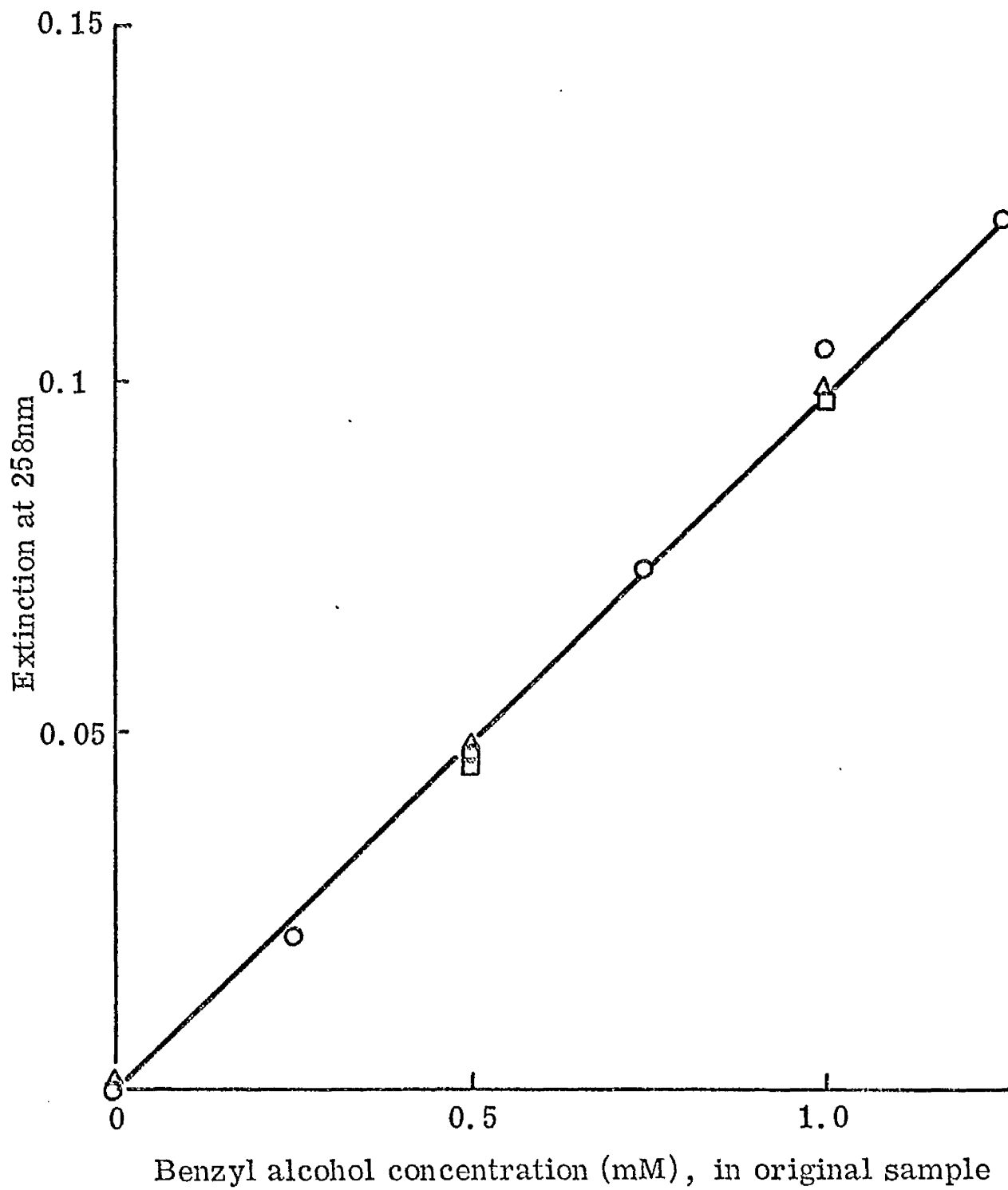
These assays were done by Dr. C.A. Fewson.

To a 6 x  $\frac{3}{4}$  in test tube containing 0.1ml M-NaOH and 10.0ml

Fig. 7 . Standard curve for benzyl alcohol estimation,  
including the effects of succinate and benzoate.

Suspensions of cells grown in succinate-salts medium (i.e. cells lacking benzyl alcohol dehydrogenase) were prepared in reaction media containing different concentrations of benzyl alcohol (Methods, p. 60 ). Similar suspensions were prepared with benzoate or succinate present at 1.0mM. A 7.5ml sample from each reaction medium was deproteinised with 1.5ml chilled 30% (<sup>w</sup>/<sub>v</sub>) perchloric acid and after centrifugation an 8.0ml portion was made alkaline with 1.5ml 10M-KOH (Methods, p. 67 ).  $KClO_4$  was removed by centrifugation and the benzyl alcohol in an 8.0ml portion of the supernatant was extracted into 5ml chloroform. The chloroform was then treated with anhydrous  $Na_2SO_4$  and the extinction read against a chloroform blank at 258nm in a Unicam SP800 Ultraviolet Spectrophotometer set to an expansion factor of 5.

○ Benzyl alcohol alone in the reaction medium  
and, △ with the additional presence of 1.0mM-  
benzoate or, □ with the additional presence of  
1.0mM-succinate.





n-hexane (28488; British Drug Houses) was added 1.0ml solution of unknown benzaldehyde concentration. After vortex mixing for 30s, the extinction of the top phase was read against a hexane blank at 240nm on a Unicam SP800 Ultraviolet Spectrophotometer and converted to mMolarity benzaldehyde by reference to a standard curve (Fig. 8) constructed using BDH AnalaR benzaldehyde. Neither benzyl alcohol nor mandelate interfered with this assay at the concentrations employed (1-1.5mM).

### 3. 5. Statistical methods.

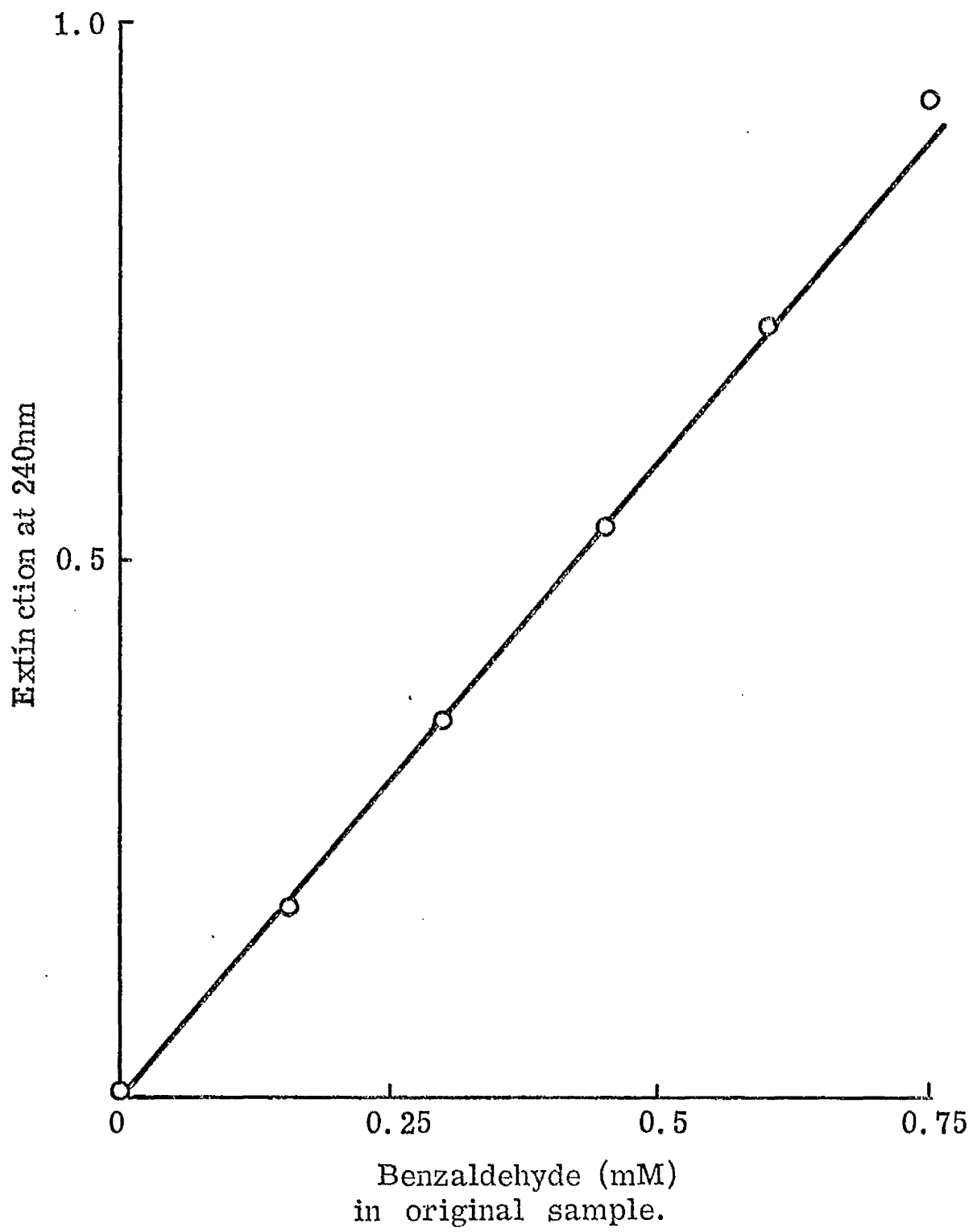
Analysis of variance was determined with the aid of a Canon-Canola 163 calculator (British Agents: Block & Anderson, Banda House, Hammersmith, London W.6.).

### 3. 6. Measurement of pH.

The pH values of solutions were determined by means of an E.I.L. direct reading pH meter (Model 23A; Electronic Instruments, Ltd.,) which was calibrated daily with standard pH buffer tablets (Burroughs, Wellcome & Co., London).

Fig. 8 . Standard curve for benzaldehyde estimation.

Benzaldehyde was prepared at different concentrations in salts medium. 1.0ml Portions were added to  $6 \times \frac{3}{4}$ in test tubes containing 0.1ml M-NaOH and 10.0ml n-hexane. After mixing, the hexane layer was read against a hexane blank at 240nm in a Unicam SP800 Ultraviolet Spectrophotometer as described in Methods (p. 68 ).



4. MATERIALS.

All reagents were the best grade which could be obtained commercially. With the exceptions of the compounds listed below, reagents were obtained from the sources indicated by Fewson (1967a) and Kennedy & Fewson (1968a,b).

British Drug Houses Ltd., Poole, Dorset.

Toluene (A.R.); Ammonium carbonate (A.R.).

Burroughs, Wellcome & Co. London.

Ethanol (fermented, absolute).

Koch-light Laboratories Ltd., Colnbrook, Bucks.

2, 5-Diphenyloxazole (puriss).

Mann Research Laboratories Inc., New York, N.Y.10006, U.S.A.

Methyl- $\beta$ -D-thiogalactopyranoside.

Packard Instrument Co. Ltd., Downers Grove, Illinois, U.S.A.

Toluene- $^{14}\text{C}$ , radioactive reference material (batch 7,  $5.10 \times 10^5$  d.p.m./g, 1968).

New England Nuclear Corp., Boston Mass. U.S.A.

$\beta$ -Methyl- $^{14}\text{C}$ thiogalactoside (lot 292-164, 8mCi/mmol);  
[methoxy- $^3\text{H}$ ]inulin (lot 334-227, 128mCi/g).

Mallinckrodt Nuclear, St. Louis, Mo., U.S.A.

p-Fluoro[carboxy- $^{14}\text{C}$ ] benzoic acid (control No.1295, 1mCi/mmol);  
DL-[carboxy- $^{14}\text{C}$ ] mandelic acid (control No.3518, 1mCi/mmol).

The Radiochemical Centre, Amersham, Bucks.

[Carboxy- $^{14}\text{C}$ ] benzoic acid (batches, 16, 17, and 18; 30, 56 and 56mCi/mmol); [ring- $^{14}\text{C}$ (U)] benzoic acid (batches 4, 5, 6 and 7; 48, 45, 45 and 45mCi/mmol); [carbinol - $^{14}\text{C}$ ] benzyl alcohol (batches 9 and 10; 2 and 5mCi/mmol); [ring- $^3\text{H}$ (G)] catechol

(batch 4, 500mCi/mmol); D-fructose- $^{14}\text{C}$ (U) (batch 45, 3mCi/mmol);  
D-fructose- $^{14}\text{C}$ (U)-1, 6-diphosphate (batch 1, 3mCi/mmol); D-  
glucose- $^{14}\text{C}$ (U) (batch 154, 3mCi/mmol); D-glucose-5- $^3\text{H}$ (n)  
(batch 3, 2Ci/mmol); n-hexadecane-1, 2- $^3\text{H}$ , radioactive reference<sup>e</sup>  
material (batch 8, 2.47 $\mu\text{Ci/g}$  on 1st August 1968); o-hydroxy  
[carboxy- $^{14}\text{C}$ ] benzoic acid (batch 8, 31mCi/mmol); inulin  
[carboxylic acid- $^{14}\text{C}$ ](batch 4, 3mCi/g); D-ribose- $^{14}\text{C}$ (U) (batch  
19, 4mCi/mmol); potassium cyanide[ $^{14}\text{C}$ ](batch 29, 45mCi/mmol).

Radioactively labelled mandelic acid became commercially  
available only towards the end of the work and all the early  
experiments were done with DL-[carboxy- $^{14}\text{C}$ ]mandelate synthesised  
by a micropreparative technique devised from the methods of  
Collins (1955) and Quickfit and Quartz (1962).

Purified benzaldehyde bisulphite addition complex  
(220 $\mu\text{mol}$ ) prepared according to Vogel (1948) was contained in  
0.6ml water in a 25ml Erlenmeyer flask and slowly stirred with a  
10mm glass-coated magnet by means of a magnetic stirrer. KCN  
(163 $\mu\text{mol}$  containing 3mCi-[ $^{14}\text{C}$ ]) was added in a total volume,  
including washes, of 0.5ml. 1.0ml Ether was added after 10min.  
The ether was removed by Pasteur pipette to a Q. & Q.B10/19  
25ml separating funnel containing 1.0ml water. Four further  
washings with 0.5ml ether were carried out over 40min.  
The bulked extract, after washing with water and 1.0ml

saturated sodium chloride solution, was added to a 5ml round-bottomed B10/19 flask set up for distillation and containing 0.6ml 5M-HCl and a boiling stone. The ether was evaporated off on a steam bath into a chilled receiver. The residue was subjected to a 2h acid hydrolysis under reflux. The hydrolysate was transferred by Pasteur pipette to a long, narrow, stoppered test tube. 0.5ml Ether, first used to wash the hydrolysis flask, was added to the test tube, vortex mixed and transferred to a 1ml B10/19 collecting vessel. This extraction was repeated with 4x0.3ml samples of ether.

Whatman 3MM chromatography paper, 46 x 57cm (W. & R. Balston Ltd., England), was marked, folded and crimped for descending chromatography and positioned in a "Panglas 500 Chromatank" (Shandon Scientific Co., London S.W.7). 200ml Distilled water was run through the paper. This process removed 70% of the u.v. absorbing material that otherwise appeared in the final elution. Further washes at this time removed only small proportions of the residual u.v. absorbing material. The washed paper was dried in a gentle air current at 20°C.

DL- $\left[ \text{Carboxy-}^{14}\text{C} \right]$  mandelate in the bulked ether extract was purified by descending chromatography for 26h on washed Whatman 3MM paper in the organic phase of n-butanol saturated with ammonia buffer (Fewster & Hall, 1951). The ammonia buffer consisted of 75ml 0.880 ammonia and 78.5g ammonium carbonate equivalent made up to 1l with distilled water without adjustment of pH. Mandelate on the

chromatogram was located as a band of u.v. absorption and radioactivity in comparison with an authentic sample of DL-mandelate. The paper containing this band was cut out and one end tapered to an arrowhead; a "wick" was sewn to the other end with platinum wire. The material on the paper was eluted with water in a Panglas 500 Chromatank lined with wet Whatmen 3MM paper to maintain the water vapour pressure. The eluate of about 3ml was collected in a Sterilin Universal Container P105/C (Sterilin Ltd., Richmond, Surrey). No radioactivity was detected on the eluted paper. An ultraviolet spectrum of the eluted material on a Unicam SP800 Ultraviolet Spectrophotometer indicated a yield of about 58µmol (35%). A radiochemical assay indicated a 40% yield.

Benzaldehyde was redistilled under nitrogen before use and stored under nitrogen. Benzyl alcohol was redistilled before use.

All solutions were made up in glass-distilled water, with the exception of the latex suspensions used as turbidity standards (Methods p. 44) when deionised water was used.

The filter membrane chosen for use in experiments on the uptake of radioactivity into cell suspensions (Results.p.146) was the 0.22µm pore size, 25mm diameter, weight constant cellulose nitrate filter, catalogue number 11307025 of the Sartorius Membranfilter GmbH, Göttingen, Germany.

## R E S U L T S.

### 1. CARBOHYDRATE UTILISATION BY BACTERIUM NCIB 8250.

#### 1. 1. Assimilation and dissimilation of five sugars and sugar phosphates.

Fewson (1967b) observed that carbohydrates neither supported growth of bacterium NCIB 8250 nor increased yields during growth on alternative carbon sources. This work has now been extended to determine whether certain sugars and sugar phosphates could be assimilated or dissimilated by cultures of bacterium NCIB 8250 (Table 2).

When these cultures were harvested by filtration, radioactivity retained on the filter was between zero and fifteen c.p.m. above background for every 100 $\mu$ l of culture filtered. The maximum value, which was for 1mM-fructose in a 2mM-benzoate-salts culture, represented 0.3% of the radioactivity applied to the filter. This could account, at most, for 0.3 % of the cell carbon. However, the filter membranes were heavily loaded and later experiments showed washing to be incomplete (Table 8), especially under conditions of heavy loading. Thus even these low values were probably overestimates of the true assimilation.

Radioactivity in acidified fractions of all media tested remained constant throughout the experiment (Table 2). Thus no carbon dioxide was formed from these sugars and no dissimilation of these sugars



Table 2. Assimilation and dissimilation of sugars and sugar phosphates by bacterium NCIB 8250 during growth on different sources.  
carbon

Glucose, fructose, ribose, glucose-6-phosphate and fructose-1, 6-diphosphate, at 1mM and 6 $\mu$ M, were present during growth of bacterium NCIB 8250 in nutrient broth or in salts medium containing 5mM-succinate, 5mM-glutamate or 2mM-benzoate. The growth medium, 50ml, was prepared in a 250ml Erlenmeyer flask. 1 $\mu$ Ci Of one sterile, uniformly <sup>14</sup>C-labelled sugar was added and each condition was done in duplicate. A 2% (v/v) nutrient broth inoculum was used and the cultures were grown on an orbital shaker (Methods, p.46). Under these conditions cultures normally reached stationary state within 12h. Cultures were sampled at 0, 12 and 24h. 100 $\mu$ l of this sample was acidified with 1.0ml 0.1M-HCl and 100 $\mu$ l of this acidified fraction was counted by liquid scintillation spectrometry. 200 $\mu$ l Of culture, or 500 $\mu$ l of a benzoate-salts culture, were harvested on a filter membrane (0.22 $\mu$ m pore, 25mm diameter) under vacuum, washed four times with 4ml chilled, distilled water and the filter membrane immersed in liquid scintillation fluid in a scintillation vial.

NT=Not tested.

Growth medium	Nutrient broth	% Assimilation		5mM - Succinate-salts	% Assimilation		% Activity remaining in medium		5mM-Glutamate-salts	% Assimilation		% Activity remaining in medium	
		12h	24h		12h	24h	12h	24h		12h	24h	12h	24h
Sugar	Assimilation	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h
		12h	24h	12h	24h	12h	24h	12h	24h	12h	24h	12h	24h
Glucose	1mM	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	NTP	NTP	
	6µM	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	NTP	NTP	
Fructose	1mM	0.1	0.2	0.2	0.2	0.2	0.3	0.2	0.3	0.2	0.3	0.2	
	6µM	0.1	0.2	0.2	0.2	0.1	0.3	0.2	0.3	0.2	0.2	0.2	
Ribose	1mM	0.1	0.2	0.1	0.2	0.2	0.2	0.1	0.2	0.1	0.1	0.1	
	6µM	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
Glucose-6-phosphate	1mM	NTP		NTP		0.2	0.2	98	102	0.1	0.1	103	103
	6µM	NTP		NTP		0.2	0.2	100	97	0.1	0.1	101	102
Fructose-1, 6-diphosphate	1mM	NTP		NTP		0.3	0.3	98	99	0.3	0.3	100	104
	6µM	NTP		NTP		0.3	0.3	96	100	0.2	0.2	101	105

could be detected. As no significant assimilation of sugar occurred, the remaining radioactivity presumably represented unchanged sugars and sugar phosphates, although no attempt was made to check the identity of the radioactive compounds.

A gluconeogenic pathway was presumed to operate in this non-saccharolytic organism. Therefore, the very low assimilation of fructose-1, 6-diphosphate promoted the idea that there was a permeability barrier to that compound. It was possible that one reason for the inability of bacterium NCIB 8250 to utilise carbohydrates was a general permeability barrier to their entry into the cell.

Much of the work described in this thesis uses cell water as a basis for the calculation of results (Results, p. 154). The presumed permeability barrier to carbohydrates was, therefore, used to determine the cell water content of bacterium NCIB 8250.

## 1. 2. Cellular water spaces and excluded volumes in cell pellets.

### 1. 2. 1. Development of a method to measure excluded volumes in cell pellets.

The technique (Methods, p. 54) was developed with E. coli ML 308 and [methoxy-<sup>3</sup>H]inulin, following the procedure of Winkler & Wilson (1966). Cell suspensions of known bacterial dry weight were prepared in a radioactive medium, and, after centrifugation in a tared tube, the pellet weight was determined. Total radioactivity in the cell pellet, with the radioactivity per unit volume of the

supernatant fluid, gave an estimate of the extracellular fluid in the pellet and the intracellular water was calculated knowing also the pellet weight and the dry weight (Methods, p. 56). A value of 2.86 $\mu$ l cell water/mg dry wt. was observed in comparison with 2.7 $\mu$ l cell water/mg dry wt. reported by Winkler & Wilson (1966).

Certain points of technique were not stressed by Winkler & Wilson (1966).

It was imperative that the radioactively labelled inulin was in a true solution. Particles of undissolved radioactive inulin were precipitated on centrifugation and gave spuriously high values of extracellular water in the cell pellet, with a correspondingly low value for cell water/dry wt. The low specific radioactivity of inulin [carboxylic acid-<sup>14</sup>C] coupled with the low solubility of inulin at 0<sup>o</sup>C made that label unsuitable for these experiments, and the high specific radioactivity [methoxy-<sup>3</sup>H] inulin solution used was heated before use to ensure a true solution.

Another problem concerned the breakdown of [methoxy-<sup>3</sup>H]-inulin on storage. A sample of this material was dissolved in distilled water, stored at -10<sup>o</sup>C and subjected to repeated thawing, heating and freezing during preliminary experiments. This sample was analysed by gel filtration chromatography on Sephadex-G25 (Cohen, 1969) and found to contain a major peak close to the excluded volume and corresponding to material of molecular weight about 5200; there was also a long and marked trailing of the major peak. This residual material accounted for some 30% of the

radioactivity present and corresponded to a range of small oligosaccharides. A freshly prepared sample contained only high molecular weight material found in two major peaks close to the excluded volume. Breakdown of inulin would allow small molecules to penetrate the cell wall (see later) and hence give a low value for cell water/dry wt. Freshly prepared solutions of [methoxy-<sup>3</sup>H]-inulin were used in the experiments quoted in this section.

These experiments were carried out on a relatively large scale in order to do both cell water and dry weight estimation on the one cell suspension. This had the disadvantage that there was a long time lag (about 30min) before cells could be separated from the suspension buffer, and time courses of uptake were not possible.

#### 1. 2. 2. Cellular water spaces in bacterium NCIB 8250.

When succinate-grown cells were harvested from suspension in salts medium, an excluded volume of 3.09 $\mu$ l/mg dry wt. to [methoxy-<sup>3</sup>H] inulin was observed. As cell suspension assays elsewhere in the thesis were done in a phosphate buffer, excluded volumes (Table 3) were estimated with succinate-grown cells resuspended in 0.05M- $\text{KH}_2\text{PO}_4 \cdot \text{Na}_2\text{HPO}_4$  pH7.0. The same excluded volume to methoxyinulin was observed in the phosphate buffer and in salts medium, and 3.09 $\mu$ l cell water/mg dry wt. was assumed to represent total cell water including that present in the cell wall.

The excluded volumes to monosaccharides were similar to one another and about half that to inulin (Table 3). The most studied monosaccharide was glucose and the average excluded volume of 1.65 $\mu$ l

Table 3. Excluded volumes to different carbohydrates  
in bacterium NCIB 8250.

Two 5l batches of bacterium NCIB 8250 were grown in 10mM-succinate-salts medium to a turbidity of 0.6, harvested, washed, resuspended to 30mg wet wt./ml in 0.05M- $\text{KH}_2\text{PO}_4 \cdot \text{Na}_2\text{HPO}_4$  pH 7.0 and stored on ice (Methods, p.51 ). 3 x 35ml Portions of the stock cell suspension were used to estimate the bacterial dry wt. present (Methods, p.53). Another 3 x 35ml portions were bulked, radioactively labelled carbohydrate was added in a 3ml volume and the suspension was thoroughly mixed. 3 x 35ml Samples of this radioactively labelled cell suspension were centrifuged in tared 50ml centrifuge tubes. The pellet wet wt. was estimated. The total radioactivity in the pellet and the radioactivity/unit volume of supernatant were measured (Methods, p.55 ). The crude data were manipulated (Methods, p.56 ) to units of  $\mu\text{l}$  cell water/mg dry wt.

Radioactively labelled molecule	Concentration mM	Cell water $\mu\text{l}/\text{mg}$ dry wt.
[Methoxy - <sup>3</sup> H]-inulin	$4 \times 10^{-4}$	3.09
Glucose - <sup>14</sup> C(U)	$5 \times 10^{-2}$	1.58
Ribose - <sup>14</sup> C(U)	$4 \times 10^{-2}$	1.33
Fructose - <sup>14</sup> C(U)	$4 \times 10^{-2}$	1.51
Glucose - <sup>14</sup> C(U)	10.9	1.79
Glucose - <sup>14</sup> C(U)	1.1	1.56
Glucose - <sup>14</sup> C(U)	$1.3 \times 10^{-1}$	1.74
Glucose - <sup>14</sup> C(U)	$2 \times 10^{-2}$	1.52
		mean 1.65

cell water/mg dry wt. was assumed to represent the true cell water (i.e. excluding that present in the cell wall) in subsequent calculations (Results, p.154).

In contrast to the positive excluded volumes to carbohydrates, no excluded volume to benzoate was detected. Negative values of about  $-0.2\mu\text{l}$  cell water/mg dry wt. were observed. These values were unaffected by the order of addition of carrier benzoate and radiochemical label or by the specific radioactivity of the benzoate present, so they presumably represented complete penetration of the succinate cell by benzoate.



## 2. SUBSTRATE UTILISATION AND GROWTH OF BACTERIUM NCIB 8250.

### 2. 1. Development of conditions for the analysis of growth and substrate utilisation.

#### 2. 1. 1. Growth and benzoate utilisation in benzoate-salts medium.

General conditions have been published for the growth of bacterium NCIB 8250 in carbon-limited batch culture (Fewson, 1967b). Though these conditions gave reproducible results, they did have disadvantages when combined with a radiochemical assay of substrate utilisation.

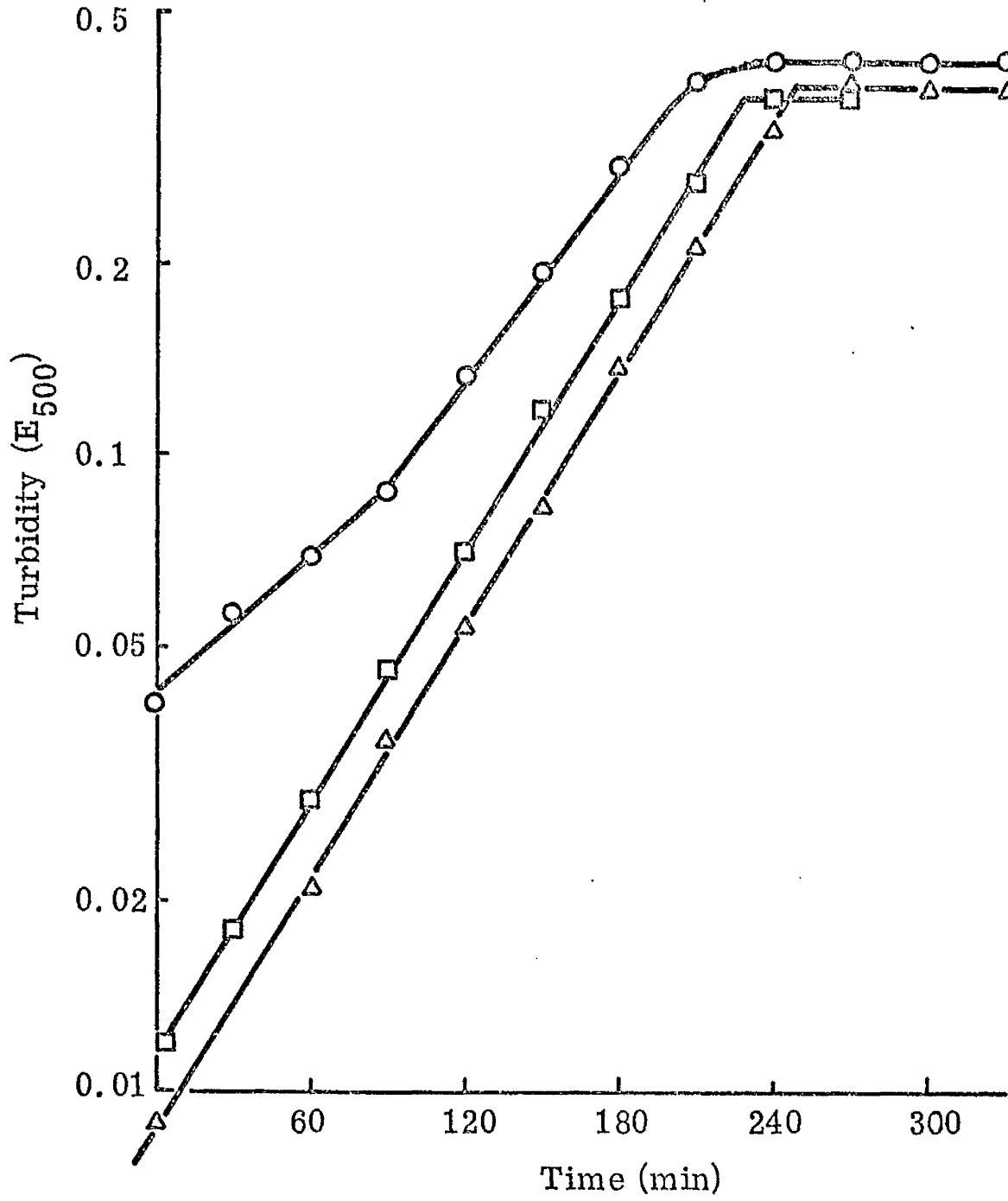
The first problem was economic. Preliminary experiments showed that a reduction in culture volume from 800ml (Fewson, 1967b) to 300ml had no effect on the growth curve of bacterium NCIB 8250 in benzoate-salts medium from a nutrient broth inoculum; the specific growth rate ( $\mu$ , Dawes, 1967) attained after 1h remained  $0.78\text{h}^{-1}$  as in Fig. 9. Forced aeration had no observable effect on the growth of bacterium NCIB 8250 in benzoate-salts medium under these conditions and all subsequent experiments were done without forced aeration to prevent loss of volatile intermediates.

A nutrient broth inoculum (Fewson, 1967b) raised problems in the interpretation of data. As illustrated in Fig. 9, growth in benzoate-salts medium from a nutrient broth inoculum was more complex and slower ( $\mu = 0.78\text{h}^{-1}$ ) than growth from an inoculum preinduced to growth in benzoate-salts medium ( $\mu = 0.92\text{h}^{-1}$ ). The

Fig. 9 .        The effect of inoculum on the growth of  
bacterium NCIB 8250 in benzoate-salts medium.

Bacterium NCIB 8250 was grown from different inocula in 1.4mM-benzoate-salts medium. Growth was measured turbidimetrically as described in Methods (p. 44).

- Nutrient broth inoculum (4%<sup>v</sup>/v) direct from a 24h nutrient broth culture.
- △ Preinduced inoculum (8%<sup>v</sup>/v) direct from a culture of turbidity 0.2 growing exponentially in 2mM-benzoate-salts medium.
- Preinduced inoculum (2%<sup>v</sup>/v) harvested (Methods, p. 47) from a culture of turbidity 0.2 growing exponentially in 2mM-benzoate-salts medium.



preinduced inoculum had to come from a growing culture. Lags of up to 1h and low subsequent growth rates ( $\mu \geq 0.58\text{h}^{-1}$ ) were observed after inoculation of benzoate-salts medium with cultures up to 2h into stationary state in benzoate-salts medium. In subsequent experiments cultures for inoculation were used when one generation from stationary state. The standard preinduction culture was grown in 2mM-benzoate-salts medium and used at a turbidity of 0.2. To prevent carry-over of carbon source from the preinduction culture and to reduce the volume of the inoculum, the inoculum was harvested and resuspended in chilled basal medium (Livingstone, 1970) immediately before use. After this treatment, cells grew immediately and at the same rate ( $\mu = 0.92\text{h}^{-1}$ ) as cells inoculated directly from the preinduction culture (Fig. 9).

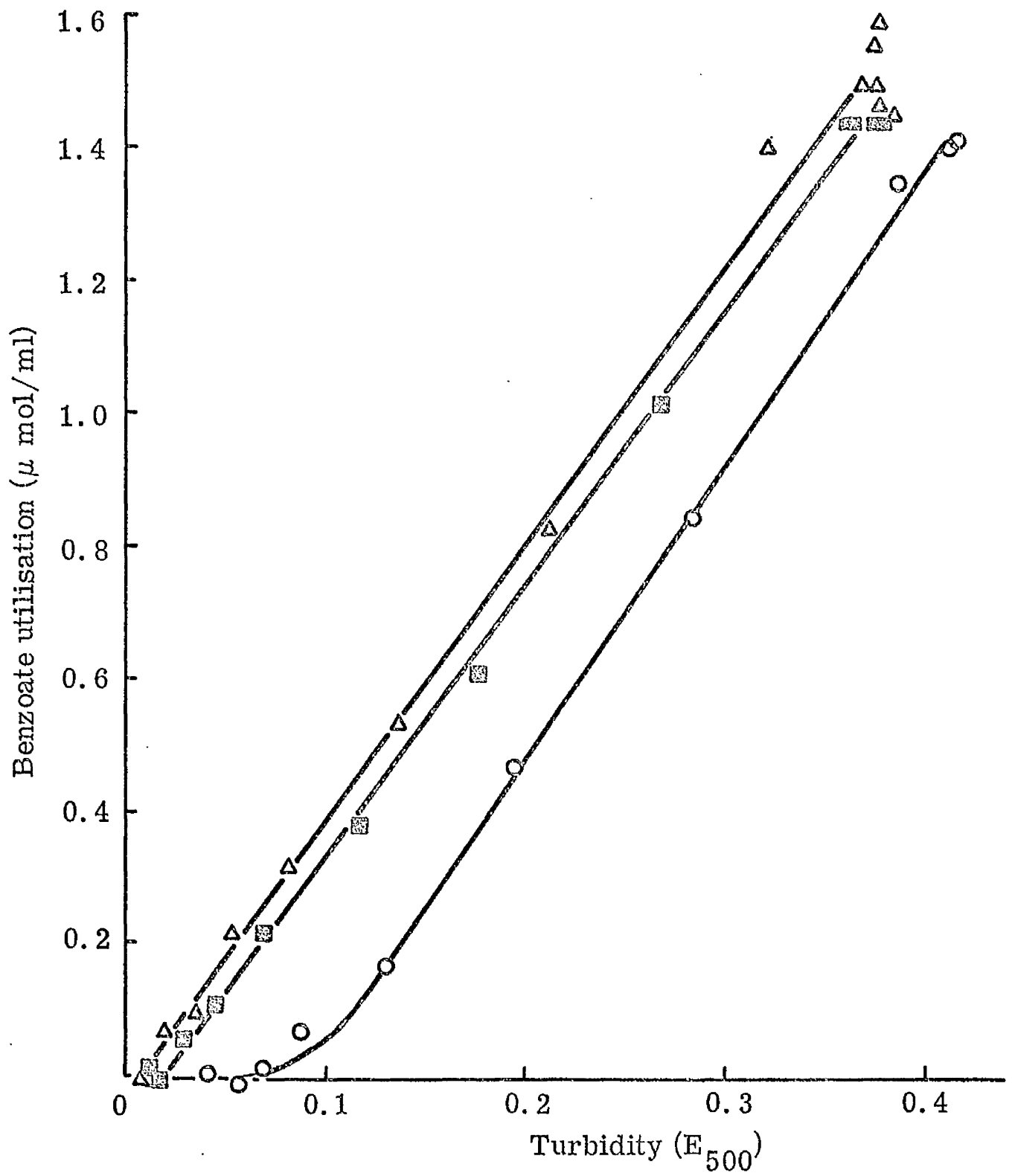
Benzoate utilisation by cultures of bacterium NCIB 8250 was calculated from the decarboxylation of [carboxy- $^{14}\text{C}$ ]benzoate by the first catabolic enzyme, benzoate oxidase. The radioactivity remaining in a sample, after the removal of  $^{14}\text{CO}_2$  by acidification, could be converted to a benzoate concentration in the growth medium and expressed as benzoate utilisation in nmol/ml culture (Methods, p. 50).

The effect of a nutrient broth inoculum on the growth of bacterium NCIB 8250 in benzoate-salts medium (Fig. 9) was reflected in the relationship between benzoate utilisation and growth (Fig. 10, the same experiment). The nutrient broth inoculum grew first on the carry-over of nutrients in the inoculum, with a gradual induction

Fig. 10 ,        The effect of inoculum on benzoate utilisation  
                  during growth of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown from different inocula in 1.4-mM-benzoate-salts medium. Samples were taken at intervals to measure growth turbidimetrically and benzoate utilisation radiochemically as described in Methods (p. 47).

- Nutrient broth inoculum (4% <sup>v</sup>/v) direct from a 24h nutrient broth culture.
- △ Preinduced inoculum (8% <sup>v</sup>/v) direct from a culture of turbidity 0.2 growing exponentially in 2mM-benzoate-salts medium.
- Preinduced inoculum (2% <sup>v</sup>/v) harvested (Methods, p. 47) from a culture of turbidity 0.2 growing exponentially in 2mM-benzoate-salts medium.



of benzoate utilisation. The preinduced inoculum, on the other hand, displayed a linear relation between benzoate utilisation and growth, throughout growth (Fig.10). The gradient of this line could be used to calculate the molar growth yield (Monod, 1942; as modified by Bauchop & Elsdon, 1960) on benzoate. The correlation between turbidity and bacterial dry weight (Methods, p.56) allowed the yield to be expressed as  $\mu\text{g dry wt./}\mu\text{mol}$ . An average value of  $70\mu\text{g dry wt./}\mu\text{mol}$  benzoate was observed with a standard error of the mean of 3% for the four determinations. The rate of benzoate utilisation during growth was calculated as the product of the specific growth rate and the inverse of the molar growth yield. At a growth rate of  $0.92\text{h}^{-1}$  and a yield of  $70\mu\text{g dry wt./}\mu\text{mol}$  benzoate, the benzoate utilisation rate was  $13\text{mmol benzoate/}\mu\text{g dry wt./h}$  or  $220\text{mmol/mg dry wt./min}$ .

The time course of substrate utilisation during growth, illustrated for benzoate in Fig.11 (experiment of Figs.9 and 10), proved a very useful graphical representation of data in this thesis. For reasons to be discussed later, it was usually more informative about patterns of substrate utilisation than the variation of substrate utilisation with growth, and it was more flexible than the corresponding semilog plot (Fig. 12) which required correction to a finite initial value as described in the Legend to Fig. 12 .

### 2. 1. 2. Growth and benzyl alcohol utilisation in benzyl alcohol-salts medium.

The growth of bacterium NCIB 8250 in benzyl alcohol-salts medium varied with the inoculum. In experiments with nutrient broth

Fig. 11 ,      Kinetics of benzoate utilisation during  
                  growth of bacterium NCIB 8250.

The culture of bacterium NCIB 8250 used for inoculation was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, resuspended and used immediately as described in Methods, (p. 47). The experiment was done in 1.4mM-benzoate-salts medium. At intervals samples were taken for the radiochemical estimation of benzoate utilisation (Methods, p. 47).



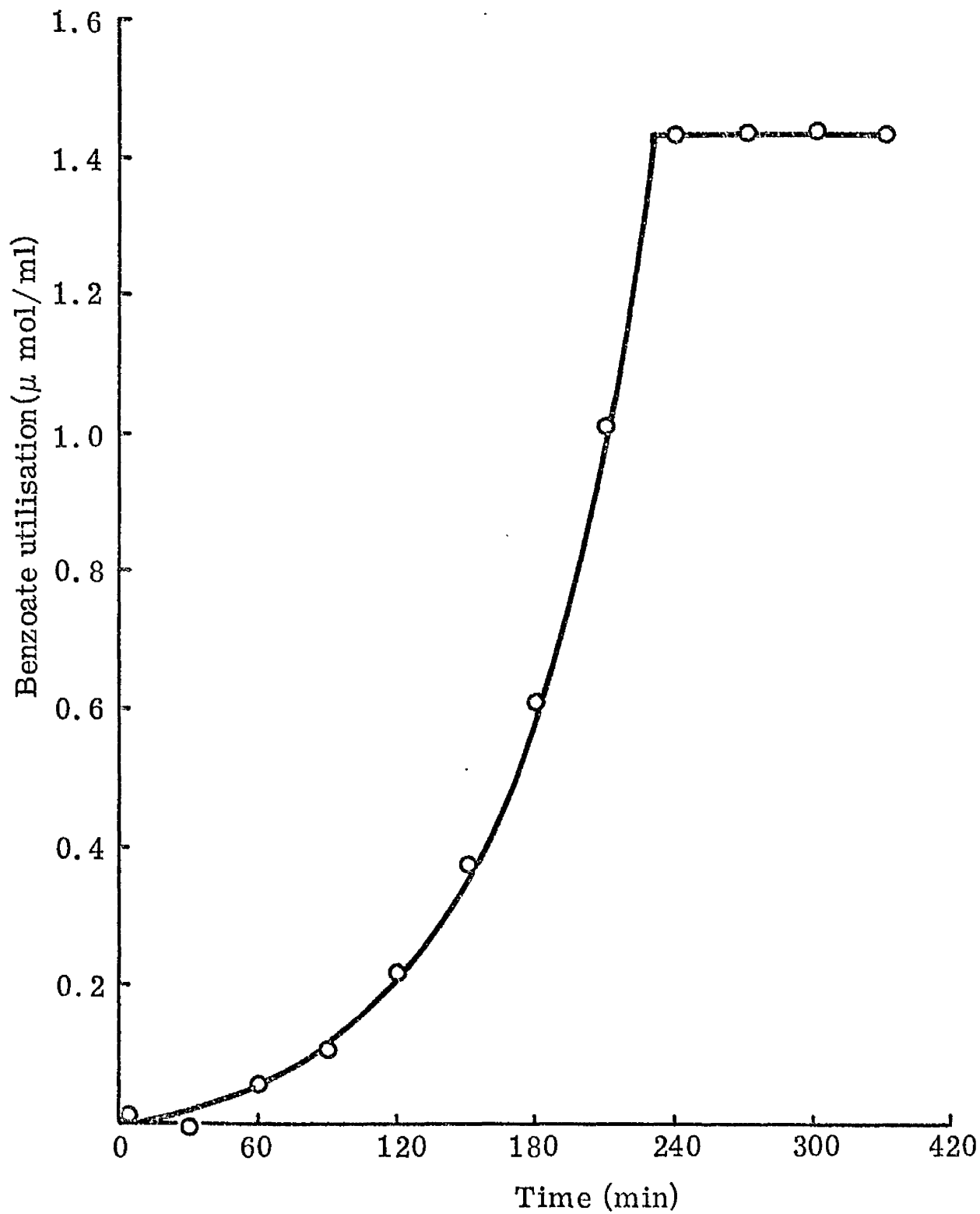
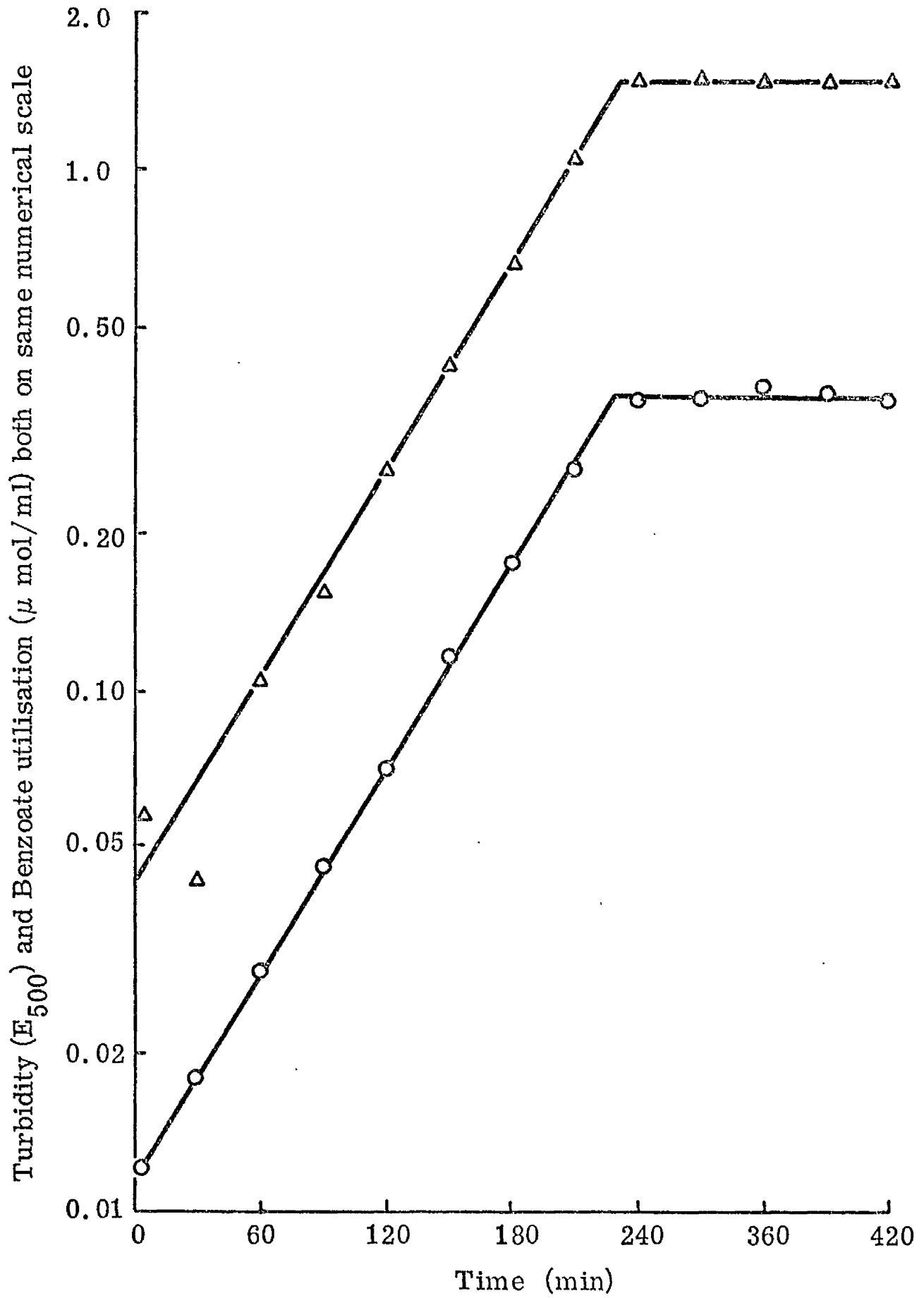


Fig. 12 .      Kinetics of benzoate utilisation and growth  
                 of bacterium NCIB 8250.

The culture of bacterium NCIB 8250 used for inoculation was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, resuspended and used immediately as described in Methods (p. 47). The experiment was done in 1.4mM-benzoate-salts medium. Samples were taken at intervals to measure growth turbidimetrically and to estimate benzoate utilisation radiochemically (Methods, p. 47). In plotting substrate utilisation on a logarithmic scale, allowance must be made for the absence of a "zero" in logarithms. A figure, representing the amount of benzoate required to produce the inoculum, must be added to the actual benzoate utilisation. This figure is obtained from the slope of the differential plot, Fig. 10.

○ Growth in benzoate-salts medium.

△ Adjusted benzoate utilisation during growth.



inocula, the results were complicated by a carry-over of nutrient in the inoculum and subsequent work was done with preinduced inocula. The routine preinduced inoculum was harvested at a turbidity of 0.4 from a culture growing in 5mM-benzyl alcohol-salts medium (Fig.13). On occasion these cells took up to one generation to attain their maximum observed growth rate ( $\mu = 0.88h^{-1}$ ), Fig.29. The reason for this acceleration was not understood. No correlation between lag (Monod, 1942) and storage was observed in an experiment in which cells for inoculation were stored as suspensions and as pellets for periods between 5 and 30min. Suspensions of preinduced cells showed no loss of benzyl alcohol dehydrogenase activity when stored on ice for three hours.

Growth of bacterium NCIB 8250 in benzyl alcohol-salts medium from two other inocula is illustrated (Fig.13). The simple lag displayed by the benzoate inoculum was interpreted as the induction of enzymes required to grow in a different medium. In contrast, the extended period of slow growth observed with a mandelate inoculum was very surprising. The same batch of cells could oxidise benzyl alcohol in the presence of mandelate (Fig.28) and cultures of bacterium NCIB 8250 grown in mandelate-salts medium have always been observed to contain the enzymes of benzyl alcohol oxidation (Livingstone, 1970 ; Kennedy & Fewson, 1968a,b).

The radiochemical estimation of benzyl alcohol utilisation in growth media was calculated (Methods, p.50) from the decarboxylation of benzoate derived from  $\left[ \text{carbinol-}^{14}\text{C} \right]$  benzyl alcohol.

Fig. 13 ,      The effect of inoculum on the growth of  
bacterium NCIB 8250 in benzyl alcohol-salts  
medium.

Bacterium NCIB 8250 was grown from different inocula in 1.4  
mM-benzyl alcohol-salts medium. Growth was measured turbidimetrically  
as described in Methods (p. 44).

- Preinduced inoculum, harvested at a turbidity of  
0.4 (Methods, p. 47) from a culture growing  
exponentially in 5mM-benzyl alcohol-salts medium.
- △ Inoculum from a 10mM-DL-mandelate-salts culture,  
harvested at a turbidity of 0.1.
- Inoculum from a culture growing in 2mM-benzoate-  
salts medium, harvested at a turbidity of 0.2.

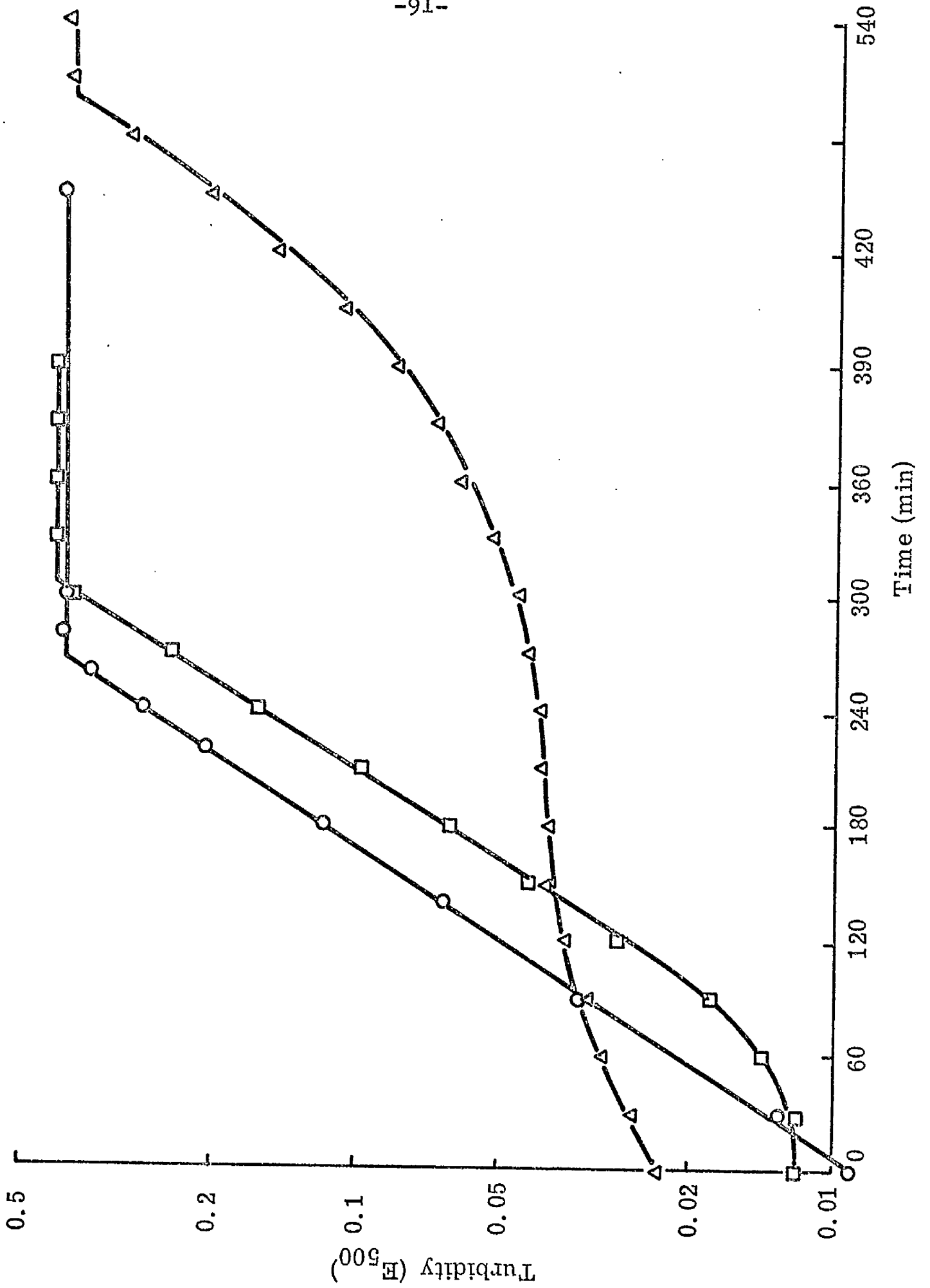
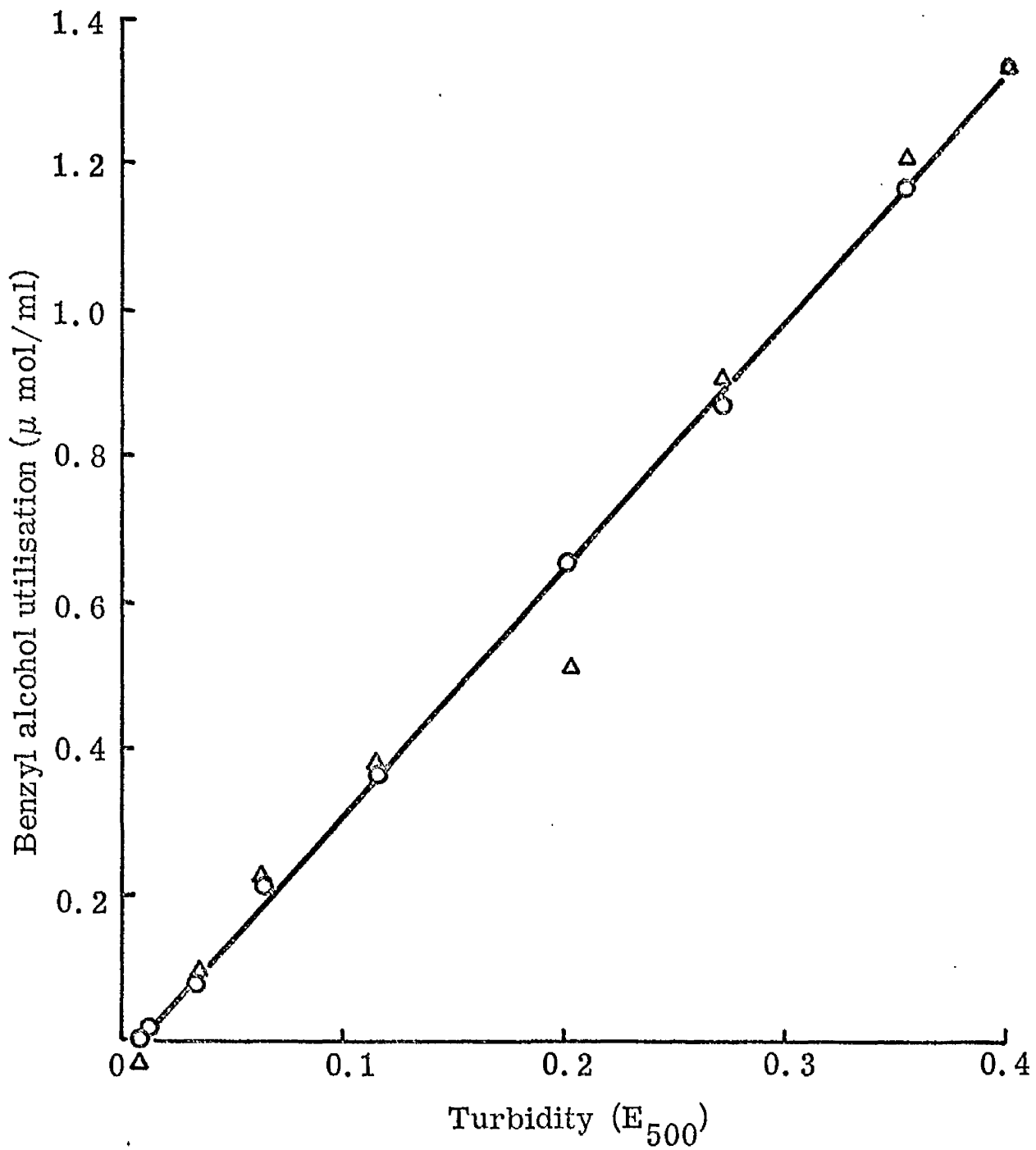


Fig. 14 . Radiochemical and spectrophotometric assays  
to follow the utilisation of benzyl alcohol  
during growth of bacterium NCIB 8250.

The culture of bacterium NCIB 8250 used for inoculation was grown in 5mM-benzyl alcohol-salts medium, harvested when the turbidity reached 0.4, resuspended and used immediately as described in Methods (p. 47). The experiment was done in 1.4mM-benzyl alcohol-salts medium. Samples were taken at intervals to measure growth turbidimetrically and to measure benzyl alcohol utilisation both radiochemically (Methods, p. 47) and spectrophotometrically (Methods, p. 67).

- Utilisation of benzyl alcohol measured as benzyl alcohol decarboxylation.
- △ Utilisation of benzyl alcohol from the direct spectrophotometric assay of benzyl alcohol disappearance.





Benzyl alcohol decarboxylation and benzyl alcohol disappearance were coincident (Fig. 14; these data correspond to the control growth curve in Fig. 13).

Throughout growth, there was a constant linear relation between benzyl alcohol utilisation and growth. The slope of this line was expressed as a yield (Results, p. 87). An average value of 81 $\mu$ g dry wt./ $\mu$ mol benzyl alcohol was observed with a 1% standard error of the mean for the 8 determinations. The rate of benzyl alcohol utilisation during growth ( $\mu = 0.88\text{h}^{-1}$ ) was calculated (Results, p. 87) as 180nmol/mg dry wt./min.

### 2. 1. 3. Growth and mandelate utilisation in mandelate-salts medium.

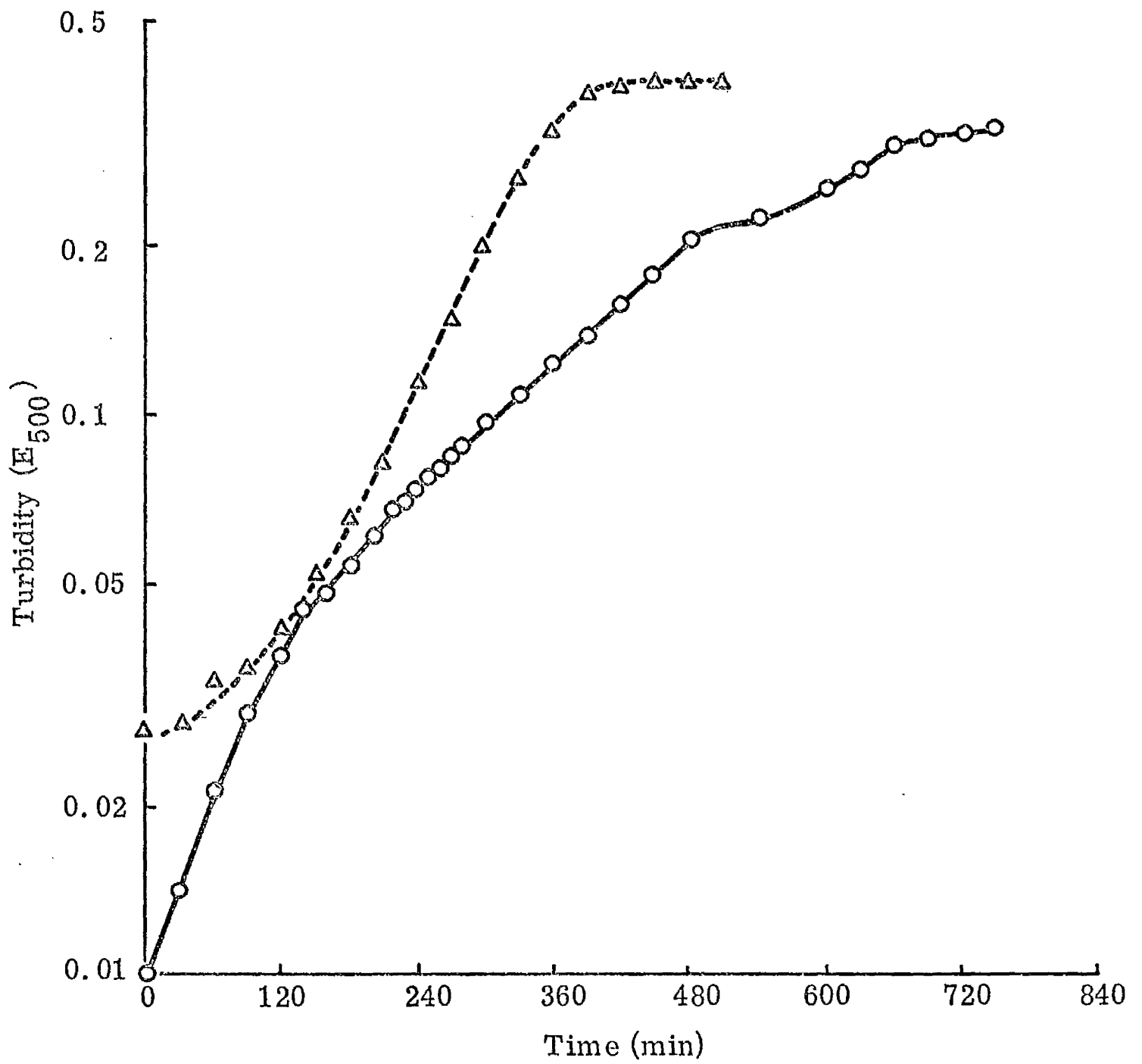
Growth of bacterium NCIB 8250 in mandelate-salts medium was complex (Fig. 15) and incompletely understood. The pattern was consistently one of initial rapid growth ( $\mu = 0.69\text{h}^{-1}$ ) for a variable time up to one generation (Figs. 23 and 27), followed by a slow fall in growth rate over two hours to a fresh exponential rate ( $\mu$  about  $0.27\text{h}^{-1}$ ) for about  $4\frac{1}{2}$ h. The growth rate then dropped to nearly zero with about one half generation remaining. Thereafter, the growth rate slowly increased for about 2h and then tailed off gradually as the culture approached stationary state. Inocula from most sources e.g. nutrient broth, benzoate- or glutamate-salts gave growth curves similar to that described above with minor variations due to carry-over of nutrient or to lags for enzyme induction. One exception, however, was an inoculum from a benzyl

Fig. 15 .      The effect of inoculum on the growth of  
bacterium NCIB 8250 in mandelate-salts medium.

Bacterium NCIB 8250 was grown from different inocula in  
2.8mM-DL-mandelate-salts medium. Growth was measured turbidimetrically  
as described in Methods (p. 44).

○ Preinduced inoculum, harvested at a turbidity  
of 0.1 (Methods, p. 47) from a 10mM-DL-mandelate  
salts culture.

△ Inoculum harvested at a turbidity of 0.4 from  
a culture growing exponentially in 5mM-benzyl  
alcohol-salts medium.



alcohol-salts culture (Fig. 15). After a lag, presumably to induce the necessary enzymes of mandelate oxidation, growth was relatively fast ( $\mu = 0.57\text{h}^{-1}$ ) for all but the last hour, when the growth rate fell steadily to zero.

The radiochemical estimation of mandelate utilisation in growth medium was calculated from the loss of  $^{14}\text{C}$  from medium containing DL- $\left[\underline{\text{carboxy}}\text{-}^{14}\text{C}\right]$ mandelate which was in fact the decarboxylation of phenylglyoxylate derived from the mandelate. Bacterium NCIB 8250 grows only on L-mandelate, and D-mandelate cannot support growth (Kennedy & Fewson, 1968b), but the reported data did not exclude the possibility of an increase of up to 1% in the yield on L-mandelate in the presence of D-mandelate. A limited decarboxylation of D-mandelate could be detected in the present experiments during growth on L-mandelate if the D-mandelate concentration was low (Table 4). The quantity of D-mandelate decarboxylated tended to a maximum and a double reciprocal plot (reciprocal change in D-mandelate concentration against reciprocal initial D-mandelate concentration) indicated a maximum change in D-mandelate concentration of about  $10\mu\text{M}$ . This decarboxylation became undetectable when the concentration of D-mandelate carrier was raised to  $400\mu\text{M}$ . For this reason all subsequent experiments were done with DL-mandelate, whether or not the mandelate was radiochemically labelled.

There was a strong smell of benzaldehyde from cultures of bacterium NCIB 8250 growing in mandelate-salts medium. It was

Table 4 . Decarboxylation of D-mandelate by bacterium  
NCIB 8250 growing in L-mandelate-salts medium.

250ml Erlenmeyer flasks were prepared containing 1500 $\mu$ M-L-mandelate and a range of D-mandelate concentrations from zero to 6000 $\mu$ M in a volume of 30ml salts medium. 0.7 $\mu$  Ci of sterile, carrier-free DL- [carboxy-<sup>14</sup>C] mandelate was added, thus raising the D-mandelate concentration by 1.5 $\mu$ M. A 1% nutrient broth inoculum was used. Flasks were sampled after 24h and 52h to determine the radioactivity remaining (Methods, p. 48). On the assumption that all the L-mandelate had been utilised, the utilisation of D-mandelate was calculated from any additional loss of radioactivity from the flask over that loss of <sup>14</sup>C representing the L- [carboxy-<sup>14</sup>C] mandelate.

ND indicates that no loss of radioactivity, above that representing utilisation of L-mandelate, could be detected, thus no loss of D-mandelate could be measured.

Initial D-mandelate $\mu$ Molar	Change in $\mu$ Molarity	
	After 24h	After 52h
1.5	1.11	1.28
3.0	1.62	1.86
4.5	2.09	2.32
7.5	2.15	2.55
13.5	3.21	3.52
24.0	5.70	7.00
51.5	3.21	6.48
101.5	6.06	4.88
401.5	ND	ND
751.5	ND	ND
1001.5	ND	ND
1501.5	ND	ND
3001.5	ND	ND
6001.5	ND	ND

believed that the accumulation of such a toxic metabolisable intermediate could explain some unusual features in the mandelate growth curve. Thus, mandelate utilisation and benzaldehyde accumulation in the medium were measured during growth in mandelate-salts medium.

The time course of mandelate utilisation during growth was unusual (Fig.16; data from control growth curve, Fig.15). Though utilisation at first accelerated the rate dropped steadily after 360min with  $\frac{1}{3}$  of the mandelate still remaining. Presented differently, mandelate utilisation resembled a sigmoidal function of growth (Fig.17; same experiment as above). Mandelate was exhausted about one half generation before growth ceased. This pseudo-sigmoidal relation of mandelate utilisation to growth was in contrast to the strictly linear relations between benzoate utilisation and growth (Fig.11), between benzyl alcohol utilisation and growth (Fig.14) and even between mandelate utilisation and growth from a benzyl alcohol inoculum (Fig.17).

Although a considerable quantity of benzaldehyde was accumulated in the medium, benzaldehyde exhaustion coincided with mandelate exhaustion (Fig.17). So the last phase of growth in a "mandelate" culture must represent yet another excreted product or products.

The contribution of excreted benzaldehyde to the complexity of the mandelate growth curve was gauged by a comparison of Figs. 17 and 15. The fall-off in initial growth rate from  $\mu = 0.69\text{h}^{-1}$  coincided with the first detection of benzaldehyde, and the fall-off from

Fig. 16 . Kinetics of mandelate utilisation during  
growth of bacterium NCIB 8250.

The culture bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately as described in Methods (p. 47). The experiment was done in 2.8mM-DL-mandelate-salts medium. At intervals samples were taken to estimate mandelate utilisation radiochemically (Methods, p. 48).



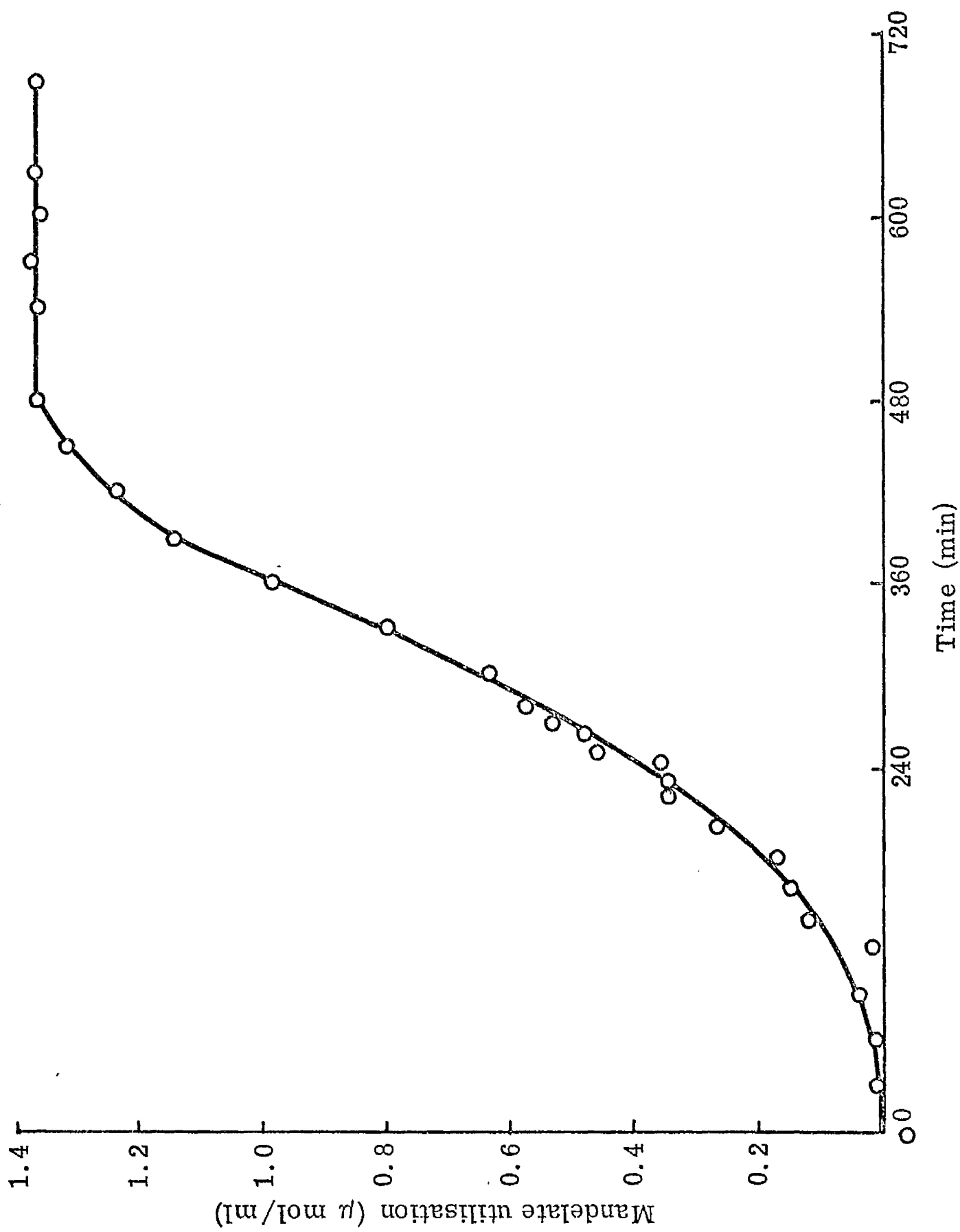


Fig. 17 . Benzaldehyde accumulation and mandelate utilisation during growth of bacterium NCIB 8250 from different inocula.

The cultures of bacterium NCIB 8250 used for inoculation were grown in 5mM-benzyl alcohol-salts medium and in 10mM-DL-mandelate-salts medium, harvested at turbidities of 0.4 and 0.1 respectively, resuspended and used immediately as described in Methods (p. 47). Experiments were done in 2.8mM-DL-mandelate-salts medium. At intervals, samples were taken to measure growth turbidimetrically, to measure mandelate utilisation radiochemically (Methods, p. 47) and to measure benzaldehyde accumulation spectrophotometrically (Methods, p. 68).

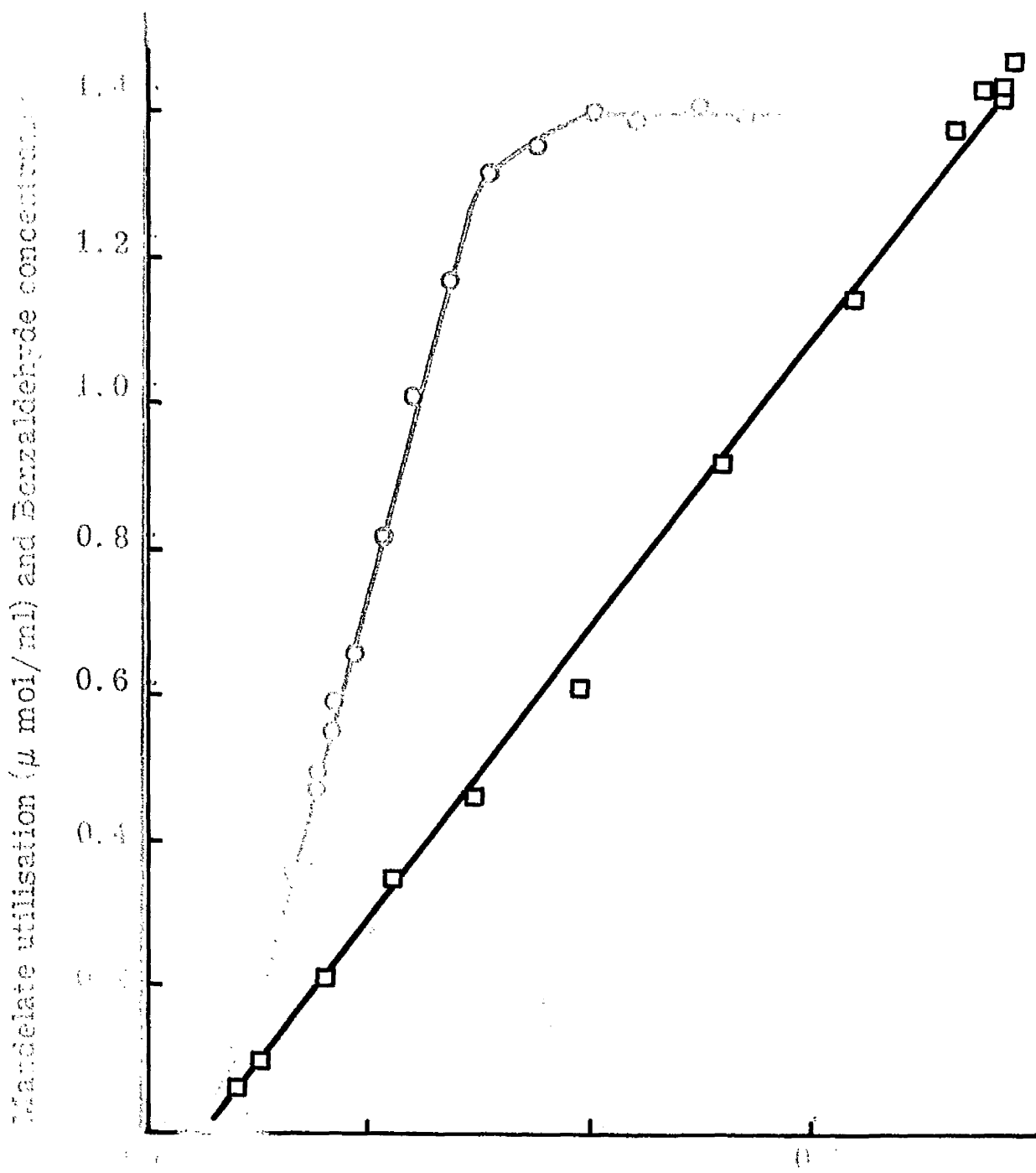
Culture grown from the preinduced mandelate inoculum:

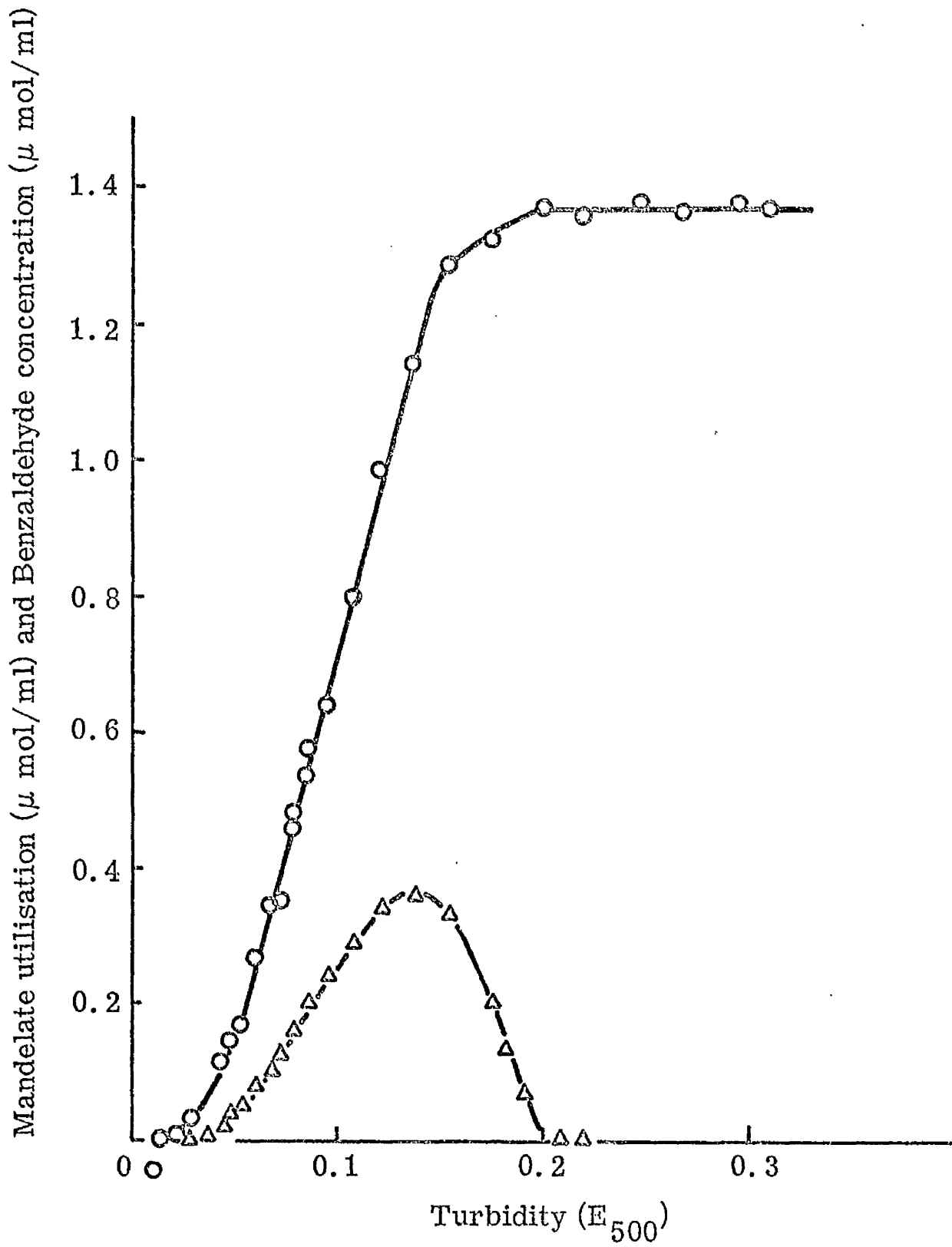
- Mandelate utilisation.
- △ Benzaldehyde concentration in the medium.

Culture grown from the benzyl alcohol-grown inoculum:

- Mandelate utilisation.

Benzaldehyde accumulation was not measured.





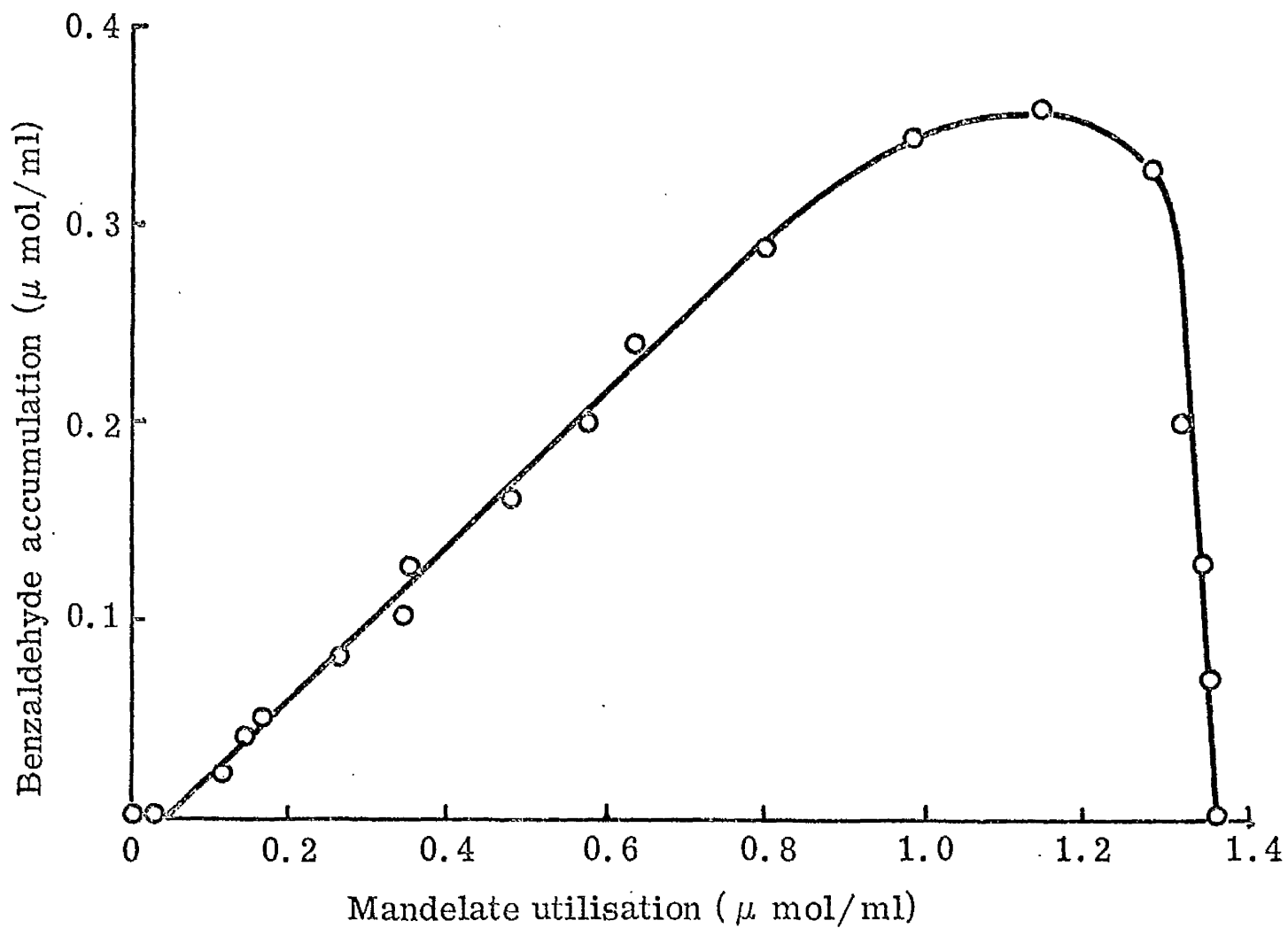
exponential growth at  $\mu = 0.25\text{h}^{-1}$  coincided with benzaldehyde exhaustion. On the other hand, cells preinduced to growth in benzyl alcohol-salts medium showed no signs of benzaldehyde toxicity during growth in mandelate-salts medium, and mandelate utilisation was a linear function of growth throughout growth, showing that no significant accumulation of intermediates occurred.

With the exception of benzaldehyde, the nature of the compounds accumulated during growth on mandelate has not been determined. A sample of the medium was taken after benzaldehyde exhaustion, deproteinised with perchloric acid and the supernate made alkaline. An ultraviolet spectrum of this alkaline extract was read from 450  $\rightarrow$  220nm on a Unicam SP800 Ultraviolet spectrophotometer. A spectrum characteristic of mandelate (presumably the D-enantiomer) was observed but there was no evidence of any other u.v. absorbing material. However, the alkaline extract was acidified and extracted into chloroform under conditions which extract benzoate (Fewson, Livingstone & Roach, 1970). The chloroform layer was extracted with base and the aqueous layer separated and neutralised. This latter solution had no u.v. absorption spectrum, read against a reagent blank, and so contained no benzoate. The culture at this point did not contain L-mandelate, phenylglyoxylate, benzaldehyde or benzoate so presumably the unidentified material remaining in the culture medium was aliphatic.

When benzaldehyde accumulation was compared with mandelate utilisation, a linear relation was observed (Fig.18). Thus,

Fig. 18 .      Variation of benzaldehyde accumulation with  
mandelate utilisation during growth of  
bacterium NCIB 8250.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately as described in Methods (p. 47). The experiment was done in 2.8mM-DL-mandelate-salts medium. At intervals samples were taken to measure mandelate utilisation radiochemically (Methods, p. 48) and to measure benzaldehyde concentration spectrophotometrically (Methods, p. 68).



benzaldehyde utilisation remained a constant fraction of the mandelate decarboxylated and hence, presumably, the ratios of the different enzymes involved in the production and removal of benzaldehyde were maintained in constant ratios to one another. The ratio of benzaldehyde accumulation to mandelate utilisation was 0.38, but the maximum benzaldehyde concentration observed, 0.36mM, was only 0.25 of the L-mandelate originally present. This discrepancy arose because the accumulation of benzaldehyde slowed down corresponding to a slight lowering in the rate of mandelate utilisation with about  $\frac{1}{3}$  of the mandelate remaining (Fig.16, same experiment). As the rate of mandelate utilisation slowly declined the benzaldehyde concentration at first remained constant and then dropped sharply to zero as mandelate was exhausted.

These characteristics of the growth of bacterium NCIB 8250 in mandelate-salts medium illustrate the difficulty in obtaining "preinduced mandelate cells" as inocula for growth experiments. There also seemed to be some variation in the exact growth pattern depending on the inoculum size; this has not been systematically studied, but the phase of growth following mandelate exhaustion seemed to comprise a larger proportion of the growth curve and benzaldehyde accumulation rose as the inoculum size was reduced. As a result of these phenomena the harvested, preinduced mandelate inocula used in growth experiments were taken from cultures before the penultimate generation (detailed in Legends to the appropriate Figures) because mandelate was exhausted in the final generation.



Most calculations of the molar growth yield on mandelate as carbon source had to be made by simple division of net growth of a culture by the quantity of substrate present, because the relation between mandelate utilisation and growth was generally non-linear (Fig.17). This was not an accurate method as there was some doubt whether all cultures had reached stationary state. The only experiment in which mandelate utilisation was a linear function of growth was growth in mandelate-salts medium from a preinduced benzyl alcohol inoculum (Fig.17). A molar growth yield of 68 $\mu$ g dry wt./ $\mu$ mol was observed with a maximum utilisation rate of 140nmol mandelate/mg dry wt./min.

## 2. 2. Growth and substrate utilisation by bacterium

### NCIB 8250 in dual substrate media.

#### 2. 2. 1. Methods of study.

To look for any possible interactions between different enzymes and regulons in the mandelate pathway of bacterium NCIB 8250, experiments were done on the utilisation of carbon sources during growth in dual substrate media.

The preinduced cultures used for inoculation in these experiments were grown in mandelate-, benzyl alcohol- or benzoate-salts medium, that is, substrates for enzymes in regulons R<sub>1</sub>, R<sub>2</sub> and R<sub>3</sub> respectively (Methods, p.46). Nutrient broth inocula were used when cells non-induced for the mandelate pathway were required. Little of the work done with nutrient broth inocula is quoted because the results were no more clear cut than the illustrated

data and defined media were used where possible. Experiments were done in single (control) and dual carbon source media with the carbon source of the preinduction culture as the common growth substrate (Methods, p.47). The dual substrate media contained appropriate, usually equimolar, mixtures chosen from mandelate, benzyl alcohol, benzoate, catechol and succinate (used at twice concentration of other carbon sources because of its low molar growth yield).

The assay for each aromatic substrate was based on the decarboxylation of a  $^{14}\text{C}$ -labelled carbon in the sidechain i.e.  $[\text{carboxy-}^{14}\text{C}]$  mandelate,  $[\text{carbinol-}^{14}\text{C}]$  benzyl alcohol and  $[\text{carboxy-}^{14}\text{C}]$  benzoate. Utilisation of each substrate in dual carbon source media was followed separately in parallel cultures differing only in the compound which was radiochemically labelled. Parallel cultures were always very similar in their growth patterns, as illustrated in Fig.19. There were slight discrepancies due to variations at inoculation but parallel flasks were never more than 10min apart at the end of growth, even in the slow growing mandelate cultures.

### 2. 2. 2. Growth of preinduced benzoate cells of bacterium NCIB 8250 in benzoate-salts media and the interactions of other intermediates.

The control culture grew exponentially in benzoate-salts medium ( $\mu = 0.92\text{h}^{-1}$ ) to a turbidity ( $E_{500}$ ) of 0.37 (Fig.19). Benzoate utilisation in the same experiment is illustrated in Fig.20.

Fig. 19 .      The effects of mandelate and of benzyl alcohol  
on the growth of bacterium NCIB 8250 in benzoate-  
salts medium.

The culture of bacterium NCIB 8250 used for inoculation was grown in 2mM-benzoate-salts medium, harvested at a turbidity of 0.2, resuspended and used immediately as described in Methods (p. 47). Experiments were done in salts medium containing 1.4mM-benzoate, 1.4mM-benzoate + 2.8mM-DL-mandelate and 1.4mM-benzoate+ 1.4mM-benzyl alcohol (Methods, p. 47). Growth was measured turbidimetrically (Methods, p. 44).

○ Control: growth in benzoate-salts medium.

□ Growth in benzoate + mandelate-salts medium.

△ Growth in benzoate + benzyl alcohol-salts medium.

Growth of parallel dual-substrate cultures shown on superimposition.

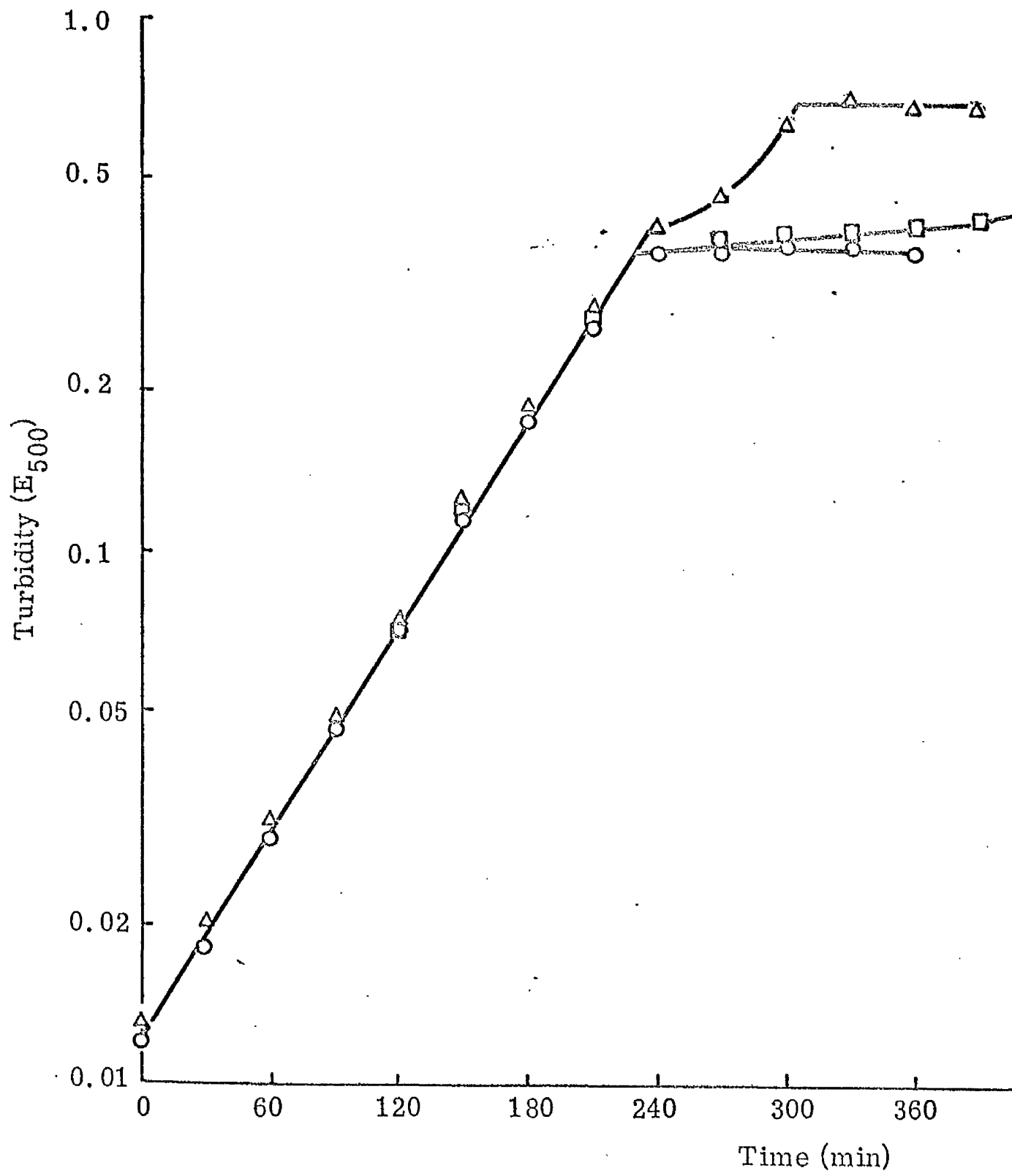
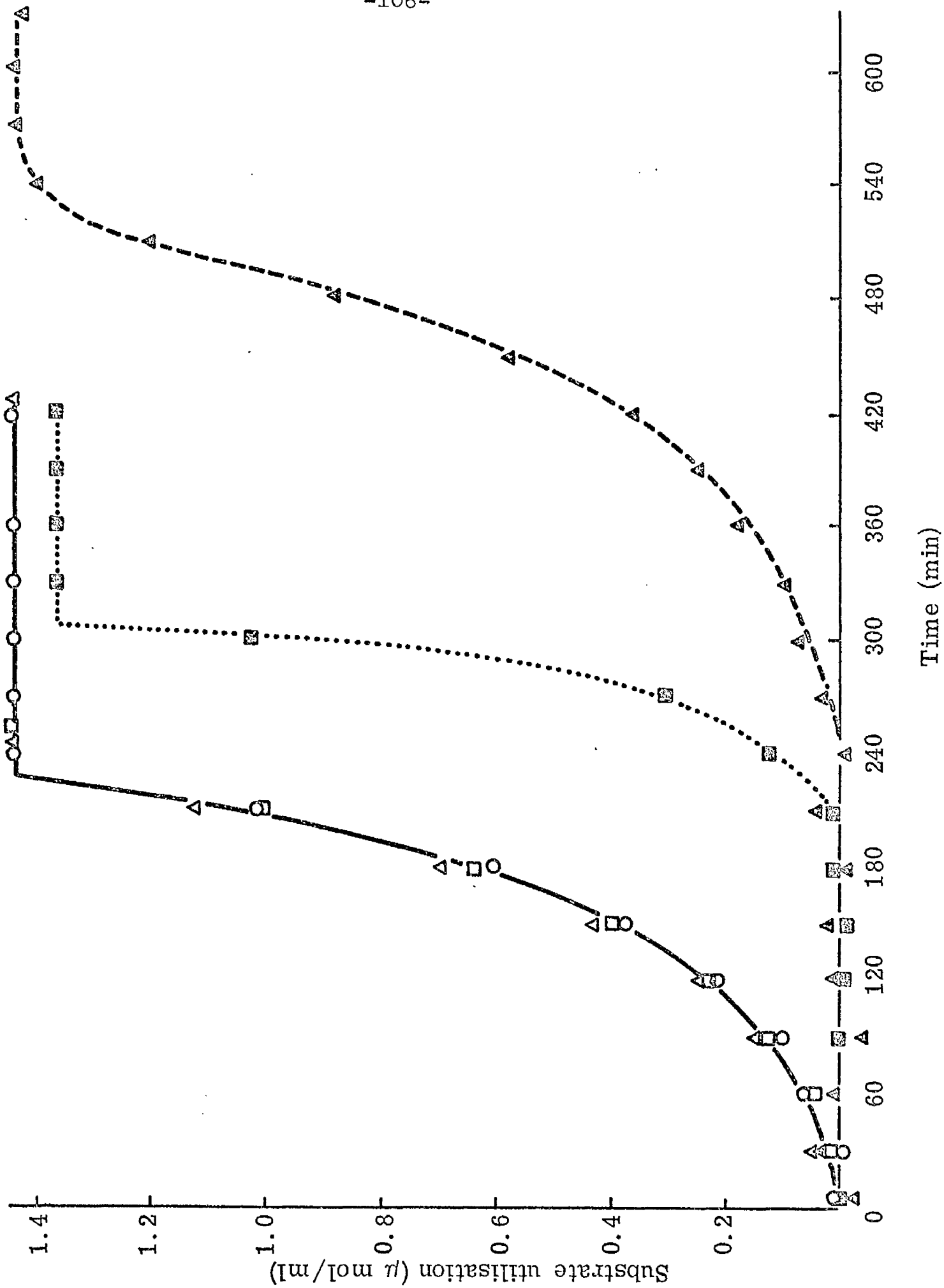


Fig. 20 . The effects of mandelate and of benzyl alcohol on benzoate utilisation by growing cultures of bacterium NCIB 8250: kinetics of the utilisation of all substrates.

The culture of bacterium NCIB 8250 used for inoculation was grown in 2mM-benzoate-salts medium, harvested at a turbidity of 0.2, resuspended and used immediately as described in Methods (p.47 ). Experiments were done in salts medium containing 1.4mM-benzoate, 1.4mM-benzoate + 2.8mM-DL-mandelate and 1.4mM-benzoate + 1.4mM-benzyl alcohol (Methods, p.47 ). At intervals substrate utilisation was estimated radiochemically (Methods, p.48 ).

- Control culture with benzoate alone: utilisation of benzoate.
- △ Utilisation of benzoate and ▲ utilisation of mandelate from parallel benzoate + mandelate-salts cultures.
- Utilisation of benzoate and ■ utilisation of benzyl alcohol from parallel benzoate + benzyl alcohol-salts cultures.



The control patterns of growth (Fig.19) and benzoate utilisation (Fig.20) were totally unaffected by mandelate in the initial stages and growth on mandelate was a slow phase following benzoate growth. There was no utilisation of mandelate during growth on benzoate (Fig.20). Mandelate utilisation was first detected at 270min i.e. 45min after benzoate exhaustion.

Benzyl alcohol only marginally affected the control patterns of growth (Fig.19) and benzoate utilisation (Fig.20), and growth on benzyl alcohol-salts occurred after a steep drop in initial growth rate. There was in fact an increased growth of 0.02 turbidity units over the control before the drop in growth rate. During growth on benzoate, no benzyl alcohol was used until some 20min before benzoate exhaustion (Fig.20). The amount of benzyl alcohol utilised in this time would account for 0.02 turbidity units of growth, and the low level of the benzyl alcohol oxidising enzymes present was indicated by the drop in growth rate on benzoate exhaustion.

The growth rate of bacterium NCIB 8250 in benzoate-salts medium ( $\mu = 0.92\text{h}^{-1}$ ) was markedly reduced ( $\mu = 0.66\text{h}^{-1}$ ) by the presence of catechol (Fig.21). Benzoate exhaustion had little effect on the culture. When these two growth curves were expressed as functions of benzoate utilisation (Fig.22a) parallel and almost coincident lines were obtained indicating no net contribution of catechol to growth on benzoate during benzoate utilisation. This was surprising as catechol lies down the catabolic pathway from

Fig. 21.        The effect of catechol on the growth of  
                 bacterium NCIB 8250 in benzoate-salts medium.

The culture of bacterium NCIB 8250 for inoculation was grown in 2mM-benzoate-salts medium and used without harvesting when the turbidity reached 0.2 (Methods, p. 46). Experiments were done in 1.5mM-benzoate-salts medium with and without 1.3mM-catechol, as described in Methods (p.47 ). Growth was measured turbidimetrically (Methods, p. 44).

○ Control: growth in benzoate-salts medium.

△ Growth in benzoate + catechol-salts medium.



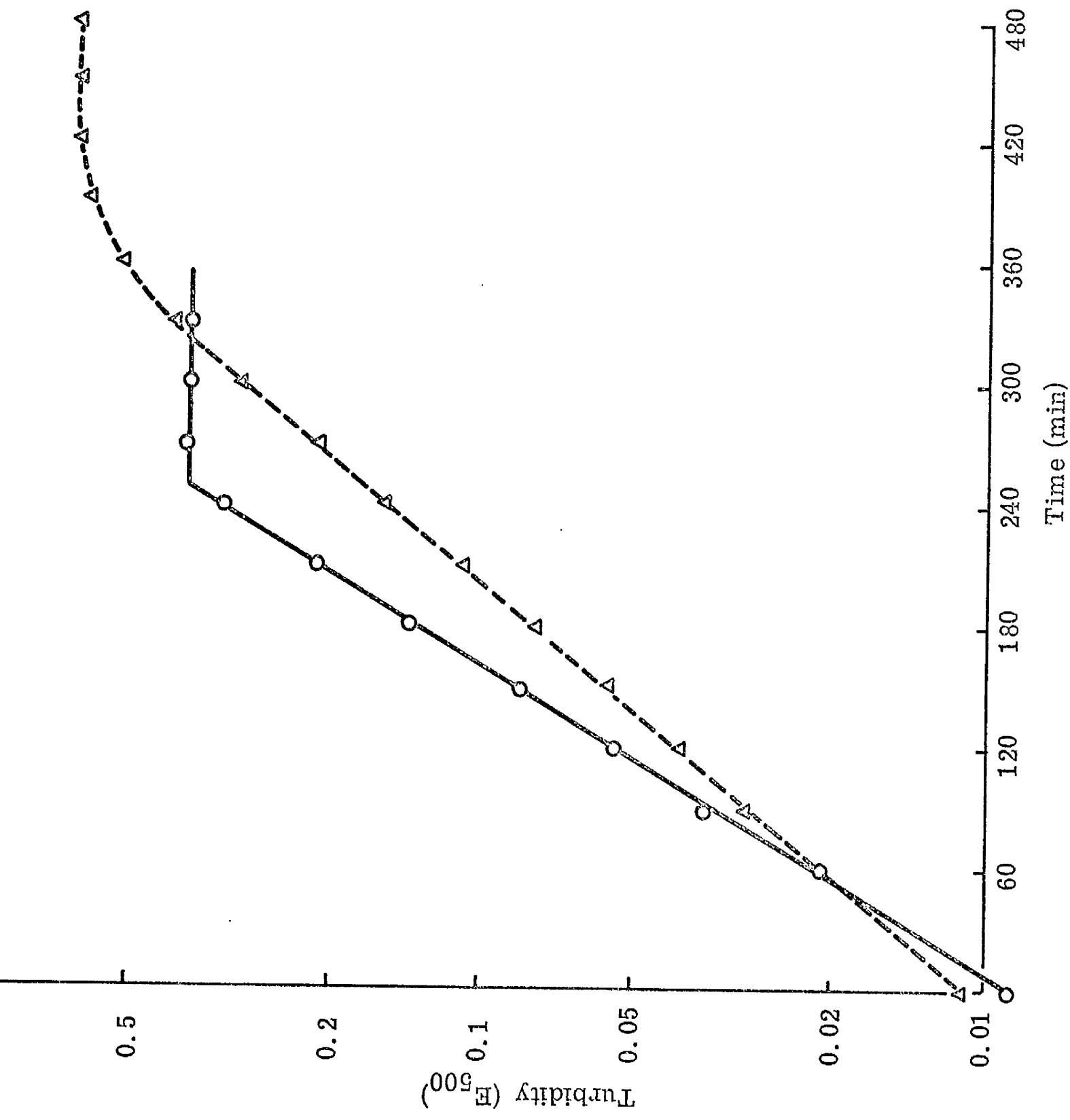
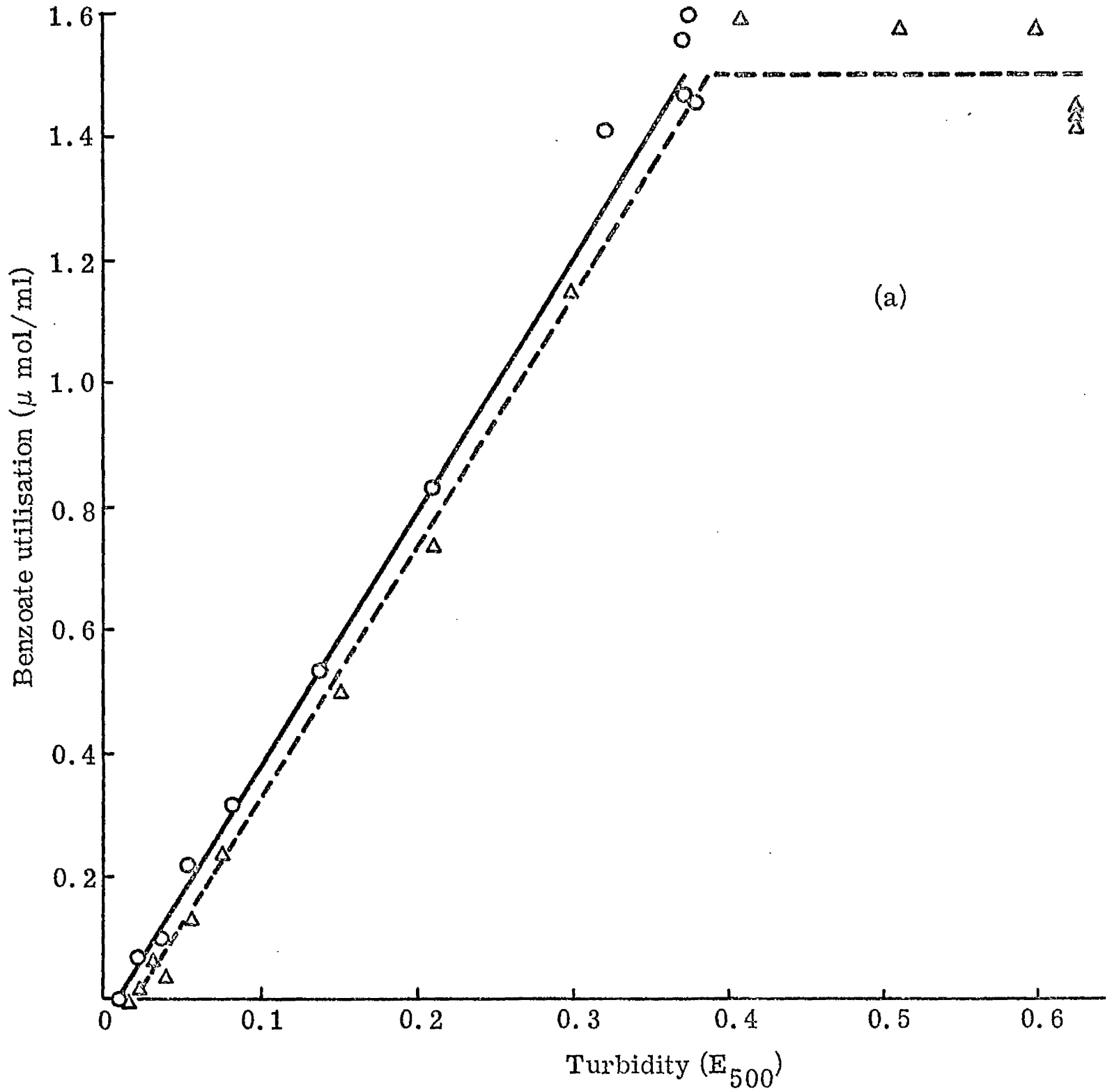


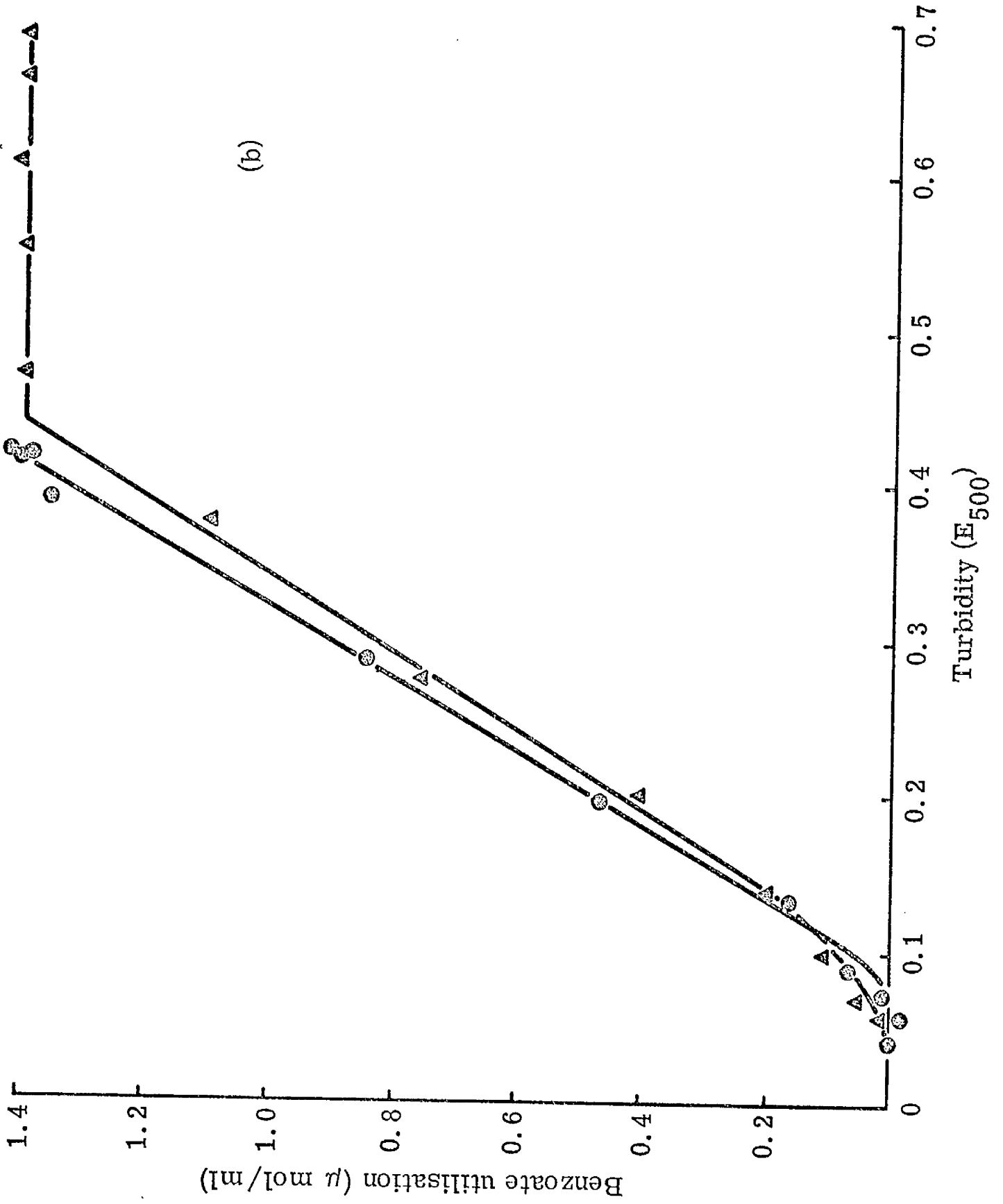
Fig. 22. The effect of catechol on the utilisation of benzoate by bacterium NCIB 8250 growing from different inocula.

The culture of bacterium NCIB 8250 for inoculation a) was grown in 2mM-benzoate-salts medium and used without harvesting when the turbidity reached 0.2 (Methods, p.46 ). These experiments were done in 1.5mM-benzoate-salts medium with and without 1.3mM-catechol (Methods, p.47 ). In b), a nutrient broth inoculum was used and these experiments were done in 1.4mM-benzoate-salts medium with and without 1.4mM-catechol. At intervals samples were taken for turbidimetric measurement of growth and radiochemical estimation of benzoate utilisation (Methods, p.47 ).

- ● Controls: benzoate utilisation during growth in benzoate-salts medium.
- △ ▲ Benzoate utilisation during growth in benzoate + catechol-salts medium.

The open symbols, graph a), represent a preinduced inoculum and the closed symbols, graph b), represent a nutrient broth inoculum.





benzoate and its catabolism is controlled by a separate regulon (see Introduction, P.11 ). This phenomenon was repeated when nutrient broth cells were used as an inoculum for benzoate-salts and benzoate + catechol-salts media (Fig.22b). The relation between benzoate utilisation and turbidity in the presence of catechol indicated only a marginal net contribution of catechol to growth on benzoate. The induction of benzoate oxidase was thus not detectably affected by the presence of catechol.

Bacterium NCIB 8250 grew exponentially ( $\mu = 0.88\text{h}^{-1}$ ) in benzoate + succinate-salts medium. Though the growth rate declined steadily in the last generation no sharp breaks in slope were observed. When benzoate utilisation was expressed as a function of growth, a straight line of lower slope than the control was obtained. Similar co-utilisation of benzoate and succinate was observed when a nutrient broth inoculum was used.

### 2. 2. 3. Growth of preinduced mandelate cells of bacterium NCIB 8250 in mandelate-salts medium and the interactions of other intermediates.

The control culture displayed the characteristic growth curve (Fig. 23) and mandelate utilisation pattern (Fig. 24) already detailed (Results, p. 93).

The presence of benzoate changed these patterns. Growth (Fig. 23) accelerated in the presence of benzoate to a rate ( $\mu = 0.83\text{h}^{-1}$ ) which was maintained till benzoate exhaustion at about 260min (Fig. 24), and there followed a phase of mandelate-

Fig. 23. The effect of benzoate on the growth of bacterium NCIB 8250 in mandelate-salts medium.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately as described in Methods (p. 47). Experiments were done in 2.8mM-DL-mandelate-salts medium with and without 1.4mM-benzoate (Methods, p. 47). Growth was measured turbidimetrically (Methods, p. 44).

○ Control: growth in mandelate-salts medium.

△ Growth in mandelate + benzoate-salts medium.

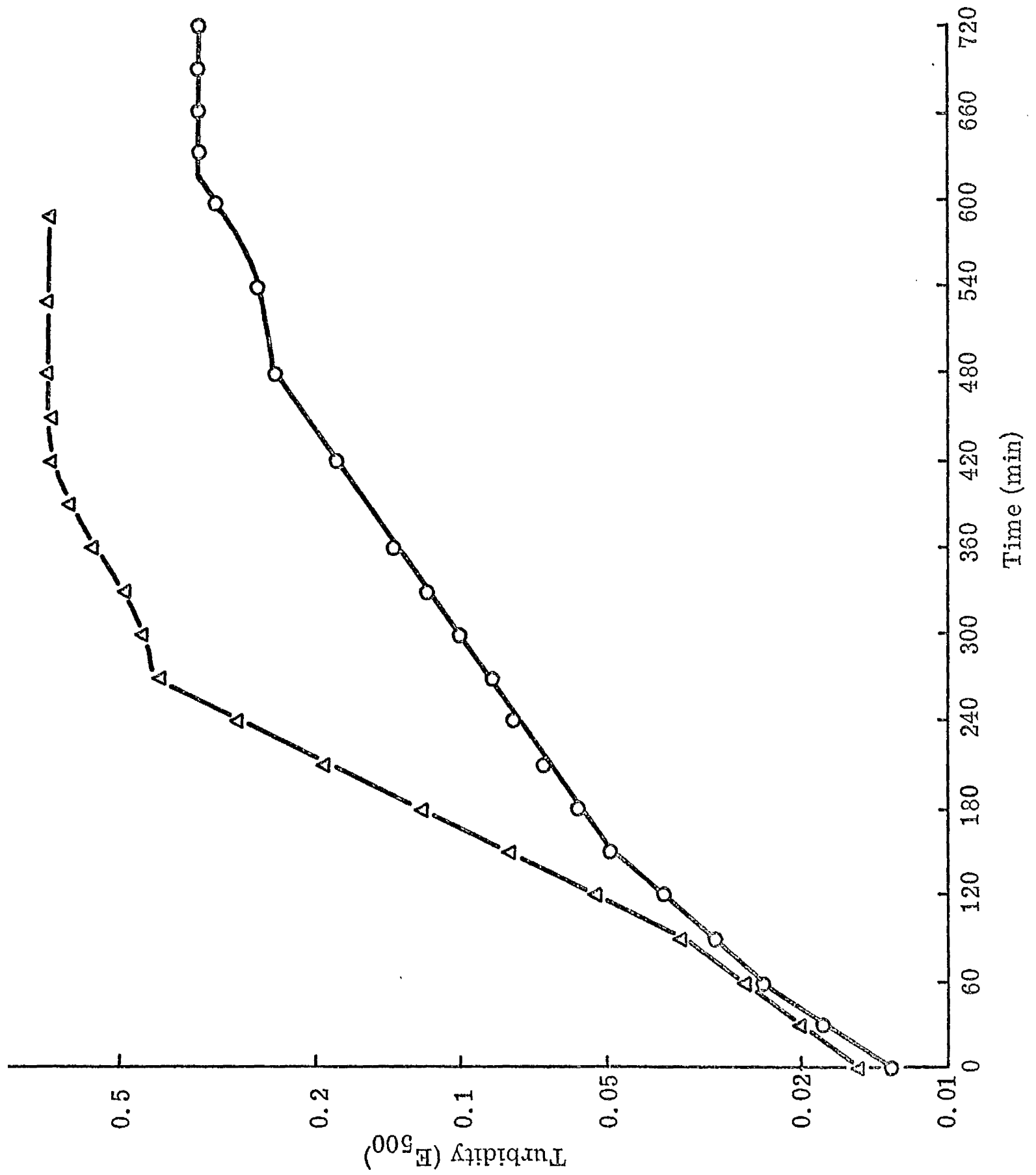
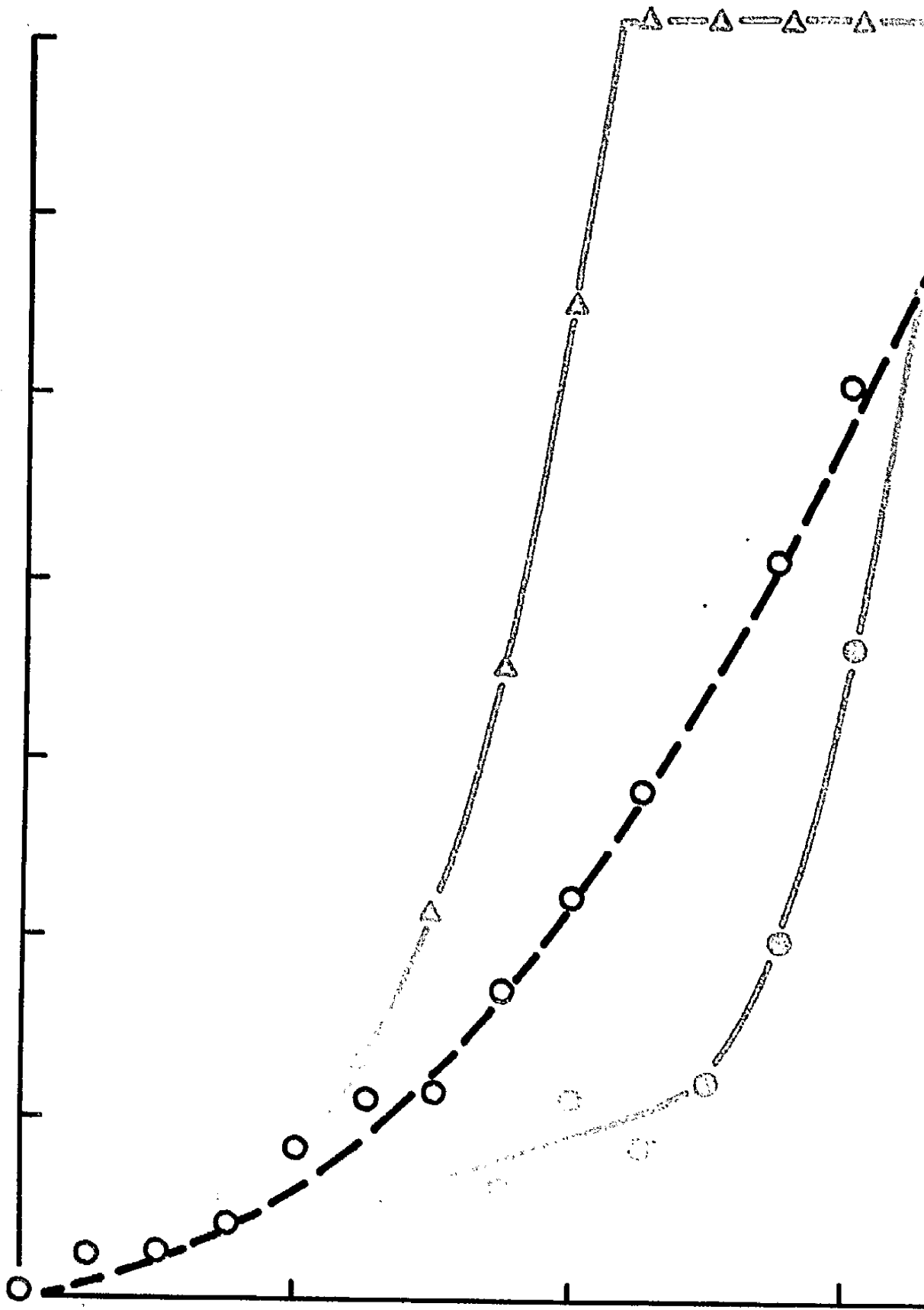


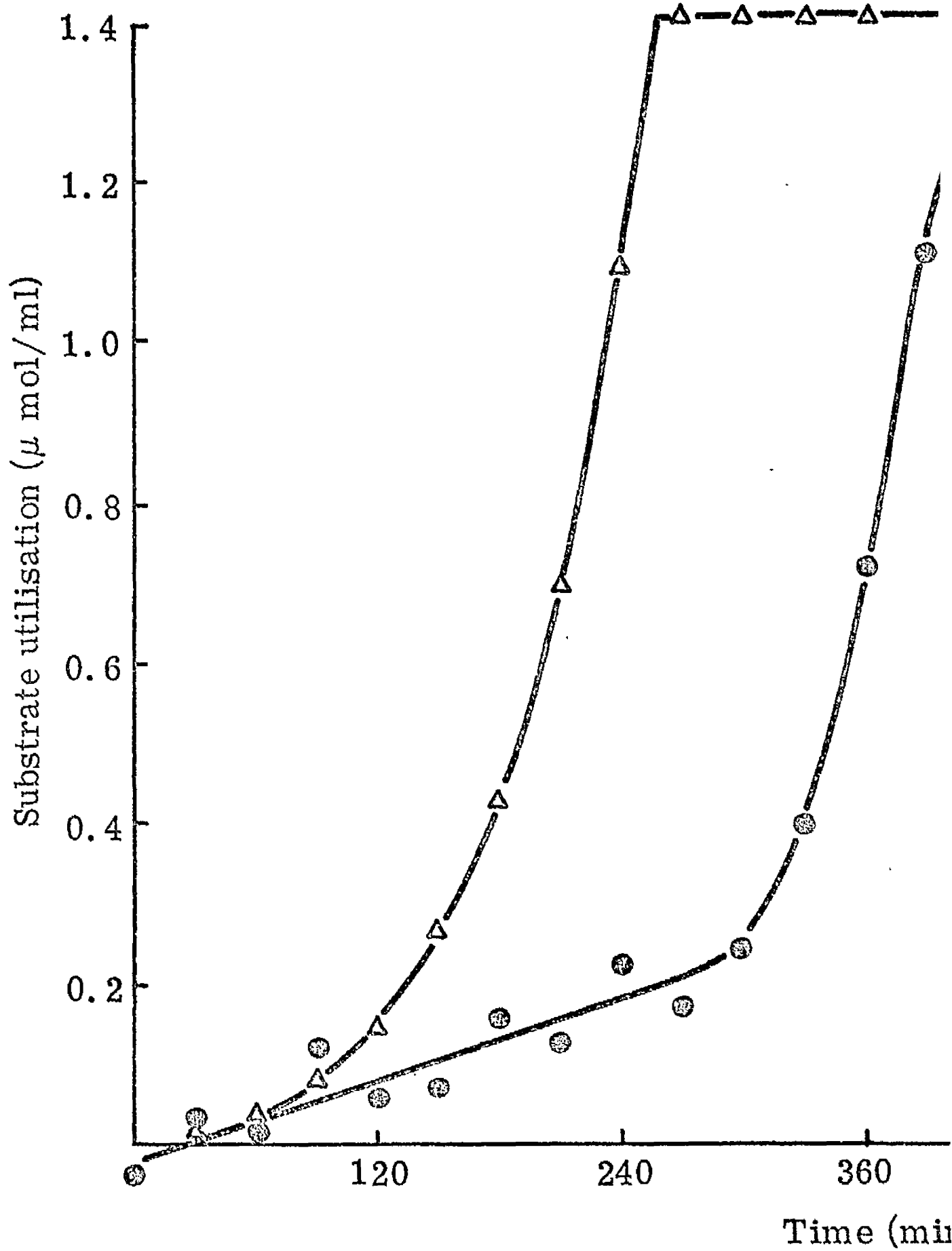
Fig. 24. The effect of benzoate on mandelate utilisation  
by growing cultures of bacterium NCIB 8250:  
kinetics of the utilisation of both substrates.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately (Methods, p. 47). Experiments were done in 2.8mM-DL-mandelate-salts medium with and without 1.4mM-benzoate as described in Methods (p. 47). At intervals substrate utilisation was estimated radiochemically (Methods, p. 48).

- Control culture with mandelate alone:  
utilisation of mandelate.
- ⊙ Utilisation of mandelate and  $\triangle$  utilisation  
of benzoate from parallel mandelate + benzoate-  
salts cultures.







supported growth. During benzoate utilisation, mandelate utilisation did occur (Fig.24) and this mandelate utilisation was linear with time. Thus the activity of the  $R_1$  enzymes remained constant despite a 27-fold increase in bacterial mass over this period.

The results did not permit an accurate comparison of mandelate utilisation rates in the two cultures at zero time. To determine whether benzoate altered the activity of pre-existing  $R_1$  enzymes, benzoate was added to a culture growing in mandelate-salts medium. Growth accelerated to  $\mu = 0.74h^{-1}$  on the addition of benzoate (Fig.25). This increased rate was maintained for a period corresponding to the utilisation of the 1.6mM benzoate added (allowing for utilisation of mandelate), after which growth reverted to a mandelate-type curve. During the period corresponding to benzoate utilisation (220-390min, Fig.25), mandelate utilisation was linear with time (Fig.26). Thus mandelate decarboxylation in the culture continued at approximately the same rate it had achieved immediately before the addition of benzoate, whereas the rate in the control culture continued to rise. The repression of mandelate oxidising activity in these cells is emphasised by the fact that at 360min, when the rate of decarboxylation of mandelate in the control culture was much faster than in the culture to which benzoate had been added (Fig.26). the latter culture contained 2.5x as much dry wt./ml (Fig.25).

The presence of benzyl alcohol caused a slight though

Fig. 25. The effect of the addition of benzoate to a culture of bacterium NCIB 8250 growing in mandelate-salts medium.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately as described in Methods (p. 47). Experiments were done in 2.8mM-DL-mandelate-salts medium with the addition (  $\downarrow$  ) of water (control) or 450 $\mu$ mol benzoate (Methods, p. 47).

- Control: growth in mandelate-salts medium.
- △ Growth in mandelate-salts medium to which benzoate was added.

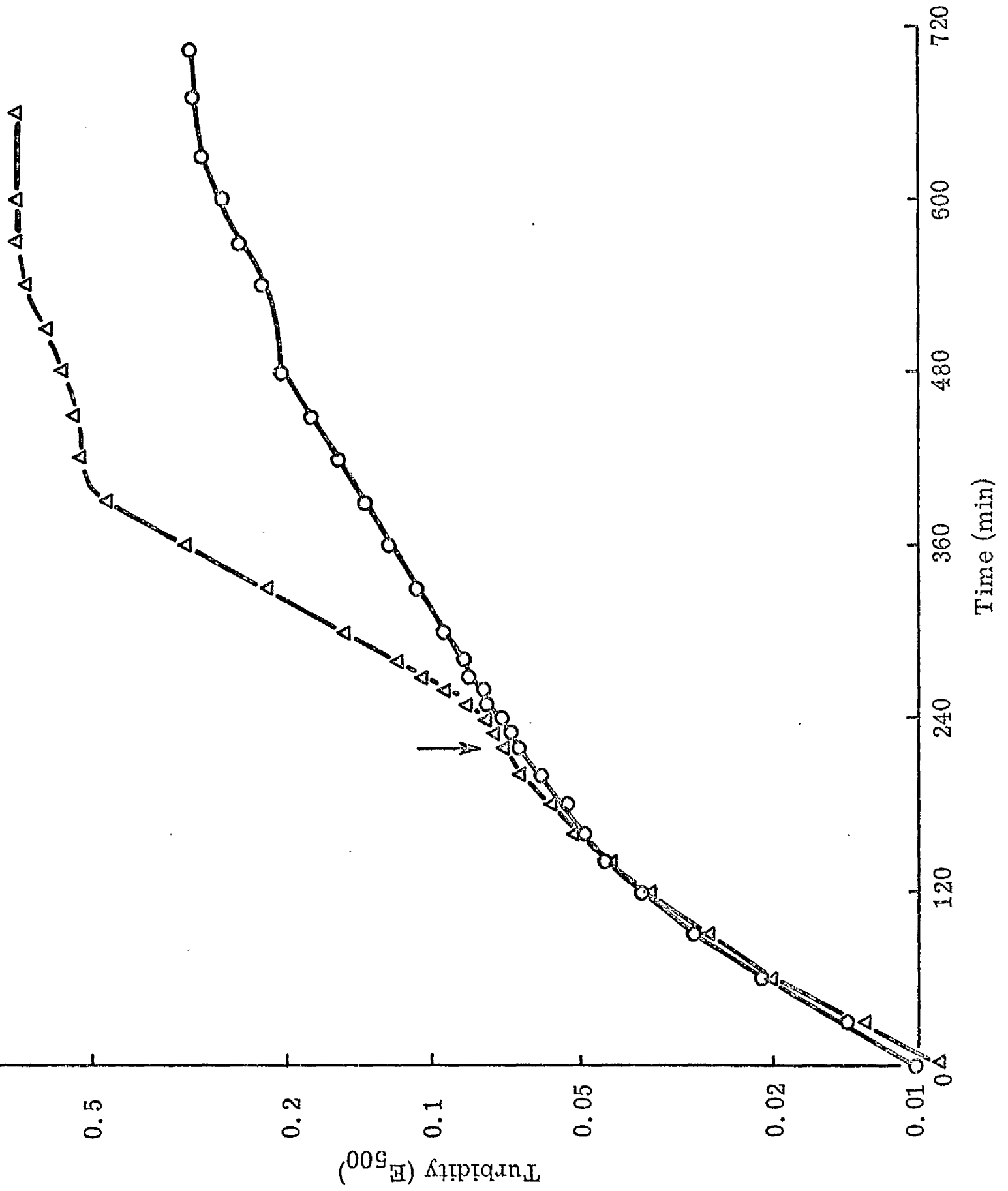
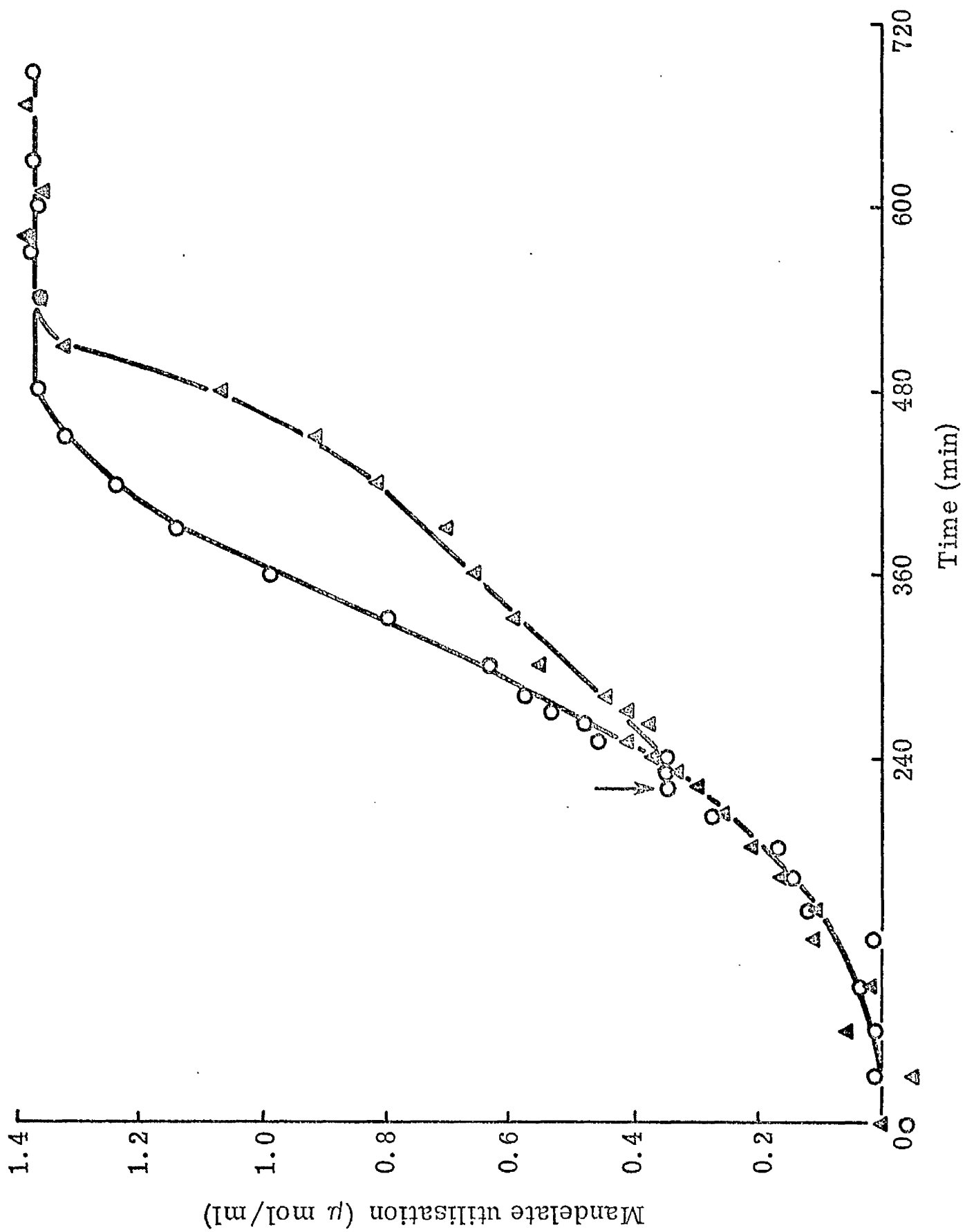


Fig. 26. The effect of benzoate, added during growth, on the utilisation of mandelate by bacterium NCIB 8250.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately as described in Methods (p. 47). Experiments were done in 2.8mM-DL-mandelate-salts medium with the addition ( ↓ ) of water (control) or 450µmol benzoate (Methods, p. 47). At intervals, samples were taken for radiochemical estimation of mandelate utilisation (Methods, p.48 ).

○ Control culture with mandelate alone:  
utilisation of mandelate.

▲ Utilisation of mandelate in a culture to which  
benzoate was added.



reproducible inhibition of growth in mandelate-salts medium (Fig.27). This marginal inhibition did not mask the characteristic growth of bacterium NCIB 8250 in mandelate-salts, after which growth accelerated till stationary state. However, although the presence of benzyl alcohol had no effect on mandelate utilisation (Fig.28), between  $\frac{1}{3}$  and  $\frac{1}{2}$  of the benzyl alcohol originally present was decarboxylated during mandelate utilisation (Fig.28).

The utilisation of benzyl alcohol probably commenced at zero time and accelerated for about 2h after which utilisation remained linear with time. Approximately half an hour before mandelate exhaustion, benzyl alcohol utilisation declined (360min, Fig.28) and a lower rate of benzyl alcohol utilisation was maintained during the kink at the end of growth on mandelate after which (about 540min) utilisation accelerated till benzyl alcohol exhaustion. At 540min approximately 0.7mM-benzyl alcohol remained (Fig. 28), enough to support 0.2 turbidity units of growth (Fig. 14). However, the culture turbidity at this time was 0.33 and the stationary state turbidity was 0.66 (Fig. 27). Presumably this discrepancy arose from the benzyl alcohol utilised in the first phase of growth without apparent contribution to growth. The accumulation of some intermediate would explain the results.

Some experiments were done with catechol as the second substrate in mandelate-salts medium. When a nutrient broth inoculum grew in catechol + mandelate-salts medium, initial rapid growth settled at a rate  $\mu = 0.69\text{h}^{-1}$  during which mandelate



Fig. 27. The effect of benzyl alcohol on the growth of bacterium NCIB 8250 in mandelate-salts medium.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately (Methods, p. 47). Experiments were done in 2.8mM-DL-mandelate-salts medium with and without 1.3mM-benzyl alcohol (Methods, p. 47). Growth was measured turbidimetrically as described in Methods (p. 44).

○ Control: growth in mandelate-salts medium.

△ Growth in mandelate + benzyl alcohol-salts medium.

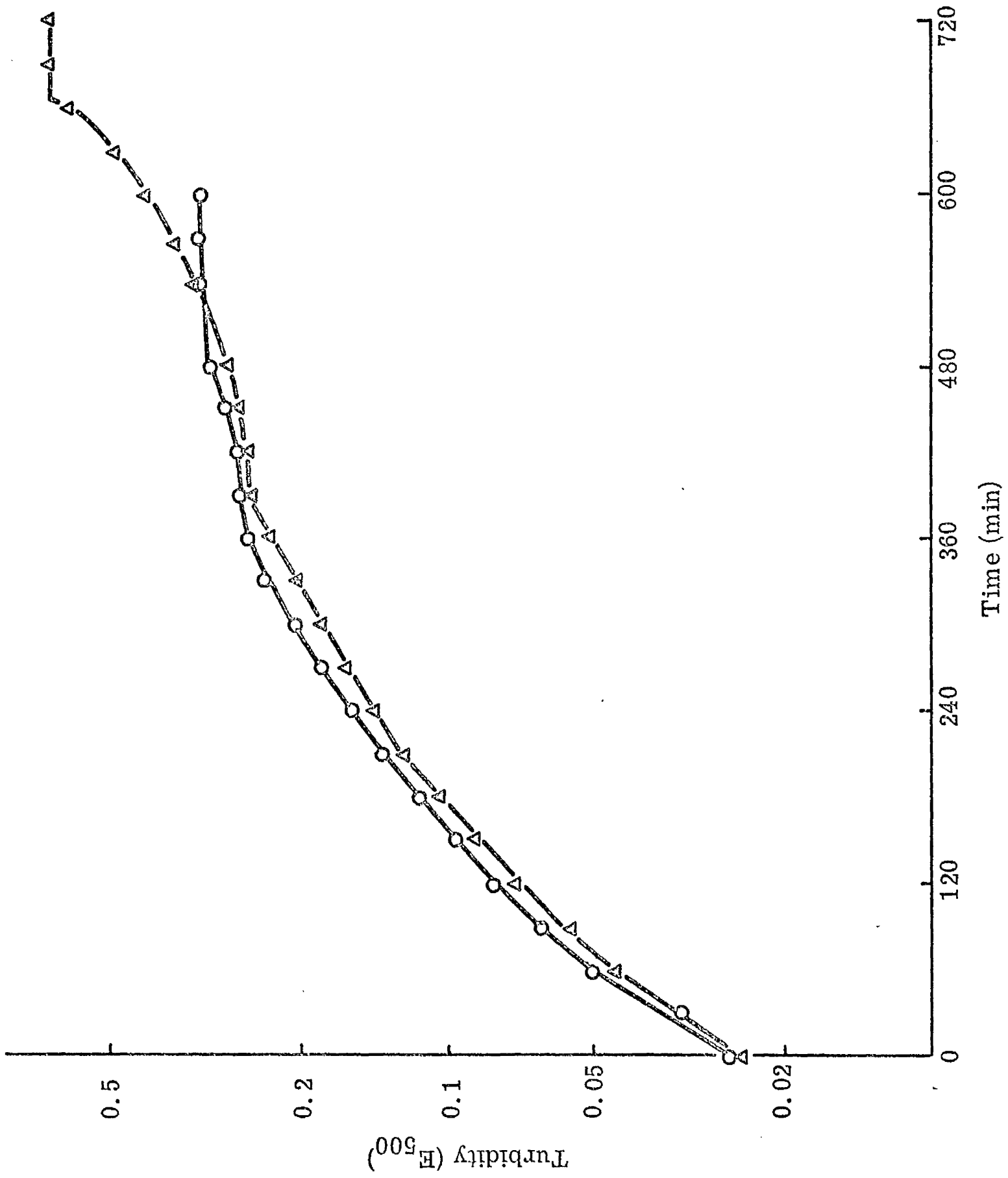
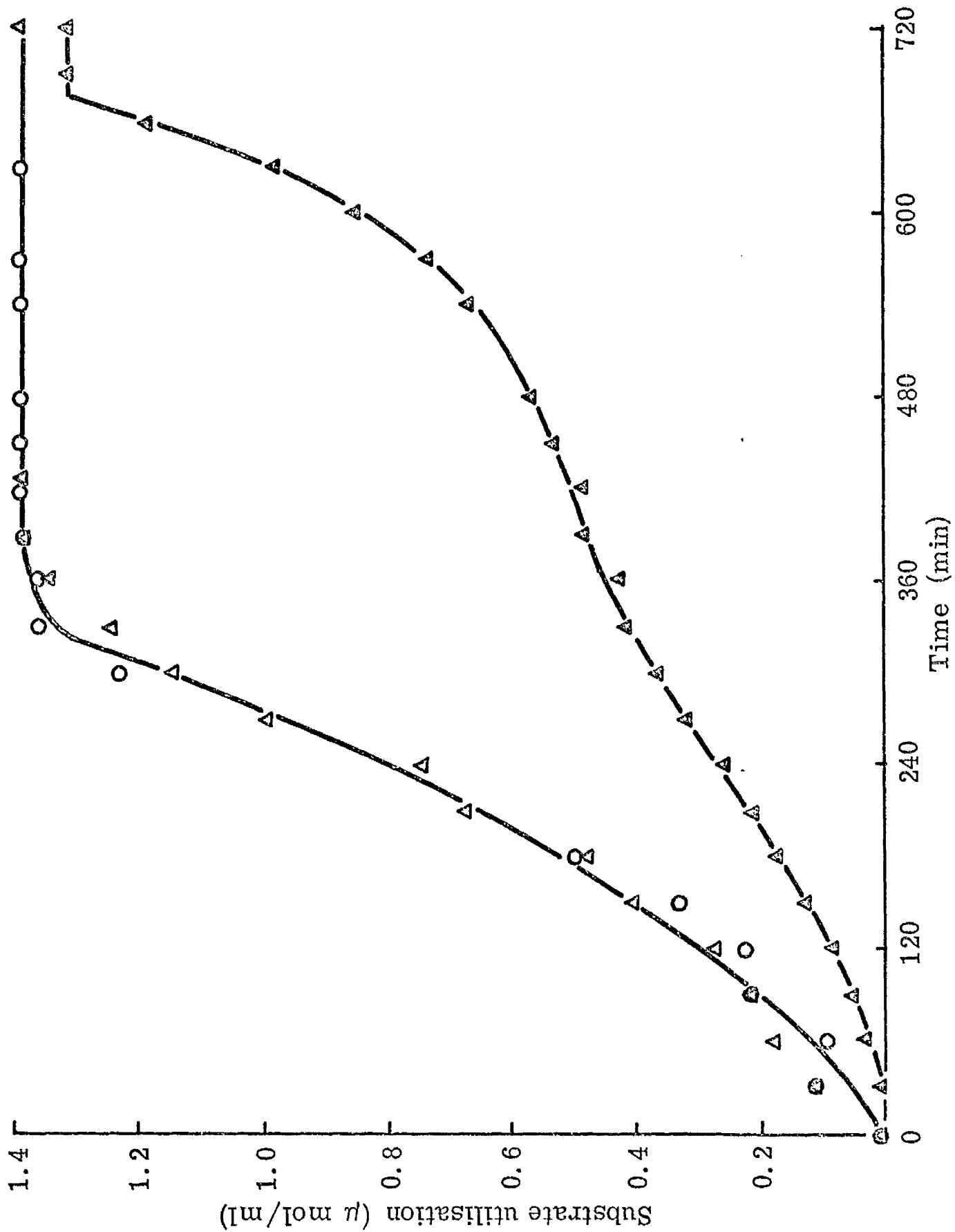


Fig. 28. The effect of benzyl alcohol on mandelate utilisation by growing cultures of bacterium NCIB 8250: kinetics of the utilisation of both substrates.

The culture of bacterium NCIB 8250 used for inoculation was grown in 10mM-DL-mandelate-salts medium, harvested at a turbidity of 0.1, resuspended and used immediately as described in Methods (p. 47). The experiments were done in 2.8mM-mandelate-salts medium with and without 1.3mM-benzyl alcohol (Methods, p. 47). At intervals samples were taken for radiochemical substrate estimation as described in Methods (p.48 ).

○ Control culture with mandelate alone: utilisation of mandelate.

△ Utilisation of mandelate and ▲ utilisation of benzyl alcohol in parallel mandelate+ benzyl alcohol-salts cultures.



utilisation was very low, possibly zero. A sharp drop in growth rate at a turbidity of 0.44 coincided with the onset of significant mandelate utilisation. When a preinduced mandelate inoculum grew in catechol + mandelate-salts medium, initial growth at a rate  $\mu = 0.66\text{h}^{-1}$  was accompanied by a low linear rate of mandelate utilisation. A marked drop in growth rate was matched by a sharp increase in mandelate utilisation at a turbidity of 0.34.

In an experiment with succinate + mandelate-salts medium, growth of a preinduced mandelate inoculum accelerated for 3h to a rate  $\mu = 0.88\text{h}^{-1}$ . At a turbidity of 0.44 there was a sharp drop in the growth rate and a period of slow growth continued to stationary state. However, the pattern of mandelate utilisation observed in the control was virtually unaltered by the presence and metabolism of succinate.

2. 2. 4. Growth of preinduced benzyl alcohol cells of bacterium NCIB 8250 in benzyl alcohol-salts medium, and the interactions of other intermediates.

The control growth curve accelerated through the first generation to a rate  $\mu = 0.87\text{h}^{-1}$  which was maintained until stationary state (Fig. 29). Benzyl alcohol utilisation in this culture is shown in Fig. 30.

In the presence of benzoate, the culture took fractionally longer than the control to attain a similar rate  $\mu = 0.86\text{h}^{-1}$  (Fig. 29). There was no break of slope in this growth curve though the growth

Fig. 29,           The effect of benzoate on the growth of  
                  bacterium NCIB 8250 in benzyl alcohol-salts  
                  medium.

The culture of bacterium NCIB 8250 used for inoculation was grown in 5mM-benzyl alcohol-salts medium, harvested when the turbidity reached 0.4, resuspended and used immediately (Methods, p. 47). Experiments were done in 1.4mM-benzyl alcohol-salts medium with and without 1.4mM-benzoate as described in Methods (p. 47). Growth was measured turbidimetrically (Methods, p. 44).

○ Control: growth in benzyl alcohol-salts medium.

△ Growth in benzyl alcohol + benzoate-salts medium.

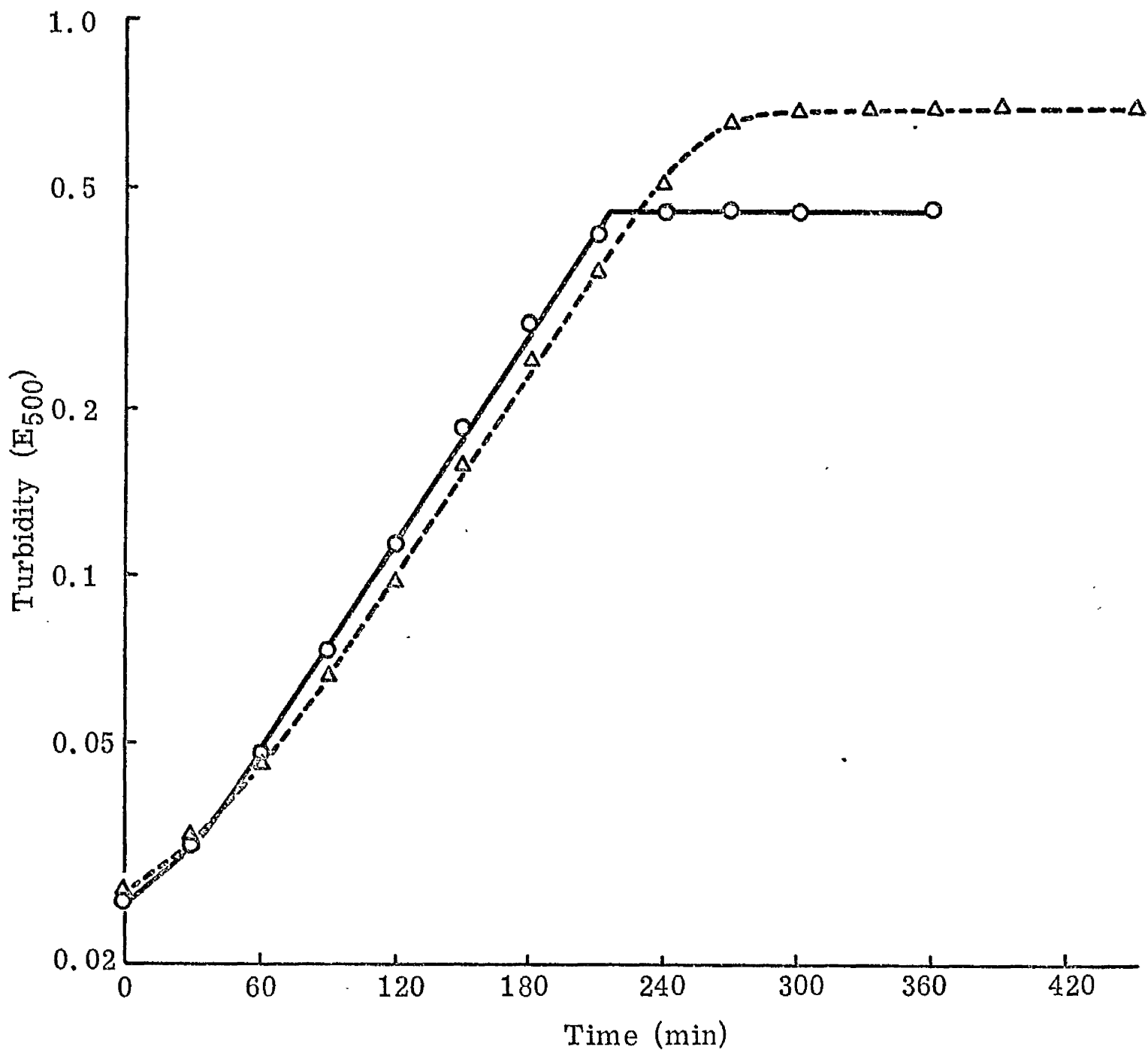
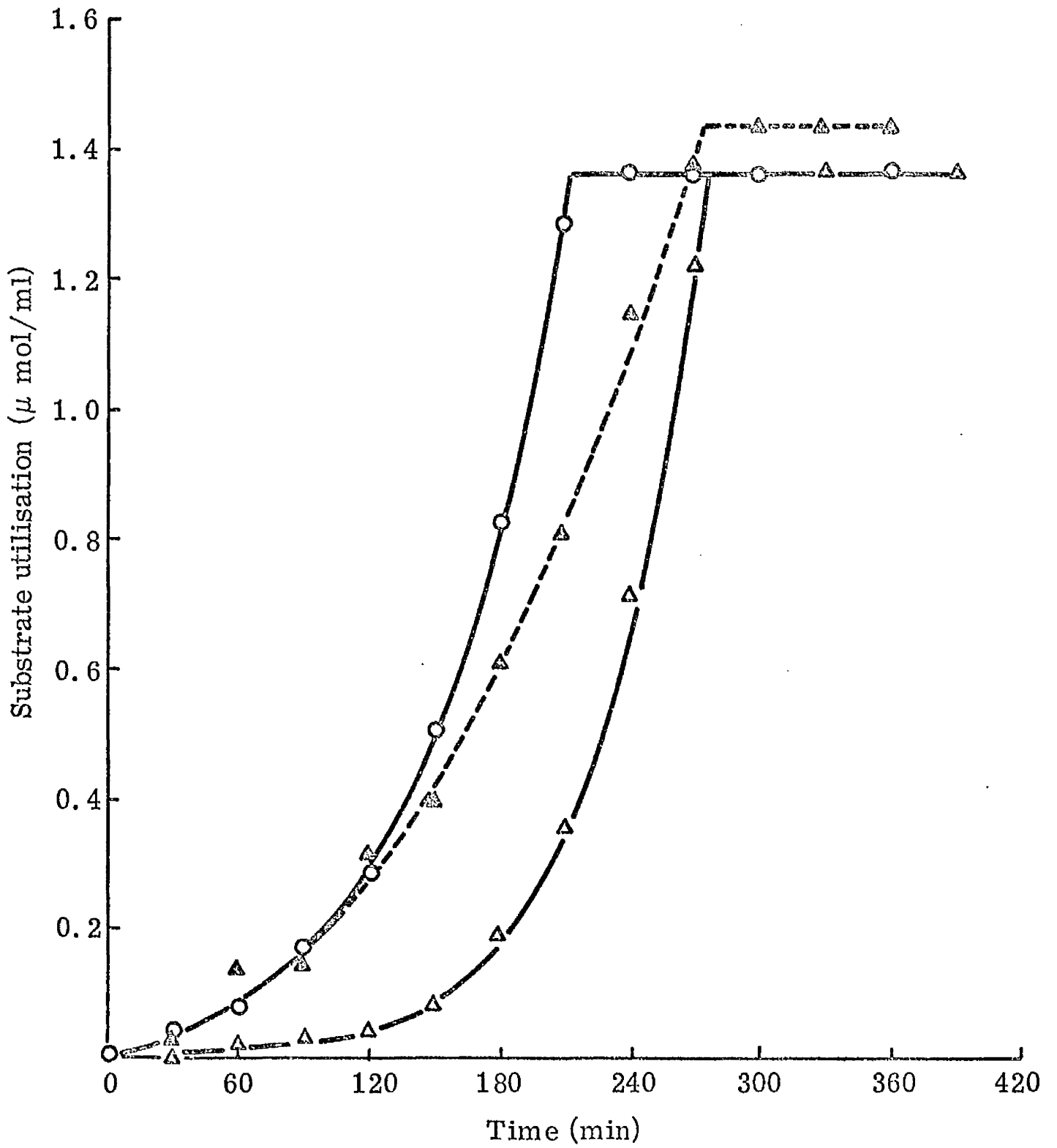


Fig. 30. The effects of benzoate on benzyl alcohol utilisation by growing cultures of bacterium NCIB 8250: kinetics of the utilisation of both substrates.

The culture of bacterium NCIB 8250 used for inoculation was grown in 5mM-benzyl alcohol-salts medium, harvested when the turbidity reached 0.4, resuspended and used immediately as described in Methods (p. 47 ). The experiments were done in 1.4mM-benzyl alcohol-salts medium with and without 1.4mM-benzoate (Methods, p. 47 ). At intervals samples were taken for radiochemical substrate estimation (Methods, p. 48).

- Control culture with benzyl alcohol alone:  
utilisation of benzyl alcohol.
- △ Utilisation of benzyl alcohol and ▲ utilisation  
of benzoate in parallel benzyl alcohol+ benzoate  
salts cultures.





rate fell steadily in the last generation. The time course of substrate utilisations in this culture (Fig. 30) was indicative of dual substrate utilisation throughout growth. Fig. 30 may be interpreted only qualitatively because benzyl alcohol utilisation was measured as benzoate decarboxylation (Results, p. 90), and benzoate derived endogenously from benzyl alcohol could equilibrate with exogenously derived benzoate thus invalidating the quantitative manipulation of data used (Methods, p. 50). Equilibration of endogenous and exogenous benzoate is seen (Fig. 30) between 150 and 210min where the sum of "benzoate" + "benzyl alcohol" utilisations corresponded to the control benzyl alcohol utilisation and the two cultures were essentially identical (Fig. 29).

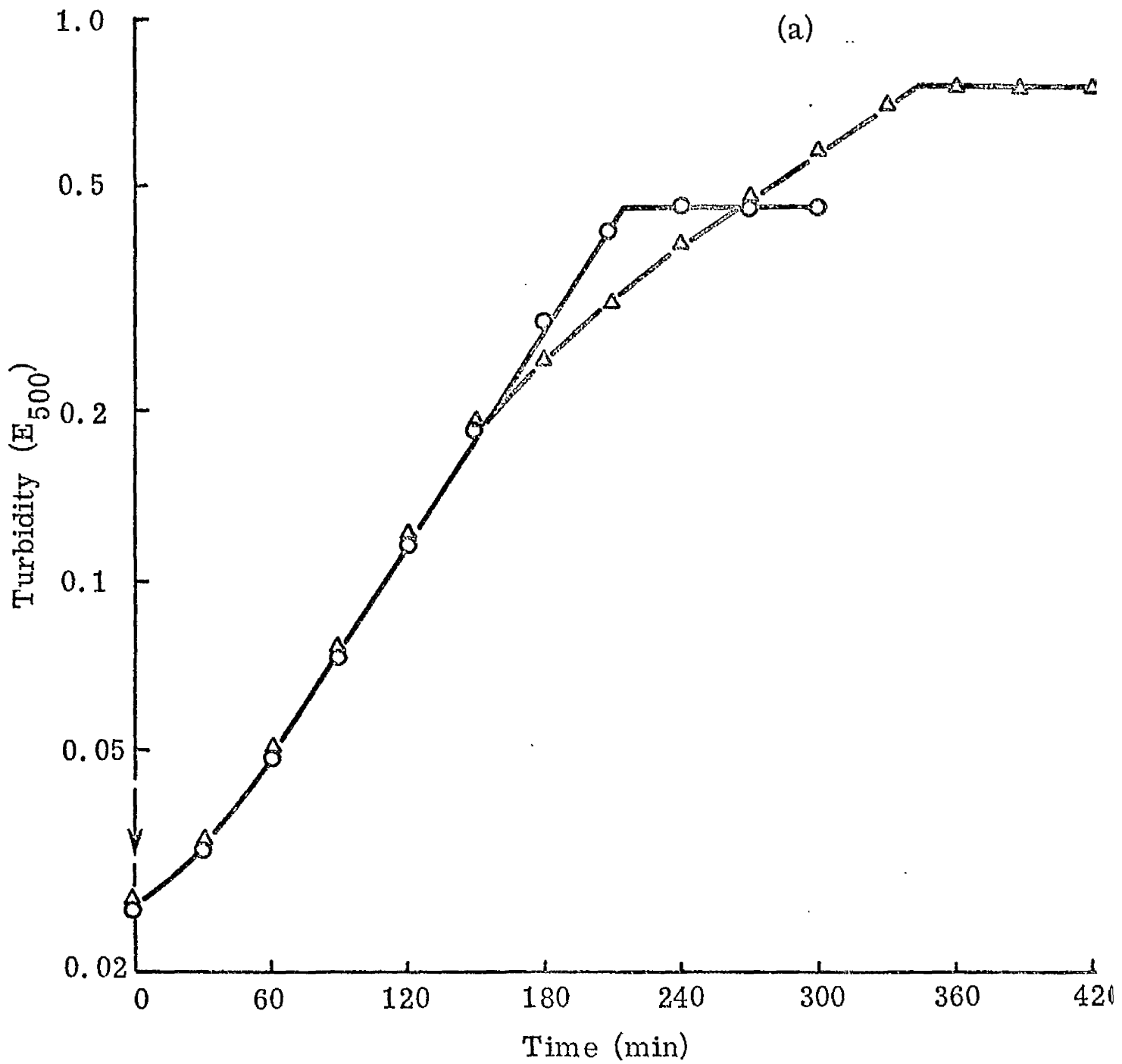
Benzyl alcohol utilisation accelerated during and probably throughout growth in benzoate + benzyl alcohol salts medium (Fig. 30). Thus the quantity of benzyl alcohol oxidising enzymes increased during growth in the presence of benzoate. A similar pattern of increasing capacity to oxidise benzyl alcohol during growth in the presence of benzoate was observed when a nutrient broth inoculum was used. Thus the presence of benzoate was not in itself a sufficient condition to repress the induction of the enzymes of benzyl alcohol oxidation.

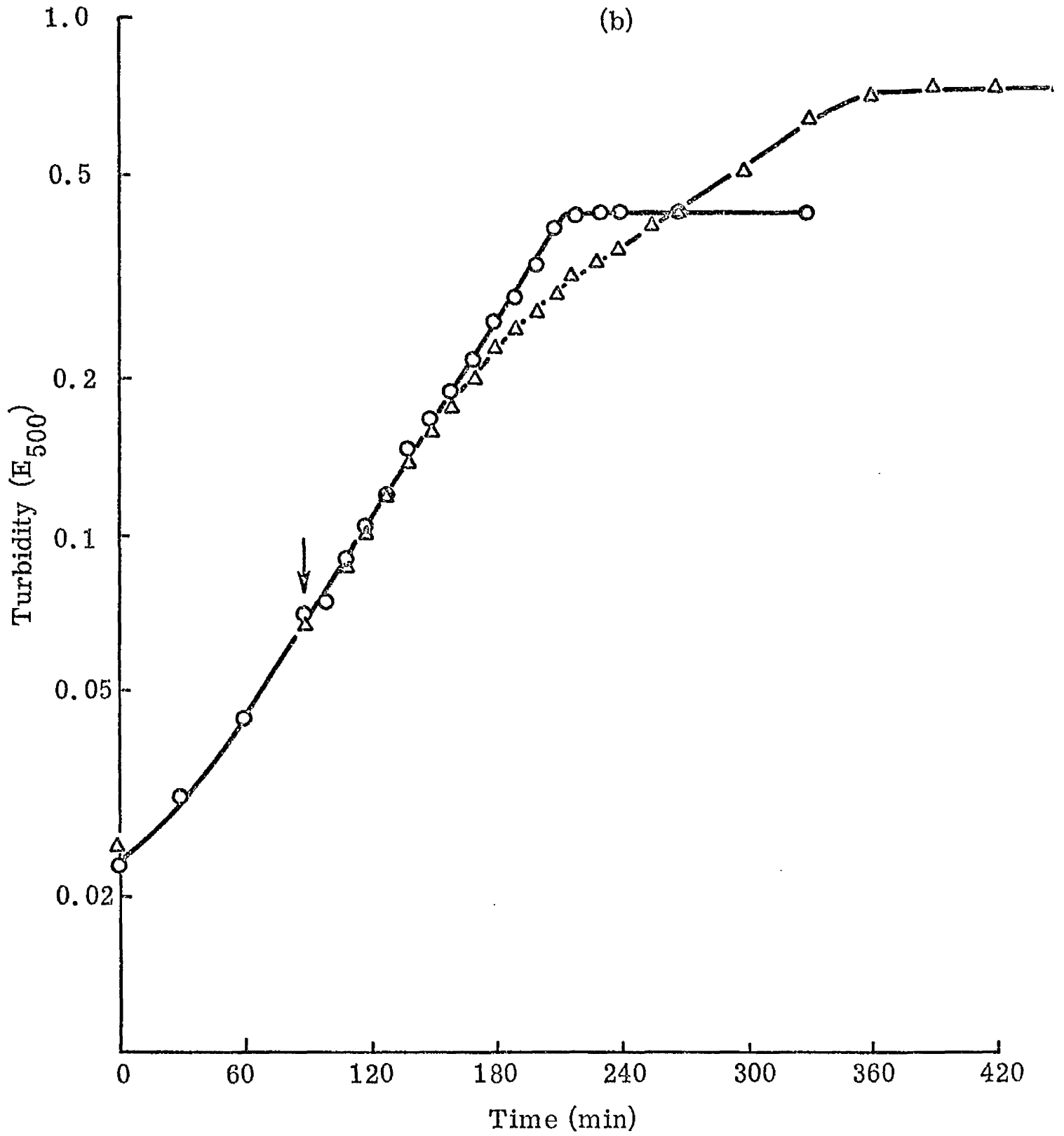
The presence of DL-mandelate initially had no effect on the growth of preinduced benzyl alcohol cells in benzyl alcohol salts medium, whether the mandelate was present from inoculation or added later in growth (Fig. 31abc). However, after varying periods

Fig. 31. The effect of mandelate on the growth of bacterium NCIB 8250 in benzyl alcohol-salts medium.

The cultures of bacterium NCIB 8250 used for inoculation were grown in 5mM-benzyl alcohol-salts medium, harvested when the turbidity reached 0.4, resuspended and used immediately (Methods, p. 47). The experiments were done in 1.4mM-benzyl alcohol-salts medium as described in Methods (p.47 ). DL-Mandelate to approximately 2.8mM was added ( ↓ ) to different cultures:- graph a) before inoculation, graph b) at a turbidity of 0.07 and graph c) at a turbidity of 0.15. Growth was measured turbidimetrically (Methods, p. 44).

- Appropriate control: growth in benzyl alcohol-salts medium.
- △ Growth in benzyl alcohol-salts medium to which mandelate was added at the times indicated.





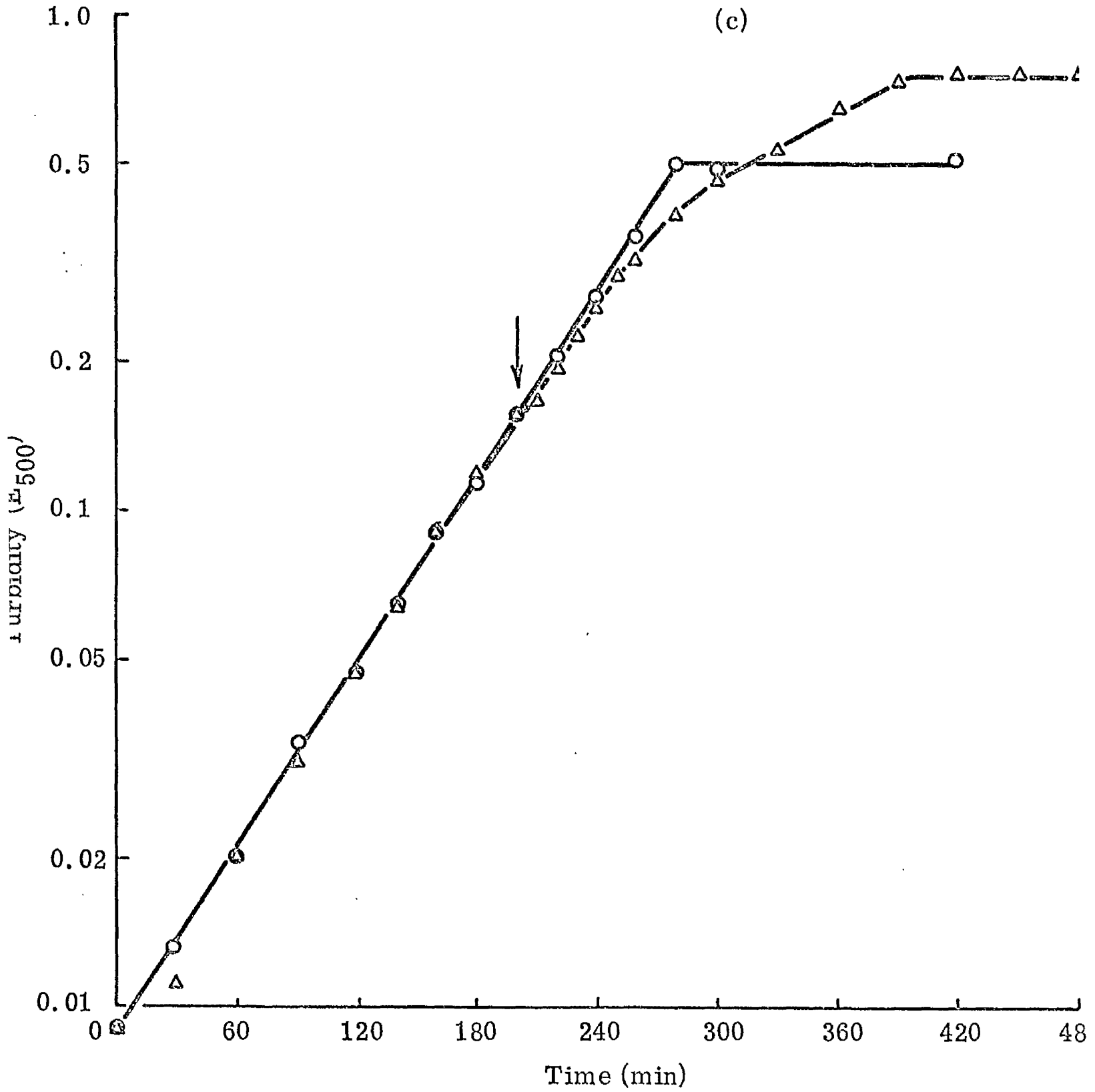
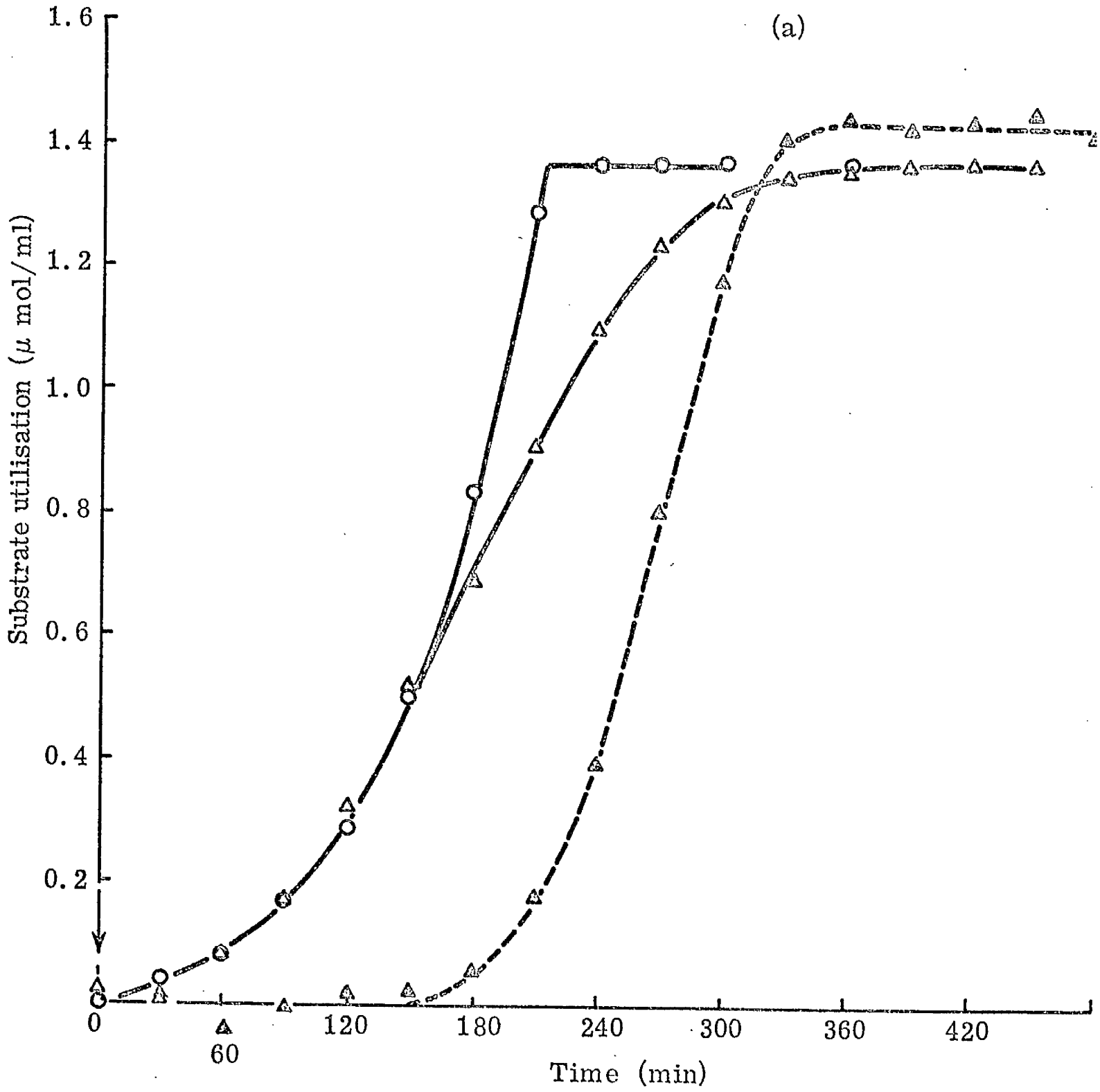


Fig. 32. The effects of mandelate on benzyl alcohol utilisation by growing cultures of bacterium NCIB 8250: kinetics of the utilisation of both substrates.

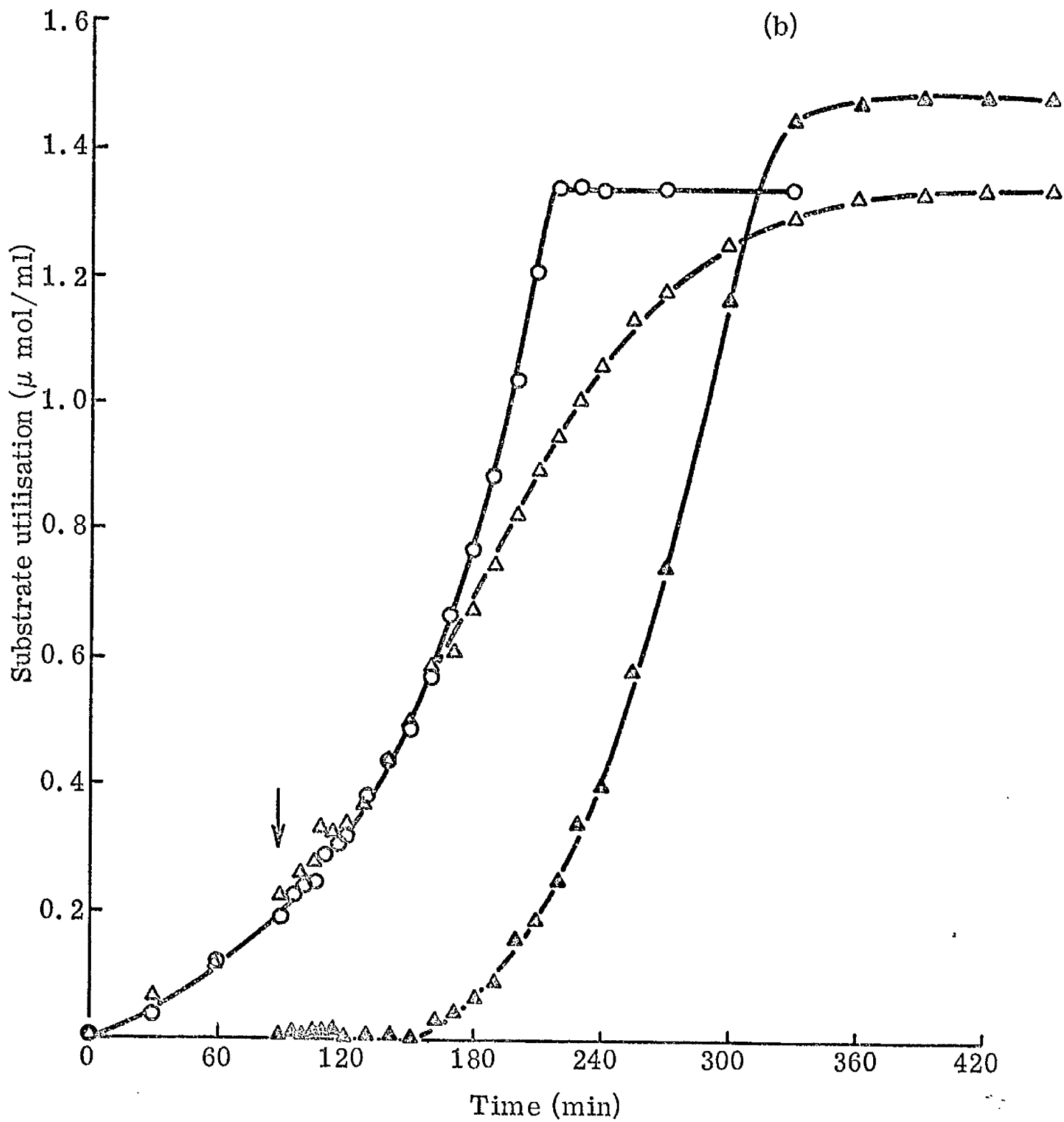
The cultures of bacterium NCIB 8250 used for inoculation were grown in 5mM-benzyl alcohol-salts medium, harvested when the turbidity reached 0.4, resuspended and used immediately (Methods, p. 47). The experiments were done in 1.4mM-benzyl alcohol-salts medium as described in Methods (p. 47). DL-Mandelate to approximately 2.8mM was added (  $\downarrow$  ) to different cultures: graph a) before inoculation, graph b) at a turbidity of 0.07 and graph c) at a turbidity of 0.15. At intervals samples were taken for radiochemical substrate estimation (Methods, p. 48).

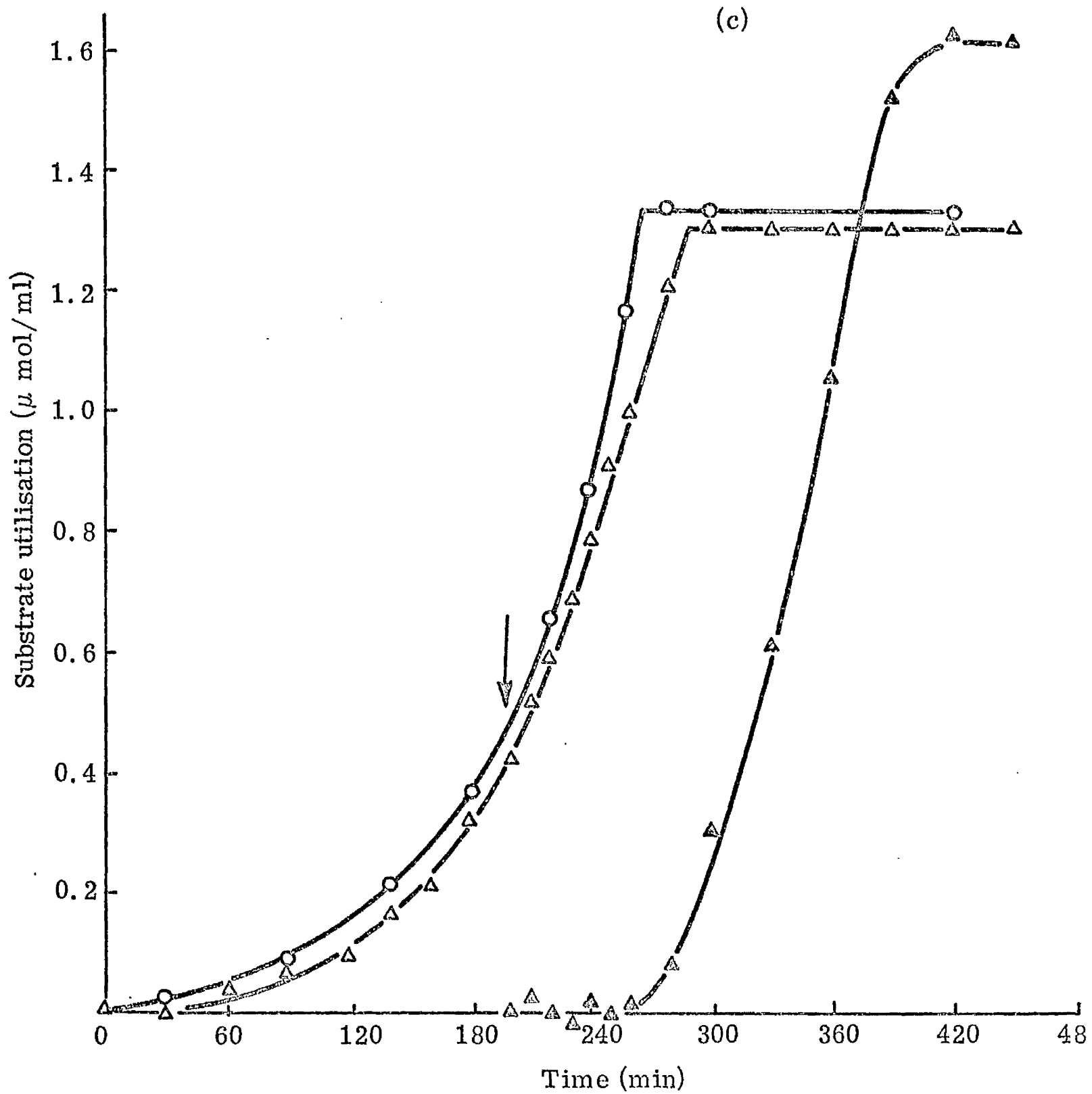
○ Appropriate control: utilisation of benzyl alcohol.

△ Utilisation of benzyl alcohol and ▲ utilisation of mandelate in parallel benzyl alcohol + mandelate-salts cultures.









of time, not less than 1h, the growth rate fell gradually from  $\mu = 0.87\text{h}^{-1}$  to  $\mu = 0.37\text{h}^{-1}$  (Fig. 31a,b) or  $\mu = 0.30\text{h}^{-1}$  (Fig. 31c). The corresponding time courses of substrate utilisations (Fig. 32 a,b,c) show that the fall in growth rate was coincident with a fall in the rate of benzyl alcohol utilisation from exponential to linear with time and coincident with the start of mandelate utilisation. The linear rate of benzyl alcohol utilisation was maintained until mandelate utilisation reached its maximum rate; the rate of benzyl alcohol utilisation then fell off steadily and both substrates were exhausted simultaneously and coincidentally with the end of growth (except in c where benzyl alcohol exhaustion occurred in the linear utilisation phase).

Experiment c of this series was assayed for benzaldehyde. None was detected. In this culture, at least, the fall in growth rate was not due to benzaldehyde toxicity and was presumably a direct result of the linear rate of benzyl alcohol utilisation.

The overall effect observed in Figs. 31 and 32 was ascribed to the presence of the L-enantiomer of mandelate. Further dual substrate experiments were done with preinduced benzyl alcohol inocula growing in D-, DL- or L-mandelate + benzyl alcohol-salts media. The D-enantiomer had no effect on growth in benzyl alcohol-salts medium but the effects of DL- and L- mandelate were identical to those described above. The same general patterns of growth and benzyl alcohol utilisation were observed in the presence of phenylglyoxylate.

When a preinduced benzyl alcohol inoculum grew in catechol + benzyl alcohol-salts medium, the data indicated simple dual substrate utilisation. There was no sharp drop in the growth rate at any time during growth and benzyl alcohol utilisation accelerated throughout growth. A similar pattern of dual substrate utilisation was observed when a preinduced benzyl alcohol inoculum grew in succinate + benzyl alcohol-salts medium.

The growth experiments indicated that the synthesis of several groups of enzymes in the mandelate pathway was subject to repression. Regulon  $R_1$  (mandelate oxidation) was subject to repression in the presence of benzoate (Results, pp.114 and 117), catechol (Results, p.117) and succinate (Results, p.120), but not in the presence of benzyl alcohol (Results, p.114). Regulon  $R_2$  (benzyl alcohol oxidation) was less sensitive to repression than regulon  $R_1$ . However, repression of regulon  $R_2$  was observed in preinduced benzoate cells in the presence of benzoate (Results, p.114) though the presence of benzoate was not a sufficient condition for repression (Results, p.120), and repression by L-mandelate was observed after a long delay (Results, p.123). No severe repression of regulon  $R_3$  (benzoate oxidation) was observed.

It was possible that some of these effects were in fact indirect effects of enzyme inhibitions and not interactions involving the control of enzyme synthesis. This was studied in cell-free enzyme assays and in whole cells (Results, Section 3).

Alternatively, interactions could occur as compounds entered the cell and this possibility was also explored (Results, Section 4).

3. THE CELL-FREE AND WHOLE CELL ACTIVITIES OF CERTAIN  
MANDELATE PATHWAY ENZYMES IN THE PRESENCE OF OTHER  
INTERMEDIATES.

3. 1. Cell-free enzyme assays.

Growth and substrate utilisation of bacterium NCIB 8250 in dual substrate media indicated few interactions between control systems at the level of enzyme activity (Results, Section 2). However, to verify that the activities of the mandelate pathway enzymes were unaffected by other intermediates, L-mandelate dehydrogenase, phenylglyoxylate carboxy-lyase, the labile and stable benzaldehyde dehydrogenases and benzyl alcohol dehydrogenase were assayed in cell-free extracts in the presence of DL-mandelate, phenylglyoxylate, benzaldehyde, benzyl alcohol, benzoate and succinate. The cell-free assay for benzoate oxidase (Fewson et. al., 1970) represents only 3% of the activity observed in whole cells and was not studied. As most of the growth experiments were done at equimolar substrate concentrations, the intermediates were present in the enzyme assays at concentrations equimolar with the optimum substrate concentration.

L-mandelate dehydrogenase activity (Table 5) was not significantly affected by benzyl alcohol, benzaldehyde, benzoate or by succinate. An assay for L-mandelate dehydrogenase, done in the absence of substrate, showed a very low, extract-dependent reduction of dye, about 0.4% of the control rate of mandelate dehydrogenase

Table 5.        The activities of mandelate pathway enzymes  
                  in the presence of other intermediates.

Bacterium NCIB 8250 was grown in salts medium on the indicated carbon sources and cell-free extracts prepared by ultrasonic disruption as described in Methods (pp.50 and 67). Enzyme activities in the cell-free extracts were measured spectrophotometrically at the indicated wavelengths (Methods, p. 65). L-Mandelate dehydrogenase was measured by the reduction of 2, 6-dichlorophenol-indophenol to the leuco form concomitant with the oxidation of L-mandelate to phenylglyoxylate (Methods, p. 66). Phenylglyoxylate carboxy-lyase was assayed by direct measurement of substrate disappearance (Methods, p. 66). Both benzaldehyde dehydrogenases were measured as benzaldehyde-dependent reductions of  $\text{NAD}^+$  and benzyl alcohol dehydrogenase was assayed as a benzyl alcohol-dependent reduction of  $\text{NAD}^+$ . Each group of assays in the spectrophotometer included a control in the absence of added intermediate, a complete assay mixture plus intermediate under study and an assay mixture with added intermediate but no substrate. Added intermediates were equimolar with enzyme substrate.

ND = Not determined

\* 5- and † 10- fold increase in concentration  
of potential inhibitor gave the same result.

Enzyme	Carbon source on which cells grown.	Wavelength at which extinction followed (nm)	Control values of $\Delta E/min$	Reaction rate as % control activity in the presence of the following intermediates.						
				Mandelate	Phenyl-glyoxylate	Benzaldehyde	Benzyl alcohol	Benzoate	Succinate	
Mandelate dehydrogenase	5mM-L-mandelate	600	0.170	ND	ND	102*	101	101	101	
Phenyl-glyoxylate carboxylase	5mM-L-mandelate	334	0.0021	ND	ND	93*	106	102	100	
Heat-stable benzaldehyde dehydrogenase	5mM-glutamate+1mM-thiophenoxycetate.	340	0.072	ND	ND	ND	108†	110	100†	
Heat-labile benzaldehyde dehydrogenase	5mM-benzyl alcohol	340	0.120	97†	103†	ND	ND	100	106†	
Benzyl alcohol dehydrogenase	5mM-benzyl alcohol	340	0.055	99	98	ND	ND	95	95	



activity. None of the intermediates under test affected this low rate of dye reduction.

Phenylglyoxylate carboxy-lyase activity was measured by the relatively insensitive assay in which phenylglyoxylate disappearance is followed at 334nm (Methods, p. 66), rather than the much more sensitive assay in which benzaldehyde dehydrogenase is used as a coupling enzyme (Kennedy & Fewson, 1968b; Livingstone, 1970). This was to ensure that any effects could be attributed to phenylglyoxylate carboxy-lyase itself and not to complications introduced by the presence of a coupling enzyme. Enzyme activity was not significantly affected by benzyl alcohol, benzaldehyde, benzoate or succinate (Table 5). No enzyme activity was detected in the absence of substrate.

The activity of the heat-stable benzaldehyde dehydrogenase was negligibly affected by benzyl alcohol, benzoate and succinate (Table 5). These extracts gave increased activities with mandelate and phenylglyoxylate, presumably because enzymes for their metabolism were present.

Neither mandelate, phenylglyoxylate, benzoate nor succinate had any significant effect on the activity of the heat labile benzaldehyde dehydrogenase (Table 5).

Benzyl alcohol dehydrogenase was not significantly affected by DL-mandelate, phenylglyoxylate, benzoate or succinate (Table 5). In assay mixtures lacking benzyl alcohol there was no reduction of  $\text{NAD}^+$  in the presence of the materials under test.

### 3. 2. Substrate disappearance from cell suspensions of bacterium NCIB 8250 and the effects of other intermediates.

Growth experiments and cell-free enzyme assays (Results, Sections 2 and 3. 1.) indicated minimal direct interactions between the substrate of one enzyme and other enzymes in the mandelate pathway. To establish this lack of direct interactions in whole cells, substrate disappearance was assayed under non-proliferating conditions (Methods, p. 62 and Results, Section 4. 1.) in the presence of the appropriate intermediates. It was felt that this technique might also show up small effects not detected in growth experiments.

#### 3. 2. 1. The effects of other intermediates on mandelate decarboxylation by cell suspensions harvested from mandelate-salts medium.

The rate of mandelate decarboxylation was linear in cell suspensions (Fig. 33). This rate,  $84\text{nmol/mg dry wt./min}$ , was lower than the computed value for mandelate decarboxylation during growth (Results, p.103) but a direct comparison is invalid because of the unusual growth and substrate utilisation kinetics observed in mandelate-salts media (Results, Section 2. 1. 3.).

Neither benzyl alcohol nor benzoate significantly altered mandelate disappearance (Table 6). In the presence of catechol, mandelate decarboxylation accelerated from a very low rate to a linear rate 67% of the control (Fig. 33). Succinate caused a lowering in the mandelate disappearance rate (Table 6).

Fig. 33.

The effect of catechol on mandelate decarboxylation by cell suspension of bacterium NCIB 8250 harvested from mandelate-salts medium.

Bacterium NCIB 8250 was grown in 10mM-DL-mandelate-salts medium, harvested when the turbidity reached 0.2, washed, re-suspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). The experiment was done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with the 10ml final volume reaction media containing DL- $[\text{carboxy-}^{14}\text{C}]$ mandelate, 20 $\mu$ mol (1 $\mu$ Ci/ $\mu$ mol). Incomplete (7.5ml) reaction medium and a portion of the stock cell suspension were preincubated separately and the reaction initiated by the addition of 2.5ml of the cell suspension (Methods, p. 60). At intervals 100 $\mu$ l samples were taken on to 1.0ml 0.1M-HCl and the radioactivity in this acidified suspension measured as described in Methods (p.48).

- ● Mandelate decarboxylation (in duplicate)  
in the absence of added intermediate.
- △ Mandelate decarboxylation in the  
presence of 1mM-catechol.

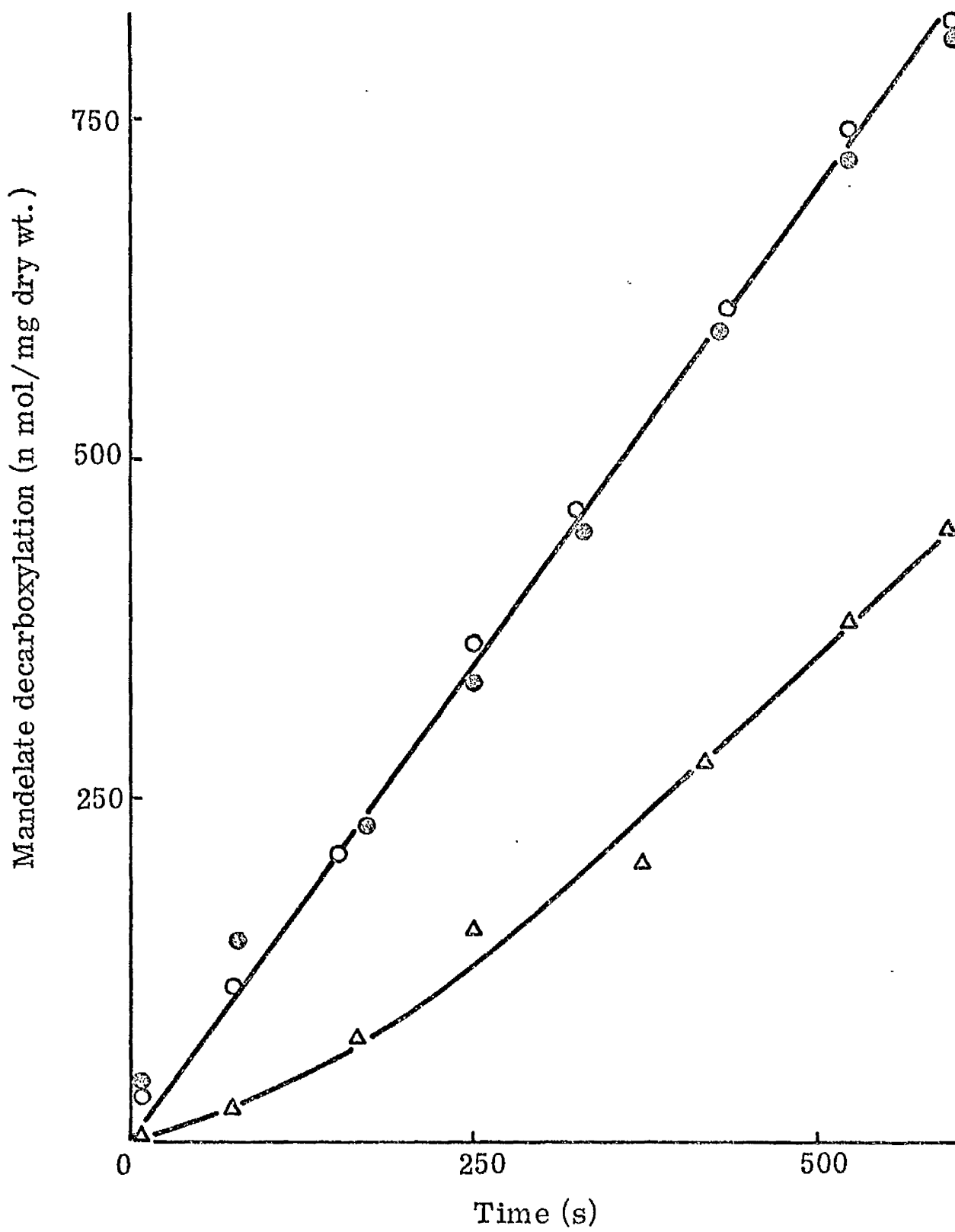


Table 6. The effects of various compounds on the disappearance of mandelate, benzyl alcohol and benzoate from cell suspensions of bacterium NCIB 8250.

Bacterium NCIB 8250 was grown in 10mM-DL-mandelate, 5mM-benzyl alcohol- or 2mM-benzoate-salts medium, harvested at turbidities of 0.1, 0.4, and 0.2 respectively, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing DL- $\left[ \text{carboxy-}^{14}\text{C} \right]$  mandelate, 20 $\mu\text{mol}$  (1 $\mu\text{Ci}/\mu\text{mol}$ ),  $\left[ \text{carboxy-}^{14}\text{C} \right]$  benzoate, 10 $\mu\text{mol}$  (0.1 $\mu\text{Ci}/\mu\text{mol}$ ) or benzyl alcohol, 10 $\mu\text{mol}$  (sometimes  $\left[ \text{carbinol-}^{14}\text{C} \right]$ -labelled at 0.1 $\mu\text{Ci}/\mu\text{mol}$ ). Other intermediates were present at 1mM except for 2mM-DL-mandelate. Incomplete (7.5ml) reaction media and portions of stock cell suspensions were preincubated separately and the reaction was initiated by the addition of 2.5ml cell suspension (Methods, p. 60). At intervals samples were taken as indicated in Legends to Figs. 33 to 36 to estimate substrate concentration remaining.

Results are tabulated as a percentage of the activity in the absence of added intermediates. The figures in brackets represent decarboxylation or disappearance rates in nmol/min/mg dry wt.

NT = Not tested

\* = No second rate : see Fig. 36

Additional intermediates	Cells harvested from salts-media containing :-					
	Mandelate	Benzyl Alcohol		Benzoate		
		Spectrophotometric assay	Radiochemical assay	Initial Rate	Second Rate	
None	100 (84)	100 (135)	100 (27)	100 (59)	100 (43)	
Mandelate	NT	NT	96	105	107	
Phenylglyoxylate	NT	NT	96	109	99	
Benzyl alcohol	96	NT	NT	88	100	
Benzoate	105	91	NT	NT	NT	
Catechol	67	NT	NT	Acceleration	89	
Succinate	73	78	NT	46	*	

3. 2. 2. The effects of other intermediates on benzyl alcohol disappearance from cell suspensions harvested from benzyl alcohol-salts medium.

3. 2. 2. 1. Comparison of radiochemical and spectrophotometric assays of benzyl alcohol disappearance in cell suspensions.

A direct spectrophotometric assay of benzyl alcohol was developed to measure benzyl alcohol disappearance in the presence of benzoate (Methods, p. 67). This assay was very tedious and the radiochemical assay was used where possible. However, the rate of benzyl alcohol disappearance was at least twice the rate of benzyl alcohol decarboxylation (Fig. 34). This discrepancy, which was not observed when cells were growing in benzyl alcohol-salts medium (Fig. 14), was not due to the accumulation of benzaldehyde (Fig. 34). A compound with the same u.v. spectrum as benzoate accumulated during the assay (Fig. 35). The effect was not due to loss of enzyme activity on storage. A stock cell suspension was stored on ice for 4h. The fresh suspension was assayed spectrophotometrically and radiochemically (after varying preincubation times of 5 to 30min) and the results compared with those after storage. Neither time of storage nor preincubation time altered the results.

The rate of benzyl alcohol disappearance in the spectrophotometric assay, 135nmol/mg dry wt./min, was 75% of the utilisation rate calculated for growing cells in a different experiment (Results, p. 93).

Fig. 34 Comparison of radiochemical and spectrophotometric assays of benzyl alcohol utilisation by cell suspensions of bacterium NCIB 8250 harvested from benzyl alcohol-salts medium.

Bacterium NCIB 8250 was grown in 5mM-benzyl alcohol-salts medium, harvested at a turbidity of 0.4, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice as described in Methods, (p. 60). The experiment was done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [carbinol-<sup>14</sup>C] benzyl alcohol, 10µmol (0.1µCi/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions were initiated by the addition of 2.5ml cell suspension (Methods, p. 60). Reaction media were incubated for different intervals and sampled for a) benzyl alcohol decarboxylation, radiochemically (100µl on to 1.0ml 0.1M-HCl, Methods, p. 62) b) benzaldehyde, spectrophotometrically (1.0ml on to 0.1ml M-NaOH in 10ml n-hexane, Methods, p. 68) and c) benzyl alcohol disappearance, spectrophotometrically (7.5ml on to 1.5ml chilled 3M-perchloric acid, Methods, p. 67). Sample c) was further treated as illustrated in Fig. 35.

- ● Spectrophotometric assay: benzyl alcohol utilisation, in duplicate.
- △ ▲ Radiochemical assay: benzyl alcohol utilisation, in duplicate.
- ■ Benzaldehyde accumulation in reaction media, in duplicate.



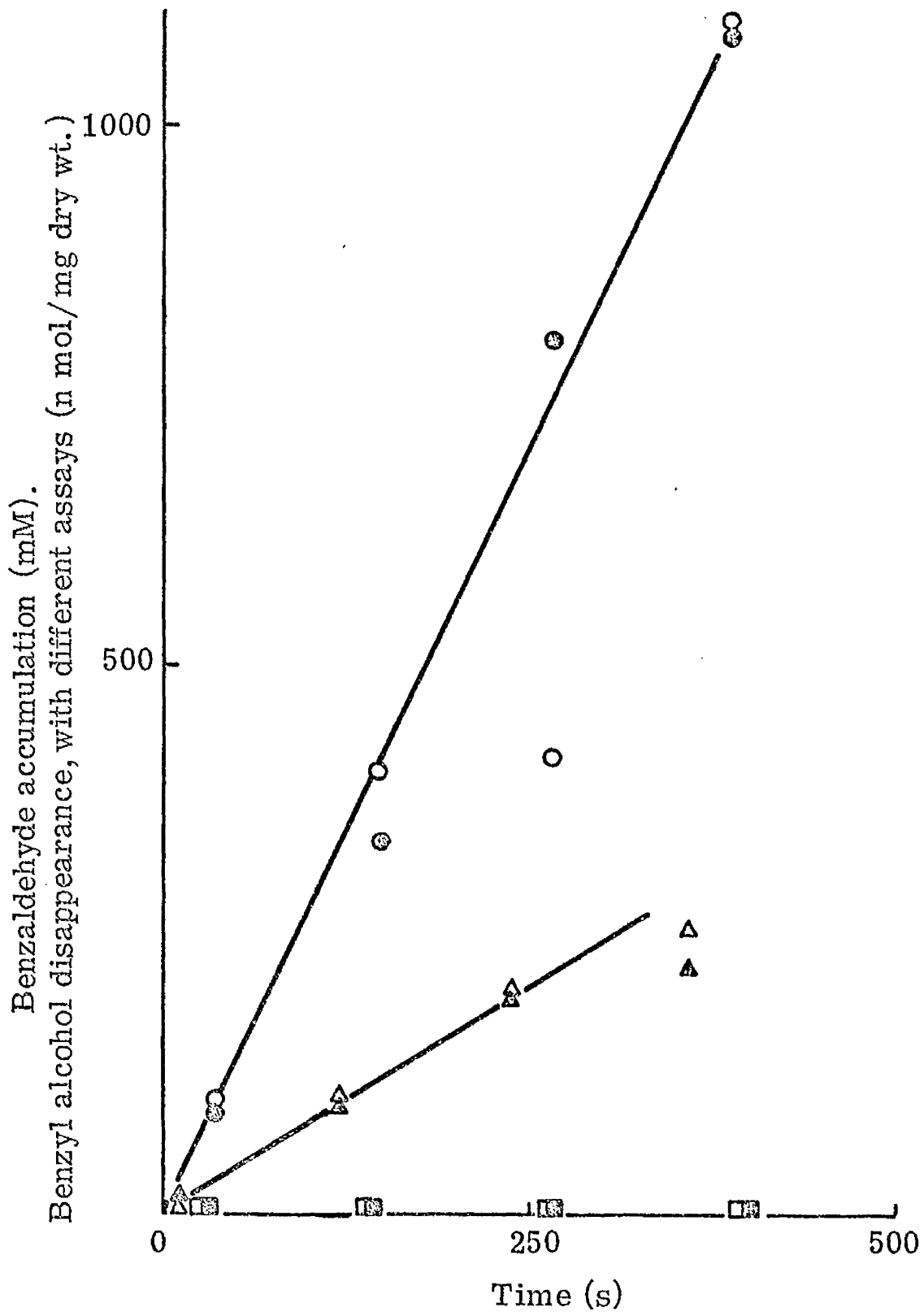


Fig. 35. Ultraviolet spectra of material accumulated in reaction media during benzyl alcohol utilisation by cell suspensions of bacterium NCIB 8250 harvested from benzyl alcohol-salts medium.

These spectra were obtained from samples in the experiment illustrated in Fig. 34 . The samples had first been assayed for benzyl alcohol by extraction into chloroform under alkaline conditions (Methods, p. 67). This alkaline aqueous sample was then acidified and extracted with chloroform. This chloroform layer was extracted with alkali. The alkaline portion was acidified after which the u.v. spectrum of the solution was read on a Unicam SP800 Ultraviolet Spectrophotometer.

- Reagent blank in the absence of benzyl alcohol and benzoate and also the spectrum of the 45s sample from Fig. 34
- ..... 385s sample from Fig. 34
- An authentic sample of benzoic acid (0.17mM) in 0.1M-HCl.

2.0

1.6

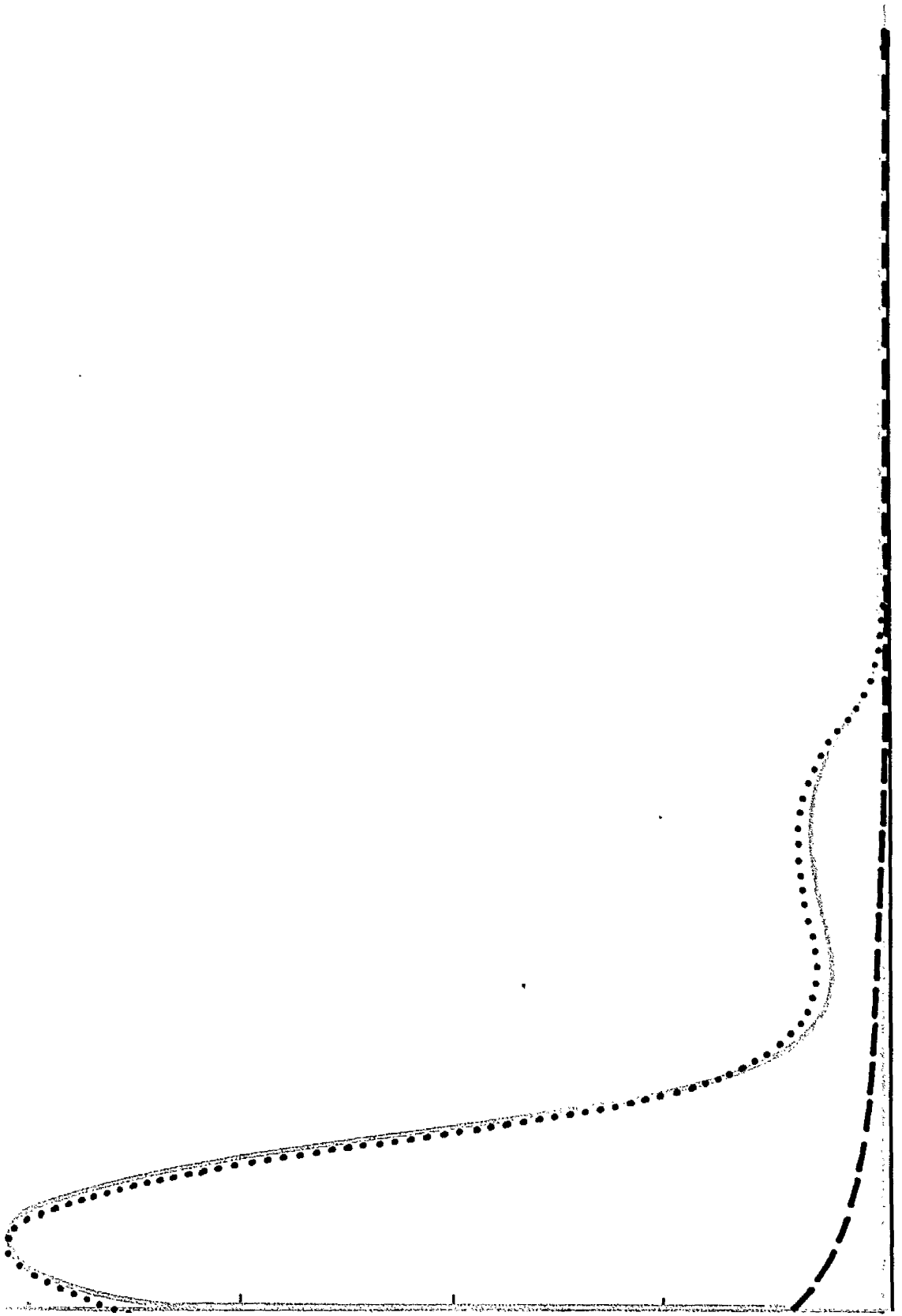
1.2

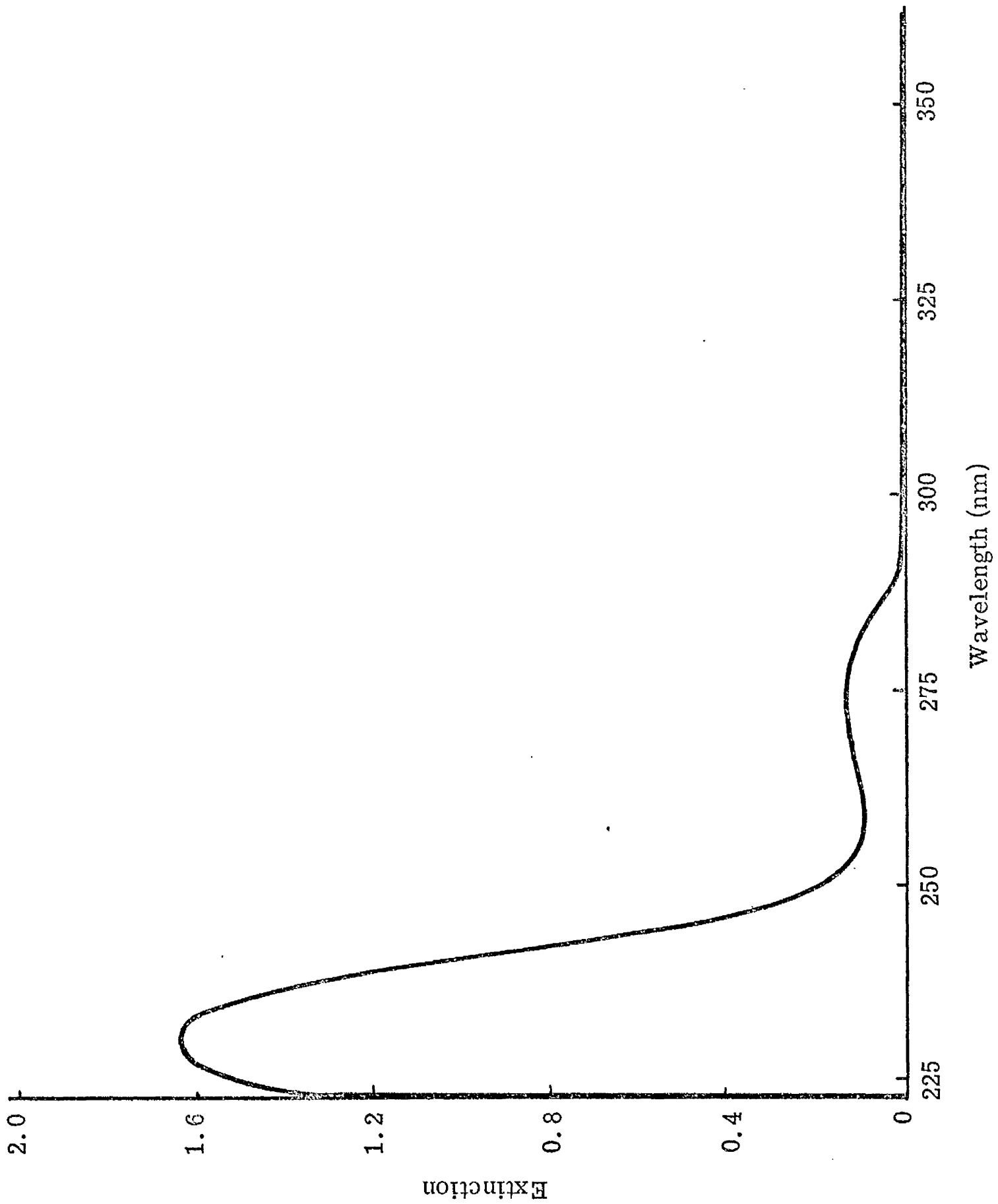
0.8

0.4

0

Extinction





3. 2. 2. 2. The effects of other intermediates on benzyl alcohol disappearance from cell suspensions.

No significant interaction of mandelate or of phenylglyoxylate on benzyl alcohol decarboxylation was observed (Table 6). Inhibitions of benzyl alcohol disappearance were detected in the presence of benzoate and succinate (Table 6). The effect of benzoate was slight.

3. 2. 3. The effects of other intermediates on benzoate disappearance from cell suspensions harvested from benzoate-salts medium.

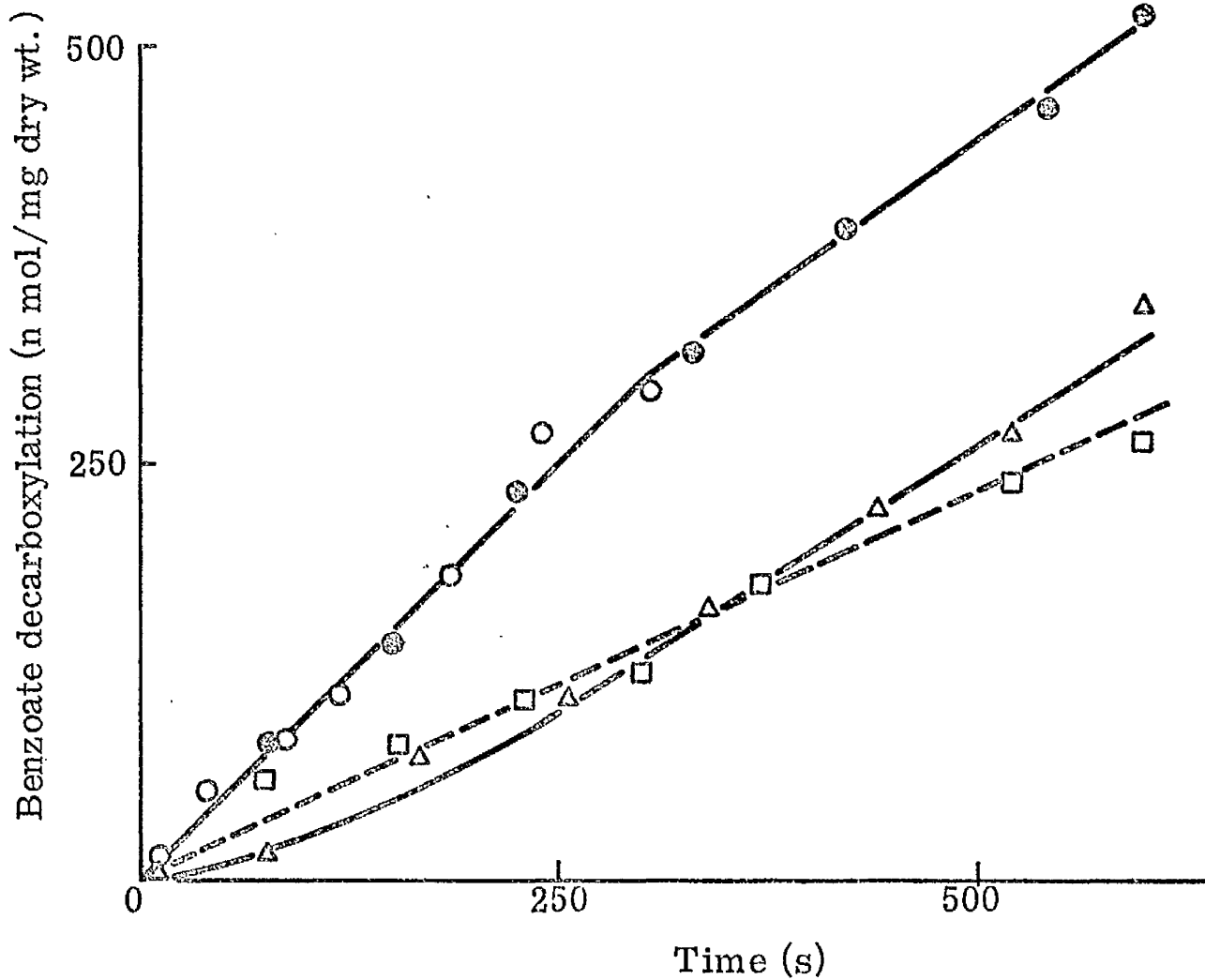
Benzoate decarboxylation was biphasic in cell suspensions (Fig. 36). The initial rate of 59nmol/mg dry wt./min was 27% of the rate in exponentially growing cells in a different experiment (Results, p. 87). After one third of the benzoate had been removed (after 300s) the rate of benzoate decarboxylation fell to 43nmol/mg dry wt./min.

This biphasic pattern was unaltered by mandelate, phenylglyoxylate and by benzyl alcohol none of which had any consistent effect on benzoate decarboxylation (Table 6, last two columns). On the other hand, the presence of catechol enforced a totally different pattern (Fig. 36) in which benzoate disappearance increased steadily for 300 seconds to a linear rate 90% of the second phase control rate of benzoate decarboxylation. In the presence of succinate, a lowered linear rate of benzoate decarboxylation was maintained throughout (Fig. 36).

Fig. 36 .        The effects of catechol and succinate on  
benzoate decarboxylation by cell suspensions  
of bacterium NCIB 8250 harvested from benzoate-  
salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). The experiment was done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [carboxy-<sup>14</sup>C] benzoate, 10µmol (0.1µCi/µmol). Incomplete (7.5ml) reaction medium and a portion of the stock cell suspension were preincubated separately and the reaction initiated by the addition of 2.5ml cell suspension (Methods, p. 60). At intervals 100µl samples were taken on to 1.0ml 0.1M-HCl and the radioactivity in this acidified suspension measured as described in Methods (p. 62).

- ● Benzoate decarboxylation (in duplicate) in the absence of added intermediates.
- △ Benzoate decarboxylation in the presence of 1mM-catechol.
- Benzoate decarboxylation in the presence of 1mM-succinate.



4. SUBSTRATE UTILISATION, INCORPORATION INTO  
MACROMOLECULE AND SUBSTRATE POOL SIZES IN CELL SUSPENSIONS  
OF BACTERIUM NCIB 8250.

The results reported so far suggested that there was little direct interaction (feedback inhibition) by intermediates of the mandelate pathway on the enzymes tested (Results, Sections 2 and 3) but that repression of enzyme synthesis probably occurred in some situations (Results, p. 131). Repression could be due to effects on transcription or translation or on permeation. The widespread specificity of repression in this and other (Mandelstam & Jacoby, 1965) mandelate pathways made permeability a probable locus. The bulk of the work in this project was aimed at examining the uptake of aromatic compounds by bacterium NCIB 8250 and looking for interactions between the uptake of pairs of compounds. This involved a good deal of developmental work, which will be described at some length as it was found that precise attention to detail was important in order to obtain satisfactory results.

4. 1. Total uptake of material into cells and  
incorporation into macromolecule.

4. 1. 1. Development of techniques for uptake and  
incorporation experiments.

"Uptake" of material refers to the total amount of material inside the cell, while "incorporation" refers to the amount of material incorporated into macromolecule. The difference between



uptake and incorporation is taken to represent the total "pool" of small molecules in the cell. All assays of uptake and incorporation were done radiochemically on cell suspensions and measured as the radioactivity retained on the filter membrane after harvesting and washing.

#### 4. 1. 1. 1. Choice of conditions for cell suspension studies.

The conditions used in these experiments were originally based on experience with manometric techniques with this organism (Kennedy & Fewson, 1968a). Experiments were done with cell suspensions (called "reaction media") contained in 50ml Erlenmeyer flasks and shaken in a water bath (Methods, p. 60). Erlenmeyer flasks were chosen to produce a large surface area for aeration and a reaction medium volume of 10ml was chosen to give sufficient depth to permit reliable sampling. For speed, samples were taken by Eppendorf pipette. The routine sample size was 100 $\mu$ l so that removal of ten samples should not markedly alter conditions in the reaction medium. Cells were suspended in phosphate buffer without added nitrogen supply, but no further steps were taken to prevent macromolecular synthesis as it was feared that the presence of compounds such as chloramphenicol might have undesirable and unknown side effects.

#### 4. 1. 1. 2. Apparatus for harvesting cells from cell suspensions.

Preliminary experiments on the kinetics of the uptake of

[ring-<sup>14</sup>C(U)] benzoate by benzoate grown cells of bacterium NCIB 8250 showed that a permanent assembly was required to harvest cell suspensions and process the samples reproducibly, rapidly, cleanly and simply. The apparatus (Methods, p. 57) was designed to be used by one operator for the rapid and complete processing of each sample individually. Samples were completely processed because individual pool sizes were small (see later) and catabolism occurred in the cells on the filter membrane when samples were not immediately immersed in scintillation fluid.

The low profile of the assembly allowed use of space behind the apparatus. Individual vacuum taps for each flask allowed independent control of each sample, made preparation for experiments simple and allowed easy change-over of flasks. However, the most important change from the individual commercial components was the replacement of the Millipore funnel with a short perspex funnel. The short funnel permitted open access to the filter membrane. This allowed samples from the Eppendorf pipette to be streaked accurately and reproducibly on the filter membrane itself. The short funnel was easily handled; perspex was chosen for its toughness and resistance to chipping.

4. 1.1. 3. The choice of a filter membrane for uptake studies and the retention of radioactivity on filter membranes.

"Reagent blanks" with [carboxy-<sup>14</sup>C] benzoate in the absence of cells indicated that radioactivity was retained on Millipore

filter membranes (Table 7). With experience the day to day variation in the radioactivity retained on the filters was minimised. The dampening of Millipore filter membranes with liquid homologous with the reaction medium (but containing neither cells nor radioactivity) did little to reduce the bound material, whether the membranes were steeped for two hours or dampened immediately before use (Table 7). To simulate more closely the conditions of a cell suspension assay, polystyrene latex similar in size to the cells under study (0.7  $\rightarrow$  1.3 $\mu$ m diameter) was added to the "reaction medium". This did not markedly alter the reagent blank recorded (Table 7). The reagent blank was not due to radioactivity picked up from the sintered glass filter base. After the above experiments, filter membranes were positioned on bases and washed without the addition of radioactivity, and no radioactivity was associated with these membranes.

Filter membranes from several commercial sources were screened in an attempt to find a type that did not retain radioactivity (Table 7). Sartorius "weight constant" filters retained the least radioactivity and the 0.22 $\mu$ m pore size was chosen in view of the small size of bacterium NCIB 8250. In the course of this work several radiochemicals were used and each was found to bind to the Sartorius filter membrane (Table 8).

#### 4. 1. 1. 4. Measurement of incorporation of radioactivity into macromolecules in cell suspensions of bacterium NCIB 8250.

The incorporation of radioactivity into the macromolecular

Table 7. Variation in retention of radioactivity from [carboxy-<sup>14</sup>C] benzoate on filter membranes under conditions simulating cell suspension assays.

Simulated reaction media were prepared in 50ml Erlenmeyer flasks in the absence of cells in 10ml volumes containing:  $\text{K}_2\text{H}_2\text{P}_2\text{O}_7 \cdot \text{Na}_2\text{HPO}_4$ , pH 7.0, 500 $\mu\text{mol}$ ; [carboxy-<sup>14</sup>C] benzoate, 10 $\mu\text{mol}$  (1 $\mu\text{Ci}/\mu\text{mol}$ ); polystyrene latex (0.7 - 1.3 $\mu\text{m}$  diameter), zero or 10-10mg dry wt. Reaction media were shaken in a water bath at 30°C and 100 $\mu\text{l}$  samples were taken on to filter membranes under vacuum, rapidly washed and the filter membranes immediately immersed in scintillation fluid in scintillation vials (Methods, p.60). The samples were washed either twice with 1ml chilled, distilled water or with 1ml of a chilled solution of the same composition as the reaction medium but lacking radioactivity and cells (medium wash).

Each result is the mean of eight values; the standard error of the mean as a percentage of the mean was between 2 and 5%.

Brand name	Pore diameter $\mu\text{m}$	Wetting of filter	Special conditions	Radioactivity re- tained on filter expressed as $\mu\text{l}$ reaction medium	
				Water wash	Medium wash
Millipore	0.22	H <sub>2</sub> O	Estimation on different days	0.15	0.19
		H <sub>2</sub> O		0.14	0.22
		H <sub>2</sub> O		0.16	0.31
		H <sub>2</sub> O	Latex in reaction medium	0.13	0.22
		H <sub>2</sub> O		0.16	0.27
	Steep in medium wash Medium wash		0.16	0.22	
		0.17	0.22		
		H <sub>2</sub> O	No radioactivity applied to filter	0.000	0.000
	0.45	H <sub>2</sub> O		0.17	0.31
Gelman	0.20	H <sub>2</sub> O		0.13	0.22
Oxoid		H <sub>2</sub> O		0.20	0.40
Sartorius	0.22	H <sub>2</sub> O	Estimated on different days	0.14	0.21
		H <sub>2</sub> O		0.09	0.12
	medium wash			0.13	0.19
	0.45	H <sub>2</sub> O	Estimated on different days	0.15	0.17
		H <sub>2</sub> O		0.09	0.15
Medium wash				0.13	0.18
	0.45	H <sub>2</sub> O	Filter variant containing plasticiser	0.18	0.26

Table 8. Reagent blanks for different radiochemicals on Sartorius filter membranes under conditions simulating cell suspension assays.

The different compounds, at the activities and concentrations indicated, were dissolved in 0.05M- $\text{KH}_2\text{PO}_4 \cdot \text{Na}_2\text{HPO}_4$  pH 7.0 in 10ml volumes contained in 50ml Erlenmeyer flasks. These simulated reaction media, which contained no cells, were shaken in a water bath at 30°C. 100 $\mu$ l Samples were taken on to filter membranes under vacuum, rapidly washed and the filter membrane immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60). Samples were washed either with 2 x 1ml chilled distilled water (Results, p. 147) or with 1ml chilled solution of the same composition as the corresponding reaction medium but without radioactivity (and without cells ; medium wash).

Each result is the mean of eight values. Standard error of the mean as a percentage of the mean is indicated in brackets.

NT = Not tested.

Radiochemical	Concentration mM	Activity $\mu$ Ci/ml	Radioactivity re- tained on filter membrane expressed as $\mu$ l of reaction medium	
			Water wash	Medium wash
[Carboxy- $^{14}$ C] benzoate	1.0	1.0	0.11(5)	0.15(4)
[Ring- $^{14}$ C(U)] benzoate	1.0	0.3	0.13(4)	0.14(5)
p-Fluoro [carboxy- $^{14}$ C] benzoate	1.0	1.0	NT	0.08(5)
p-Methoxy [carboxy- $^{14}$ C] benzoate	1.0	1.0	NT	0.30(3)
o-Hydroxy [carboxy- $^{14}$ C] benzoate	1.0	1.0	NT	0.31(1)
DL- [Carboxy- $^{14}$ C] mandelate	2.0	1.0	NT	0.02(4)
[Carbinol- $^{14}$ C] benzyl alcohol	1.0	1.0	NT	6.5
[Carbinol- $^{14}$ C] benzyl alcohol after filtration through 0.20 $\mu$ m pore filter membrane.	1.0	1.0	NT	6.5
[Ring- $^3$ H(G)] catechol	1.0	2.5	3.08(8)	NT
Glucose-5- $^3$ H(n)	0.1	2.5	0.02(5)	0.02(6)
[Methoxy- $^3$ H] inulin	$4 \times 10^{-4}$	1.5	0.05(9)	0.07(14)

fraction of bacterium NCIB 8250 in cell suspensions was estimated as the material insoluble in 2M-perchloric acid. When trichloroacetic acid was used (0.3M and 0.6M) longer extraction times were required than with 2M-perchloric acid. Reliable perchloric acid extractions of filter harvested cells were very tedious and unsuited for routine use in rapid time-course assays, so incorporation into macromolecule was measured as the water insoluble material on the filter membrane, Table 9 (Britten & McClure, 1959). This method of washing and pool extraction by chilled water was quick, reliable and reproducible. In early experiments, one x 1ml chilled water was occasionally inadequate, and the wash routinely used was 2 x 1ml volumes of chilled water dispensed by Zippette. Extraction with perchloric acid in addition to water washing had no further effect until four perchloric acid extractions were done. Water washes were done at 0°C; at 30°C the extraction of pools was unreliable. No other conditions were tested.

4. 1. 1. 5. Measurement of total uptake of radioactive material into cells and estimation of "pool" sizes.

The choice of a method to estimate total uptake of radioactivity into cells on a filter membrane was very much an arbitrary decision, with the criterion that the most radioactivity that could be reproducibly measured would be considered as "total uptake". The method employed was based on that of Britten & McClure (1959) and Winkler & Wilson (1966) in which cells, harvested



Table 9 . The effects of washing on the measurement of incorporation of [ring-<sup>14</sup>C(U)] benzoate into macromolecule in cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice as described in Methods (p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [ring-<sup>14</sup>C(U)] benzoate, 10µmol (0.3µCi/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions were initiated with 2.5ml cell suspension (Methods, p. 60). At 300s, 100µl samples were taken on to filter membranes under vacuum, washed as indicated and the filters immediately immersed in scintillation fluid in scintillation vials.

Washes all at 0°C	Radioactivity incorporated mmol benzoate/l cell water (glucose)
1ml H <sub>2</sub> O	25.4
2x1ml H <sub>2</sub> O	21.5
3x1ml H <sub>2</sub> O	21.5
1ml H <sub>2</sub> O+4.5ml 2M-perchloric acid	25.4
1ml H <sub>2</sub> O+3x4.5ml 2M-perchloric acid	24.5
1ml H <sub>2</sub> O+4x4.5ml 2M-perchloric acid	20.0

by filtration, were washed with a chilled solution homologous with reaction medium (but without cells and radioactivity). This solution was called a "medium-wash". The composition of the wash was chosen to eliminate osmotic effects on washing. The medium-wash was chilled in an attempt to limit enzyme reactions till the sample was immersed in scintillation fluid.

Some modifications of the medium-wash were tried (Table 10). A second chilled 1ml medium-wash caused a slight decrease in the pool size (6%) suggesting either that the first wash was giving a true reading or that a labile pool had been completely removed. A third chilled 1ml medium-wash caused a 25% reduction in pool size, showing that pools were not permanently protected under these washing conditions. Attempts to prevent a possible temperature shock by washing at 30°C showed a lower retention of radioactivity on the filters and a second wash at 30°C reduced the pool size to 50% of the chilled 1ml medium-wash value (Table 10). This latter effect could be due to metabolism in cells on the filter. When the osmotic pressure of the wash was raised (Table 10, 5% NaCl) a 5% increase in pool size resulted, but this liquid filtered more slowly than the simple medium-wash.

The routine medium-wash used throughout was 1 x 1ml, chilled, homologous with the appropriate reaction medium and dispensed by Zippette.

Table 10. The effects of washing on the measurement of total uptake of radioactivity from [ring-<sup>14</sup>C(U)] benzoate in cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice as described in Methods (p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [ring-<sup>14</sup>C(U)] benzoate, 10µmol (0.3µ Ci/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions were initiated with 2.5ml cell suspension (Methods, p. 60). At 300s, 100µl samples were taken on to filter membranes under vacuum, washed as indicated, and the filters immediately immersed in scintillation fluid in scintillation vials. The pool size was calculated by subtraction of incorporation (water wash, Table 9) from uptake.

Washing conditions	Total uptake mmol benzoate/ l cell water (glucose)	Relative pool size
1 x 1ml medium at 0°C	42.5	100
2 x 1ml medium at 0°C	41.5	94
3 x 1ml medium at 0°C	37.5	75
1 x 1ml medium at 30°C	40.0	88
2 x 1ml medium at 30°C	32.0	50
3 x 1ml medium at 30°C	29.5	38
1 x 1ml (medium + 5% <sup>w/v</sup> NaCl) at 0°C	44	105
2 x 1ml H <sub>2</sub> O at 0°C	21.5	0

4. 1. 1. 6. Calculation of pool sizes in cell suspensions of bacterium NCIB 8250.

Materials inside the cell were usually calculated as concentrations. In preliminary experiments on the uptake and incorporation of [ring-<sup>14</sup>C(U)] benzoate, these concentrations must have represented many components as carbon from the [ring-<sup>14</sup>C(U)] benzoate was incorporated into macromolecule. On the other hand, radioactivity from [carboxy-<sup>14</sup>C] benzoate within the cell should represent only benzoate as the first metabolic reaction removes the radioactive moiety from the molecule.

In the manipulation of data, three variables were required in addition to the gross radioactivity retained on the filter membrane:

- (a) The reagent blank for the radioactive material under study (Results p.146). The blanks required for any experiment were done during that experiment.
- (b) The radioactivity per unit volume of reaction medium (Methods, p. 61). The specific radioactivity of the compound under test was then calculated from its molarity.
- (c) The turbidity of the stock cell suspension which was the measure of intracellular water space (Methods, p. 56) in the reaction medium. Intracellular concentration was calculated using the following calculation

$$I = \frac{(G - a) \cdot 100}{b \cdot c}$$

where I = intracellular concentration, nmol/ $\mu$ l cell water; G= total radioactivity on the filter membrane from a 100 $\mu$ l sample, pCi; a = reagent blank, pCi; b = specific radioactivity of material entering cell, pCi/nmol; c = intracellular water,  $\mu$ l/10ml reaction medium; and 100 x 100 $\mu$ l samples/10ml reaction medium.

4. 1. 1. 7. A test of the uptake apparatus and procedures with the lac permease of Escherichia coli.

ML 308 is a strain of Escherichia coli which synthesises constitutively the enzymes of the lactose (lac)operon (Monod & Cohn, 1952). A mutant of E. coli ML 308, presumably deficient in the lac permease, was derived in this laboratory by Dr. A. G. Robertson. This mutant grew normally on glucose ( $\mu = 0.88\text{h}^{-1}$ ) and synthesised  $\beta$ -galactosidase constitutively at the same specific activity as the parent strain (5.3 enzyme units/mg dry wt.) but this strain grew very slowly on lactose and failed to hydrolyse o-nitrophenyl- $\beta$ -galactoside in cell suspension.

Thiomethyl- $\beta$ -galactoside is a substrate for the lac permease but is not hydrolysed by  $\beta$ -galactosidase (Cohen & Rickenberg, 1955). The uptake of thiomethylgalactoside by E. coli ML 308 (Fig. 37) was very rapid and reached a steady state internal concentration of 47mmol/l cell water (inulin) within 200s. This massive uptake reduced the external concentration of substrate and the ratio of internal to external thiomethylgalactoside concentrations was 117:1. The patterns of the duplicates were identical (Fig. 37) and matched those of the thiomethylgalactoside

Fig. 37. Uptake of thiomethylgalactoside by cell suspensions of Escherichia coli ML 308 and by a mutant of E. coli ML 308 deficient in the lac permease.

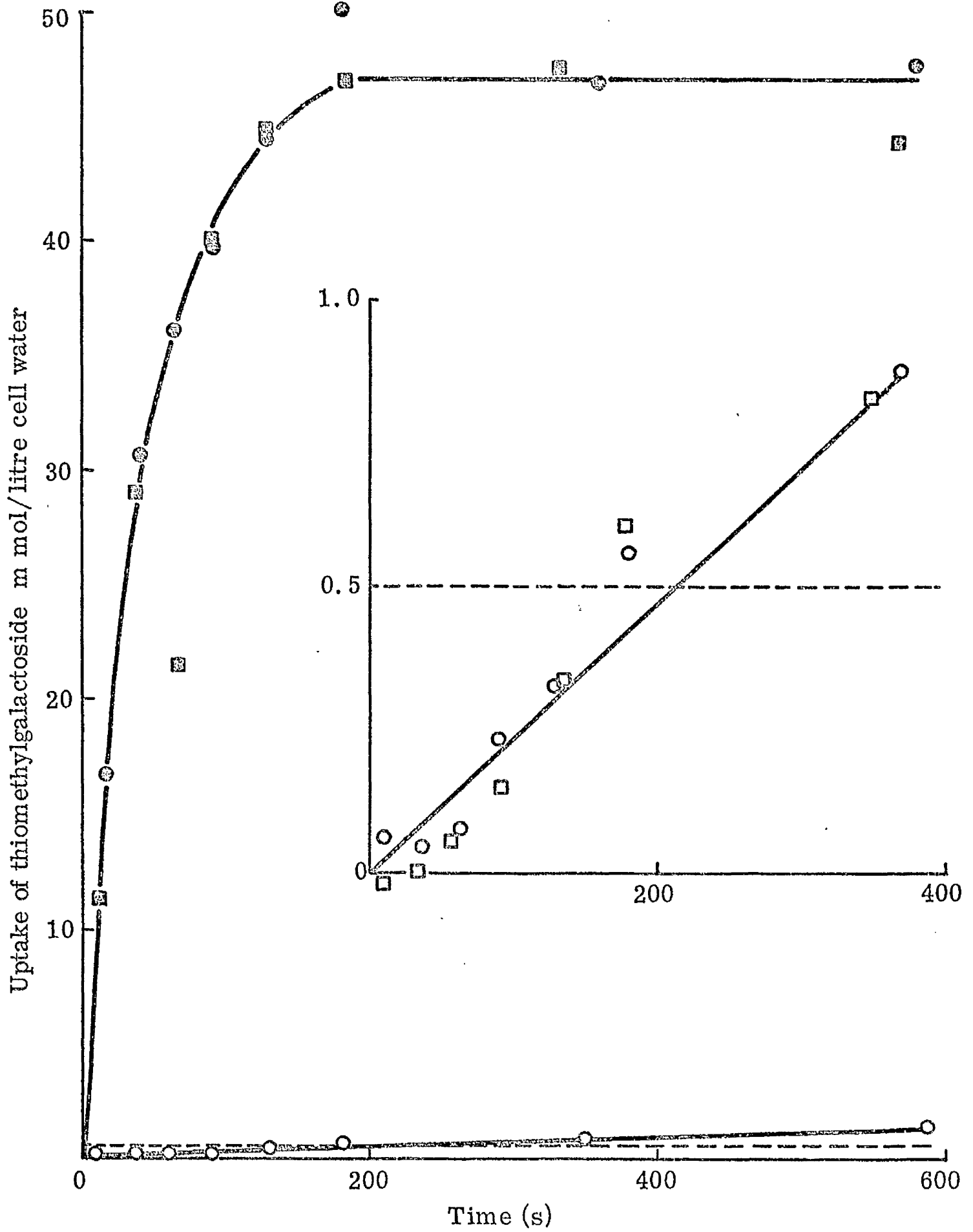
E. coli ML 308 and the permease negative mutant were grown separately in 20mM-glycerol-salts medium (Methods, p. 52) harvested when the turbidity reached 1.0, resuspended to 20mg wet wt./ml in the appropriate salts medium and stored on ice (Methods, p. 60). The experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing thiomethyl [<sup>14</sup>C]-β-D-galactopyranoside, 5μmol (0.6μ Ci/μmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions were initiated by the addition of 2.5ml of cell suspension (Methods, p. 60). At intervals 100μl samples were harvested on filter membranes under vacuum, washed with 1ml chilled "medium wash" and the filter immediately immersed in liquid scintillation fluid in a liquid scintillation vial (Methods, p. 62).

● ■ Thiomethylgalactoside uptake by E. coli ML 308 (in duplicate).

○ □ Thiomethylgalactoside uptake by permease negative strain derived from E. coli ML 308.

----- Concentration of thiomethylgalactoside in the reaction medium at zero time.





uptake in E. coli ML 308 observed by Rickenberg, Cohen, Buttin & Monod (1956) and Winkler & Wilson (1966). The intracellular concentration of 47mM or 2.8% dry wt. of bacteria is higher than the 2.2% dry wt. of bacteria quoted by Rickenberg et al. (1956) but the temperature and reaction media were both different. In contrast to the parent strain of E. coli ML308, the mutant strain showed a very low uptake of thiomethylgalactoside (Fig. 37 ), which confirms that in this mutant the lac permease has very little activity.

These data demonstrate that the assay technique is capable of resolving a very wide range of uptake rates, including those with very low levels of substrate in the cell.

#### 4. 1. 1. 8. Modification of the assay technique for "slow filtering" cell types.

The technique for estimating intracellular concentrations was developed with cell suspensions harvested from benzoate-salts medium. Samples of these suspensions filtered rapidly on harvesting and reproducible results were readily obtained. Similar rapid filtration of bacterium NCTB 8250 was obtained with cells of different sizes (growth rates varying from  $\mu = 0.92\text{h}^{-1}$  to  $0.27\text{h}^{-1}$ ) harvested from nutrient broth, benzyl alcohol- and mandelate-salts media. Cell suspensions of E. coli also filtered easily under a variety of conditions. However, data were required from cell suspensions non-induced for the mandelate pathway enzymes but grown in defined media and succinate-grown cells were

chosen because of their rapid growth ( $\mu = 0.92\text{h}^{-1}$ ) to a high yield (turbidity about 1.0). Surprisingly, these cells did not filter quickly. Up to 30s was required for liquids to disappear from the filter membrane. In addition the results showed a very wide and random scatter which could not be interpreted in terms of intracellular substrate concentrations.

Cell density was reduced, the phase of growth from which the cells were harvested was varied, composition of the reaction medium was altered and washings were changed, all without effect. The use of cells grown in glutamate-salts medium (another known non-induced cell type) also gave an uninterpretable scatter in results. The scatter seemed worse if filtration was especially poor, but an improvement in vacuum with a corresponding speeding of filtration did not significantly alter the results.

It was considered possible that reaction medium was somehow being trapped between cells on the filter membrane, and that effective washing of this material would remove most intracellular pools present. This idea was supported by phase contrast microscopy where the normal pattern (e.g. benzoate-salts cultures) of pairs of cells (Fewson, 1967a) was largely replaced by small clumps in succinate-salts cultures. The reaction medium was, therefore, supplemented with glucose-5-<sup>3</sup>H, a compound that could not enter the cell (Results, p. 80) and would measure extracellular reaction medium on filter membranes.

This modification of the assay technique required an

additional step in the calculation of intracellular concentrations. Consider the entry of [carboxy- $^{14}\text{C}$ ] benzoate into cells harvested from a succinate-salts culture (Fig. 38a,b). Net  $^{14}\text{C}$  and  $^3\text{H}$  on the filter were expressed as  $\mu\text{l}$  reaction medium. The volume of reaction medium, indicated by the tritiated glucose as extracellular space, was subtracted from the reaction medium equivalent volume of  $^{14}\text{C}$  on the filter. The residual "volume" of  $^{14}\text{C}$  was then expressed as  $\text{mmol benzoate/l cell water (glucose)}$ , Fig. 38b. A water wash of samples from the same reaction medium showed that no radioactivity was incorporated into macromolecule. No decarboxylation of benzoate was detected.

The correction of data for extracellular material was essential. The scatter of individual points, uncorrected for material presumably trapped between cells, is seen in Fig. 38 a. If these values were taken as a true representation of intracellular concentration, the uninterpretable scatter described on the previous page would be seen in Fig. 38b when the units were converted. The correction is valid because glucose does not enter these cells at a significant rate (Discussion, p. 188). The acquirement of an interpretable result by a legitimate correction made further work practicable.

#### 4. 2. Metabolism and utilisation of benzoate in cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

As already observed, benzoate decarboxylation in cell suspensions of benzoate-grown cells was biphasic (Fig. 36 ).

Fig. 38 . Intracellular concentration of benzoate  
in a cell suspension of bacterium NCIB 8250  
harvested from succinate-salts medium.

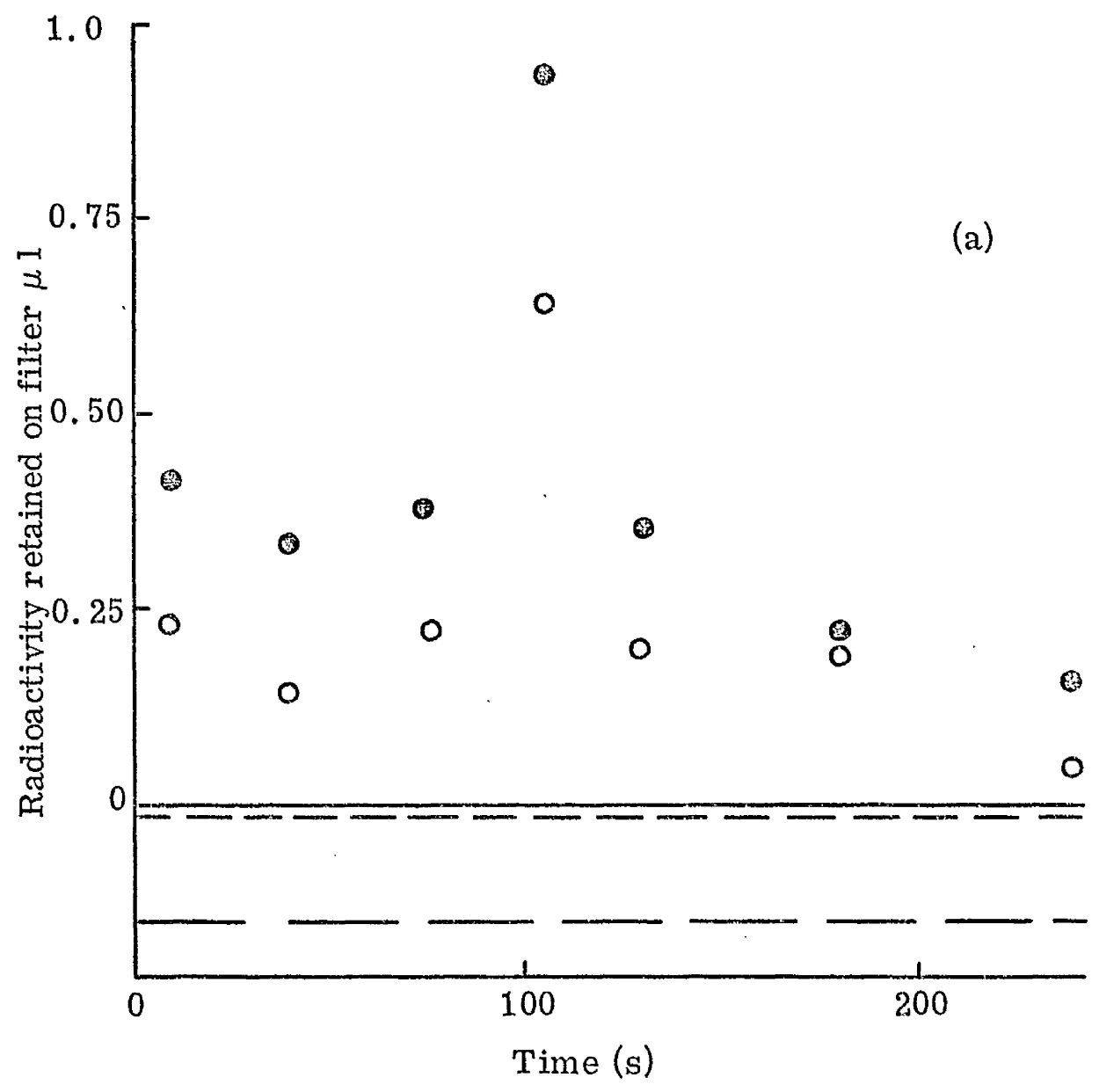
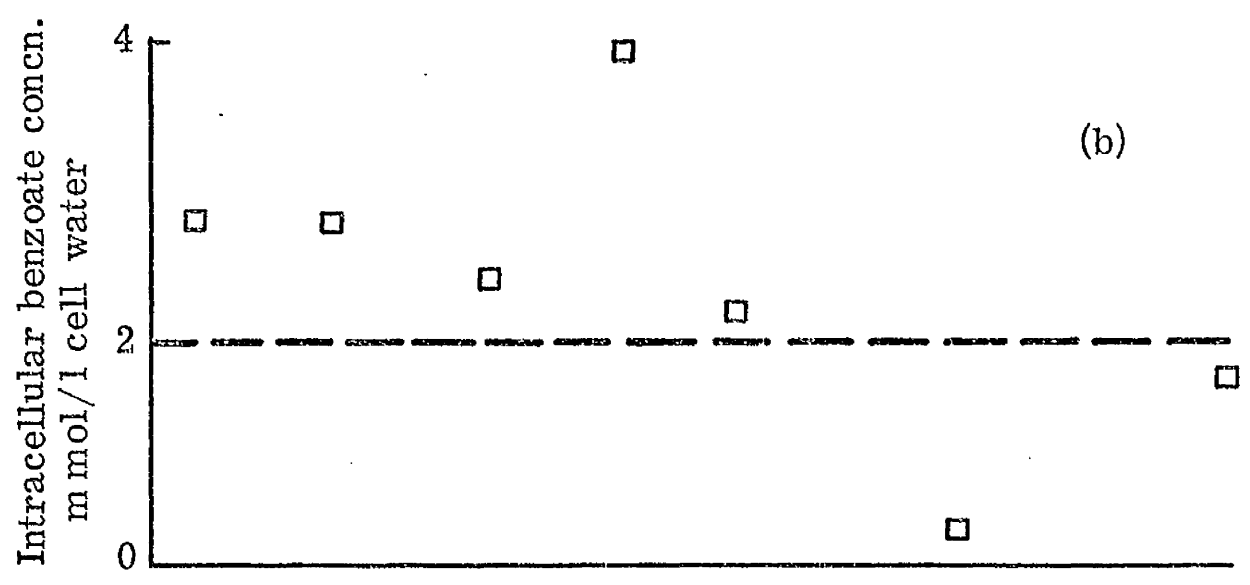
Bacterium NCIB 8250 was grown in 5mM-succinate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60 ). The experiment was done in a shaking water bath at 30°C in a 50ml Erlenmeyer flask with a 10ml final volume reaction medium containing [carboxy-<sup>14</sup>C] benzoate, 20µmol (0.5µCi/µmol) and glucose-5-<sup>3</sup>H, 1µmol (25µCi/µmol). Incomplete (7.5ml) reaction medium and a portion of the stock cell suspension were preincubated separately and the reaction was initiated with 2.5ml cell suspension. At intervals 100µl samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60).

Graph a);      and      represent the reagent blanks for [carboxy-<sup>14</sup>C] benzoate and glucose-5-<sup>3</sup>H respectively; ● [carboxy-<sup>14</sup>C] benzoate and ○ glucose-5-<sup>3</sup>H retained on the filter expressed as volumes of reaction medium.

Graph b) is a further manipulation of the data in graph a) (Results, p159 ).

□ Intracellular benzoate.

Extracellular benzoate concentration.



The incorporation of radioactivity from  $\left[ \text{ring-}^{14}\text{C}(\text{U}) \right]$  benzoate proceeded from zero time (Fig. 39a ). The rate of incorporation fell steadily with time and accounted for only 11% of the benzoate decarboxylated at 800s, presumably because there was no exogenous nitrogen supply in the reaction medium.

#### 4. 2. 1. The effect of catechol on benzoate metabolism.

Catechol influenced the growth of bacterium NCIB 8250 in benzoate-salts medium (Results, p.107 ) and slightly inhibited decarboxylation of benzoate by cell suspensions (Results, p.142 ). This work has been extended to include the effect of catechol on benzoate incorporation in cell suspensions (Fig. 39 a,b).

The presence of catechol caused a delay in benzoate decarboxylation (Fig. 39 b) but within 100s the reaction attained and maintained a rate of 38nmol/mg dry wt./min, the control second phase rate of benzoate decarboxylation. In contrast to its relatively small effect on benzoate decarboxylation, catechol caused a 90% reduction in the incorporation of  $^{14}\text{C}$  from benzoate in macromolecule (Fig. 39 a). However, overall incorporation into macromolecule was not greatly affected by the presence of catechol. Radioactivity from  $\left[ \text{ring-}^3\text{H}(\text{G}) \right]$  catechol was rapidly detected in macromolecule (Fig. 39 a). The large reagent blank for catechol nullified exact comparisons of total incorporation in the presence and absence of catechol.

When suspensions of catechol-grown cells were studied,

Fig. 39. The effect of catechol on benzoate decarboxylation and incorporation by cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

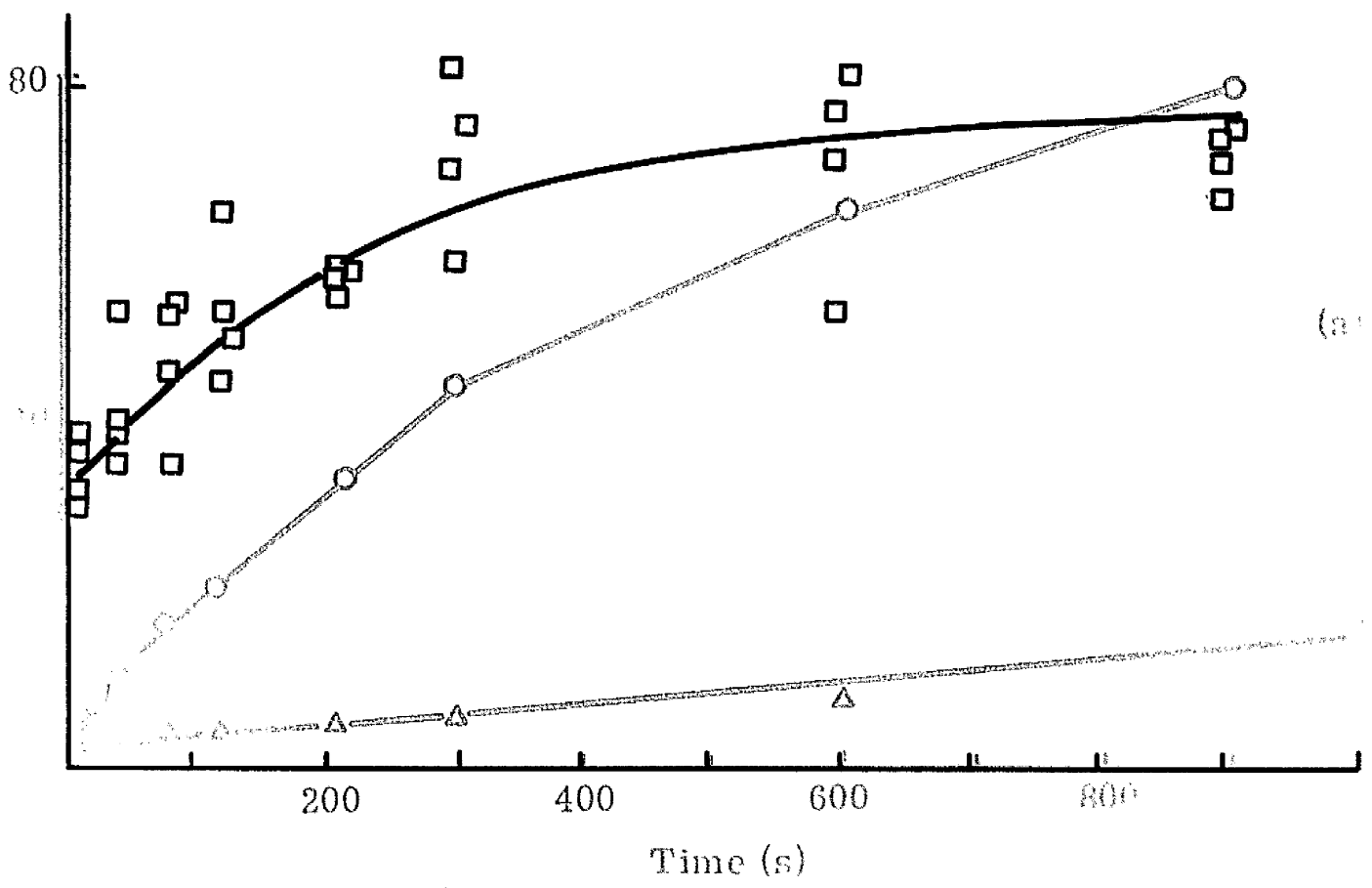
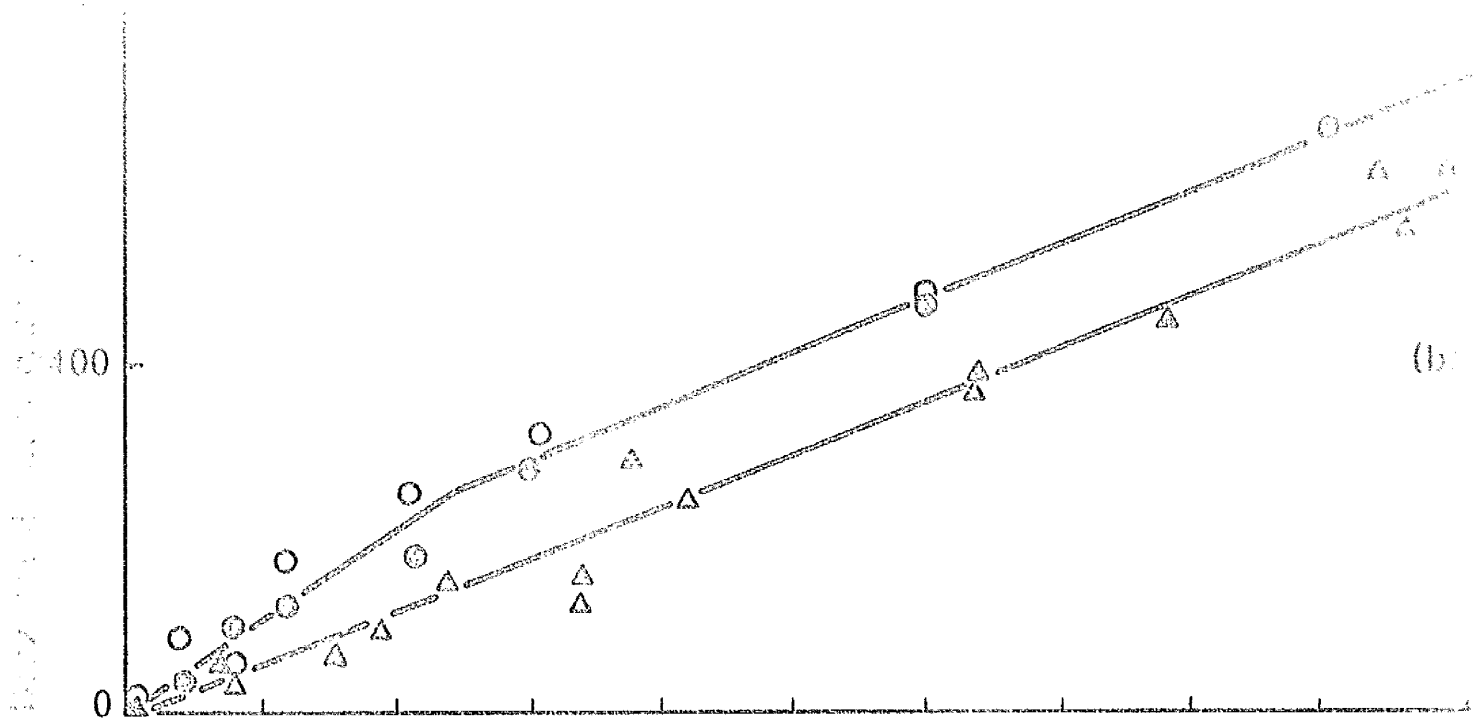
Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [carboxy-<sup>14</sup>C] - or [ring-<sup>14</sup>C(U)] benzoate, 10µmol (0.1 and 0.3µCi/µmol respectively); and [ring-<sup>3</sup>H(G)] catechol, zero or 10µmol(2.5µCi/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and the reaction initiated by the addition of 2.5ml cell suspension. At intervals, 100µl samples were harvested on filter membranes under vacuum, washed with 2 x 1ml chilled distilled water and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60). In addition, those reaction media containing [carboxy-<sup>14</sup>C] - benzoate were further sampled (100µl) at intervals on to 1.0ml 0.1M-HCl to estimate benzoate utilisation (Methods, p. 62).

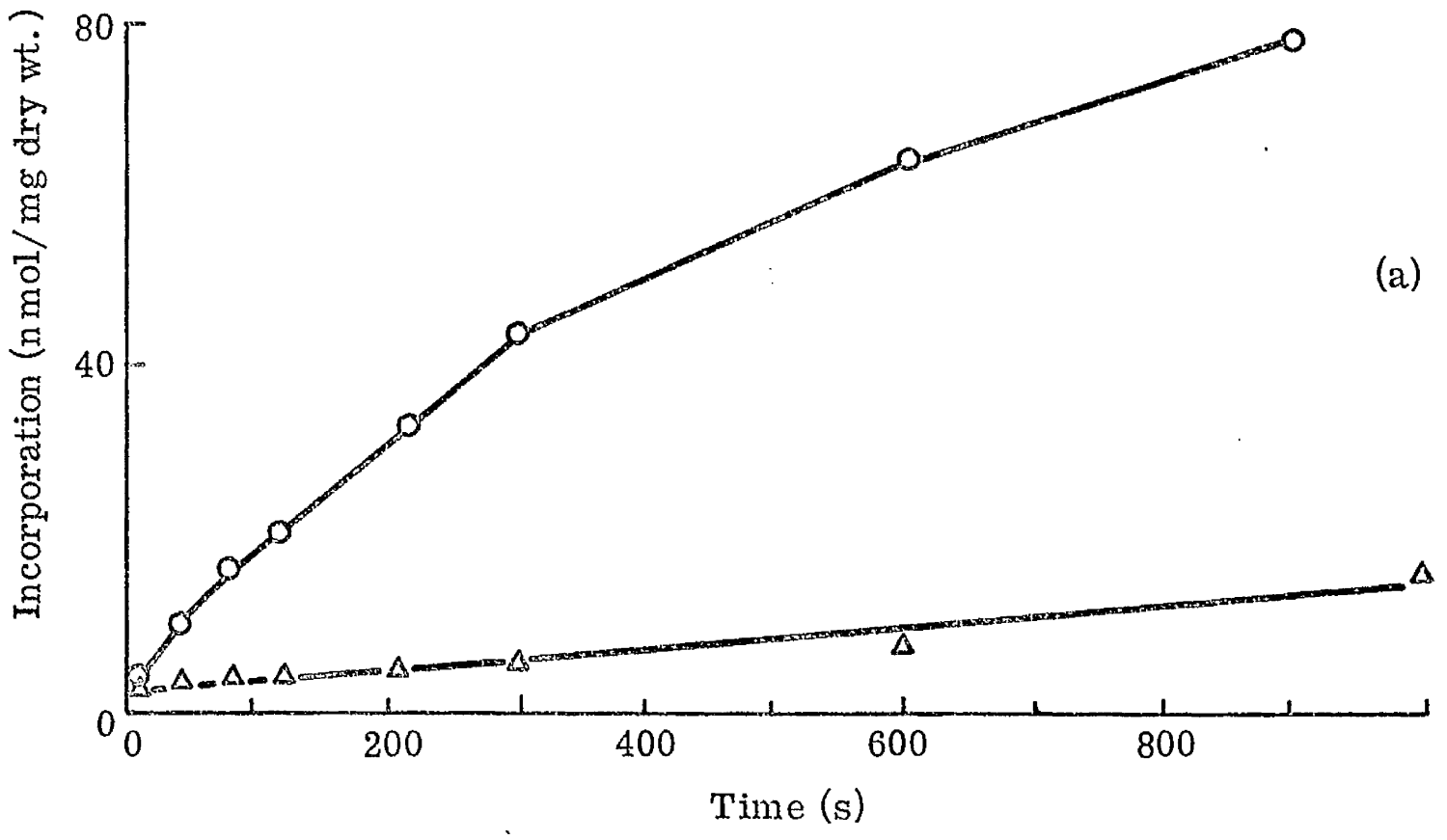
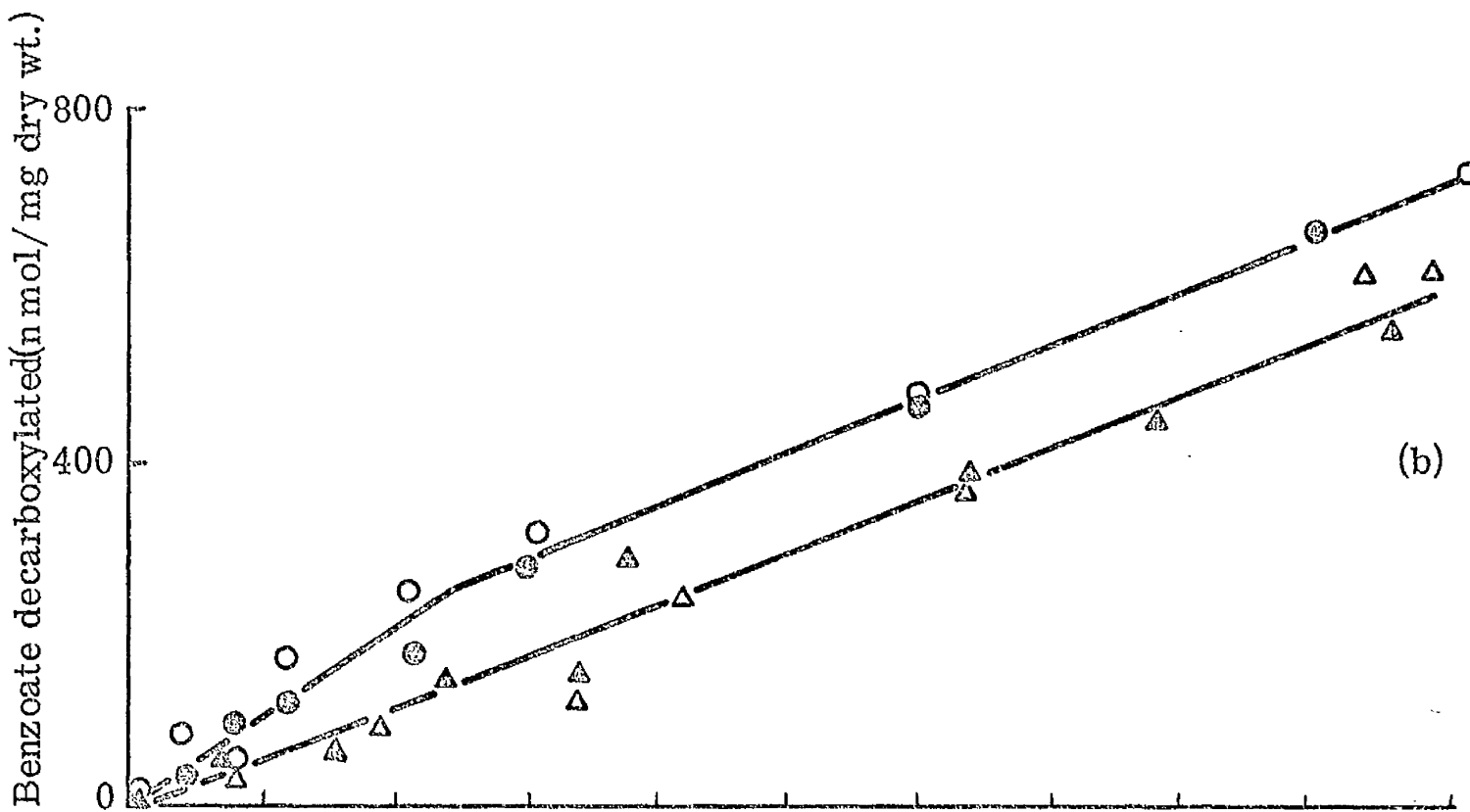
Graph a)incorporation into macromolecule and graph b) decarboxylation of benzoate.

- ● Benzoate alone in reaction medium.
- △ ▲ Effect of catechol on benzoate utilisation and decarboxylation.
- Catechol incorporation in the presence of benzoate.

Incorporation data have not been corrected for blanks.







the incorporation of catechol was similar to its incorporation in benzoate-grown cells (Fig. 39 a). In the presence of catechol, benzoate decarboxylation and incorporation rose between 300s and 900s from zero to about 2% of the values in benzoate-grown cells.

#### 4. 2. 2. The decarboxylation of benzoate and certain substituted benzoates.

##### 4. 2. 2. 1. Inhibition of benzoate decarboxylation.

42 Compounds, substituted benzoates and analogues of benzoate, were screened for their ability to inhibit benzoate decarboxylation in cell suspension. None of these compounds was found to have any substantial effect when present at 1mM, equimolar with benzoate: o-, m- and p-aminobenzoate, o-, m- and p-hydroxybenzoate, o-, m- and p-fluorobenzoate, o-, m- and p-chlorobenzoate, o-, m- and p-bromobenzoate, o-, m- and p-iodobenzoate, o-, m- and p-nitrobenzoate, o-, m- and p-methoxybenzoate, o-, m- and p-toluate, benzenesulfonate, phenylphosphinate, phenylphosphonate, benzamide, anisole, cyclohexanecarboxylate, p-chloromercuribenzoate, methyl benzoate, phenyl benzoate, phthalate, benzene-1,3-dicarboxylate, terephthalate, diphenate, acetophenone and acetyl salicylate. However, three of these compounds were chosen for further study because their utilisation had been studied previously and they were now available radiochemically labelled. p-Fluorobenzoate is oxidised by benzoate-grown cells (Fewson,

Kennedy & Livingstone, 1968) while o-hydroxybenzoate is not (Kennedy & Fewson, 1968a). p-Methoxybenzoate does not support growth (Fewson, 1967b).

p-Fluorobenzoate, p-methoxybenzoate and o-hydroxybenzoate all had slight inhibitory effect on benzoate decarboxylation.

Benzoates present in reaction medium each at 1mM	Relative benzoate decarboxylation rates.	
	First phase	Second phase
Benzoate alone	100	100
Benzoate + <u>p</u> -fluorobenzoate	84	81
Benzoate + <u>p</u> -methoxybenzoate	87	83
Benzoate + <u>o</u> -hydroxybenzoate	97	88

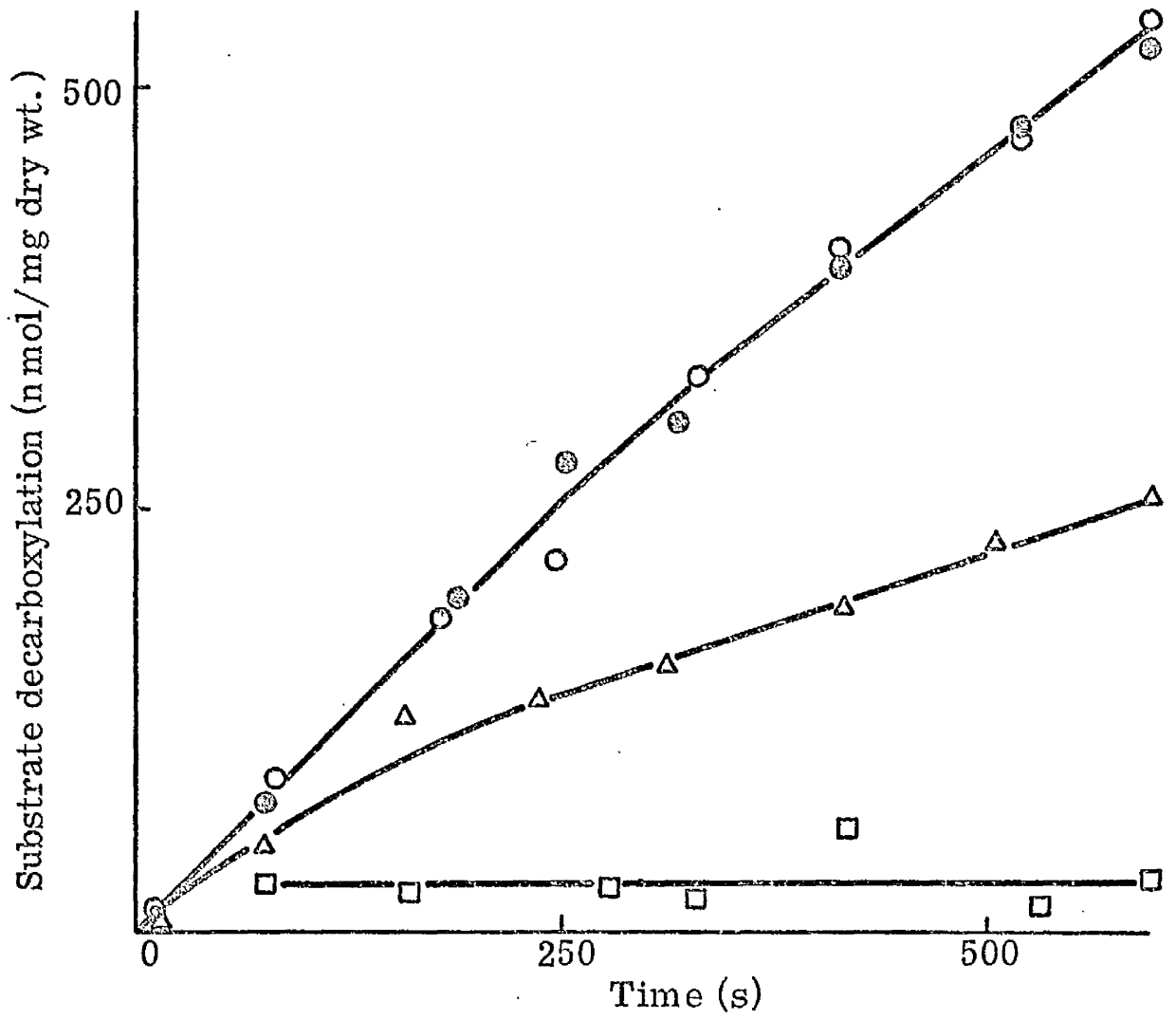
4. 2. 2. 2. The decarboxylation of substituted benzoates present alone and in the presence of benzoate.

Of the substituted benzoates under test, only p-fluoro [carboxy-<sup>14</sup>C] benzoate was decarboxylated (Fig. 40). This decarboxylation was abolished in the presence of benzoate (Fig. 40 ). Presumably benzoate prevented p-fluorobenzoate from acting as a substrate for benzoate oxidase. None of the substituted benzoates was co-metabolised with benzoate.

Fig. 40. Decarboxylation of benzoate and of p-fluorobenzoate by cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [carboxy-<sup>14</sup>C] benzoate, 10µmol (0.1µCi/µmol) or p-fluoro [carboxy-<sup>14</sup>C] benzoate, 10µmol (0.1µCi/µmol) with benzoate, zero or 10µmol. Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and the reaction initiated by 2.5ml cell suspension. At intervals, 100µl samples were taken on to 1.0ml 0.1M-HCl and the radioactivity in this acidified suspension measured (Methods, p. 62).

- ● Benzoate decarboxylation, in duplicate.
- △ p-Fluorobenzoate decarboxylation in the absence of other intermediates.
- p-Fluorobenzoate decarboxylation in the presence of equimolar benzoate.



[Carboxy- <sup>14</sup> C] benzoates present in reaction medium at 1mM	Decarboxylation rates, nmol/mg dry wt./min		
	Present individually		Additional presence of 1mM-benzoate
	Initial Rate	Second Rate	
Benzoate	61	45	-
p-fluorobenzoate	non-linear	20	0
p-methoxybenzoate	0	0	0
o-hydroxybenzoate	0	0	0

#### 4. 3. Benzoate uptake and pool size in cell

suspensions of bacterium NCIB 8250.

##### 4. 3. 1. Benzoate uptake and pool size in cell

suspensions harvested from benzoate-salts medium.

The uptake of radioactivity from [ring-<sup>14</sup>C(U)] benzoate was immediate and rapid in benzoate-grown cells (Fig. 41). The initial rate of uptake (67nmol/mg dry wt./min) was maintained for about 35s after which the rate dropped to 8nmol/mg dry wt./min. The kink in uptake of [ring-<sup>14</sup>C(U)] benzoate as the rate changed was regularly observed.

An estimate of the overall pool of small molecules in the cell was calculated as the difference between uptake (total radioactivity in the cell; Results, p. 150) and incorporation values (perchloric acid insoluble material, measured as water insoluble material; Results, p. 147), Fig. 41. This pool,

Fig. 41. Uptake and incorporation of [ring-<sup>14</sup>C(U)] benzoate into cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

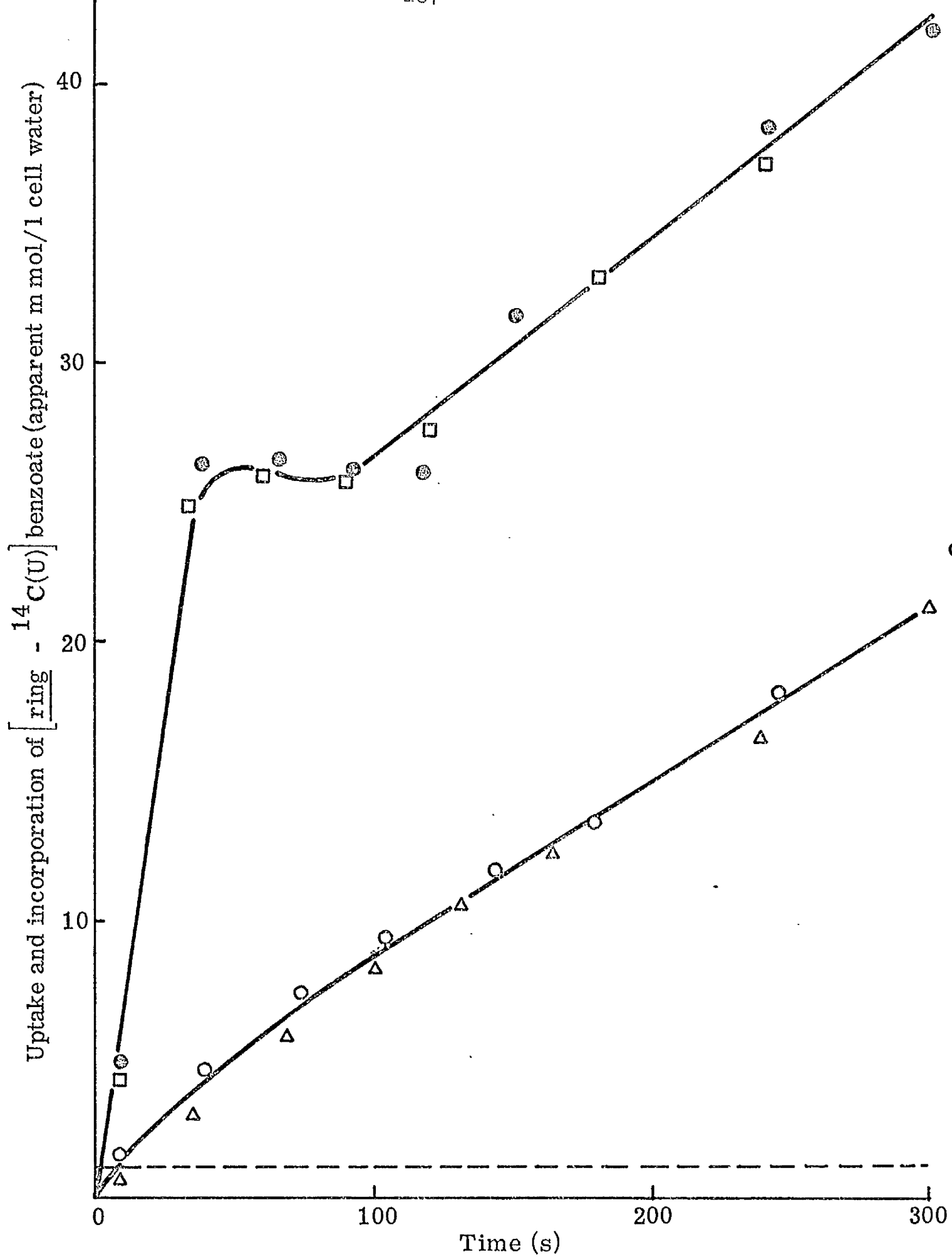
Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [ring-<sup>14</sup>C(U)] benzoate, 10µmol (0.3µCi/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were pre-incubated separately and reactions were initiated by the addition of 2.5ml cell suspension. At intervals 100µl samples were harvested on filter membranes under vacuum, washed and the filter immediately immersed in scintillation fluid in a liquid scintillation vial (Methods, p. 60). The washes used were either 2 x 1ml chilled distilled water or 1ml medium wash. Reagent blanks were done in the absence of cells and data were corrected by subtraction for the appropriate blank (Results, p.154).

○ △ Incorporation of radioactivity into macromolecule.

□ ● Total uptake of radioactivity from [ring-<sup>14</sup>C(U)] benzoate.

— — — — — Extracellular benzoate concentration.





presumably the summation of individual pools in catabolic, amphibolic and anabolic pathways as macromolecule was synthesised, was the equivalent of 21mM-benzoate inside the cell. Experiments were done to estimate the contribution of benzoate itself to the overall pool. Radioactivity from [carboxy- $^{14}\text{C}$ ] benzoate inside the cell was assumed to be benzoate as the first metabolic reaction was decarboxylation of the radioactive carbon atom. No intracellular benzoate could be detected in benzoate-grown cells (Fig. 42). Presumably any intracellular benzoate had been decarboxylated before the reaction was stopped (Results, p.152).

Experiments were done in the presence of 0.3mM-puromycin, 0.5mM-2, 4-dinitrophenol or of 1mM- $\alpha, \alpha'$ -bipyridyl (an iron chelator and an inhibitor of benzoate oxidase, Fewson et al., 1970) but before the assay technique had been fully developed. In no case was [carboxy- $^{14}\text{C}$ ] benzoate present inside the cell at a concentration above that outside the cell. The pre-incubation time used was 5min. Puromycin had little effect on the uptake and incorporation of [ring- $^{14}\text{C(U)}$ ] benzoate, 2, 4-dinitrophenol caused a delay of about 60s before a slightly inhibited uptake and incorporation occurred and  $\alpha, \alpha'$ -bipyridyl severely inhibited both uptake and incorporation.

#### 4. 3. 2. Benzoate uptake and pool size in cells

non-induced for the mandelate pathway enzymes.

Benzoate readily entered non-induced cells of bacterium NCIB 8250 (Figs.38 and 43) rapidly reaching and remaining constant

Fig. 42. Intracellular benzoate concentration in cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml  $[ \text{carboxy-}^{14}\text{C} ]$  benzoate, 10 $\mu$ mol (1 $\mu$ Ci/ $\mu$ mol) and glucose-5- $^3\text{H}$  (25 $\mu$ Ci/ $\mu$ mol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and the reactions initiated by the addition of 2.5ml cell suspension. At intervals 100  $\mu$ l samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60).

Graph a); \_\_\_\_\_ and ———— represent the reagent blanks for  $[ \text{carboxy-}^{14}\text{C} ]$  benzoate and glucose-5- $^3\text{H}$  respectively;  $\odot$   $[ \text{carboxy-}^{14}\text{C} ]$  benzoate and  $\Delta$  glucose-5- $^3\text{H}$  retained on the filter and expressed in units of volume of reaction medium.

Graph b) is a further manipulation of the data in graph a) (Results, p.159).

$\odot$  Intracellular benzoate.

————— Extracellular benzoate concentration.

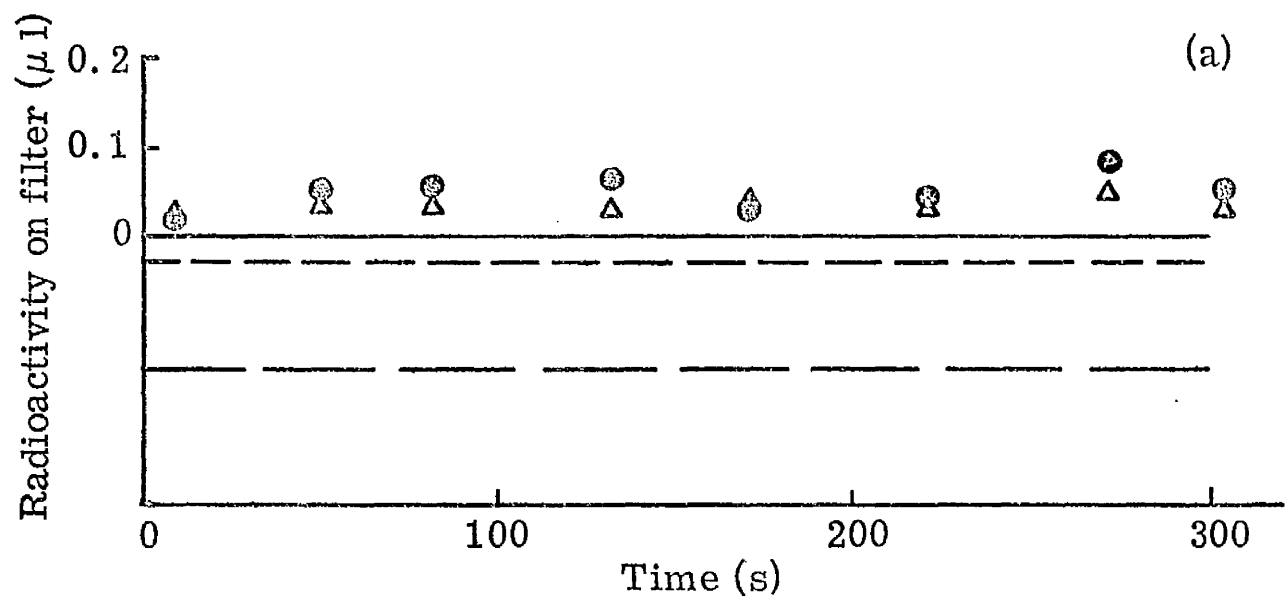
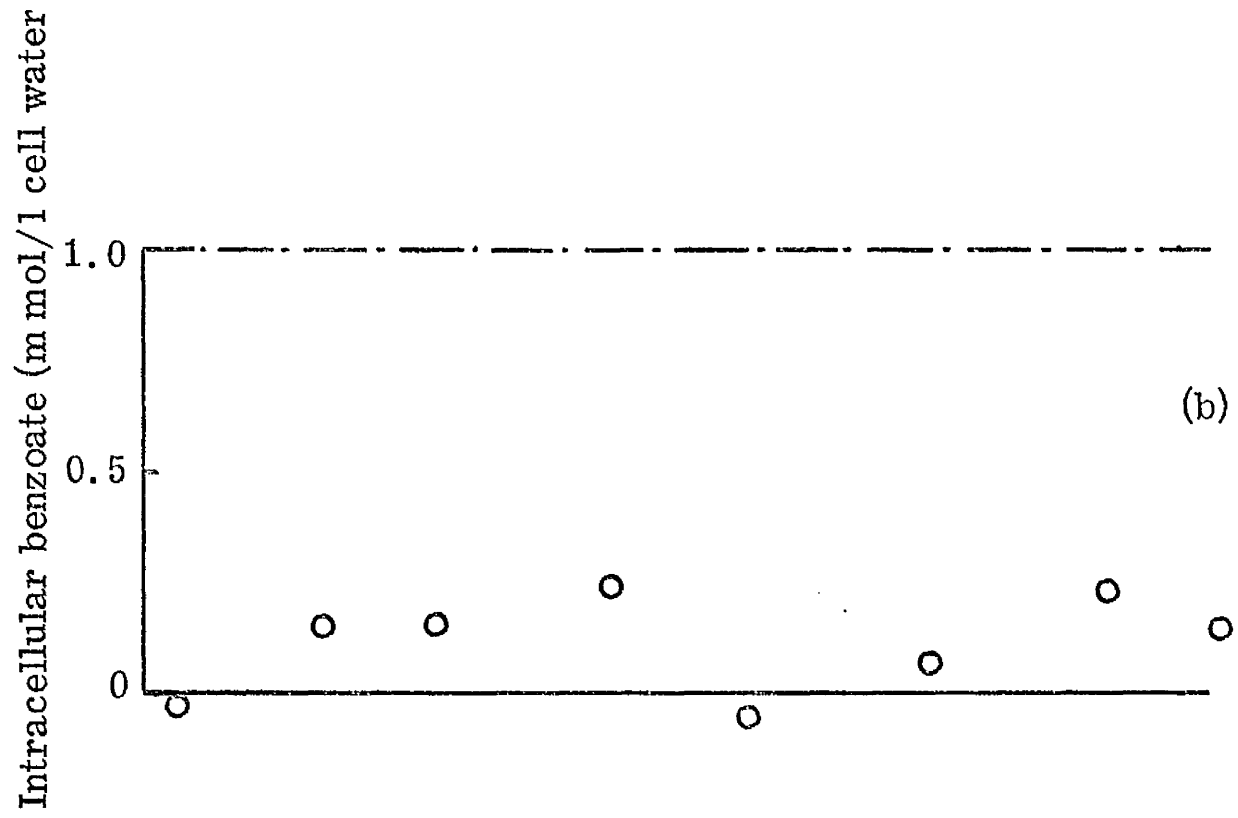


Fig. 43.  $[\text{carboxy}]$  intracellular benzoate concentration in cell suspensions of bacterium NCIB 8250 harvested from succinate-salts medium.

Bacterium NCIB 8250 was grown in 5mM-succinate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml  $\text{Er}[\text{carboxy-}^{14}\text{C}]$  benzoate, 10 $\mu\text{mol}$  (1 $\mu\text{Ci}/\mu\text{mol}$ ) and glucose-5- $^3\text{H}$ , 1 $\mu\text{mol}$  [ $\text{c.}(25\mu\text{Ci}/\mu\text{mol})$ ]. Incomplete (7.5ml) reaction media and portions of (25 $\mu\text{Ci}/\mu\text{mol}$ ). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions were initiated by the addition of 2.5ml cell suspension. At intervals 100 $\mu\text{l}$  samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60).

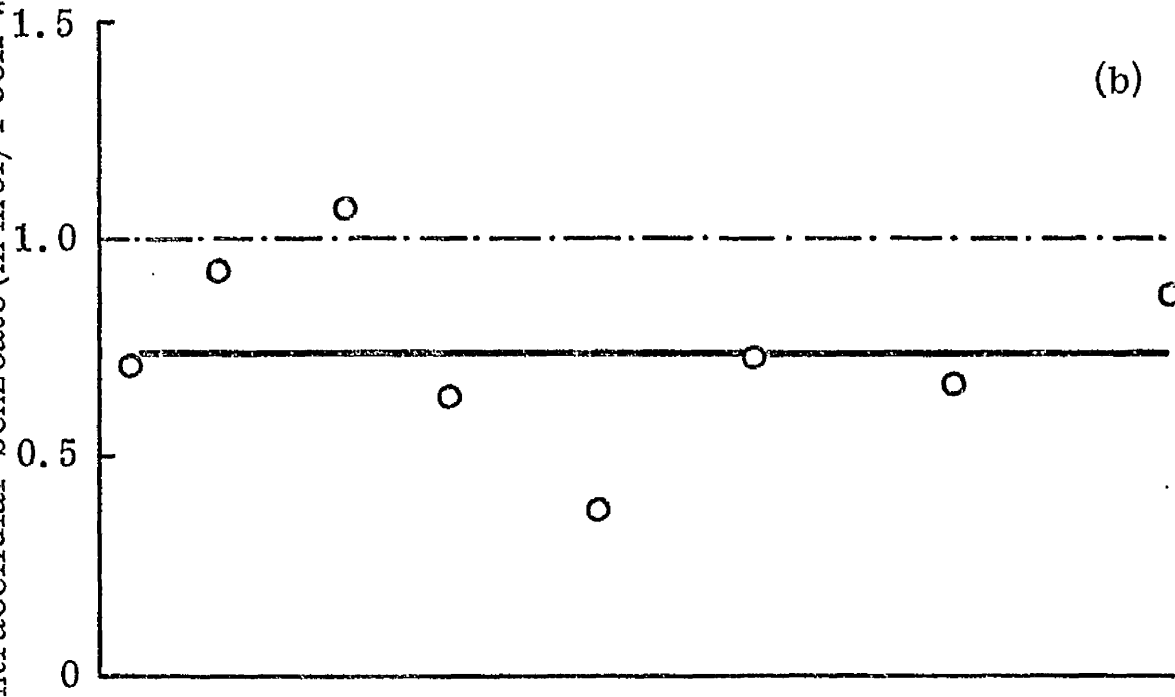
Graph a);      and      represent the reagent blanks for  $[\text{carboxy-}^{14}\text{C}]$  benzoate and glucose-5- $^3\text{H}$  respectively;  $\odot$   $[\text{carboxy-}^{14}\text{C}]$  benzoate and  $\Delta$  , glucose-5- $^3\text{H}$  retained on the filter expressed as volumes of reaction medium.

Graph b) is a further manipulation of the data from graph a) (Results, p.159).

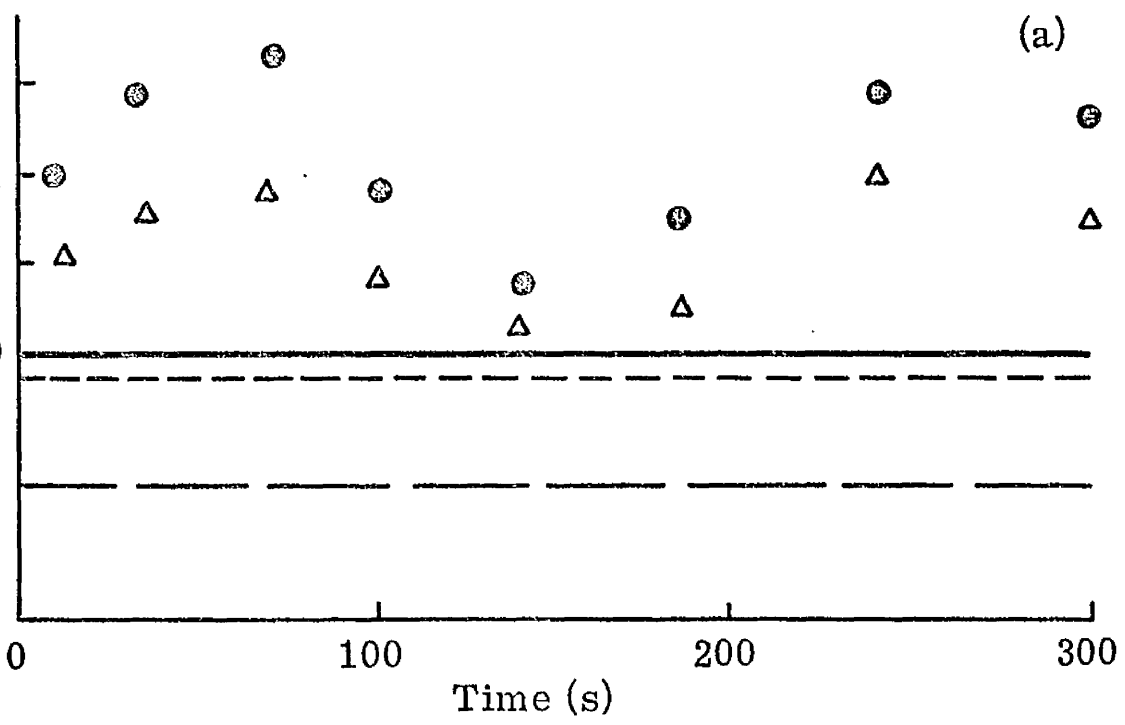
$\odot$  Intracellular benzoate.

     Extracellular benzoate concentration.

Intracellular benzoate (mmol/l cell water)



Radioactivity on filter ( $\mu$ l reaction medium)



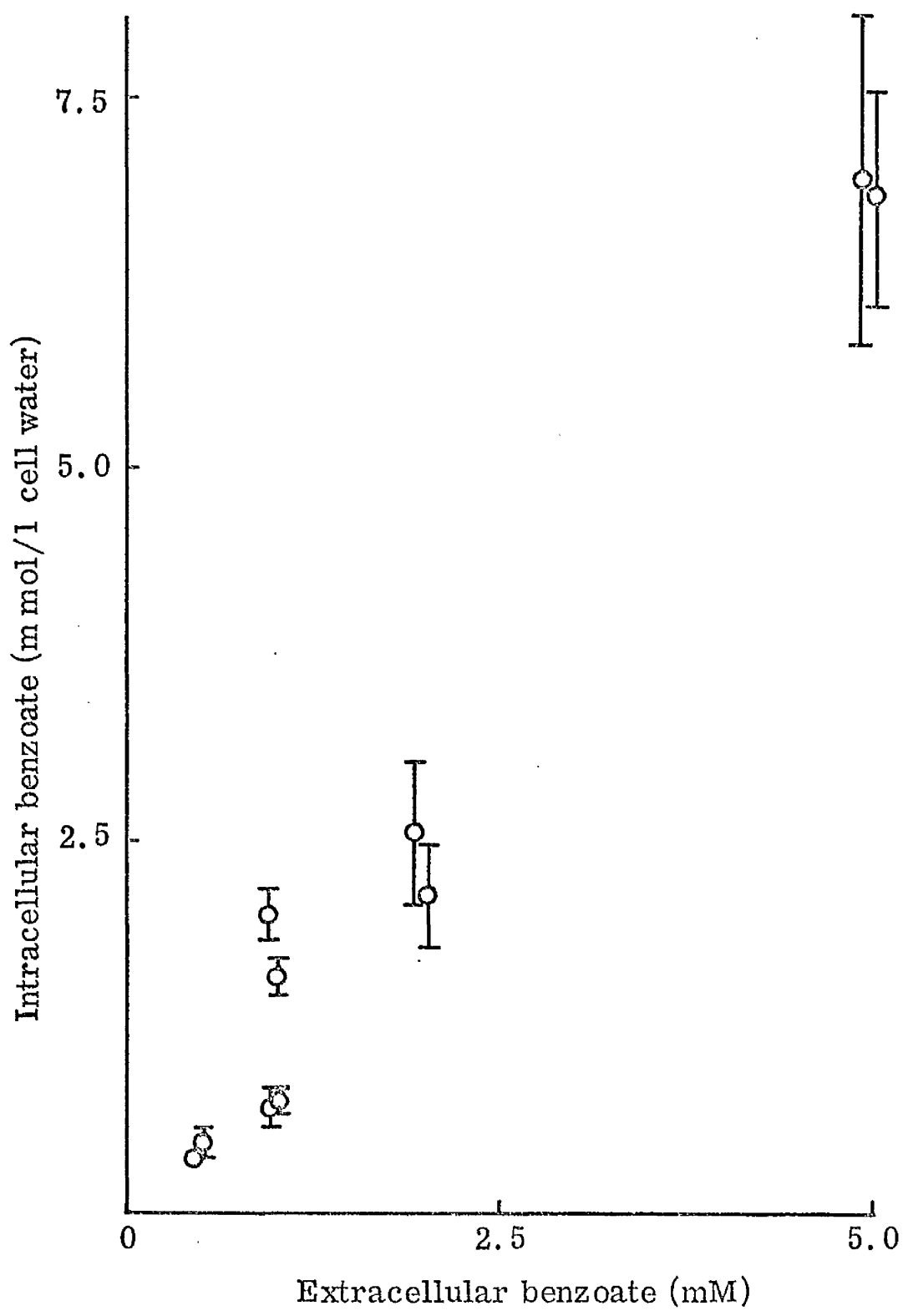
at the extracellular concentration. Similar results were obtained with nutrient broth-grown cells. Over a range of benzoate concentrations (0.5-5.0mM) the intracellular concentration rapidly attained a concentration close to the extracellular concentration (Fig. 44). There was a discrepancy at the highest benzoate concentration where the internal level exceeded the external level. It is not known whether this was a fault in the determination of pool size or an effect of benzoate at a concentration at which it is inhibitory to growth.

In all these experiments, the equilibrium intracellular benzoate concentration was attained by the time the first sample was taken (10s). Thus, at 1mM-benzoate in a typical reaction medium containing 50mg wet wt. of cells (i.e. about 9mg dry wt. and 15 $\mu$ l cell water (glucose) ), the minimum flux of benzoate into these cells was 15nmol/9mg dry wt./10s or 10nmol/mg dry wt./min. This is a minimum value for flux because the "time" used in the calculation (10s) is the maximum required to attain equilibrium. If equilibrium is attained within 10s, the value for flux will rise above 10nmol/mg dry wt./min. In contrast, the decarboxylation rate of benzoate in suspensions of benzoate-grown cells is 59nmol/mg dry wt./min (Results, p. 142). So if the flux of benzoate into non-induced cells was a 6-fold underestimate, the rate of entry of benzoate into non-induced cells would equal the decarboxylation rate in suspensions of induced cells. These calculations however, give no information on the mechanism of entry of benzoate into

Fig. 44,      Variation of intracellular benzoate concentration with extracellular benzoate concentration in cell suspensions of bacterium NCIB 8250 harvested from succinate-salts medium.

Bacterium NCIB 8250 was grown in 5mM-succinate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing [carboxy-<sup>14</sup>C] benzoate (1μCi/ml) at the indicated concentration and glucose-5-<sup>3</sup>H, 1μmol (25μCi/μmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and the reaction initiated by the addition of 2.5ml cell suspension. At intervals 100μl samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60). Individual sets of data were processed as indicated in Results (p.159). Values are plotted with the variance in that estimation.





bacterium NCIB 8250 and a further study was made on the interactions of benzoate and substituted benzoates in induced cells.

4. 3. 3. The effects of benzoate on the entry of o-hydroxybenzoate and p-fluorobenzoate into cell suspensions harvested from benzoate-salts media.

Benzoate-grown cell suspensions did not decarboxylate o-hydroxybenzoate (Results, p.166). Benzoate decarboxylation was little affected by o-hydroxybenzoate (Results, p.164). o-Hydroxybenzoate readily entered benzoate grown cells (Fig. 45 a) and within 60s attained and maintained the extracellular concentration. This pattern was much altered in the presence of benzoate (Fig. 45 b) when entry spiked to a level possibly above the external concentration and then fell over 250s to 15% of the extracellular level. In parallel assays with [carboxy-<sup>14</sup>C] benzoate, no intracellular benzoate was detected.

p-fluorobenzoate was decarboxylated by benzoate-grown cell suspensions (Fig. 40) and so presumably entered the cell, though no intracellular p-fluorobenzoate was detected under these conditions (Fig.46 a). However, even when p-fluorobenzoate decarboxylation was prevented by benzoate (Fig. 40), no intracellular p-fluorobenzoate could be detected (Fig.46 b). Benzoate apparently prevented p-fluorobenzoate decarboxylation by preventing its entry into the cell.

4. 4. The entry of mandelate into cell suspensions of bacterium NCIB 8250.

Fig. 45. The effect of benzoate on the entry of o-hydroxybenzoate into cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested when the turbidity reached 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing o-hydroxy [carboxy-<sup>14</sup>C] benzoate, 10µmol (1µ Ci/µmol); benzoate, zero or 10µmol; and glucose-5-<sup>3</sup>H, 1µmol (25µ Ci/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions were initiated by the addition of 2.5ml cell suspension. At intervals 100µl samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filters immediately immersed in scintillation fluid in scintillation vials (Methods, p. 60).

Graph a), no benzoate in reaction medium.

○ △ Intracellular o-hydroxybenzoate (in duplicate).

Graph b), 1mM-benzoate present in reaction medium.

● ▲ Intracellular o-hydroxybenzoate (in duplicate).

— — — — — Extracellular o-hydroxybenzoate concentration.

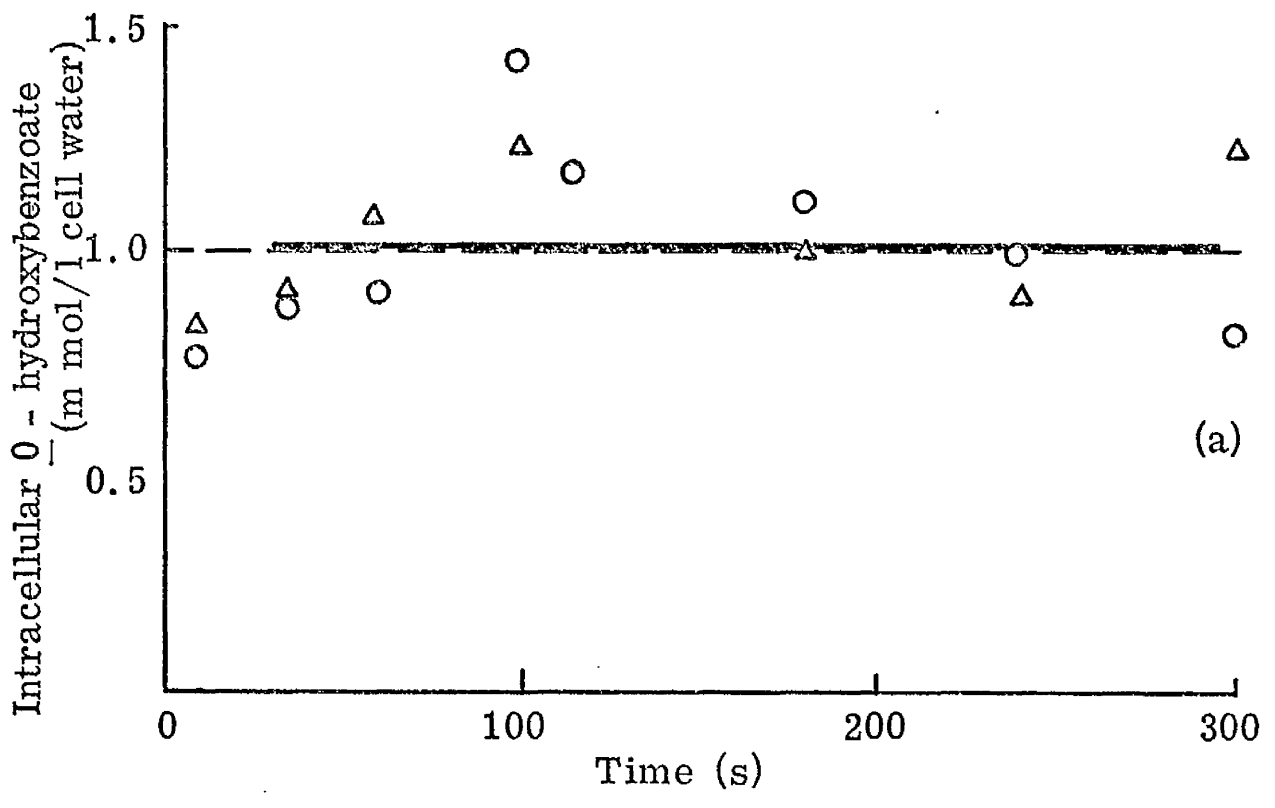
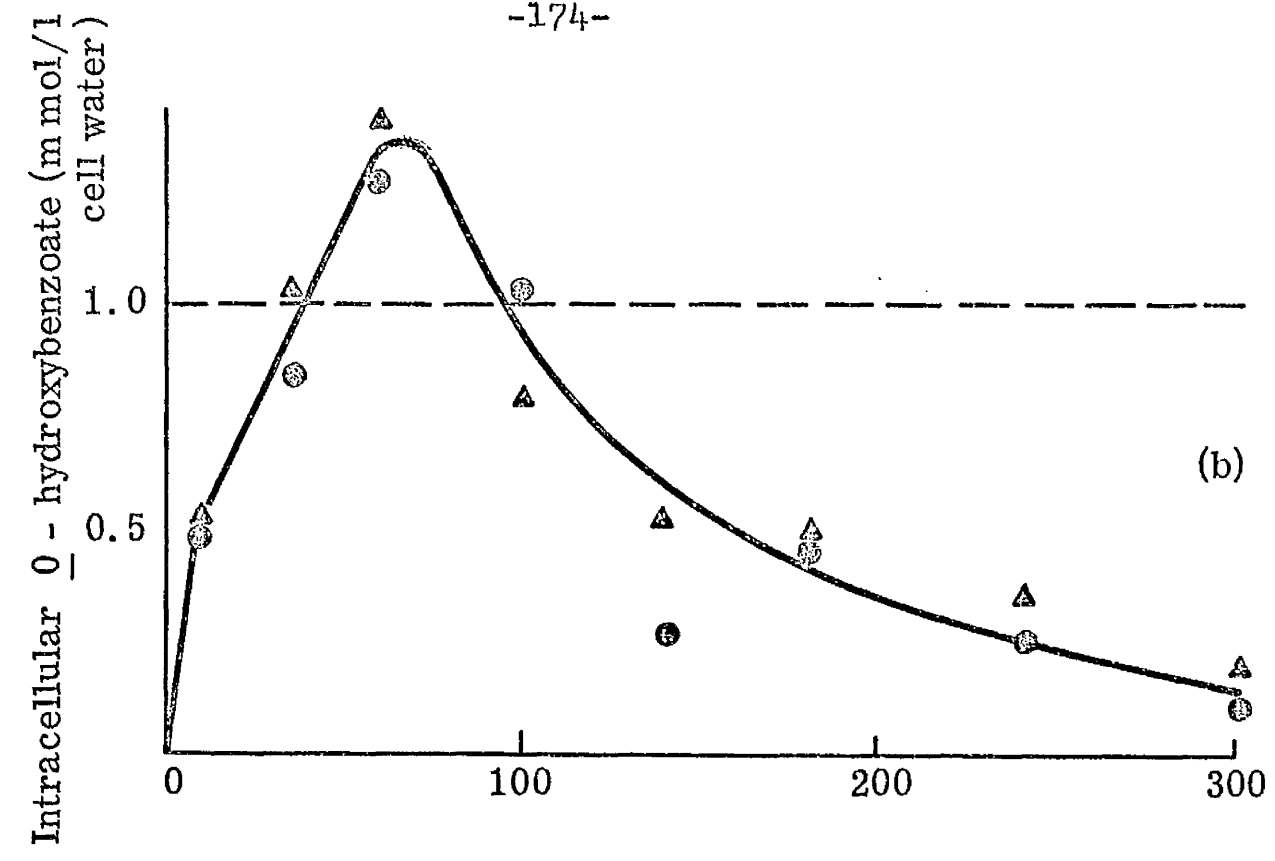


Fig. 46. The effect of benzoate on the level of p-fluorobenzoate in cell suspensions of bacterium NCIB 8250 harvested from benzoate-salts medium.

Bacterium NCIB 8250 was grown in 2mM-benzoate-salts medium, harvested at a turbidity of 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing p-fluoro [carboxy-<sup>14</sup>C] benzoate, 10µmol (1µCi/µmol); benzoate, zero or 10µmol; and glucose-5-<sup>3</sup>H, 1µmol (25µCi/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions initiated by the addition of 2.5ml cell suspension. At intervals 100µl samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60).

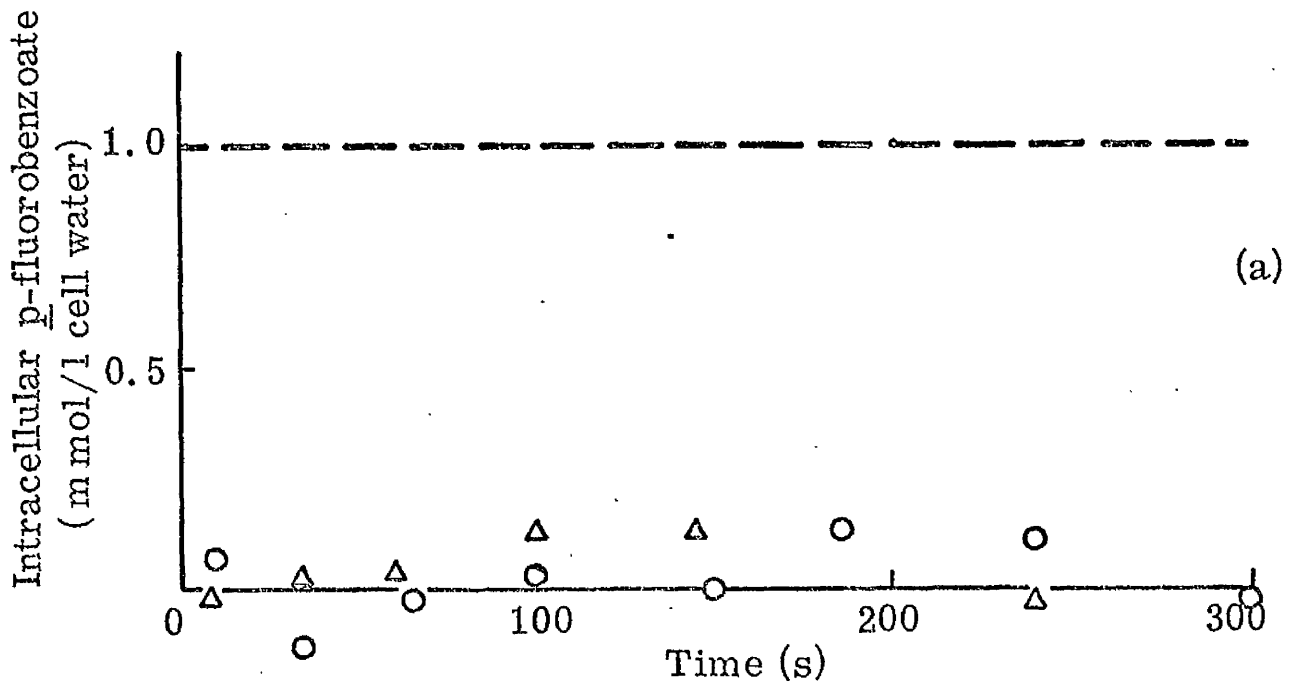
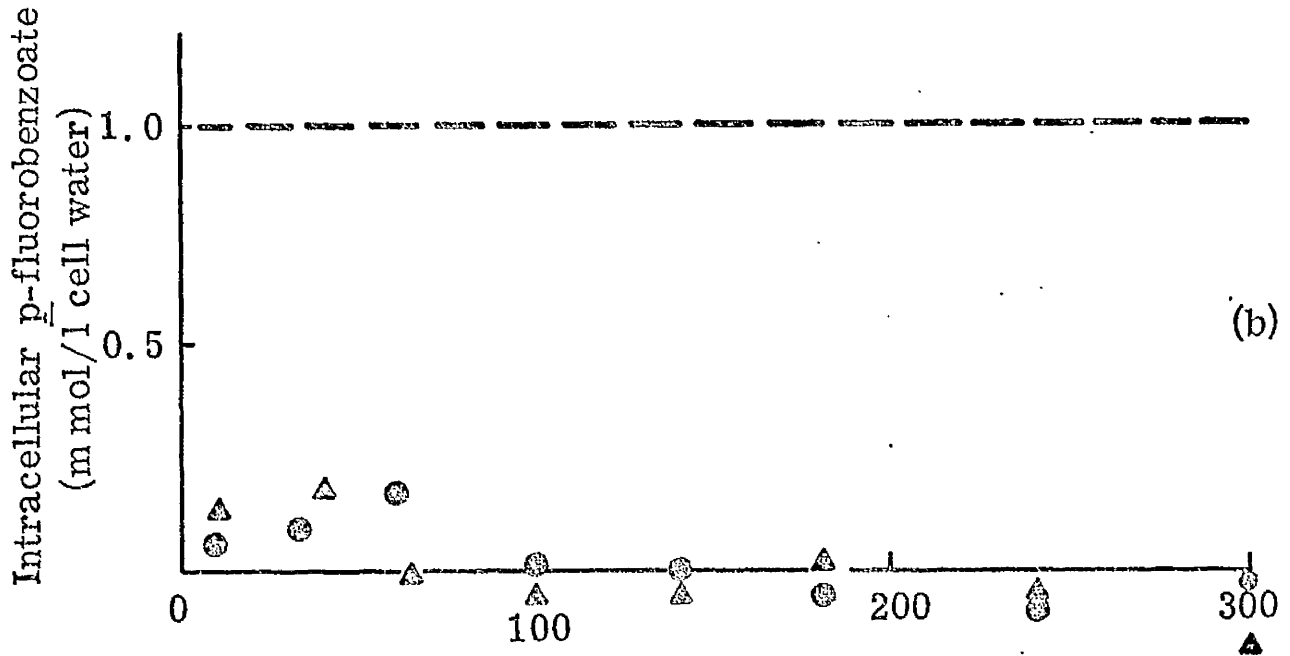
Graph a), no benzoate in reaction medium.

○ △ Intracellular p-fluorobenzoate.

Graph b), 1mM-benzoate in reaction medium.

● △ Intracellular p-fluorobenzoate.

— — — — — Extracellular p-fluorobenzoate concentration.



#### 4. 4. 1. Mandelate pool sizes in cell suspensions

harvested from phenylglyoxylate-salts media.

Wild type bacterium NCIB 8250 and a mutant strain deficient in mandelate dehydrogenase (NF 1408, Livingstone, 1970) were grown in phenylglyoxylate-salts media. In cell suspension the wild type organism decarboxylated mandelate (58nmol/mg dry wt./min) but no intracellular mandelate was detected (Fig.47 a). On the other hand, the mutant strain displayed no significant capacity to decarboxylate mandelate (no loss of radioactivity from DL-[carboxy-<sup>14</sup>C] mandelate over 500s) and mandelate could be detected entering the cell (Fig. 47 b). The intracellular equilibrium level of mandelate was attained in about 60s and did not exceed the extracellular mandelate concentration. Some trouble was experienced with the numerical value of "zero" intracellular mandelate concentration, presumably an artifact of the corrections (Table 8) made to the crude data (Results, pp154 and 158).

#### 4. 4. 2. The entry of mandelate into suspensions of cells non-induced for mandelate enzymes.

DL-Mandelate entered nutrient broth-grown cells slowly (Fig. 48a). The initial rate of entry (0.4 $\mu$ mol DL-mandelate/mg dry wt./min) was 0.6% of the decarboxylation rate in mandelate grown cells in cell suspension. After 600s the internal concentration was about 85% of the external concentration and the entry rate had dropped to 0.09 $\mu$ mol DL-mandelate/mg dry wt./min. Neither benzoate nor benzyl alcohol had a significant effect on mandelate entry into

Fig. 47 . DL-Mandelate concentrations in cell suspensions of wild type and mandelate dehydrogenase-deficient strains of bacterium NCIB 8250 harvested from phenylglyoxylate-salts media.

A mutant strain of bacterium NCIB 8250 deficient in mandelate dehydrogenase (NF 1408) and the wild type organism were grown separately in 5mM-phenylglyoxylate-salts media, harvested at a turbidity of 0.2, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing DL-  $\left[ \begin{array}{c} \text{carboxy-}^{14}\text{C} \\ \text{carboxy-}^{14}\text{C} \end{array} \right]$  mandelate, 20µmol (0.5µ Ci/µmol) and glucose-5- $^3\text{H}$ , 1µmol (20µ Ci/µmol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and reactions initiated by the addition of 2.5ml cell suspension. At intervals 100µl samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60).

Graph a), wild type organism.

○ △ Intracellular DL-mandelate (in duplicate).

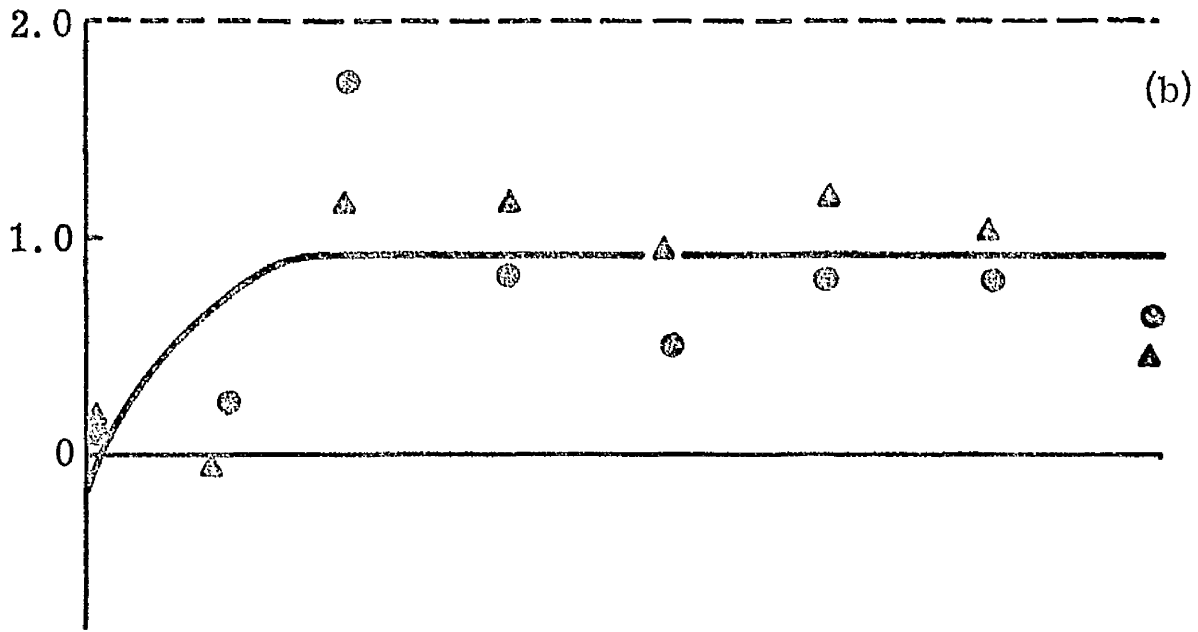
Graph b), mutant strain.

⊙ ▲ Intracellular DL-mandelate (in duplicate).

----- Extracellular DL-mandelate concentration.



Internal mandelate (mmol/l cell water)



Internal mandelate (mmol/l cell water)

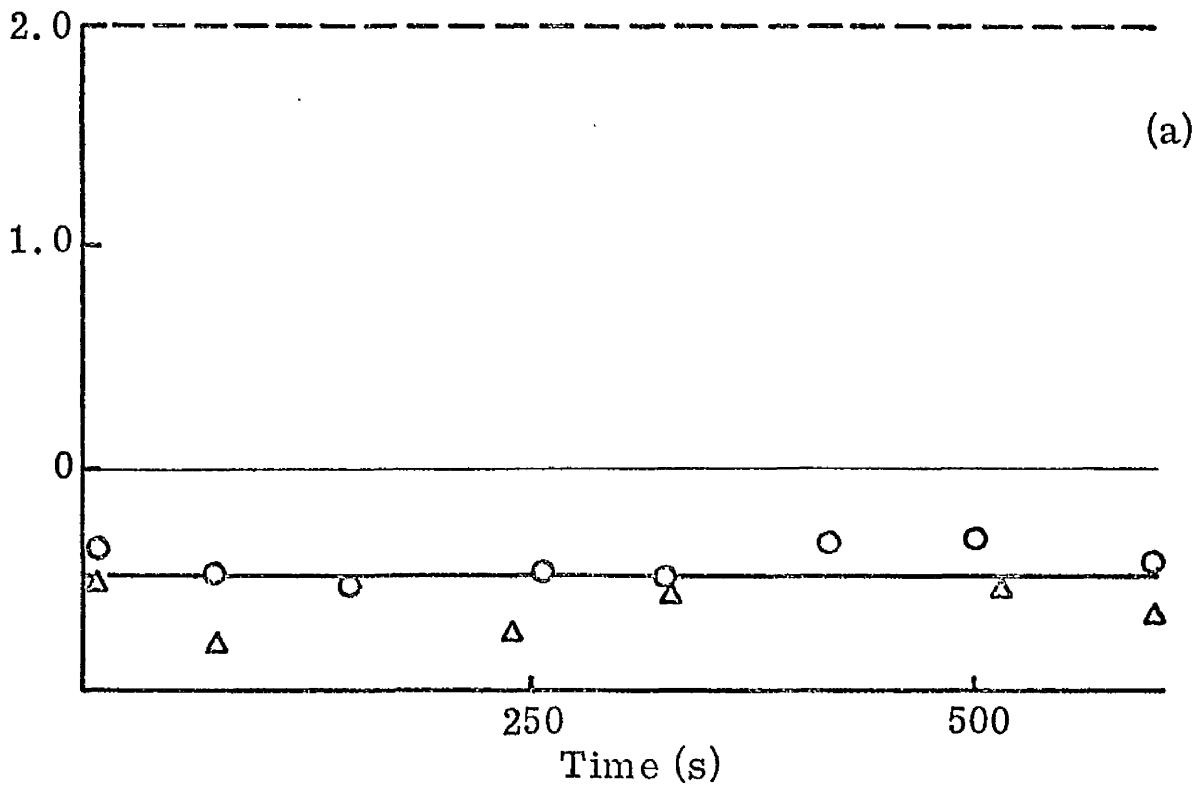


Fig. 48. The effect of benzoate and of benzyl alcohol on the entry of DL-mandelate into cell suspensions of bacterium NCIB 8250 non-induced for the mandelate oxidising ( $R_1$ ) enzymes.

Bacterium NCIB 8250 was grown in nutrient broth, in 2mM-benzoate-salts or in 5mM-benzyl alcohol-salts medium, harvested at turbidities of 0.4, 0.2 and 0.4 respectively, washed, resuspended to 20mg wet wt./ml in phosphate buffer and stored on ice (Methods, p. 60 ). Experiments were done in a shaking water bath at 30°C in 50ml Erlenmeyer flasks with 10ml final volume reaction media containing DL- [carboxy- $^{14}C$ ] mandelate, 20 $\mu$ mol (0.5 $\mu$ Ci/ $\mu$ mol); benzoate, zero or 10 $\mu$ mol; benzyl alcohol, zero or 10 $\mu$ mol; and glucose-5- $^3H$ , 1 $\mu$ mol (25 $\mu$ Ci/ $\mu$ mol). Incomplete (7.5ml) reaction media and portions of the stock cell suspension were preincubated separately and the reactions initiated with 2.5ml cell suspension. At intervals 100 $\mu$ l samples were harvested on filter membranes under vacuum, washed with 1ml medium wash and the filter immediately immersed in scintillation fluid in a scintillation vial (Methods, p. 60).

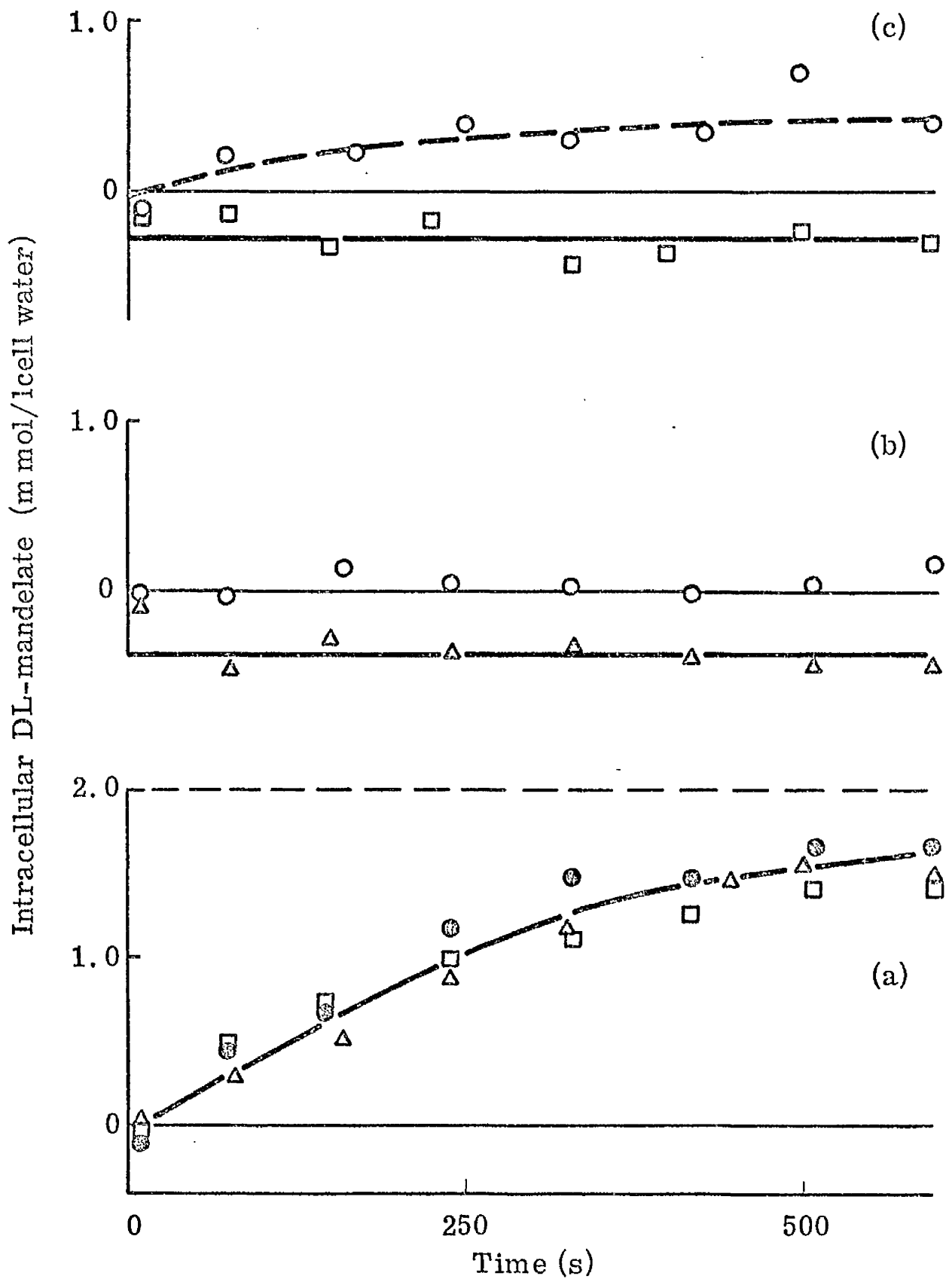
Graph a), nutrient broth-grown cells; graph b), benzoate-grown cells; graph c), benzyl alcohol-grown cells.

○ ● Mandelate only in reaction medium.

△ Benzoate present in addition to mandelate.

□ Benzyl alcohol present in addition to mandelate.

----- Extracellular DL-mandelate concentration.



nutrient broth-grown cells (Fig. 48 a).

Mandelate did not enter benzoate-grown cells in the presence of benzoate (Fig. 48 b) and in the absence of benzoate the internal mandelate concentration only rose to 20% of the external level. In a similar manner, mandelate did not penetrate benzyl alcohol-grown cells in the presence of benzyl alcohol (Fig. 48 c) though in the absence of benzyl alcohol, intracellular mandelate rose to 35% of the external concentration.

## DISCUSSION.

### 1. DEVELOPMENTAL WORK.

The present project was designed to determine whether intermediates of the mandelate pathway entered the cell by passive diffusion or by specific processes and also to find what contribution to the control of the mandelate pathway enzymes was made by the permeability of the cell membrane. In practice, however, it was a study of the control mechanisms operating during growth in dual substrate media (Results, Section 2. 2.) that led to a meaningful study of the permeability of the cell membrane.

#### 1. 1. Growth and substrate analysis.

The development of conditions to study substrate utilisation during growth was basically an extension of techniques already in use in the laboratory (Kennedy & Fewson, 1968 a,b; Harvey et al., 1968). The use of a simple and reliable radio-chemical assay, which involved the minimum of manipulation before liquid scintillation counting (Methods, p. 48) made large scale experiments practicable. Direct spectrophotometric assays of these aromatic compounds, mandelate, benzyl alcohol and benzoate, were relatively complex (cf. Methods, p. 67) although essential in some cases.

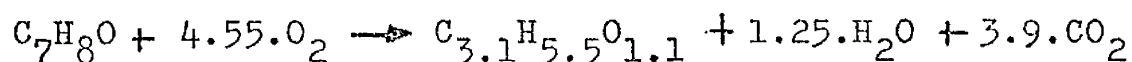
A careful choice of inocula for growth experiments was

found to aid considerably in the interpretation of data. Most of the detailed results quoted in this thesis came from experiments with harvested inocula preinduced to one of the pair of substrates in the dual substrate experiments. This was because subsequent growth and utilisation of radiochemically labelled carbon sources were immediate (e.g. Figs. 9, 13 and 15). These reproducible conditions (Fig.19) overcame the interference from growth on material carried over in unharvested nutrient broth inocula (e.g. Fig. 9) and eliminated problems of interpreting possibly varying lag times when non-induced inocula were employed (e.g. Fig. 13). On the other hand, the carry-over of material in unharvested nutrient broth inocula could be exploited to demonstrate effects masked by preinduced inocula, for example the co-utilisation of benzoate and benzyl alcohol from a nutrient broth inoculum (Results, p.123). The use of harvested non-homologous preinduced cells could serve to highlight unusual aspects of metabolism like the poor growth in benzyl alcohol-salts medium of cells containing some benzyl alcohol oxidising ability (Results, p. 90 ).

When benzoate or benzyl alcohol served as sole carbon and energy sources for growth from homologous preinduced inocula, growth was exponential and was a linear function of substrate utilisation (e.g. Figs. 10 and 14). On exhaustion of the defined carbon source, growth ceased. This is the classical pattern of carbon limited bacterial growth on a single carbon source (e.g.

Monod, 1942).

The values of molar growth yield derived from these cultures (cf. Results, p. 87) may be manipulated in different ways. An estimate of the P/o ratio can be calculated when the growth yield is expressed in terms of oxygen consumption,  $Y_o = \mu\text{g dry wt.}/\mu\text{g atom oxygen}$ . The most studied molar growth yield in this project is that in benzyl alcohol-salts medium,  $81\mu\text{g dry wt.}/\mu\text{mol}$  (Results, p. 93). With the cellular composition (Methods, p. 57) the following equation for benzyl alcohol utilisation was derived:



The gross oxygen uptake shown was corrected by  $2\text{O}_2$  for the direct oxygenation of benzoate and catechol (e.g. Payne, 1970) but no allowance was made for maintenance energy (cf. Pirt, 1965; van Uden, 1969). These results gave  $Y_o = 15.9\mu\text{g dry wt.}/\mu\text{g atom oxygen}$ . A growth yield ( $Y_{\text{ATP}}$ ) of 10.5 (range 8.3 - 12.6) $\mu\text{g dry wt.}/\mu\text{mol ATP}$  has been observed by Bauchop & Elsdon (1960) for anaerobic growth of Streptococcus faecalis, Saccharomyces cerevisiae and P. lindneri. Determination of  $Y_{\text{ATP}}$  values for aerobic growth has proved much more difficult; the results for different organisms are sometimes difficult to interpret and occasionally the evidence is conflicting (reviews by Stouthamer, 1969; Forrest & Walker, 1971). The recent observation of Hempfling (1970) that

-105-

<sup>P</sup>/o ratios in E. coli vary (0.2 - 3.3) under conditions of varying catabolite repression has added another difficulty to this sort of observation. Nevertheless, if the  $Y_{ATP}$  is constant for all bacteria, a comparison of the  $Y_o$  and the  $Y_{ATP}$  shows a <sup>P</sup>/o ratio of about 1.4 for bacterium NCIB 8250.

Payne (1970) reported a  $Y_o$  of 12.8 $\mu$ g dry wt./ $\mu$ gatom oxygen for Pseudomonas C<sub>12</sub>B grown on benzoate. The molar growth yield of bacterium NCIB 8250 growing on benzoate is 70 $\mu$ g dry wt./ $\mu$ mol. The corresponding  $Y_o$  is 16.7 $\mu$ g dry wt./ $\mu$ gatom oxygen.

The radiochemical assays of substrate utilisation were sensitive and simply done (Methods, p. 48) but there were certain drawbacks in the method and some minor problems in converting loss of radioactivity from the culture medium into utilisation of substrate (mol/l). The drawbacks arose from the non-specific nature of the assay. Mandelate disappearance was in fact measured as phenylglyoxylate decarboxylation (Results, p. 95). Benzyl alcohol was measured as benzoate decarboxylation (Results, p. 90) and this was particularly inconvenient when benzoate itself was the second carbon source (e.g. Results, p. 123). Because the assay was indirect, simply a measure of radioactivity per unit volume, the specific radioactivity of the substrate (pCi/ $\mu$ mol) was required. This depended on an accurate measure of the volume of the liquid in the growth flask and it was known that some liquid was lost on autoclaving (Methods, p. 43). The volume of liquid



in a flask, autoclaved in the same batch as the flask under study, was measured and additions to and removals from the flask were noted. This potential source of inaccuracy in the measurement of substrate concentration, a constant factor throughout growth, could have no significant effect on the patterns of substrate utilisation for which the experiment was designed. The slight incorporation into cell material of radioactivity from carboxyl groups in the sidechain (1.5 - 2.0%, e.g. Methods, p. 49) was another potential source of error. Presumably this represents heterotrophic carbon dioxide fixation (e.g. Wood & Utter, 1965) although it should be noted that the manufacturer only claims 99% purity. Because the differential rate of substrate utilisation during growth was constant (at least on benzoate or benzyl alcohol as carbon sources, e.g. Figs. 10 and 14) this incorporation probably did occur throughout growth as assumed for the purposes of the calculation (Methods, p. 50).

In the case of mandelate utilisation the problem of the very slight metabolism of D-mandelate during growth on L-mandelate (Results, p. 95) was masked by the presence of D-mandelate carrier. The utilisation of D-mandelate was shown up only by the sensitive radiochemical methods: it had not been suspected previously (Fewson, 1967b, Kennedy & Fewson, 1968 a,b). It is not clear why D-mandelate cannot support growth (Fewson, 1967b; Baumann, 1968) albeit at a low rate. It is possible that the slow rate of metabolism is not sufficient to satisfy the demand for maintenance energy. The mechanism of D-mandelate utilisation is not yet known.

Kennedy & Fewson (1968b) found that D-mandelate was not oxidised by L-mandelate dehydrogenase from wild type cells so presumably the oxidation of D-mandelate observed here (Table 4) was the operation of some other enzyme with a slight capacity to oxidise or decarboxylate D-mandelate. Lancaster (1971) isolated from bacterium NCIB 8250 mutant strains that grew rapidly in D-mandelate-salts medium. Cell-free extracts of these mutants contained D-mandelate dehydrogenase activity, but the nature of the mutation is not known.

The main feature of these radiochemical assays as regards experimental data was their sensitivity in elucidating detailed patterns of substrate utilisation. Provided that substrate utilisation is not inhibited, then the kinetics of substrate utilisation in the growth culture give a direct measure of total enzyme activity. Thus a constant rate, as opposed to an exponentially increasing rate of utilisation, indicates a constant level of enzyme in the culture. This latter condition in exponentially growing cells must mean that the synthesis of the enzyme concerned is subject to complete repression. Further, the fact that the enzymes of the mandelate pathway are induced in regulons (Fig. 3; Kennedy & Fewson 1968 a,b; Livingstone, 1970) presumably means that the measurement of one enzyme is a measure of the other enzymes in that regulon.

This technique is somewhat limited in application because partial repression is not easily identified and is difficult to

estimate quantitatively. However, the cell-free assays of enzymes in the mandelate pathway require sonication at a high cell density in certain cases (Livingston, 1970) and there is a very low recovery of benzoate oxidase activity on cell disruption (Fewson, et al., 1970). Therefore, the study of substrate utilisation is an attractive method for a broad screening for possible repressions or other interactions within the mandelate pathway. Indeed, growth and substrate utilisation measured in this way are influenced not only by intracellular enzyme activity but also by factors such as permeation and inhibition. Cell-free enzyme assays must always give information more limited in scope than whole cell assays, but cell-free assays are, of course, of great value in elucidating defined situations.

#### 1. 2. Assays in cell suspension.

The development of an assay specific for the uptake of individual compounds into bacterium NCIB 8250 fell into several phases. Initially the assay could be seen to work on a qualitative basis using [ring-<sup>14</sup>C(U)] benzoate and while the exact manual techniques were being improved (Methods, p. 60) a means of expressing the results quantitatively was devised.

#### 1. 2. 1. Quantitative measurement of intracellular material.

Intracellular material was expressed as a concentration

in cell water by means of the correlation between cell water and turbidity (Methods, p. 56). Winkler & Wilson (1966) used inulin to estimate cell water and their results with E. coli ML 308 showed that the intracellular concentration of thiomethylgalactoside produced by facilitated diffusion was apparently 80% of the extracellular level (Fig. 5 in Winkler & Wilson, 1966). Inulin, however, does not penetrate cell walls (Myers, Provost & Wisseman, 1967; Matula & MacLeod, 1969) while small molecules like disaccharides penetrate at least to the cell membrane. Sucrose does not rapidly penetrate the cell membrane of E. coli (Myers et al., 1967; Knowles, 1971), Rickettsia mooseri (Myers et al., 1967) or a marine pseudomonad (Buckmire & MacLeod, quoted in Matula & MacLeod, 1969). Myers et al. (1967) found that at low osmolarity sucrose penetrated 28 - 30% of the inulin space in E. coli B while MacDonald & Gerhardt (1958) considered the cell wall to comprise 20% of the inulin space of E. coli B. It thus seems likely that Winkler & Wilson (1966) were underestimating the actual intracellular concentration by some 20 - 30% by using an inulin value for cell water.

The estimation of cell water in bacterium NCIB 8250 exploited the non-saccharolytic characteristic of this organism (e.g. Fewson, 1967a). This phenomenon was further explored after a report that Veillonella spp. characteristically non-saccharolytic organisms, could assimilate D-ribose into nucleic acid (Kafkewitz & Delwiche, 1969); no ribose catabolism was detected. Bacterium

NCIB 8250 showed no significant assimilation or dissimilation of ribose or any of the sugars or sugar phosphates tested, and this enhanced the idea that the cell membrane of this organism was impermeable to carbohydrates (Results, p. 78). Passive diffusion of glucose across the cell membrane of bacterium NCIB 8250 is at an extremely low rate because the value for cell water/dry weight did not alter when suspensions were exposed to glucose for periods between 10 and 45min (results not in this thesis).

A comparison of the inulin and glucose values for cell water in bacterium NCIB 8250 shows that the cell wall is 45% of the inulin space (Results, p. 80). This figure seems a very high proportion of the total cell volume when compared with the value for E. coli at similar (low) osmotic pressure (Myers et al., 1967), but bacterium NCIB 8250 is a small organism and a "normal Gram-negative wall" would constitute a larger proportion of a small organism. An electron micrograph of a phosphotungstate-stained cell of bacterium NCIB 8250 (Hodgson & McGarry, 1968; their plate 1b, magnification 70,000) was considered for calculations as a picture of the cross-section of a cylinder with hemispherical ends. The cell measured 7.6 x 4.5cm containing a 0.5cm wall, assuming both stain densities in the wall were in fact cell wall. The volume within the cell membrane was 54% of the total cell volume. This would seem to confirm that in bacterium NCIB 8250, inulin does not penetrate the cell wall and that glucose penetrates the wall to the cell membrane. Consequently, the appropriate value

for cell water/turbidity unit is that obtained with glucose as the extracellular marker (Methods, p. 56 ).

Although the fundamental calculation in studying intracellular concentrations in cell suspensions is the expression of data in the same units as the extracellular material, there remain two other major items in the calculation. Fig. 38a gives a demonstration of the problems involved in assaying the intracellular concentration in succinate grown cells (non-induced). The reagent blank (Table 8 and e.g. Results, p. 146) for [carboxy-<sup>14</sup>C]benzoate represents the same number of d.p.m. as the equilibrium level of benzoate inside the cell. Fortunately, the reagent blank is reproducible (S.E.M. 2 - 4%) but even at the high levels of radioactivity employed ( $1\mu\text{Ci} [^{14}\text{C}]/\text{ml}$ ) there is a statistical error of  $\pm 4\%$  on the counting of each vial in the determination of the reagent blank. After subtraction of the appropriate reagent blanks the net values of radioactivity on the filter membrane must be corrected for the material presumably trapped between cells in clumps (Results, p.157). The importance of this individual correction on each result must be stressed. It can be seen that a wide range of trapping occurs (Fig. 38a). Without adjustment for this extracellular material on the filter, calculated from the amount of <sup>3</sup>H-glucose on the filter, the data in Fig. 38a could not be legitimately converted to the units of Fig. 38b, and any conclusions drawn from uncorrected data would be

totally misleading. Despite these potential sources of error, duplicate estimations were very close to one another, as evidenced by pairs of points in Fig. 44. The accuracy and versatility of the technique is seen to its best advantage where reagent blank and trapping are small, as in the uptake of thiomethylgalactoside by different strains of E. coli (Fig. 37). The occasional misplacement of the calculated zero internal concentration (Fig. 47) causes some concern as the reason for this is not clear.

#### 1. 2. 2. Washing techniques in cell suspension assays.

The manipulation of data into readily understood units is essential for this work. However, this facility can only be meaningful if the washing techniques are valid.

The use of chilled water to leach all intracellular pools was the technique of Britten & McClure (1959). The mechanism of action of the chilled water wash in rapidly removing the PCA-soluble pool is not understood. Britten & McClure (unpublished data quoted in the 1959 paper) found that "complete removal of pool compounds by the violent osmotic shock (of a water wash) is due to a very transient change in the cell. Synthesis of any significant fraction of the cell cytoplasm is not necessary to repair the damage." The removal of pool materials from bacterium NCIB 8250 seems to require chilled water (Results, p.150) but it would seem that this organism, also, suffers little damage on water

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wash. Much of the manometric work done by Kennedy & Fewson (1968a,b) used cells of bacterium NCIB 8250 resuspended in water and Fewson (personal communication) stated that resuspension in buffer offered no advantage over resuspension in water. The membrane visualised by Britten (1965) was "a sort of thixotropic gel supported on the outside by the wall.... . Under sudden stress it would behave like a layer of silly-putty and crack momentarily. It would then be capable of healing immediately". This model would not apply to some Gram-positive bacteria, for example Streptococcus faecalis, used by Gale (1947) in his studies on amino acid uptake where pools were insensitive to water washing.

The validity of the medium wash, which should leave the pools intact but remove substrate outside the cell membrane, is particularly difficult to establish (Results, p. 150). The principle of the washes used in this work is that the medium wash must be of the same composition as the solution from which the cells have been harvested, as detailed by Britten & McClure (1959) and Kepes (1971). However, Britten & McClure (1959) found that osmolarity rather than composition was the important feature in the "medium wash". A wash of higher osmolarity was of little advantage but if the osmolarity was below that of the reaction medium the observed pool was very much reduced. Kepes (1971) also noted that galactoside efflux from E. coli at 0°C was less



than at 30°C. Although no study was made with medium washes of low osmolarity with bacterium NCIB 8250 (except the extreme case of water), an increase in the osmolarity of the wash (5% NaCl, Table 10) had no marked effect on the pool size recorded. Studies on efflux at different temperatures were not done, but a wash temperature of 0°C had already been chosen to reduce metabolism on the filter membrane (Results, p. 152). Using the same criteria as employed by the workers with E. coli (Britten & McClure, 1959; Kepes, 1971) it seems likely that the pool sizes recorded for non-metabolised substrates in bacterium NCIB 8250 are a good representation of the actual intracellular concentration. One piece of supporting evidence for this hypothesis comes from a different type of experiment. In centrifugation experiments to find the cell water/dry wt. excluded to benzoate in cell suspension (Results, p. 82), benzoate was found to penetrate totally the intracellular water space of succinate-grown cells; confirmation of the results observed after harvesting by filtration (e.g. Figs 38 and 43).

When a substrate such as mandelate or benzoate was metabolised by the cells under study, no intracellular pool of that compound could be found (Figs. 47a and 42) although a pool of radioactive material (presumably metabolic intermediates) could be detected (Results, p. 166). The lack of a measurable pool could be due to the fact that there is no significant pool under steady-state metabolic conditions, or could also be due to

metabolism on the filter before washing. There was a 1 - 2s lag between disappearance of reaction medium from the filter membrane and addition of wash. In the case of benzoate, even if the intracellular concentration was equal to the external level (1mM) as the reaction medium disappeared, it would require only 1.5s to be totally decarboxylated. It is not by any means certain that the intracellular concentration of benzoate, in the presence of an active benzoate oxidase, would ever achieve the extracellular level (e.g. Results, p. 168) so the chances of detecting this pool are poor. Even if the benzoate pool size exceeded the external concentration, it is doubtful whether any direct "permease assay" would reliably detect a pool of benzoate in view of the flux of benzoate. Whole cell decarboxylation of benzoate showed a lower specific activity than that of mandelate decarboxylation or benzyl alcohol disappearance (Table 6). The rapid flux of the intermediates under study makes the direct measurement of a putative permeation system impossible in the wild type organism. Proof of the existence of a specific transport system must come from some general property of that system, some aspect of its specificity in overcoming the permeability characteristics of the cell membrane.

2. REPRESSION AND PERMEATION IN THE MANDELATE  
PATHWAY OF BACTERIUM NCIB 8250.

2. 1. Regulon R<sub>1</sub>.

L-Mandelate dehydrogenase, phenylglyoxylate carboxy-lyase and the heat-stable benzaldehyde dehydrogenase comprise the enzymes of regulon R<sub>1</sub> (Fig. 3; Kennedy & Fewson, 1968 a,b; Livingstone & Fewson, 1971).

Benzoate affects neither the cell-free activities of mandelate dehydrogenase and phenylglyoxylate carboxy-lyase (Table 5) nor the decarboxylation of mandelate by suspensions of cells of bacterium NCIB 8250 preinduced to mandelate (Table 6). Thus no part of the mandelate decarboxylation system is inhibited by benzoate, nor in fact is the heat-stable benzaldehyde dehydrogenase inhibited (Table 5). It therefore follows that the repression of the synthesis of the enzymes of mandelate decarboxylation in the presence of benzoate in growing cultures of preinduced mandelate cells (Results, p.114; Figs. 24 and 26) is a direct control system at the transcription or translation level. The effect cannot be at the level of entry of inducer into the cell, otherwise mandelate decarboxylation in cell suspensions would have been inhibited. This is why benzoate-grown cells do not metabolise mandelate till benzoate exhaustion (Fig. 20); these cells lack the R<sub>1</sub> enzymes (Kennedy & Fewson, 1968 a,b; Livingstone, 1970)

which are repressed until the benzoate has disappeared.

These experiments do not prove that benzoate itself is the repressor or the trigger of the repression mechanism; a later intermediate of the pathway or another metabolic intermediate entirely could be mediating the effect. However, in experiments arising out of this work, Fox (1970) has shown that benzoate is probably the repressor. She used a mutant strain isolated by Livingstone (1970) and which contained less than 0.2% of the wild type level of benzoate decarboxylation activity after growth in conditions which induce benzoate oxidase in the parent strain (Cook, unpublished data). The mutant was grown in glutamate-salts medium to which mandelate or mandelate + benzoate was added. After  $2\frac{1}{2}$ h a 78% repression of the specific activity of L-mandelate dehydrogenase by benzoate was observed by direct enzyme assay. On kinetic analysis it became apparent that incomplete repression was caused by a single small burst of L-mandelate dehydrogenase synthesis on the addition of inducer, thereafter the enzyme was subject to absolute repression. The only potential repressor present was benzoate which could not be further metabolised.

In the presence of catechol, mandelate decarboxylation in a culture of mandelate-grown cells was arithmetic (described without illustration in Results, p. 117) thus catechol causes complete repression of regulon  $R_1$ . A culture grown from a nutrient broth inoculum used catechol, to the exclusion of mandelate, till a

point corresponding to catechol exhaustion (Results, p.120 ). Catechol, however, has an inhibitory effect on mandelate decarboxylation by whole cells (33%, Table 6, Fig. 33). The severity of the effect decreases with time and the specificity of the inhibition is uncertain because catechol has some toxicity for the cell as evidenced by decreasing growth rates at higher concentrations (1 - 1.5mM, Kennedy & Fewson, 1968a). The inhibition of mandelate decarboxylation in whole cells (Table 6), whatever the mechanism, would be unable to cause the complete repression of the  $R_1$  enzymes observed during growth (Results, p.120 ). Thus catechol, too, represses or triggers the repression of regulon  $R_1$ .

Succinate caused no feedback inhibition of mandelate dehydrogenase or of phenylglyoxylate carboxy-lyase in cell extracts (Table 5) though a limited inhibition of mandelate decarboxylation was observed in whole cells (27%, Table 6). The inhibition of mandelate decarboxylation could be an effect on mandelate transport, or it may reflect, for example the redox balance in the cell suspensions and be an effect on L-mandelate dehydrogenase. The presence of succinate caused severe but incomplete repression of mandelate dehydrogenase during growth; the rate of mandelate utilisation increased very slowly in the presence of succinate (described but not illustrated in Results, p. 120 ). Nonetheless a 27% inhibition of mandelate decarboxylation could not explain the

observed repression of regulon  $R_1$  during growth on succinate. Thus succinate partially represses or triggers partial repression of regulon  $R_1$ .

In contrast to the effects of benzoate, catechol and succinate, no repression of regulon  $R_1$  was caused by benzyl alcohol. Neither growth (Fig. 27) nor mandelate utilisation (Fig. 28) was significantly affected by the presence of benzyl alcohol though benzyl alcohol was metabolised to a considerable extent during mandelate utilisation (Fig. 28). Fewson (personal communication) has found that benzaldehyde shows a very low or non-existent repression of L-mandelate dehydrogenase. He grew bacterium NCIB 8250 in glutamate-salts medium to which mandelate or mandelate + benzaldehyde were added. After  $2\frac{1}{2}$ h large amounts of L-mandelate dehydrogenase were detected under each condition though no  $R_2$  enzyme was detected in either condition.

The picture of the constraints on induction of regulon  $R_1$  is one of repression in the presence of intermediates which lie down the catabolic pathway and are substrates for later regulons, while the intermediates of the branch pathway do not cause repression. The repression mechanism operates at some level in messenger transcription or translation because repression is seen to occur in preinduced mandelate cells without detectable alteration of pre-existing enzyme levels or their activity.

Data culled from experiments in the presence of catechol and succinate indicate a possible contribution of a minor nature

from control mechanisms other than repression, but their importance during growth has not been determined. Thus no role (or at most a very minor role) in the observed repression can be attributed to permeability phenomena. This does not mean that a transport system for mandelate is absent or that such a system has no part in the regulation of regulon  $R_1$ .

Consider the contrast between the growth of a benzoate-grown inoculum in benzoate + mandelate-salts medium and in benzoate + benzyl alcohol-salts medium (Fig. 19) with the corresponding substrate utilisations (Fig. 20). A small amount of benzyl alcohol was utilised for growth before benzoate exhaustion while mandelate utilisation was not detected for 45min after benzoate exhaustion. This difference could possibly be ascribed to the fact that benzyl alcohol supports more rapid growth than does mandelate, but in the light of data discussed in the next section (p.203) it was considered possible that mandelate could not penetrate benzoate-grown cells.

The entry of mandelate into different preinduced cell types was examined in cell suspension. As already indicated in the Discussion (p.192) no intracellular mandelate was detected in wild type cells fully induced for the enzymes of regulon  $R_1$  (Fig. 47a) presumably because of its rapid metabolism. However, in the mutant deficient in mandelate dehydrogenase, the entry of mandelate into the cell could readily be detected (Fig. 47b). The rate of entry is low, about 3nmol/mg dry wt./min, some 4% of the rate of mandelate

utilisation in cell suspensions of the parent strain (e.g. Table 6). The entry of mandelate into wild type cells grown on nutrient broth is also easily detected (Fig. 48a) and here the rate of entry is only 0.6% the rate of mandelate decarboxylation in suspensions of cells adapted to mandelate. Benzoate neither affects the entry of mandelate into these non-induced cells (Fig. 48a) nor indeed into mandelate-grown cells in suspension (Table 6) or during growth (Fig. 26). In marked contrast, mandelate does not penetrate benzoate-grown cells in the presence of benzoate (Fig. 48b) and only slowly penetrates the membrane in the absence of benzoate (Fig. 48b). This permeability barrier to mandelate proves that the membrane of bacterium NCIB 8250 requires some specific complement to permit mandelate to penetrate the membrane. To quote Britten (1965), "Once a cell... has been clearly shown not to be rapidly penetrated by a substance we may say that the 'membrane' is impermeable to that substance... . There may be passages and doors... with a variety of active or passive properties. The point is that once one has found the doors closed their existence is clear even though they may be difficult to observe when open."

Mandelate metabolism thus appears to involve a mandelate transport system in addition to the enzymic entities already demonstrated by Kennedy & Fewson (1968a,b). The regulation of regulon  $R_1$  so far observed has not acted at the level of transport but at the level of enzyme synthesis.



## 2. 2. Regulon R<sub>2</sub>.

Benzyl alcohol dehydrogenase and the heat-labile benzaldehyde dehydrogenase comprise the enzymes of regulon R<sub>2</sub> (Fig. 3).

No diauxie was observed in benzyl alcohol + benzoate-salts medium when nutrient broth-grown or benzyl alcohol-grown cells were used as inocula (e.g. Fig. 29). Substrate co-utilisation occurred throughout growth and benzyl alcohol utilisation accelerated (Fig. 30). If there was any repression of regulon R<sub>2</sub> in the presence of benzoate this technique could not detect it, partly because of the insensitivity of the assay at low degrees of repression (Discussion, p.185) and partly because of the problems in interpreting these particular results as the disappearance of both substrates was actually measured as benzoate decarboxylation (Results, p.123). In contrast to this pattern of substrate co-utilisation, benzoate-grown cells utilised only benzoate (till just before benzoate exhaustion) to the exclusion of benzyl alcohol (Fig. 20). Benzyl alcohol disappearance from whole cells (Table 6) and the cell-free activities of benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase (Table 5) were almost unaffected by benzoate. The paradox of the repression of the R<sub>2</sub> enzymes when no R<sub>2</sub> enzymes already exist and the low degree of repression in cells containing some, even low levels of, benzyl alcohol dehydrogenase must mean that benzyl alcohol (an inducer of

regulon R<sub>2</sub>, Livingstone & Fewson, unpublished data) is being prevented from entering the cell. Attempts to demonstrate this permeability barrier to benzyl alcohol have so far been frustrated by the very large reagent blank of [carbinol-<sup>14</sup>C]benzyl alcohol on Sartorius filter membranes (Table 8). No attempt to do centrifugation experiments of the cell water/dry wt. type (Methods, p. 54 ) has been made because of the long time lag between exposure of the cells to the radioactive material and removal of the cells from suspension. It should be stressed that the repression must arise from a permeability barrier to entry, not an inhibition of a preexisting transport system, as benzyl alcohol utilisation in whole cells is essentially unaffected by benzoate (Table 6).

Growth of bacterium NCIB 8250 in benzyl alcohol + catechol-salts or in benzyl alcohol + succinate-salts medium is not diauxic and the utilisation of benzyl alcohol indicates substrate co-utilisation throughout growth (described but not illustrated, p.131 ). No data are as yet available from experiments with, say preinduced succinate cells growing in succinate + benzyl alcohol-salts medium, so the generality of repression of regulon R<sub>2</sub> deriving from a permeability barrier to entry is not established.

The effect of mandelate on the growth of bacterium NCIB 8250 in benzyl alcohol-salts medium (Results, p.123; Fig. 31 a,b,c) is teleologically most surprising in light of the fact that benzyl alcohol supports a considerably higher growth rate ( $\mu = 0.88h^{-1}$ )

than does mandelate ( $\mu_{\max}$   $0.69\text{h}^{-1}$  dropping to  $0.27\text{h}^{-1}$ ).

Furthermore, mandelate does not affect benzyl alcohol decarboxylation in whole cells (Table 6) or benzyl alcohol dehydrogenase or benzaldehyde dehydrogenase in cell extracts (Table 5). After a time lag, but before benzyl alcohol exhaustion, exposure to mandelate causes a reduction in growth rate (Results, p.130 ;Fig. 31a,b,c). This drop in growth rate coincides with repression of regulon  $R_2$  (arithmetic utilisation of benzyl alcohol, Fig, 32a,b,c) and with commencement of utilisation of mandelate (Fig.32a,b,c). The altered growth rate is not due to benzaldehyde toxicity - at least in the one case tested (Results, p.130 ). The time lag before repression is apparent is not due to a lag in the formation of phenylglyoxylate as phenylglyoxylate itself has a similar time lag before repression occurs (mentioned without illustration, p.130 ). Repression of the  $R_2$  enzymes in the presence of mandelate is also seen when benzyl alcohol is decarboxylated during mandelate utilisation by preinduced mandelate cells of bacterium NCIB 8250 (Fig. 28). Benzyl alcohol utilisation accelerated to a maximum apparently arithmetic rate that was maintained for several hours over a wide range of mandelate concentration. The fact that the supposed preinduced mandelate cells did not already contain "equilibrium" levels of the  $R_2$  enzymes is possibly due to problems in obtaining these cells (Results, p.102 ) and the fact that the level of the  $R_2$  enzymes do actually vary during growth on mandelate.

The delay in the repression of the  $R_2$  enzymes by mandelate after its addition to cells growing on benzyl alcohol with the corresponding lag before the initiation of mandelate utilisation (Fig. 32a,b,c), led to the idea that the cell membrane of benzyl alcohol-grown cells was impermeable to mandelate. (These are the data referred to in Discussion, p. 198). This permeability barrier was tested as illustrated earlier (p.198 ). Benzyl alcohol did not affect the entry of mandelate into non-induced cells (Fig.48a) or even into induced cells (e.g. Table 6), but mandelate was unable to penetrate benzyl alcohol grown cells in the presence of benzyl alcohol (Fig. 48c). Mandelate slowly entered benzyl alcohol cells in the absence of benzyl alcohol (Fig. 48c). This demonstration of a permeability barrier to mandelate not only expands the evidence showing that specific mandelate transport exists but also illustrates that this entity is not present under all conditions of growth.

### 2. 3. Regulon B<sub>3</sub>.

Benzoate was decarboxylated at the same differential rate, with respect to growth, whether catechol was present or not and using either a preinduced benzoate inoculum or a nutrient broth-grown inoculum (Fig. 22a,b). Thus catechol does not repress benzoate oxidase though benzoate oxidase and catechol oxygenase constitute sequential regulons in the mandelate pathway (Fig. 3). The apparently exclusive use of benzoate with no contribution to growth

from catechol (Fig. 22a,b) was shown to be a balance between benzoate decarboxylated and entering the catechol pool and catechol entering the metabolic pathway from that pool (Fig. 39, Results, p.161). This also shows endogenous and exogenous catechol to be in rapid equilibrium.

The absence of repression of regulon  $R_3$  by catechol is perhaps not unusual, but the full implication of the differential plot (Fig. 22a,b) must be viewed in comparison with the lowered growth rate of bacterium NCIB 8250 growing in benzoate + catechol-salts medium (Fig. 21). This presumably means that catechol is having a specific inhibitory effect on benzoate oxidation (cf. Fig. 36). It is hoped to improve the release of benzoate oxidase from cells on disruption to allow the use of the cell-free benzoate oxidase assay to find whether this effect is at the cell membrane or an effect on benzoate oxidase itself.

The co-utilisation of succinate and benzoate during growth (described without illustration, p. 111) is such that the assay can detect no repression of regulon  $R_3$  by succinate (Discussion, p. 185). The rapid entry of benzoate into succinate cells (Figs. 38 and 43) which is unaffected by the presence of succinate (data not in thesis) indicates that there is no control by succinate at the level of entry of benzoate into the cell.

At first sight the entry of benzoate into bacterium NCIB 8250 has several aspects which imply passive diffusion as a method of entry. A wide range of analogues of benzoate and substituted

benzoates showed no convincing inhibition of benzoate decarboxylation in whole cells (Results, p. 163). This would support the idea that entry was passive because there was no competition for entry between like compounds; conversely, it could simply mean a highly specific entry not inhibited by any of the compounds tested. These compounds might still themselves enter the cell via a specific mechanism in the absence of benzoate, for example p-fluorobenzoate (Fig. 40). In non-induced cells the intracellular benzoate concentration is attained at a rate which cannot be distinguished from the rate of benzoate decarboxylation in cell suspensions of induced benzoate cells (Results, p. 171). Set against these findings, however, is the fact that benzoate was found to be a potent inhibitor of the decarboxylation of p-fluorobenzoate by whole cells (Results, p. 164) and to effect this inhibition by preventing p-fluorobenzoate from entering the cell (Fig. 46). The observations are interpreted in terms of a benzoate transport system which will transport p-fluorobenzoate but which transports benzoate preferentially when both substrates are available.

This is another case of identifying a transport system by the "shut door" technique of Britten (1965, quoted on p. 199). This preliminary evidence for the existence of a benzoate transport system is supported by the pattern of exit of o-hydroxybenzoate from cells decarboxylating benzoate (Fig. 45). This outflow is similar to the exit counterflow patterns of Wong & Wilson (1970)

and suggests the operation of a facilitated diffusion system for benzoate in bacterium NCIB 8250.

2. 4. Growth of bacterium NCIB 8250 in mandelate-salts medium.

Kennedy & Fewson (1968a,b) noted the presence of  $R_2$  enzymes during growth in mandelate-salts medium (Table 1). Livingstone (1970) verified these observations and the data shown here come from his Tables 16 and 28.

Enzymes	Carbon sources for growth		
	5mM-L-mandelate	5mM-benzyl alcohol + 10mM-glutamate	10mM-glutamate
	Specific activities mmol/mg protein/min		
$R_1$ L-mandelate dehydrogenase	187	3	2
$R_1$ phenylglyoxylate carboxy-lyase	394	4	4
$R_1$ benzaldehyde dehydrogenase	74	0	6
$R_2$ benzaldehyde dehydrogenase	42	324	2
$R_2$ benzyl alcohol dehydrogenase	15	233	7

The accumulation of benzaldehyde observed during growth on mandelate (Fig. 17) verifies that the low cell-free activity of benzaldehyde dehydrogenase is a true reflection of the capacity of

the whole cells to oxidise benzaldehyde. This benzaldehyde presumably causes the observed induction of the  $R_2$  enzymes, but the  $R_2$  enzymes are repressed by mandelate (Discussion, p. 202). The potential of the  $R_2$  benzaldehyde dehydrogenase to alleviate the effects of benzaldehyde can be seen when benzyl alcohol-grown cells grow in mandelate-salts medium (Fig. 15). After a lag, presumably to induce the  $R_1$  enzymes, these cells grew rapidly for most of the growth curve with no accumulation of benzaldehyde (mandelate utilisation was a linear function of growth, Fig. 17).

Benzaldehyde accumulation was a linear function of mandelate disappearance (Fig. 18) for a considerable time. The presumption (Results, p. 100) that the enzymes involved in benzaldehyde production and removal were in a constant balance over this period requires that more  $R_2$  benzaldehyde dehydrogenase be synthesised as the ratio of the two  $R_1$  enzymes involved should be constant. If the quantity of  $R_2$  enzymes did increase during growth, repression of regulon  $R_2$  by mandelate (Discussion, p. 202) was not in fact absolute. Thus the induction of  $R_2$  enzymes during growth in mandelate-salts medium is a balance between the induction response of the cell to the accumulation of benzaldehyde and the repression of regulon  $R_2$  by mandelate.

The effects of the accumulated benzaldehyde are widespread. Initially it appears to inhibit growth (Results, p. 97) and when benzaldehyde does accumulate, other material is excreted into the growth medium (Results, p. 100). The material does not appear if



benzaldehyde is not accumulated (Results, p. 100). The nature of the excreted intermediate is not known, although aromatic intermediates of the mandelate pathway (except benzyl alcohol) have been excluded (Results, p. 100). Papers on material excreted during the metabolism of aromatic compounds largely appeared between 1947 and 1953 (e.g. Dagley, Fewster & Happold, 1952) and can give little guidance on this problem as the organism employed was either *Vibrio* 01 (e.g. Evans, 1947) or uncertain (Introduction, p. 3) and the experiments were done under conditions of relatively poor aeration.

The kinetics of mandelate utilisation are unusual (Fig.16), being pseudo-sigmoidal in contrast to the exponential utilisation of for example benzoate (Fig.10). This utilisation is almost certainly a characteristic of mandelate dehydrogenase itself as it has a high  $K_m$  ( $2.4 \times 10^{-4}M$ , Kennedy & Fewson, 1968b) and benzaldehyde neither causes a substantial repression of regulon  $R_1$  (Fewson, personal communication, described p. 197) nor inhibits L-mandelate dehydrogenase or phenylglyoxylate carboxy-lyase (Table 5).

Several aspects of the rather peculiar mandelate growth curve have been explained, but more work on the nature of the material accumulated during growth is still required.

### 3. THE MECHANISMS AND IMPLICATIONS OF PERMEATION AND REPRESSION.

#### 3. 1. Permeation.

##### 3. 1. 1. Methodology.

The concept of bacterial transport systems has been extant for several years (Cohen & Monod, 1957) but even now, understanding of the mechanisms involved is sketchy (Introduction, p. 26 ). This lack of understanding basically stems from the fact that transport depends on an intact membrane structure (Introduction, p. 26 ) and that experimental techniques have been able to give only an indirect description of the phenomena. The two basic techniques used to detect permeation mechanisms have been:

- a) accumulation of the material inside the cell to a concentration far exceeding the extracellular concentration, and,
- b) crypticity, the inability of a cell to grow on a compound when all the necessary metabolic enzymes are present (e.g. Cohen & Monod, 1957). In the basic form, each technique has severe limitations.

Accumulation of material inside the cell firstly requires an active transport system to be operating and automatically excludes systems of facilitated diffusion and group translocation. Care must be taken to ascertain that the intracellular material is in fact the same chemical entity as that supplied externally. Further, accumulation depends on being able to devise conditions in which the

material under study is actually accumulated, and this usually requires either substrate analogues not metabolised by the first metabolic enzyme or a mutant strain deficient in the first metabolic enzyme. The ability to demonstrate just one of the three known types of transport mechanism in bacteria thus requires a considerable body of prior knowledge about that system, either enzymic or genetic and preferably both; in addition, failure to demonstrate accumulation does not mean that either facilitated diffusion or group translocation is absent.

The second technique, demonstration of crypticity, may involve the use of mutant strains deficient in transport or of the induction of the transport systems. This method, also, demands considerable knowledge of the system under study to justify the statement that observed lack of growth is in fact due to a lesion in transport and, further, that this lesion in transport is appropriate to the system under study (cf. the controversy over the characterising of lactose transport in E. coli, Introduction, p. 25 ).

Early in the work for this thesis, attempts were made to demonstrate the accumulation of benzoate by wild type bacterium NCIB 8250. No mutant strains were available and no non-metabolised substrate analogues were known, so it is hardly surprising that no pool of benzoate itself was recorded (Fig. 42; Discussion, p. 192 ). The great advantage of studying the uptake of intermediates at this part of the mandelate pathway is the ease with which intracellular material can be identified. No separation of pool materials is

required as the use of [carboxy-<sup>14</sup>C] benzoic acid means that intracellular radioactivity is either benzoate or the newly discovered intermediate between benzoate and catechol (3,5-cyclohexadiene -1, 2-diol-1-carboxylic acid; Reiner & Hegeman, 1971); the next metabolic reaction is decarboxylation of the radioactive moiety in the molecule. When an inhibitor of benzoate oxidase was used ( $\alpha, \alpha'$ -bipyridyl), no accumulation of benzoate against a concentration gradient was detected (see Results, p. 168). Though this was not followed up once the assay technique was improved, and the effects of  $\alpha, \alpha'$ -bipyridyl on energy transduction are unknown, the observation suggests that there is no active transport of benzoate by bacterium NCIB 8250. The fact that all the cell-free assays for mandelate pathway enzymes used the chemical entities supplied exogenously to the whole cell suggests that group translocation is not a mechanism of transport for aromatic compounds. The enzymes have, however, only been studied in crude cell extracts and the possibility that, say, activating enzymes exist has not been totally excluded (Kennedy & Fewson, 1968b). Thus, by exclusion of other possibilities, benzoate enters the cell either by facilitated diffusion or by passive diffusion and the problem simplified to distinguishing between these two methods of entry.

Facilitated diffusion systems demonstrate several features which may distinguish them from passive diffusion (e.g. Stein, 1977). Of these, some are of little use in initially establishing the existence of a transport system, for example saturation kinetics and

inhibition by enzyme poison, because the chances of finding the right conditions in an unknown system are very poor. The best test of a facilitated diffusion system, counterflow (Introduction, p. 19), is only likely to succeed after an initial indication of the correct conditions to use. Thus a simple screen for a facilitated diffusion system is likely to be either:

- a) a demonstration that the permeant enters the cell faster in the presence of the facilitated system than in its absence, or,
- b) a demonstration of competitive inhibition at the level of transport.

The filtration assay system (Discussion, p. 186) was refined to carry out these tests on the entry of aromatic intermediates into bacterium NCIB 8250. It is ironic that the development of a system of sufficient accuracy to measure directly a permeability barrier to aromatic compounds (Results, pp 154 and 157) should depend on the demonstration of a permeability barrier to carbohydrates by a form of the crypticity test (p. 209) already rejected as a means of finding the permeability barrier to aromatic compounds.

### 3. 1. 2. Mandelate transport.

To recapitulate, the evidence for mandelate transport in bacterium NCIB 8250 derives from the initial failure of mandelate to suppress the utilisation of benzyl alcohol (Fig.32 a,b,c) when mandelate is known to repress regulon  $R_2$  (Discussion, p. 201). This indirect evidence was supported by the detection of a

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permeability barrier to mandelate in benzyl alcohol-grown cells (Fig.48c). The permeability barrier to mandelate was also observed in benzoate grown cells (Fig. 48b). This barrier was a function of the membrane itself and not an inhibition of mandelate transport as neither benzyl alcohol nor benzoate inhibited whole cell decarboxylation of mandelate during growth (Figs. 26 and 28) nor in cell suspension (Table 6) and even the slow entry of mandelate into nutrient broth-grown cells was unaffected by these compounds. On the facts that mandelate was not accumulated in the mutant strain deficient in mandelate dehydrogenase (Fig.47b) and that entry into this mutant was more rapid than entry into nutrient broth-grown cells, it was assumed that the mutant retained at least a part of the mandelate transport system and that transport was not active.

Mandelate transport is envisaged as a facilitated diffusion system, inducible with regulon  $R_1$  and not subject to inhibition by benzoate or benzyl alcohol. This transport system has no direct role in the repression of regulon  $R_1$  (at least in the case of benzoate) in as much as there is no catabolite inhibition (Introduction, p. 31). The initial absence of induction of regulon  $R_1$  by mandelate during growth of bacterium NCIB 8250 in benzyl alcohol-salts medium (Fig. 32a,b,c ) when mandelate does not enter the cell (Fig. 48c) is simply the barrier function of the cell membrane.

The evidence for mandelate transport is largely based on being able to detect its absence. Although the permeability barrier

is positive evidence in support of physiological findings, the author hopes to confirm the existence of a facilitated diffusion system by counterflow experiments (Introduction, p. 20 ). Wong & Wilson (1970) have employed counterflow to determine the relative number of operative membrane carriers in the lac system of E. coli and it may be possible to demonstrate different numbers of carriers in, say, benzoate-grown ('impermeable') cells and nutrient broth-grown cells (sparingly permeable).

Work on the mandelate pathway of P. putida has largely ignored transport. This probably arises from the interpretation of the attempts by Hegeman (1966b) to demonstrate a mandelate permease in that organism. His results support the claim that there is no active transport of mandelate, and group translocation can presumably be rejected as the substrates of mandelate racemase are D- and L-mandelate (Hegeman, Rosenberg & Kenyon, 1970). There is no detail of the method employed by Hegeman (1966b), and the work in this thesis illustrates that considerable attention to detail is required in the filtration technique (Discussion, p. 190). Thus the failure to eliminate facilitated diffusion as a mechanism of transport (e.g. by counterflow) means that no distinction can be drawn between facilitated and passive diffusion on the existing evidence. In the light of the data in this thesis it seems likely that P. putida will in fact contain a mandelate transport system, unless the membrane characteristics of P. putida are very different from those of bacterium NCIB 8250. Nevertheless, if this putative transport system

for mandelate in E. putida exactly resembles that of bacterium NCIB 8250, notably as regards catabolite inhibition, the argument (Stevenson & Mandelstam, 1965) that permeation is not the site of action of repression will still be valid.

### 3. 1. 3. Benzoate transport.

The evidence for benzoate transport is largely based on the absolute preference of benzoate-grown cells to decarboxylate benzoate in the presence of p-fluorobenzoate when no p-fluorobenzoate can be detected inside the cell. This is interpreted as competition at the level of transport (Discussion, p. 205). Indirect supporting evidence comes from the pattern of loss of o-hydroxybenzoate from benzoate-grown cells in the presence of benzoate, which resembles exit counterflow (Discussion p. 205). The rapid entry of benzoate into non-induced cells (Results, p. 168) compared with the permeability barrier to a close structural analogue of benzoate, p-fluorobenzoate (Fig.46b) presumably means that benzoate transport is constitutive. The failure of induced cells to accumulate benzoate in the presence of an inhibitor of benzoate oxidase (Results, p. 168, Discussion, p. 211) and the failure of succinate-grown cells to accumulate benzoate in the presence of an energy source (succinate; data not in this thesis) suggests that the system is not active.

This preliminary evidence for benzoate transport requires support. Again, counterflow appears to be the best and most easily applied test. A suitable substrate is probably o-hydroxybenzoate



which is not metabolised by benzoate-grown cells (Results, p. 166 and Kennedy & Fewson, 1968a). Counterflow in non-induced cells would confirm the constitutivity of the system.

### 3. 1. 4. Benzyl alcohol transport.

As yet there is only indirect evidence for the existence of an inducible, benzyl alcohol transport system, based on the repression of the  $R_2$  enzymes by benzoate-grown cells in the presence of benzoate, a repression that was not detected when cells already contained the  $R_2$  enzymes (Discussion, p. 200). A somewhat similar phenomenon was observed by Hamilton & Daves (1959) working with *P. aeruginosa*. Diauxic growth in glucose + citrate-salts medium was observed when citrate-grown cells were used for inoculation, but not when glucose-grown cells were used. Hamilton & Daves (1960, 1961) provided evidence that this phenomenon involved an inducible glucose permease that was not subject to inhibition by citrate.

Direct evidence for a benzyl alcohol transport system could be obtained if a type of filter membrane with a low reagent blank (Table 8) for [carbinol- $^{14}C$ ]benzyl alcohol can be found. Alternatively the centrifugation experiments to determine cell water/dry wt. (Methods, p.54) will have to be adapted for shorter time courses, or possibly some totally new system will have to be developed. There is as yet no indication whether benzyl alcohol transport is active or not. As neither benzoate nor mandelate transport seems to be active it is probable that benzyl alcohol transport also is not

active.

### 3. 1. 5. Relevance of these transport systems.

The preliminary picture of the transport of aromatic compounds into bacterium NCIB 8250 is one of individual, specific, facilitated diffusion systems. Benzaldehyde and phenylglyoxylate have yet to be tested for their transport characteristics. Although more information is required to substantiate the existence of these systems, the fact that permeability barriers can be detected shows that these transport systems are essential to the organism if it is to grow on the appropriate compound.

The specificity of these systems has not been studied. The fact that mandelate does penetrate benzoate-grown cells very slowly in the absence of benzoate (Fig. 48b) while no penetration can be detected in the presence of benzoate presumably means that mandelate is only a very poor substrate for the benzoate transport system. A similar argument suggests that mandelate is a poor substrate for the benzyl alcohol transport system (Fig. 48c). The specificities of the enzymes in regulons  $R_1$  and  $R_2$  have been shown to be very wide as regards ring substitution (Introduction, p. 7, Kennedy & Pewson, 1968a,b) and this was interpreted in terms of economy in protein synthesis. If this conclusion is correct, it is likely that the corresponding transport systems have similarly broad substrate specificities. It may be that benzoate transport, also, is non-specific, especially when benzoate, p-fluorobenzoate and

o-hydroxybenzoate appear to share a transport system. Benzoate transport appears to be constitutive while those for mandelate and benzyl alcohol are inducible. This may reflect the common occurrence of organisms capable of utilising benzoate (e.g. Stanier et al., 1966; Baumann et al., 1968) and the corresponding requirement for rapid induction of the relevant enzymes, while mandelate and benzyl alcohol utilisation seem to be found in fewer organisms and the competition for the latter carbon sources will be less fierce than for benzoate. A constitutive benzoate transport system might also allow the escape of those non-metabolised benzoates produced by the non-specific enzymes in  $R_1$  or  $R_2$  (see Introduction, p. 9).

According to the empirical "laws" governing the magnitude of passive diffusion across cell membranes (Stein, 1967; this thesis, p. 18), if any of these aromatic compounds is going to diffuse into the cell, a molecule such as p-fluorobenzoate (or benzyl alcohol) will rapidly diffuse through the membrane. The fact that lipophilic, water-soluble compounds like p-fluorobenzoate and benzyl alcohol require transport systems makes it highly unlikely that lipophobic substances like glucose will usefully enter any cell by passive diffusion: this conclusion assumes that the permeation characteristics of all cell membranes are similar even if the chemical compositions vary. If this is a correct deduction, then papers claiming that passive diffusion is a useful method of transport (e.g. Eagon, 1971) should demonstrate directly, by for example counterflow, that

facilitated diffusion does not exist rather than giving only indirect evidence that it does not occur.

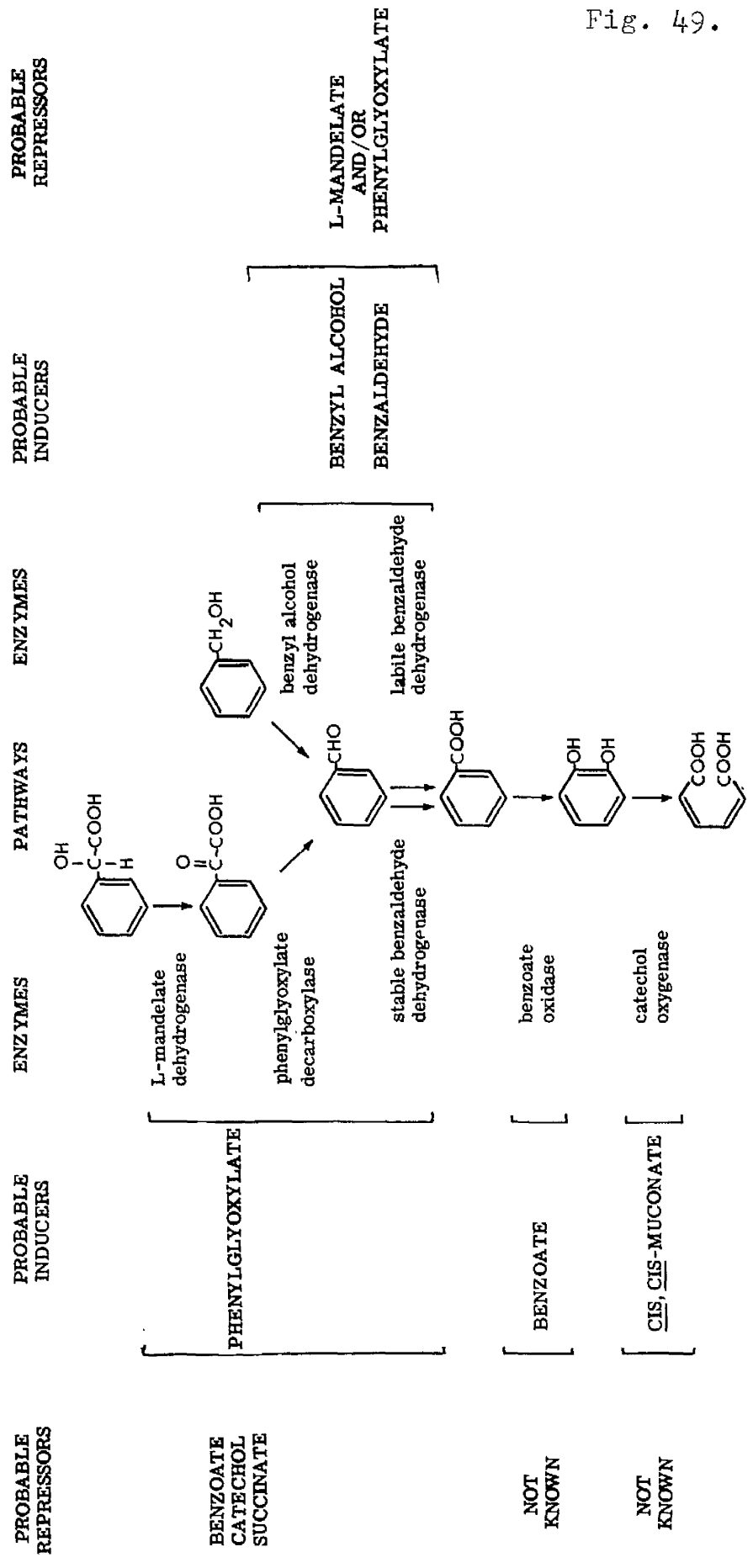
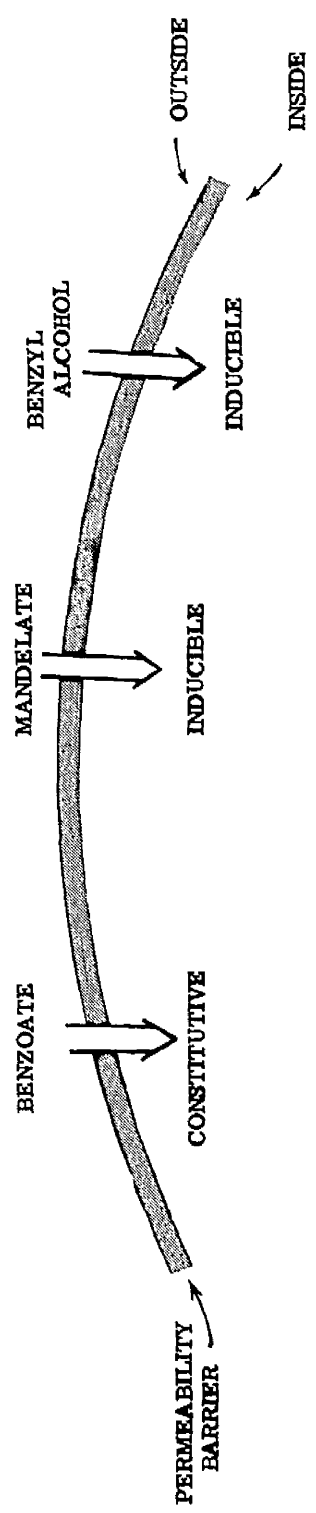
### 3. 2. Repression of enzyme synthesis.

The repressions of enzyme synthesis observed in this work have been ascribed to effects at the level of enzyme manufacture, with the exception of those effects due to a permeability barrier preventing entry of inducer into the cell. No catabolite inhibition (Introduction, p. 31) has been observed with mandelate, phenylglyoxylate, benzoate or benzyl alcohol, but it may be produced by catechol and succinate (Discussion, p. 195). Enzyme inhibition does not seem to play a role in control, though not all cell-free enzyme assays were carried out at potential inhibitor concentrations approaching those in the growth experiments. The possibility exists in other organisms that catabolite and/or enzyme inhibitions are effected by indicators of the energy charge of the cell, but the cases cited by Sanwal (1970) were all for amino acid degradations and acted at the first metabolic enzyme, which could represent a means of channelling the amino acid for anabolic and catabolic routes; this effect would be unnecessary when the growth substrate is channelled entirely down the catabolic pathway. The lack of enzyme inhibition by pathway intermediates was also observed in the mandelate pathway of P. putida (Hegeman, 1966c).

The repressions observed are summarised in Fig. 49.

Fig. 49.

REGULATION OF THE MANDELATE PATHWAY IN BACTERIUM NCIB 8250



Of the repressors of regulon  $R_1$ , only benzoate has so far been shown to be itself a potential repressor, as neither catechol nor succinate has been studied in detail (Discussion pp. 195 and 196). The repression of  $R_2$  is quite likely to be a direct effect of mandelate or phenylglyoxylate because subsequent intermediates do not cause detectable repression. Partial repressions are not readily detected by the methods employed (Discussion, p. 185) but it has been observed that benzyl alcohol (and probably benzaldehyde) does not repress regulon  $R_1$  and that catechol does not repress regulon  $R_3$ .

In this thesis, repressions have been described as "about 100%", "not detectable" or "absent". It is likely that such clear-cut distinctions will be impossible in soil, which is the natural environment of this organism. Preliminary evidence for this idea comes from the co-utilisation of benzoate and benzyl alcohol by a culture growing from a nutrient broth inoculum, showing an induction of  $R_2$  enzymes that is absent in benzoate grown cells (e.g. Discussion, p. 200). Another example comes from the work of Fox (1970; this thesis, p. 195) in which the absolute repression of mandelate dehydrogenase by benzoate was not immediate in a culture growing exponentially in glutamate-salts medium. It seems probable that different degrees of repression will occur if different proportions of inducer and repressor are present. During batch culture of *P. putida* Mandelstam & Jacoby (1963) have observed that the differential rate of synthesis of mandelate

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dehydrogenase varies with the amount of benzoate present. When P. aeruginosa was grown in continuous culture, the induction of the enzymes of glucose metabolism was found to be a balance between induction by glucose (or a metabolite) and repression by citrate (or a metabolite): this balance was related to concentration ratios rather than absolute concentrations (Ng & Daves, 1969, quoted in Clarke & Lilly, 1969). There is little doubt that continuous culture will be a valuable tool in examining quantitative aspects of repression in the mandelate pathway of bacterium NCIB 8250.

Work in this thesis has considered only the unsubstituted intermediates of the mandelate pathway. It is not clear what conditions exist in soil, especially whether substituted or non-substituted intermediates predominate (e.g. Kennedy & Fewson, 1968a,b). It is conceivable that the unusual growth characteristics found when bacterium NCIB 8250 grows on mandelate (Discussion, p. 206), especially the potentially wasteful excretion of a large quantity of benzaldehyde, will not occur when substituted mandelates are utilised.

Hegeman (1966c) argued that the observed regulons in the mandelate pathway of R. putida were the result of a balance between the utter simplicity of block induction of all enzymes regardless of the intermediate degraded with its subsequent waste in protein synthesis and the genetic complexity of the individual control of each enzyme. He recognised a further component in the equation, the ability to utilise rapidly a compound readily available in soil,

for example benzoate (Hegeman, 1966c). Mandelstam complemented these arguments, saying that repression allowed a further control over the converging pathways to economise in protein synthesis in the presence of the end product of a regulon (Stevenson & Mandelstam, 1965). Similar arguments may be advanced for the regulatory groupings observed in the mandelate pathway of bacterium NCIB 8250.

Fig. 49 is strongly reminiscent of that observed in the mandelate pathway of P. putida (Fig. 4, see p. 15 ). Hegeman (1966b) did not detect repression of the mandelate enzymes by succinate, but as he sampled late in growth, cultures were probably growing solely on the mandelate after succinate exhaustion and repression could not be detected.

The mechanism by which multisensitive end-product repression operates is unknown. The probable use of individual pathway intermediates as repressors allows a very fine control over induction to be achieved and this specificity makes translation an unlikely locus for the control mechanism. The effect, then, is most likely to occur at transcription and there is a well-defined precedent on which to base ideas - the cyclic AMP mediated repression of the lac operon of E. coli (de Crombrughe et al., 1971; this thesis, p. 34). However, multisensitive end-product repression differs in one major characteristic from the cyclic AMP mediated system: individual pathway intermediates not amphibolic intermediates



are the repressors. This fact makes the cyclic AMP system per se an unlikely candidate for this mechanism (Introduction, p. 38 ), though it too may operate. The specificity of the repressions and the fact that there are other convergent pathways probably under similar control make it likely that the repressors themselves modulate the transcription process rather than involving an intermediate system which would itself require many allosteric sites. Direct modulation of transcription could take several forms.

The most economical method, as regards protein synthesis, would be a single protein coded by a regulator gene and which contained both induction and repression sites. In this model constitutive strains would show no end-product repression. If the regulons of the mandelate pathway are under positive control, end-product repression could act by preventing the repressor (ara-C-type, Englesberg et al., 1965) from converting to the activator. An alternative model, by close analogy to the lac system, could involve one protein coded by a regulator gene and another regulatory protein apo-repressor activated by low molecular weight repressors. This would require one apo-repressor type for each regulon. All these models require multiple control sites. The metabolic versatility of the organisms, Moraxella spp. and Pseudomonas spp., means that large numbers of regulons are involved. So to have a two protein control system would be very

extravagant as regards protein synthesis, but possibly necessary as a result of the fine control needed over the multiple metabolic pathways. It seems possible that the regulation of convergent, inducible, catabolic enzymes will provide another extension of the basic model of Jacob & Monod (1961) for the regulation of gene expression.

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