METABOLISM OF PHENYLACETIC ACID BY A PSEUDOMONAS SP.: CHANGES IN THE PHYSIOLOGICAL ACTIVITY AS A FUNCTION OF GROWTH

by

David Jia-Shiuan Pan (Phan-Tri)

A thesis

submitted to

the Faculty of Graduate Studies and Research

University of Manitoba

In partial fulfilment

of the requirements for the degree of

Master of Science

April, 1969

To my former professor Dr. Howard E. Brewer whose noble character has served to stimulate and encourage me this thesis is affectionately dedicated.

ACKNOWLEDGEMENTS

The writer expresses his sincere gratitude to Dr. H. Halvorson, Department of Microbiology, University Manitoba, for his assistance and advice throughout the course of this investigation and in the preparation of the manuscript. Sincere thanks are also expressed to Dr. H. Lees for his helpful discussions. Financial assistance obtained through the National Research Council of Canada was greatly appreciated.

ABSTRACT

Whole cells and, where possible, cell free extracts of a Pseudomonas sp. grown in batch culture containing 0.2% phenylacetic acid as sole carbon source was evaluated for their enzyme activity toward phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid. Studies carried out at various cell densities over the entire range of the growth curve showed that cytoplasmic enzyme activity toward p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid changed as a function of growth while activity against phenylacetic acid did not. Preliminary studies were initiated on the characterization of the enzymes p-hydroxyphenylacetic acid monooxygenase and 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase.

The results further support the hypothesis that phenylacetic acid is metabolized by a multiple pathway in the Pseudomonas sp. and the operation of the pathway is a function of growth. It is proposed that at an early stage of growth, phenylacetic acid is converted directly to 3,4-dihydroxyphenylacetic without involving a mono-

hydroxylated intermediate. But at the exponential and late logarithmic stage of growth, the production of 3,4-dihydroxyphenylacetic acid from phenylacetic acid proceeds via p-hydroxyphenylacetic acid. 'Metabolite induction' has been excluded as a possible regulatory mechanism of the multiple pathway.

o-Hydroxyphenylacetic acid and phenylacetaldehyde were detected as metabolites in fermentation liquor using gas chromatographic methods; the concentration of both metabolites increased as growth increased. Interestingly, o-hydroxyphenylacetic acid proved to be inhibitory toward 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase under certain conditions. A plausible explanation of the significance of metabolite production is presented.

TABLE OF CONTENTS

TABLE OF CONTENTS CONT'D

LIST OF TABLES AND FIGURES CONT'D

LIST OF TABLES AND FIGURES

LIST OF TABLES AND FIGURES CONT'D

INTRODUCTION

INTRODUCTION

Blakley et al (2, 3) demonstrated that a Pseudomonas sp. was able to carry out the oxidation of phenylacetic acid by means of enzyme induction, and proposed a metabolic pathway for the degradation of phenylacetic acid to δ-carboxymethyl-α-hydroxymuconic acid. The inclusion of the monohydroxylated intermediate in this scheme was based upon results obtained with the sequential induction technique of Stanier (33). Van Caeseele (35) used the same organism for further studies on the metabolism of phenylacetic acid. He suggested that phenylacetic acid was metabolized by means of a multiple pathway, only one branch of which contains p-hydroxyphenylacetic acid, and the formation of a monohydroxylated intermediate was not obligatory for the breakdown of phenylacetic acid. His results also were obtained using the sequential induction technique. However, a criticism of the sequential induction technique is that appropriate lag periods (indicative of enzyme induction) prior to substrate metabolism might be due to production of permeases

rather than due to induction of cytoplasmic enzymes. Of even greater importance, Van Caeseele obtained evidence that the enzyme activity changed as a function of growth and phenylacetic acid was oxidized directly to 3,4-dihydroxyphenylacetic acid without the formation of monohydroxylated intermediates. Consequently, the purpose of this work was to assess, at the cell-free level the presence of a multiple pathway, and to show that cytoplasmic enzyme activity changes as a function of growth.

HISTORICAL

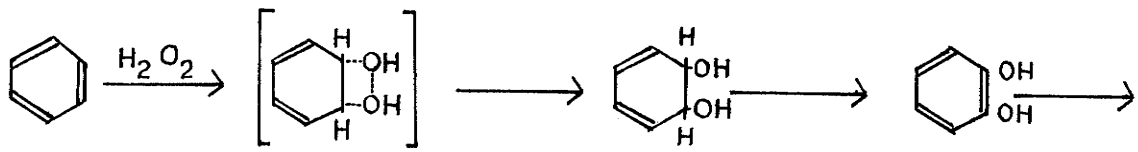
HISTORICAL

The degradation of complex organic compounds derived from plant and animal sources is carried out mainly by microorganisms. Bacteria and fungi are the most active organisms in this respect while yeasts are able to break down a more limited range of complex organic compounds. The bacteria most active in the decomposition of aromatic structures belong to the families Coccaceae, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirillaceae and Bacillaceae (13, 12, 30), while fungi include organisms in such genera as Aspergillus, Penicillium, and Neurospora (17). Saccharomyces cerevisiae, Candida tropicalis, Rhodotorula glutinis and several other yeasts can grow on some simple and a limited number of complex aromatic substrates (15). Decomposition of aromatic compounds is brought about in most organisms by the formation of a series of enzymes which take part in the conversion of aromatic compounds to yield aliphatic acids and aliphatic semialdehydes; the enzymes are formed usually through induction.

Vast numbers of aromatic compounds exist in nature.

These compounds may have one or more aromatic rings or one or more side chains of varying complexity attached to the ring(s) and the manner of degradation of the compounds varies with the structure. In order to exemplify mechanism of oxidation of aromatic compounds, some of the less complex monophenolic compounds have been chosen for illustrative purposes.

Benzene, the simplest aromatic compound is utilized by some bacteria. Marr and Stone (24) have shown that two organisms, a Pseudomonas sp. and a Mycobacterium sp. are capable of utilizing benzene as the sole source of carbon and energy. These workers proposed a metabolic pathway for the oxidation of benzene via 3,5-cyclohexadiene-1,2-diol and catechol as shown below:

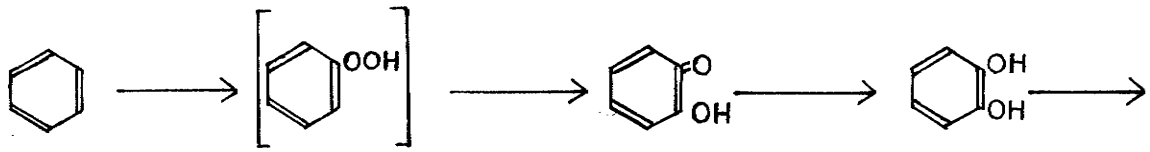


Benzene

3,5-cyclohexadiene-1,2-diol

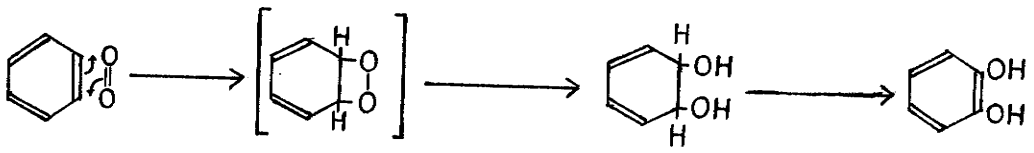
catechol

Wieland et al (18) studied the oxidation of benzene by a Norcadia sp. and suggested a modified scheme as follows:



Benzene Catechol

Recently, Gibson et al (14) suggested that catechol was produced from benzene by Pseudomonas putida grown with toluene as the carbon source, according to the mechanism as shown below:

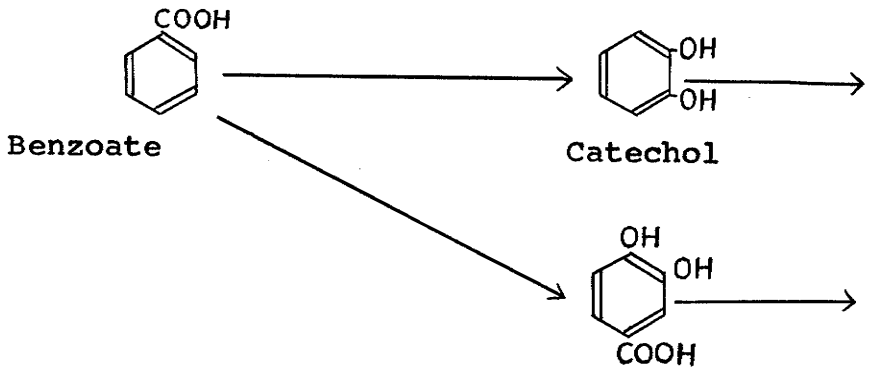


Benzene Catechol

These proposals suggest that no monohydroxylated metabolite is involved in the oxidation of benzene to catechol.

When one carboxyl group is linked to the benzenoid ring to form benzoic acid, the breakdown of the aromatic moiety by bacteria may occur by several alternate routes. For example, benzoic acid may undergo either a simultaneous

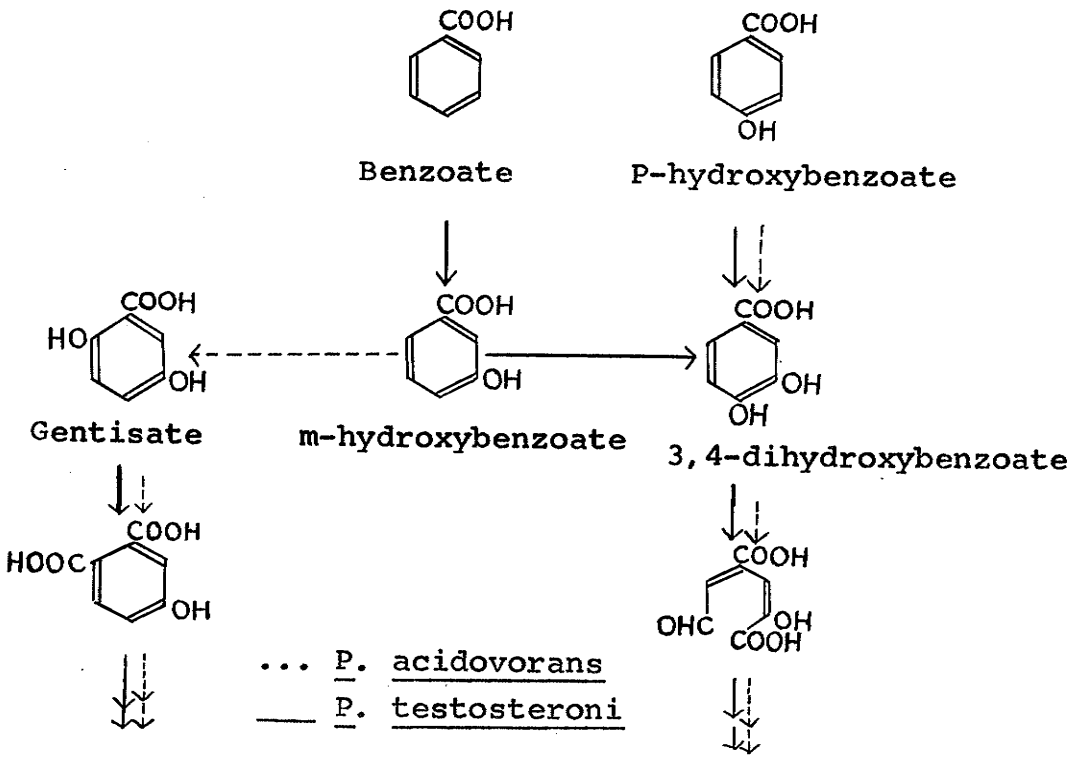
oxidative decarboxylation and hydroxylation to form catechol (27, 28, 29, 32) or may be degraded via 3,4-dihydroxybenzoic acid (26, 33) as shown below:



3,4-dihydroxybenzoate

These different mechanisms do not occur in the same species.

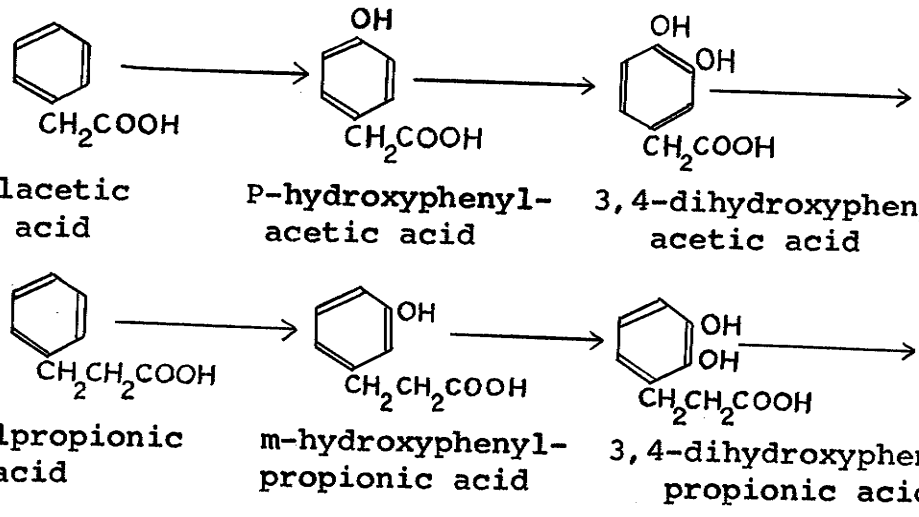
When one hydroxyl group is substituted into the ring of benzoic acid, the method of cleavage depends upon the relative position of the substituent groups. For example, Stanier et al (31, 38) demonstrated that inducible pathways for the metabolism of aromatic acids are present in two species of the acidovorans group (Pseudomonas acidovorans and Pseudomonas testosteroni), and these mechanisms are summarized below:



The pathway for the metabolism of p-hydroxybenzoic acid is identical in both species. The metabolism of m-hydroxybenzoic acid follows different routes in Pseudomonas acidovorans and Pseudomonas testosteroni determined by the position of the initial hydroxylation: Pseudomonas acidovorans synthesizes a 6-hydroxylase, and Pseudomonas testosteroni, a 4-hydroxylase. The m-hydroxybenzoate 4-hydroxylase of Pseudomonas testosteroni is specifically induced by its substrate, whereas the 6-hydroxylase of Pseudomonas acidovorans can be product-induced by gentisate. Although the metabolism of m-hydroxybenzoate by Pseudomonas

<u>testosteroni</u> proceeds exclusively through protocatechuate, the three enzymes of the gentisate pathways (gentisate oxygenase, maleylpyruvate isomerase and fumarypyruvate hydrolase) are present in these species, and are gratuitously induced to high levels by m-hydroxybenzoate. It is interesting to note that the above mechanism for the degradation of benzoate does not involve a monohydroxylated intermediate in <u>Pseudomonas testosteroni</u>.

Blakley et al (2, 3) have suggested that a <u>Pseudomonas</u> sp. metabolizes phenylacetic acid via p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid whereas the metabolism of phenylpropionic acid by the same organism involves m-hydroxyphenylpropionic acid and 2,3-dihydroxyphenylpropionic acid as intermediates. These reactions are illustrated below:

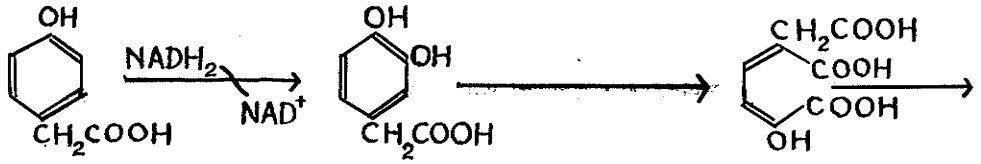


Phenylacetic acid — P-hydroxyphenylacetic acid — 3,4-dihydroxyphenylacetic acid

Phenylpropionic acid — m-hydroxyphenylpropionic acid — 3,4-dihydroxyphenylpropionic acid

The above results give ample evidence to illustrate that the side chain of aromatic compounds plays an important role in determining the mechanism of hydroxylation of aromatic compounds.

Hiroshi (1, 18) demonstrated that the degradation of p-hydroxyphenylacetic acid by a Pseudomonas sp. was carried out by a monooxygenase which converted p-hydroxyphenylacetic acid via 3,4-dihydroxyphenylacetic acid to α-carboxymethyl-cis,cis-muconic acid as shown below:

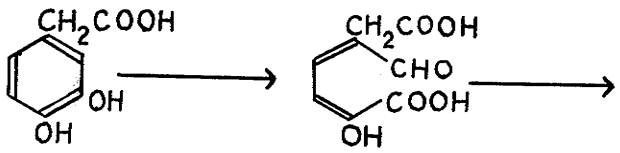


p-hydroxyphenyl-acetic acid | 3,4-dihydroxy-phenylacetic acid | α-carboxymethyl-cis,cis-muconic acid

He elucidated the metabolic pathway of p-hydroxy-phenylacetic acid with partially purified enzyme preparations. The significance of this result is that it shows NADH + H^+ is required for the introduction of one hydroxyl group into the aromatic ring; the simultaneous introduction of two hydroxy groups, for example, the conversion of benzene to catechol, does not.

Dagley et al (7, 8, 9) found that a Pseudomonas sp. isolated from soil, metabolized 3,4-dihydroxyphenylacetic acid, and the fission of the nucleus appeared to take place between C_4 and C_5, as in the case of protocatechuic acid. He suggested a general sequence of reaction of 3,4-dihydroxyphenylacetic acid (mechanism I), which is different from the mechanism suggested by Blakley et al (mechanism II) as shown below:

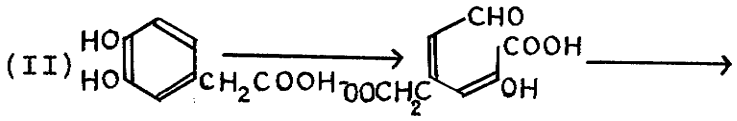
(I)



Formate + pyruvate + succinate semialdehyde

3,4-dihydroxy-phenylacetic acid

(II)

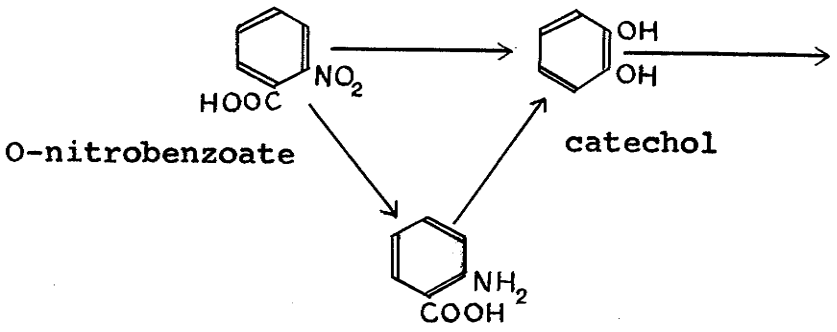


Formate + pyruvate + acetoacetate

3,4-dihydroxy-phenylacetic acid

In a study of the oxidation degradation of o-nitrobenzoate by Nocardia opaca, Cain et al (4) showed the organism not only oxidized o-nitrobenzoate directly to catechol but also reduced small amounts of it to

anthranilate as illustrated below:



Anthranilic acid

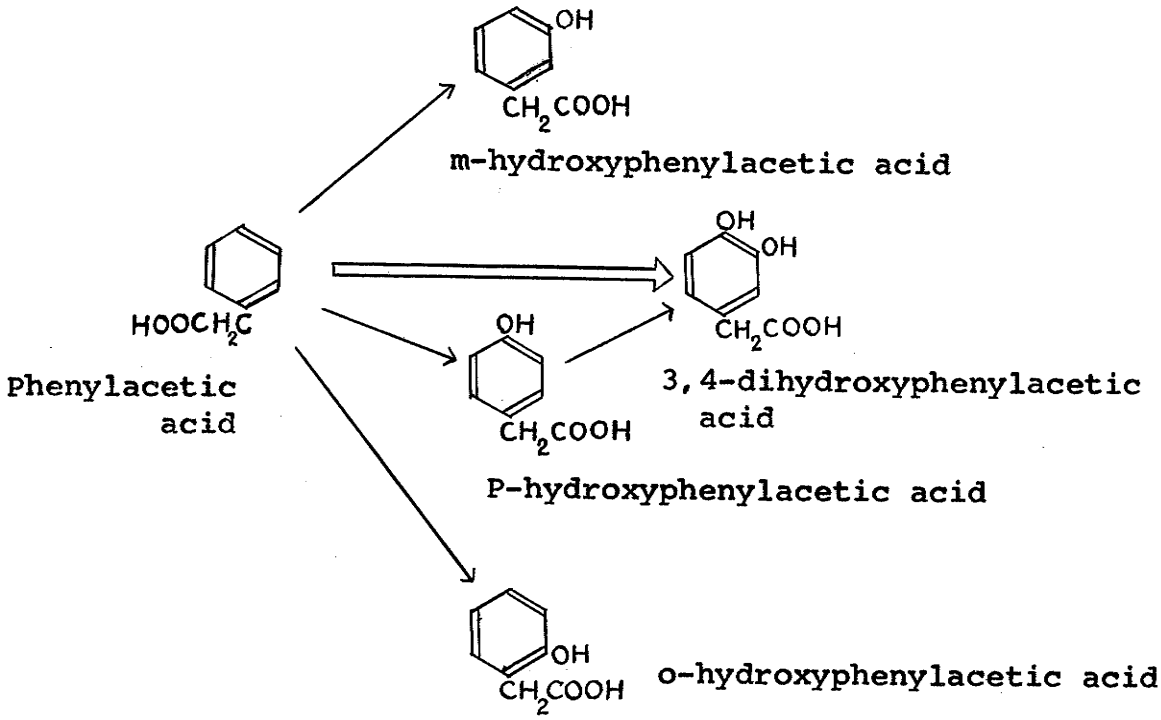
The anthranilate accumulated in fermentation liquor until it reached a concentration sufficiently high to serve as an enzyme inducer, and the metabolite subsequently was depleted from the medium. This phenomenon has been termed "metabolite induction" by Cain. However, the accumulation and the degradation of anthranilate by this organism shows the oxidative decomposition of o-nitrobenzoate can proceed by more than one route; the demonstration of a multiple pathway was dependent upon the time at which the organism was harvested with respect to growth.

Dawson (9, 10, 11) investigated the intracellular amino acid pool of Candida utilis during growth in batch and continuous flow culture and showed that the pattern of

metabolism of this organism in the culture, as reflected by the intracellular amino acid pool, is dependent upon the medium and the growth rate. These results are exemplary of many reports which show that changes in the physiology of cells occur as a function of growth.

In the majority of cases, the enzyme systems responsible for degradation of aromatic acid in microorganism are oxidative reaction. However, Farmer et al (17) found that certain substituted aromatic acids such as, o-, m-, and p-methoxybenzoic, 3,4-dimethoxybenzoic, p-naphthoic, and benzoic acid were reduced either to the corresponding aldehyde or alcohol by Polystictus versicolor. Thus reduction products may be formed as metabolites by microorganism.

Van Caeseele (35) suggested the induced enzyme activity of a Pseudomonas sp. grown on phenylacetic acid as sole carbon source changed as a function of growth. An overall mechanism for the oxidation of phenylacetic acid by this Pseudomonas sp. was proposed as shown below:



This scheme was proposed on the basis of the pattern of induced enzyme formation that was obtained with intact cells taken from various phases of the growth curve.

Macleod (25) questioned the validity of the interpretation of the results on the premise that variation in activity of resting cell suspensions with respect to growth might be due to substrate specific permease induction rather than due to cytoplasmic enzyme induction. Consequently, this investigation was undertaken to add substance to the view that decomposition of phenylacetic acid occurs via a multiple pathway, and to confirm at the

cell free level that the metabolic activity of the Pseudomonas sp. changed as a function of growth. Further, the regulation of a multiple pathway via metabolite induction seemed worthy of study.

MATERIALS AND METHODS

MATERIALS AND METHODS

Culture

The organism used throughout this study was a *Pseudomonas* sp. obtained through the courtesy of Dr. E. R. Blakley, Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan. This organism has been used by Blakley et al (2, 3) to study mechanisms of decomposition of aromatic compounds including phenylacetic acid.

Maintenance of Culture

Stock cultures of this species were grown on slants of Bacto L Agar (Difco), incubated for 24 hours at 28° C, then kept in the cold until required.

Growth Medium

For growth, an aqueous mineral salts solution of the following composition was used:

K_2HPO_4	0.1%
KH_2PO_4	0.1%
NH_4NO_3	0.1%
$MgSO_4 \cdot 7H_2O$	0.002%
$CaCl_2 \cdot 2H_2O$	0.002%
$FeCl_3$	0.002%

These salts with 0.2% phenylacetic acid as sole carbon source were dissolved in distilled water, then adjusted to pH 6.8 with 1 N NaOH or the solution was sterilized by autoclaving at 121° C for 15 minutes then allowed to cool. Occasionally 0.2 to 0.4% glucose was used as carbon source; it was sterilized separately in aqueous solution and added asceptically to sterile neutral mineral salts solution.

Preparation of Inocula

Inocula were prepared by incubating the culture at 28° C for 24 hours in 1 liter of growth medium in 2 liter Erlenmeyer flasks on an Eberbach rotary shaker at 120 cycles per minute. The growth medium used for the preparation of inocula always consisted of mineral salts solution supplemented with 0.2% to 0.4% glucose.

Enzyme Activity of Intact Cells

To obtain resting cell suspensions possessing induced enzyme activity, enzyme induction was achieved in a variety of ways, by either growing cells on an inducing substrate or by exposing glucose grown resting cell suspensions to inducers for varying lengths of time. The purpose of these experiments was to determine whether the length of exposure time to an inducer affected the amount of enzyme induced. Activity was measured manometrically (see Manometric Experiments).

To obtain large amounts of cells for manometric studies cells were grown on phenylacetic acid in several 2 liter Erlenmeyer flasks containing 1 liter of sterile medium with 0.2% phenylacetate as sole carbon source. After the addition of 50 ml of fresh inoculum into the flask the culture was incubated at 28^{o} C on an Eberbach rotary shaker at 120 cycles per minute and growth was followed by measuring the cell density using a Klett-Summerson Photo-electric Colorimeter equipped with a KS-54 filter. When growth reached a cell density of approximately 10, 40, 80, and 100 Klett units (these values were selected arbitrarily

as points distributed over the entire growth curve), cells were harvested by centrifugation at a speed of 30,000 x g for 15 minutes, then washed twice with 0.05 M phosphate buffer (pH 7.4). Standardized cell suspensions for manometric studies were made by diluting the cells to a density of 400 Klett units with buffer. This preparation is referred to as "resting cell suspension". The resting cell suspensions were used immediately to measure the activity toward phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid.

To induce enzyme formation in glucose grown cells 50 ml of fresh inoculum was added to 1 liter of medium containing 0.4% glucose as sole carbon source. The cells were harvested in the late logarithmic phase of growth, washed once with 0.05 M phosphate buffer (pH 7.4), then diluted to a cell density of 400 Klett units with the same phosphate buffer, and 500 ml of the diluted cell suspension was treated with 0.5 mM of phenylacetic acid, p-hydroxy-phenylacetic acid, or 3,4-dihydroxyphenylacetic acid as inducers for a period of 50, 100, and 200 minutes, then 100 ml of induced cell suspensions were removed, and harvested by centrifugation at a speed of 30,000 x g for 15 minutes

and prepared for manometry as previously described. These cell suspensions were tested for activity against phenylacetic acid, o-, m-, and p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid.

Manometric Experiments with Intact Cells

All manometric experiments were conducted at 30° C with air as the gas phase employing a Braunwill apparatus using standard manometric methods (35). For measuring the amount of oxygen uptake by resting cell suspensions, each Warburg vessel contained: 0.2 ml of 20% KOH and a fluted filter paper in the center well, 1.0 ml of resting cell suspension in the main compartment and 10 μM of substrate in the side arm. The final volume of the reaction mixture was brought up to 3.0 ml with 0.05 M potassium phosphate buffer (pH 7.0).

Enzyme Activity in Cell Free Extracts

To prepare cell free extracts cells grown on 0.2% phenylacetic acid as sole carbon source were harvested by centrifugation and washed twice with 0.05 M potassium phosphate buffer (pH 7.5). Then 6 gm (wet weight) were

resuspended in 10 ml of the same buffer solution containing 0.1 mM glutathione (reduced form) and sonicated under an atmosphere of N_2 for 5 minutes in a water cooled (5^{o} C) Raytheon Sonic Disintegrator (10 Kc/sec). Cell debris was removed by centrifugation at 30,000 x g for 15 minutes. The red supernatant liquid referred to as "cell free extract" was used for manometric studies as well as for enzyme partial purification. The buffer used for preparing cell free extract was boiled, cooled, then saturated with nitrogen before use to prevent possible destruction of the enzyme by oxygen.

Manometric Experiments with Cell Free Extract

Manometric experiments with cell free extract were carried out as with intact cells. For measuring the amount of oxygen uptake by cell free extract each flask contained: 0.2 ml of 20% KOH and a fluted filter paper in the center well, 1.0 ml of cell free extract (approximately 12 mg/ml protein), 0.4 ml of 1.5 μM of $FeSO_4 \cdot 7H_2O$, 1.5 μM of glutathione (reduced form), 0.2 ml of 1.5 μM of $NADH_2$ in the main compartment and 10 μM of substrate (p-hydroxyphenyl-acetic acid or 3,4-dihydroxyphenylacetic acid) in the side

arm. The final volume of the reaction mixture was brought up to 3.0 ml with 60 μM of potassium phosphate buffer, pH 7.4.

Partial Purification of 3,4-Dihydroxyphenylacetic Acid-2,3-Dioxygenase

Cell free extract active against 3,4-dihydroxyphenylacetic acid was treated with solid $(NH_4)SO_4$ (0.5 gm/ml) by gentle stirring at 5° C for 30 minutes, then centrifuged to remove precipitates. The supernatant liquid was retreated with solid $(NH_4)_2SO_4$ (1 gm/ml) once again. The supernatant liquid was precipitated with solid $(NH_4)_2SO_4$ (3.5 gm/ml) under the same conditions. The precipitates were recovered by centrifugation and dissolved in small volumes of 0.05 M potassium phosphate buffer (pH 7.4). The final supernatant fluid was dialyzed against 1 liter of buffer containing 1 mM glutathione (reduced form) for 15 hours, then used for enzyme assay. All experiments were carried out at 5° C.

The activity of 3,4-dihydroxyphenylacetic acid 2,3-dioxygenase was assayed spectrophotometrically in a Unicam Model SP500 spectrophotometer by measuring increase

in optical density of the absorption maximum of the reaction product at 380 mμ at 25° C (7). A typical reaction mixture contained: 0.1 M phosphate buffer, 0.185 mM $FeSO_4 \cdot 7H_2O$, a suitable amount of enzyme (60 μg of protein), and water to a final volume of 3 ml. Protein concentrations were determined according to the method of Lowry et al (22) using crystalline serum albumin as the reference protein.

Effect of pH on the Activity of p-Hydroxyphenylacetic Acid Monooxygenase

To observe the effect of pH on the activity of p-hydroxyphenylacetic acid monooxygenase, the method of enzyme assay were used as described previously, except that the buffer composition and pH values were varied from 5.4 to 9.5 (phosphate buffer), with the enzyme concentration constant.

Effect of o-Hydroxyphenylacetic Acid on the Activity of 3,4-Dihydroxyphenylacetic Acid-2,3-Dioxygenase

The effect of o-hydroxyphenylacetic acid on the activity of 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase

was determined. In each assay, the reaction mixture contains: 0.185 mM $FeSO_4 \cdot 5H_2O$, 4.0 mM o-hydroxyphenylacetic acid, 0.1 M phosphate buffer at different pH values and variable amounts of substrate (3,4-dihydroxyphenylacetic acid), with the enzyme concentration constant.

Growth of Culture in the Presence of o-Hydroxyphenylacetic Acid

To observe the effect of o-hydroxyphenylacetic acid on the growth of organisms with phenylacetic acid as major carbon source, 0.01%, 0.04%, 0.12% and 0.20% o-hydroxyphenylacetic acid were incorporated into the growth medium along with 0.2% phenylacetic acid. After a growth period of 6, 12, 21, and 28 hours, the optical density was measured using a Klett-Summerson Photoelectric Colorimeter equipped with a KS-54 filter, a suitable amount of cells was harvested by centrifugation, then usedfor manometric studies as described previously.

Extraction of Metabolites from Fermentation Liquor

To detect metabolites in fermentation liquor, cells were grown in basal salts medium with 0.2% phenylacetic acid as sole carbon source. After removing the cells by

centrifugation, the pH of 500 ml of supernatant fermentation liquor was lowered to pH 3.0 with H_2SO_4, the precipitated protein was removed by centrifugation, and the supernatant liquor was neutralized with KOH. Then 250 ml of the neutral liquor was lyophilized employing a Virtis lyophilizer. Finally, the powder was extracted with ether by gentle stirring. The ether was evaporated, and the dried material was analyzed by gas chromatography for metabolites.

Gas Chromatographic Experiments

(a) Construction of column

A Varian Aerograph series 2000 linear temperature programmed gas chromatograph was employed. The column was constructed from stainless steel tubing (6' x 3/8") containing 10% SE-52 on Chromosorb W 60/80 mesh treated with hexaethyldisilizane. Prior to construction, the steel column was cleaned thoroughly. Two gm of SE-52 were dissolved completely in chloroform and 20 gm of Chromosorb W was added to the solution. After mixing, the chloroform was gently removed on a rotary evaporator and the dried Chromosorb W was packed into the column. Before use, the column was held at 200° C overnight, then stabilized by

injecting 10-15 µl samples of Silyl 8 (Pierce Chemical Co.) until extraneous peaks disappeared and a stable base line resulted.

(b) Preparation of derivatives

Trimethylsilyl derivatives of phenylacetic acid and suspected metabolites were prepared by dissolving 1 to 3 mg of sample in 0.2 or 0.4 ml of Tri-Sil (a solvent-reagent catalyst for preparing trimethylsilyl derivatives of polar compounds for gas chromatography marketed by Chromatographic Specialties, Ltd.) in a small glass stoppered vial; the mixture was shaken and ready for analysis after a few minutes.

(c) Quantitative detection of metabolites

To make standard curves of phenylacetic acid, o-, m-, and p-hydroxyphenylacetic acid, and phenylacetaldehyde, these compounds were transformed to the trimethyl silyl derivatives as described above and increasing aliquots (1-3 µl) were injected into the gas chromatograph. A standard curve was obtained by plotting peak height against quantity of compound. A standard profile was also constructed based on elution time.

Similarly, dried material was treated to the silylation procedure and metabolites were identified from the standard profile and the quantity determined from the standard curve.

(d) Conditions for operation

For phenylacetic acid and o-hydroxyphenylacetic acid:

Column temperature	200° C
Upper limit temperature	230° C
Program rate	0.5° C/min
Injector cell temperature	230° C
Detector cell temperature	230° C
Amps/mv	10^{-11}
Carrier gas He, O_2, H_2	20 ml/min
Attenuator	64
Chart speed	1 in/min

For phenylacetaldehyde:

Column temperature	70° C
Upper limit temperature	110° C
Program rate	0.5° C/min
Injector cell temperature	120° C/min
Detector cell temperature	120° C/min
Amps/mv	10^{-11}

Carrier gas He, O_2, H_2	20 ml/min
Attenuator	64
Chart speed	1 in/min

RESULTS

RESULTS

Activity of Induced Enzyme Systems

A. Activity of intact cells and cell free extracts of cells grown on phenylacetic acid

Figs. 1-4 show the activity of standardized resting cell suspensions and cell free extracts of cells grown on phenylacetic acid and harvested at various points over the growth curve. Each preparation was tested for activity against phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid. When intact cells were used, the results are expressed as exogenous oxygen uptake; when cell free extracts were used, the results were uncorrected for endogenous respiration.

Fig. 1A shows the activity of cells harvested at a cell density of 20 Klett units. This cell density represents a very early logarithmic stage of growth. Phenylacetic acid and 3,4-dihydroxyphenylacetic acid were both metabolized readily; activity against phenylacetic acid was slightly higher than against 3,4-dihydroxyphenylacetic acid and activity against p-hydroxyphenylacetic acid was considerably

Fig. 1. Comparison of the oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by resting cell suspensions and cell free extract of a Pseudomonas sp. grown on phenylacetic acid. Cells were harvested at a cell density of 20 Klett units.

A. Resting cell suspensions. Curves are exogenous plots representative of:

1. p-hydroxyphenylacetic acid
2. phenylacetic acid
3. 3,4-dihydroxyphenylacetic acid

B. Cell free extract. Curves are endogenous plots representative of:

1. endogenous
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid

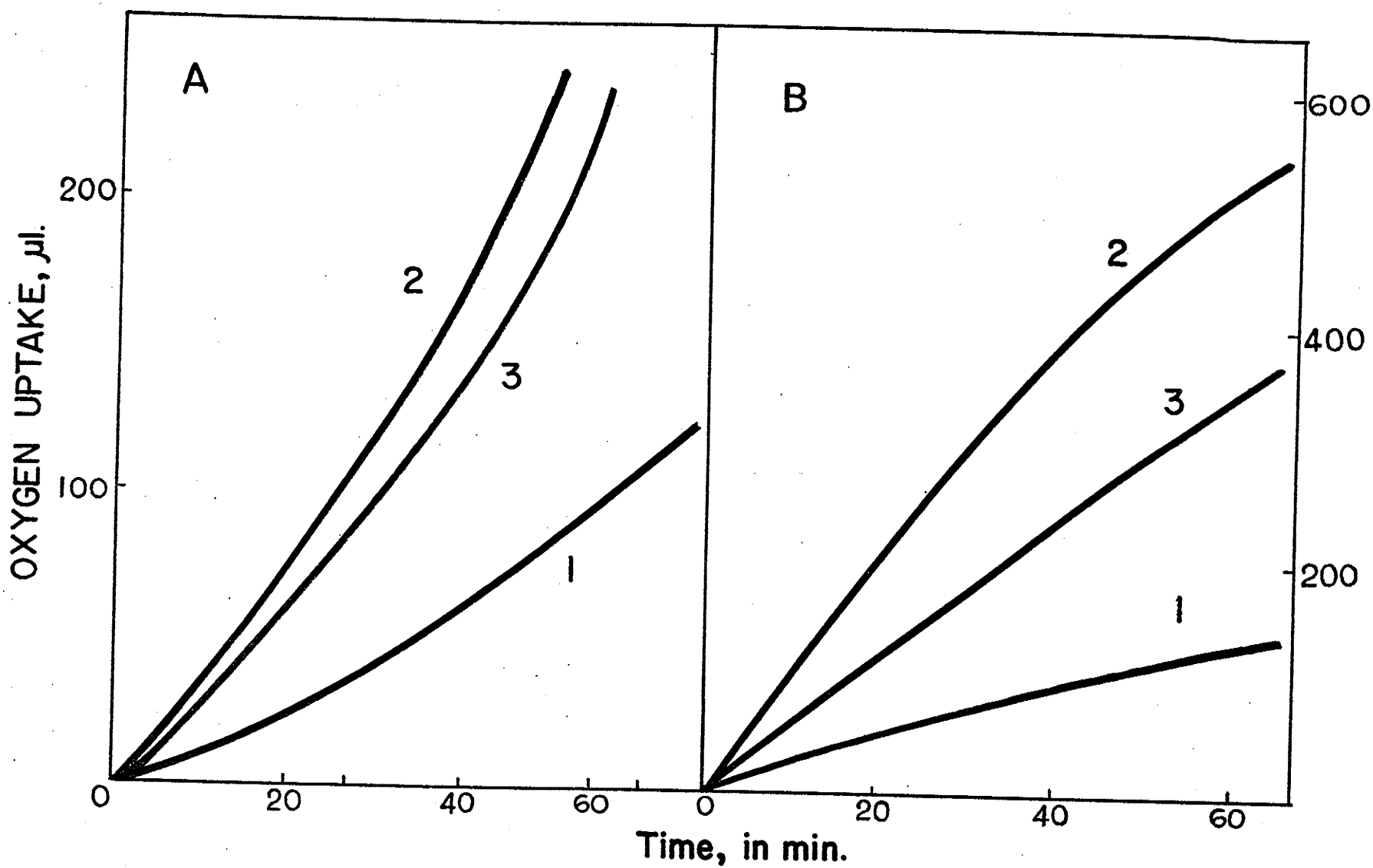

A
B
OXYGEN UPTAKE, µl.
200
100
0
600
400
200
2
3
1
20
40
60
Time, in min.

Fig. 2. Comparison of the oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by resting cell suspensions and cell free extracts of a Pseudomonas sp. grown on phenylacetic acid. Cells were harvested at a cell density of 45 Klett units.

A. Resting cell suspensions. Curves are exogenous plots representative of:

1. p-hydroxyphenylacetic acid
2. phenylacetic acid
3. 3,4-dihydroxyphenylacetic acid

B. Cell free extracts. Curves are endogenous plots representative of:

1. endogenous
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid

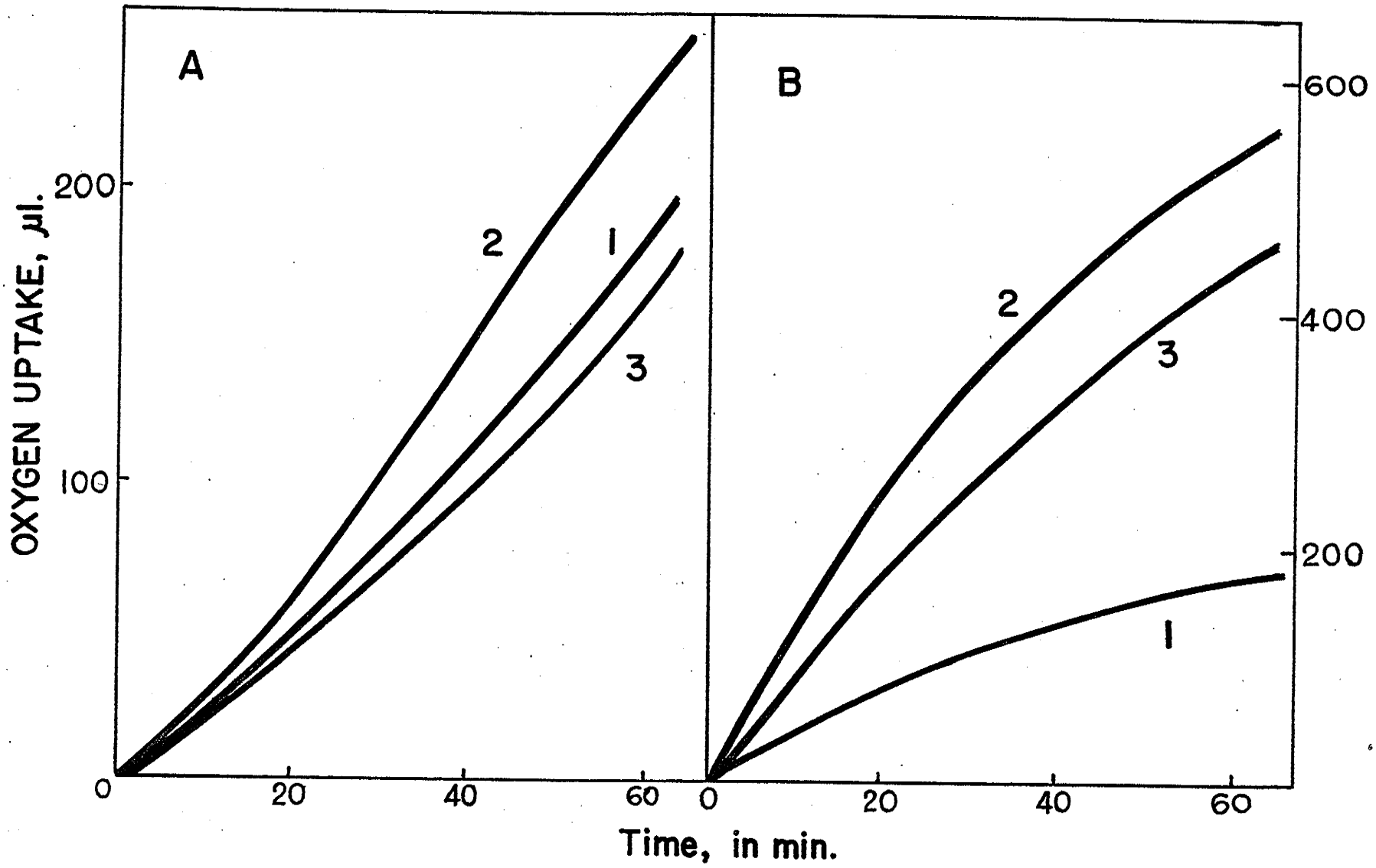

A
B
OXYGEN UPTAKE, µl.
200
100
0
600
400
200
2
1
3
2
3
1
0
20
40
60
0
20
40
60
Time, in min.

Fig. 3. Comparison of the oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by resting cell suspensions and cell free extracts of a Pseudomonas sp. grown on phenylacetic acid. Cells were harvested at a cell density of 82 Klett units.

A. Resting cell suspensions. Curves are exogenous plots representative of:

1. p-hydroxyphenylacetic acid
2. phenylacetic acid
3. 3,4-dihydroxyphenylacetic acid

B. Cell free extracts. Curves are endogenous plots representative of:

1. endogenous
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid

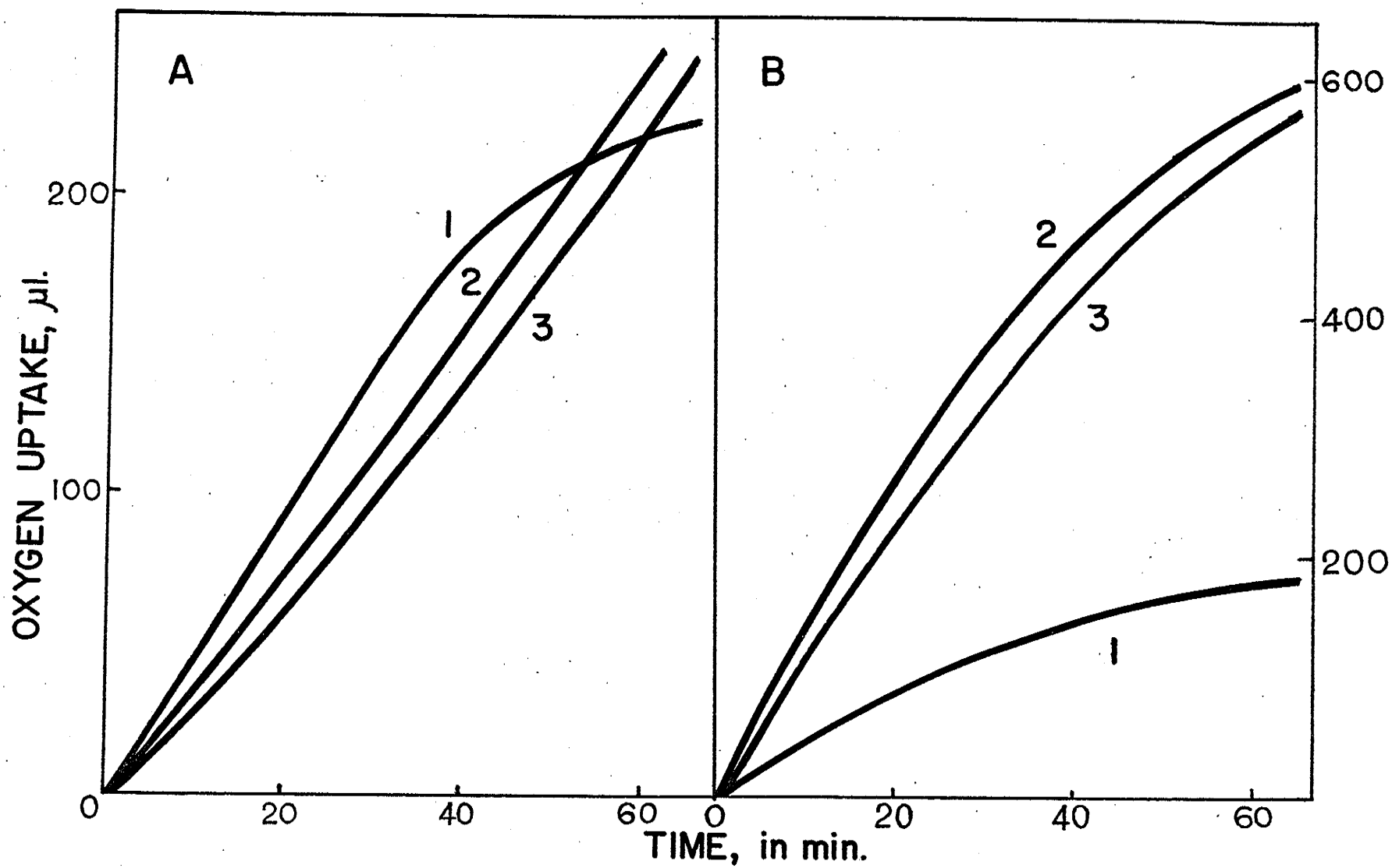

A
B
OXYGEN UPTAKE, µl.
200
100
0
600
400
200
1
2
3
20
40
60
TIME, in min.

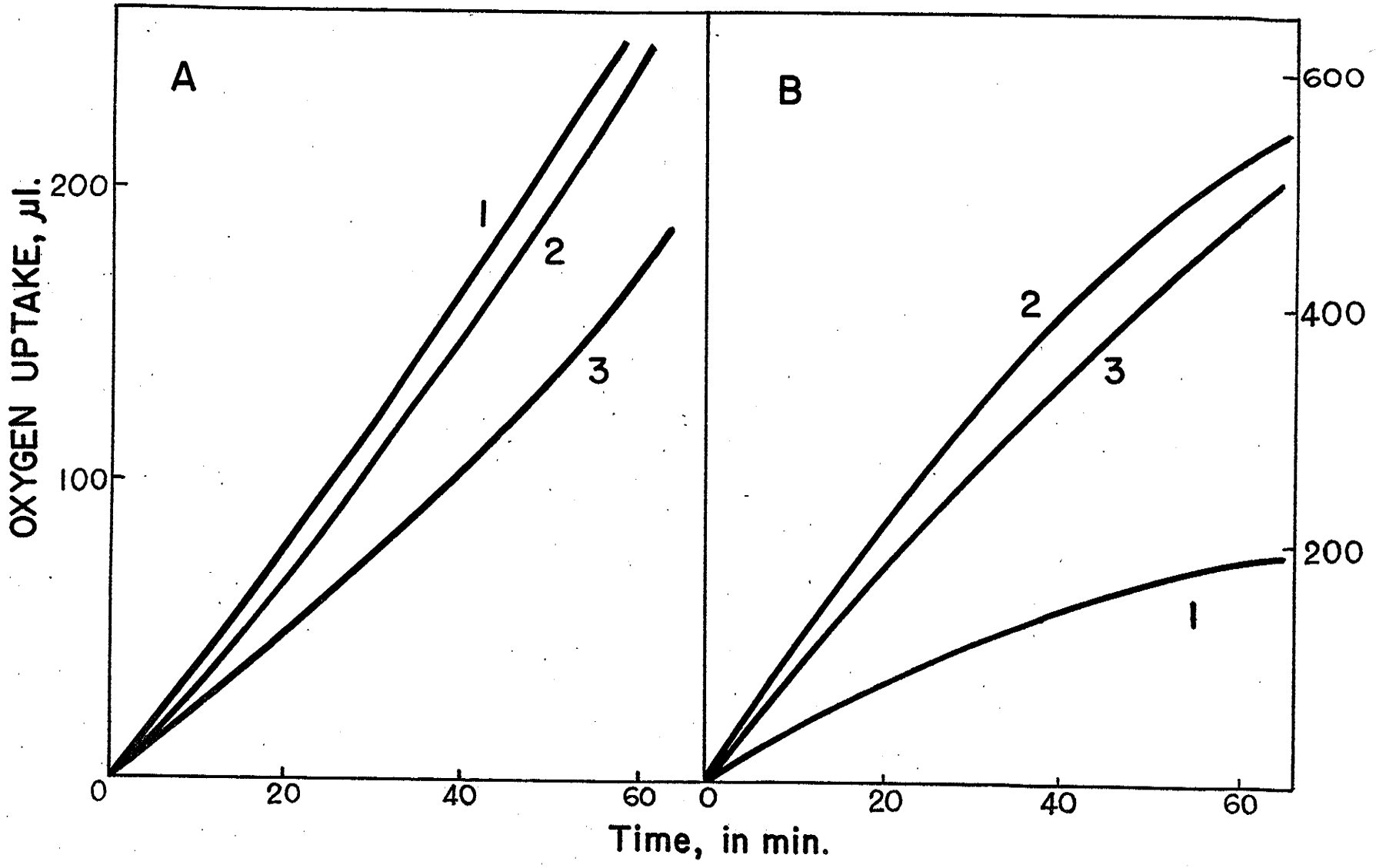
Fig. 4. Comparison of the oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by resting cell suspensions and cell free extracts of a Pseudomonas sp. grown on phenylacetic acid. Cells were harvested at a cell density of 118 Klett units.

A. Resting cell suspensions. Curves are exogenous plots representative of:

1. p-hydroxyphenylacetic acid
2. phenylacetic acid
3. 3,4-dihydroxyphenylacetic acid

B. Cell free extracts. Curves are endogenous plots representative of:

1. endogenous
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid


A
B
OXYGEN UPTAKE, µl.
200
100
0
600
400
200
1
2
3
2
3
1
0
20
40
60
0
20
40
60
Time, in min.

lower than with the other two substrates. Fig. 2A shows the activity of cells harvested at 45 Klett units. Phenylacetic acid and 3,4-dihydroxyphenylacetic acid were oxidized approximately at equal rates and remained virtually unchanged from the activity found at 20 Klett units. The activity against p-hydroxyphenylacetic acid had increased significantly as the cell density increased. When cells were harvested at 82 Klett units activity against phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid were almost the same as shown in Fig. 3A. The significance of Figs. 2A and 3A is that an increase in activity against p-hydroxyphenylacetic acid occurred concomitant with growth of the culture. Fig. 4A shows the activity of cells harvested when the cell density had reached 118 Klett units. This cell density represents a late logarithmic stage of growth. The activity against 3,4-dihydroxyphenylacetic acid had been retained but activity against phenylacetic acid and p-hydroxyphenylacetic acid was slightly reduced.

Contiguously with the results obtained with intact cells are shown in Figs. 1B-4B the results obtained with cell free extracts. Numerous results to obtain activity

against phenylacetic acid with cell free extracts were unsuccessful, therefore, only activity against p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid is shown. Endogenous oxygen uptake was sufficiently low not to interfere with interpretation of the results. Activity against 3,4-dihydroxyphenylacetic acid showed slight fluctuations in activity over the range of the growth curve but very significant changes in activity were found against p-hydroxyphenylacetic acid as growth increased. The amount of activity found in cell free extracts paralleled quite closely that found in intact cells. The results show that changes in enzyme activity which accompany growth are due to changes in cytoplasmic enzyme activity and not due to permease formation.

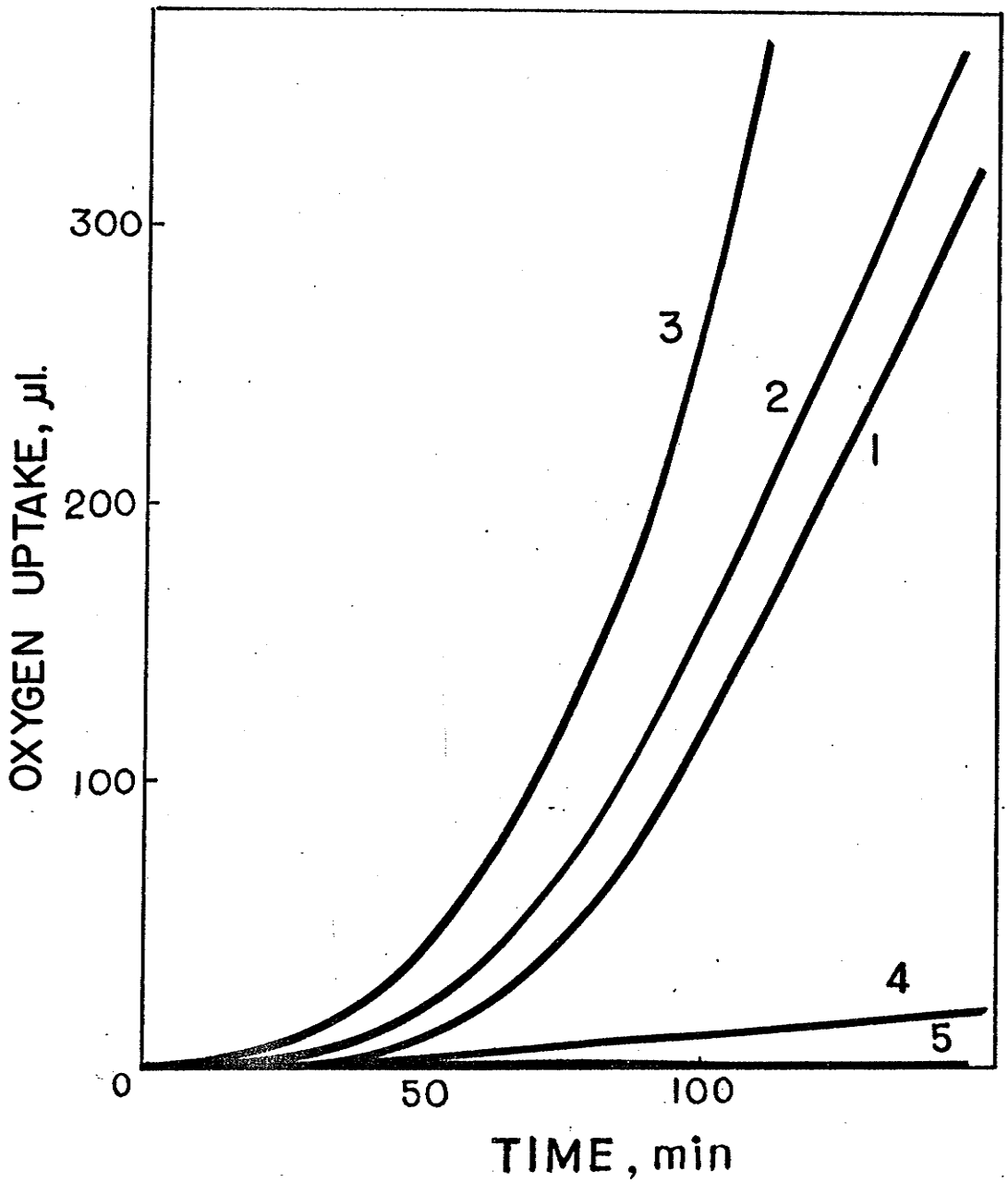
B. Activity of intact cells grown on glucose and induced to phenylacetic acids

Figs. 5-13 show the activity of resting cell suspensions of cells grown on glucose, induced for varying lengths of time toward phenylacetic acid, p-hydroxyphenylacetic acid, or 3,4-dihydroxyphenylacetic acid and tested for activity against phenylacetic acid, o-, m-, and

Fig. 5. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown phenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 50 minutes.

Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. m-hydroxyphenylacetic acid
5. o-hydroxyphenylacetic acid


OXYGEN UPTAKE, µl.
300
200
100
0
50
100
TIME, min
1
2
3
4
5

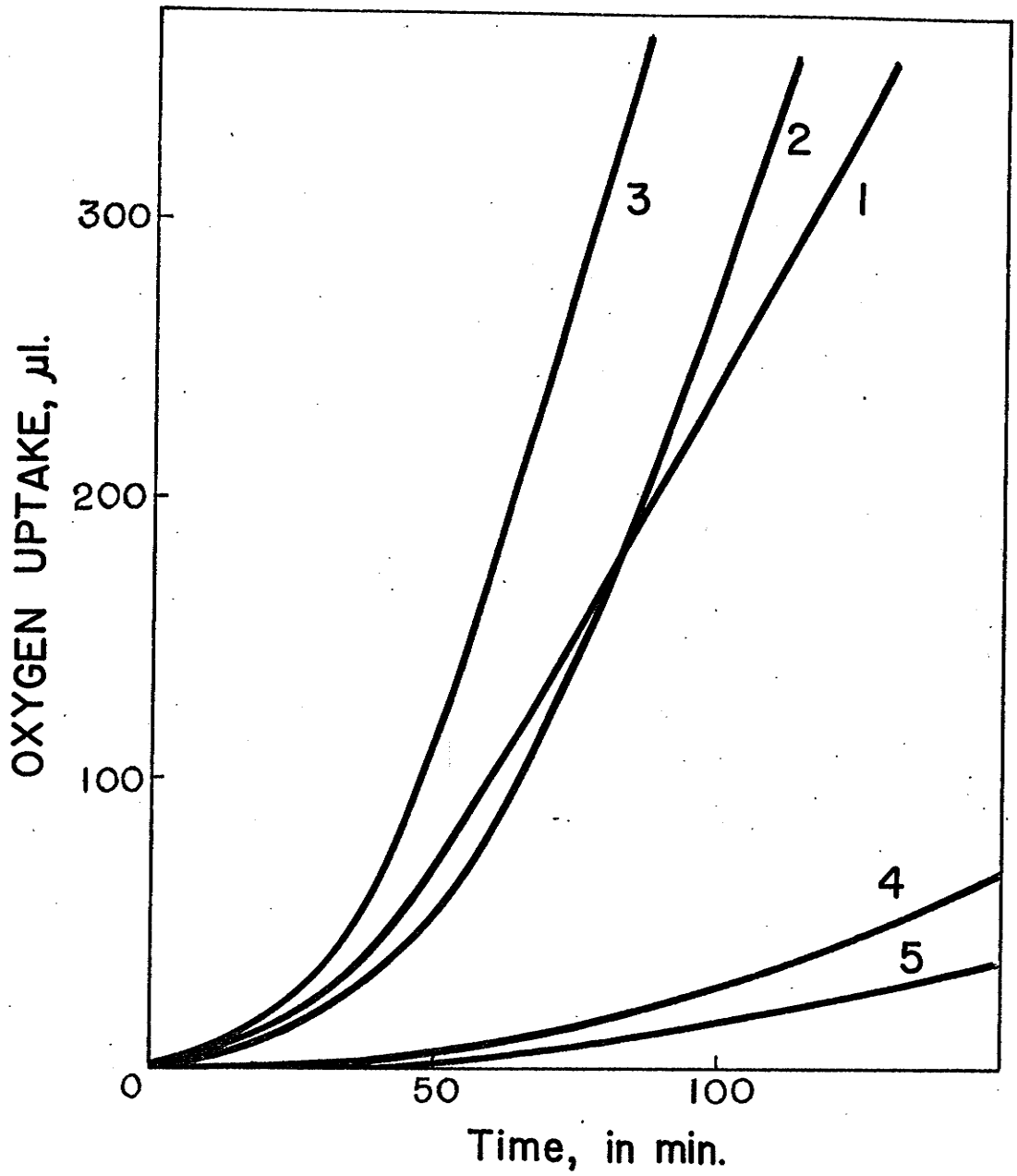

OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

Fig. 7. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown phenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 200 minutes.

Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. m-hydroxyphenylacetic acid
5. o-hydroxyphenylacetic acid

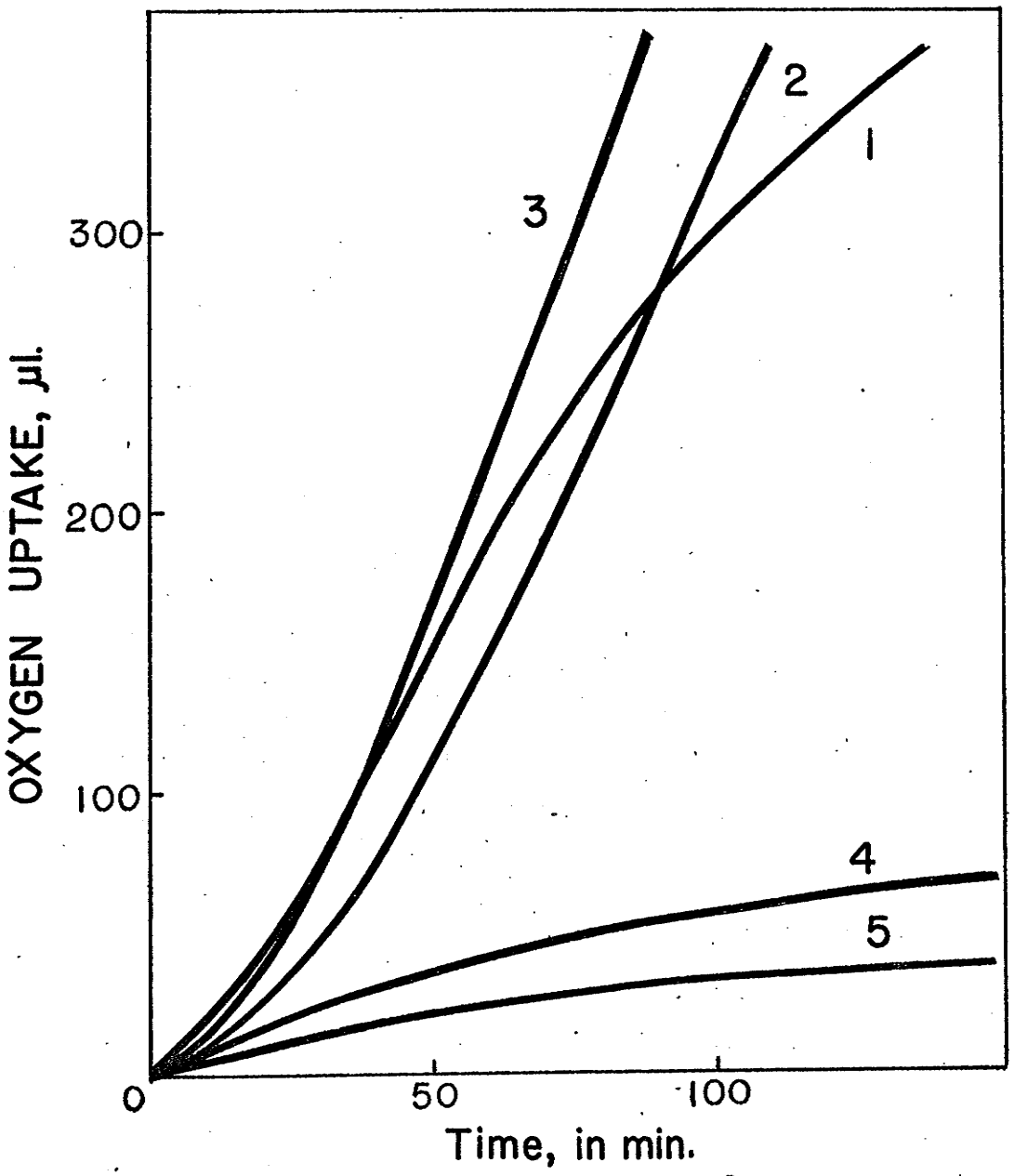

OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

Fig. 8. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown p-hydroxyphenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 50 minutes. Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. o-hydroxyphenylacetic acid
5. m-hydroxyphenylacetic acid

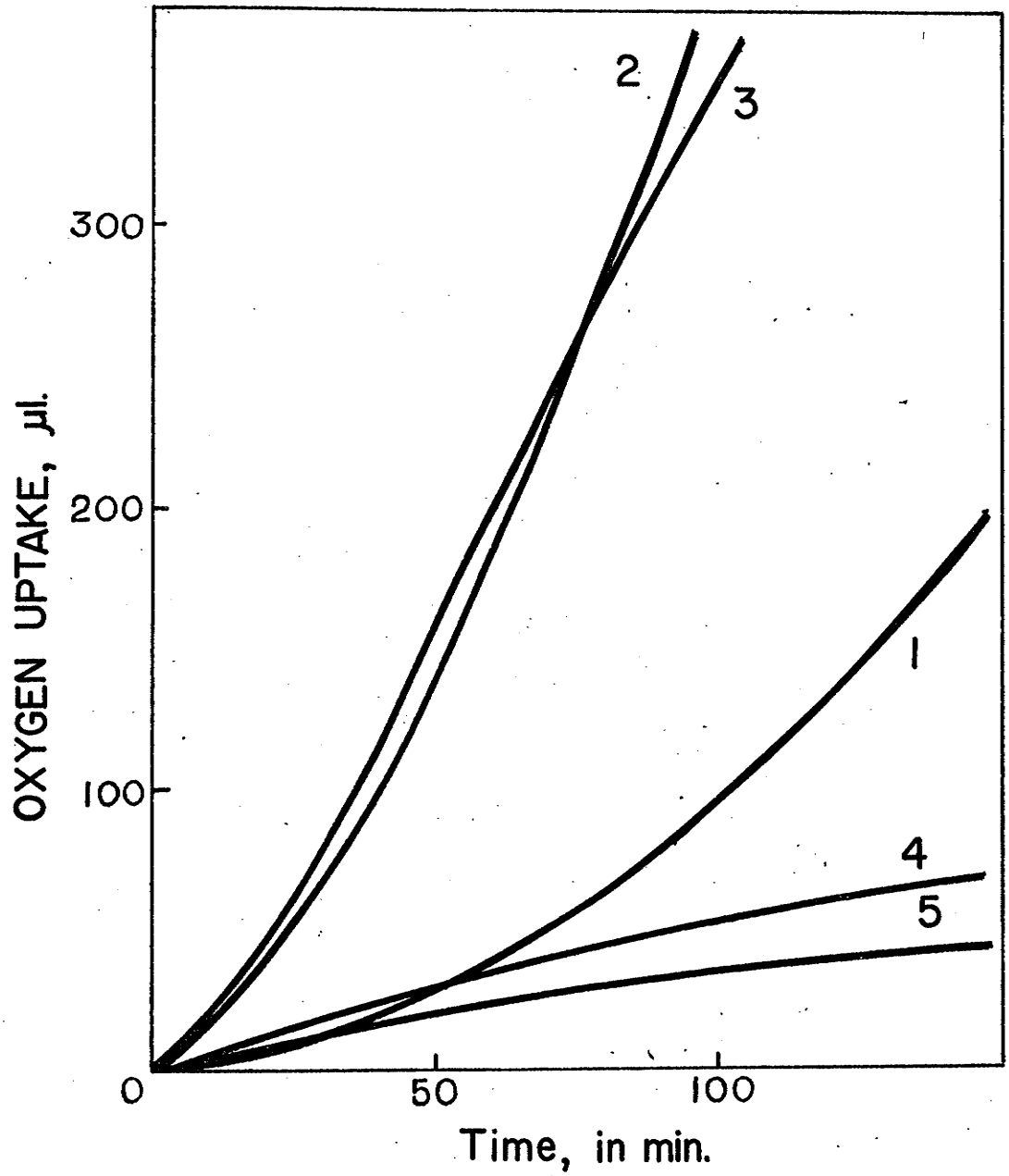

OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

Fig. 9. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown p-hydroxyphenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 100 minutes. Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. o-hydroxyphenylacetic acid
5. m-hydroxyphenylacetic acid

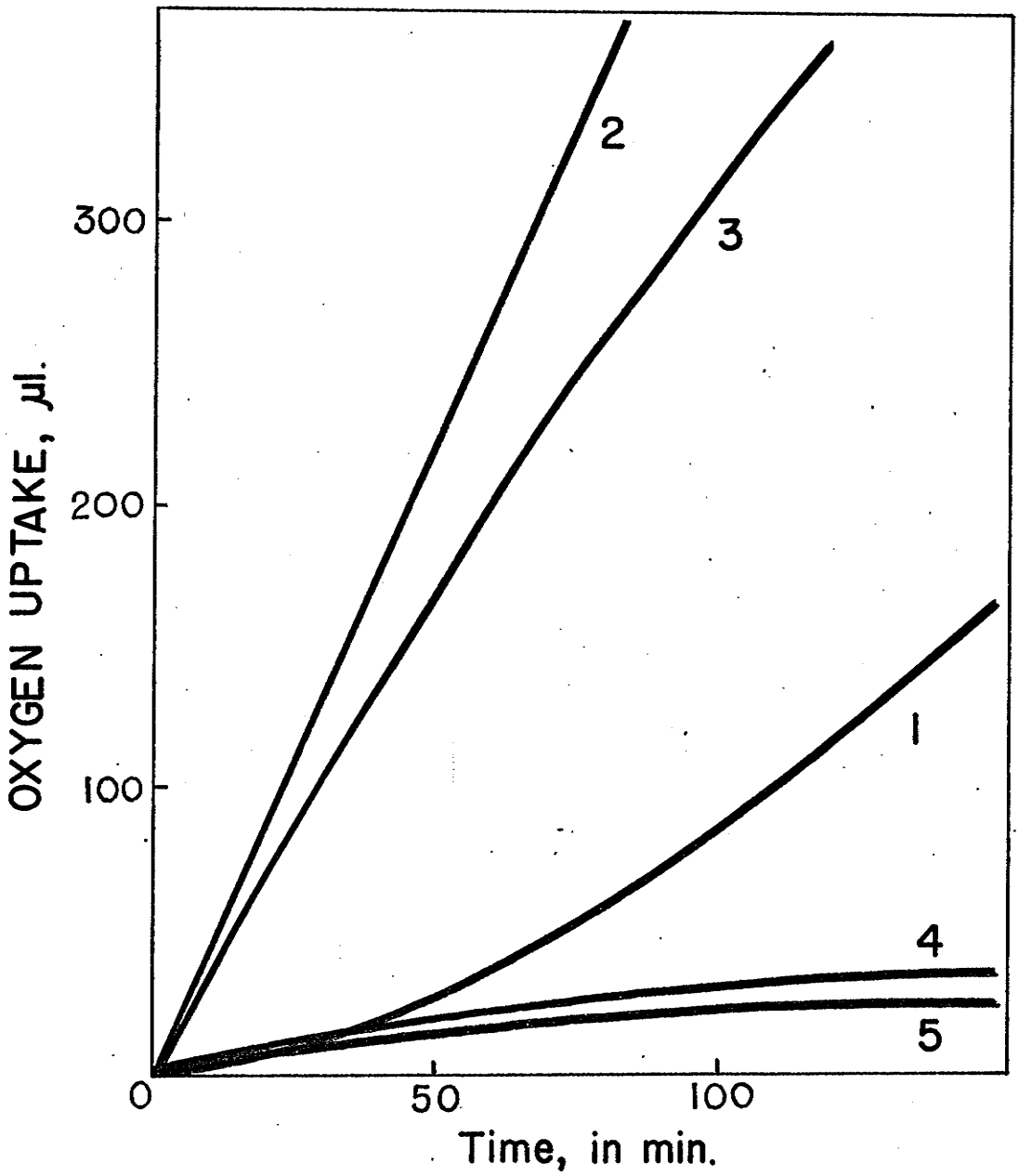
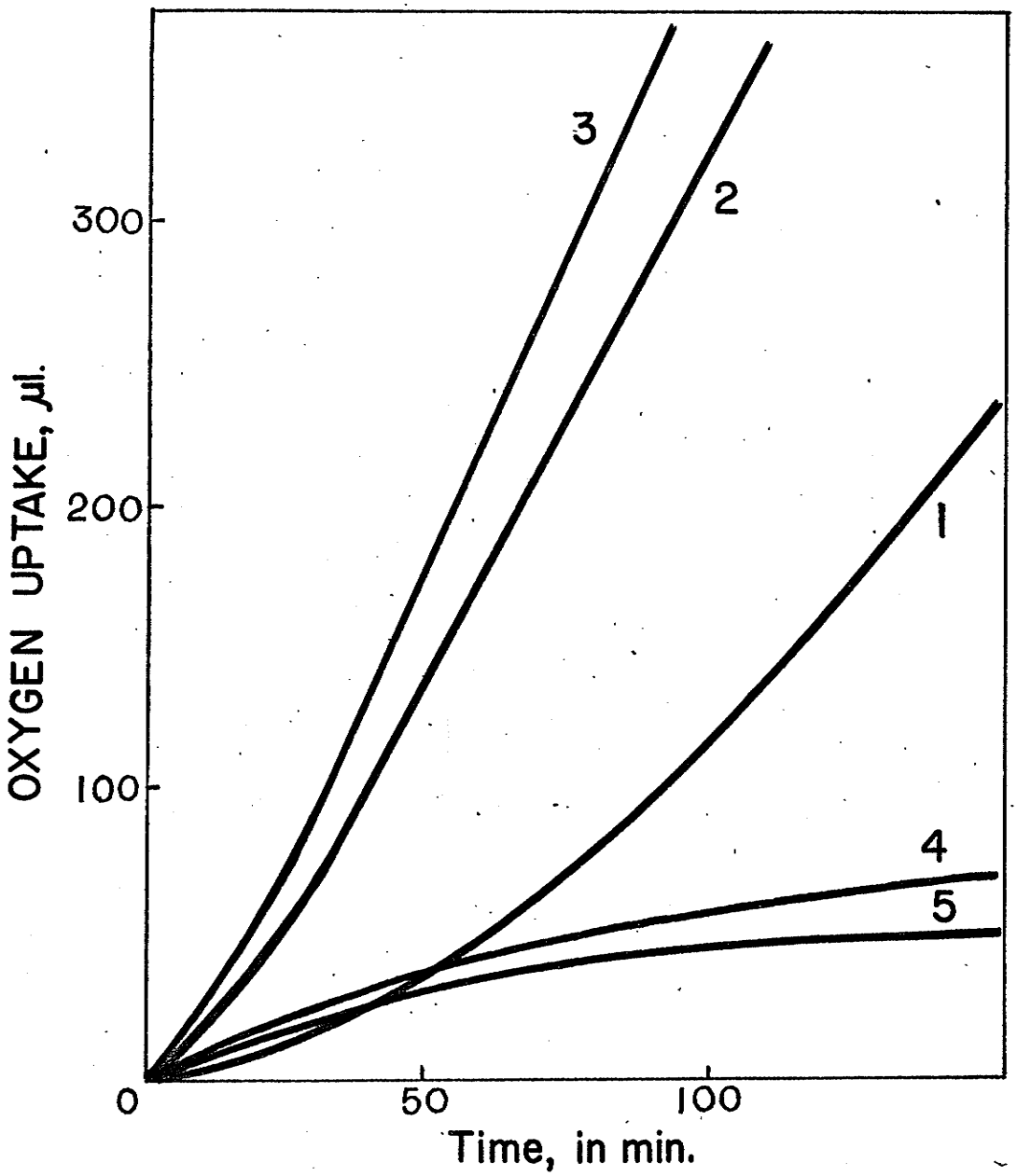

OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

Fig. 10. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown p-hydroxyphenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 200 minutes. Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. o-hydroxyphenylacetic acid
5. m-hydroxyphenylacetic acid


OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

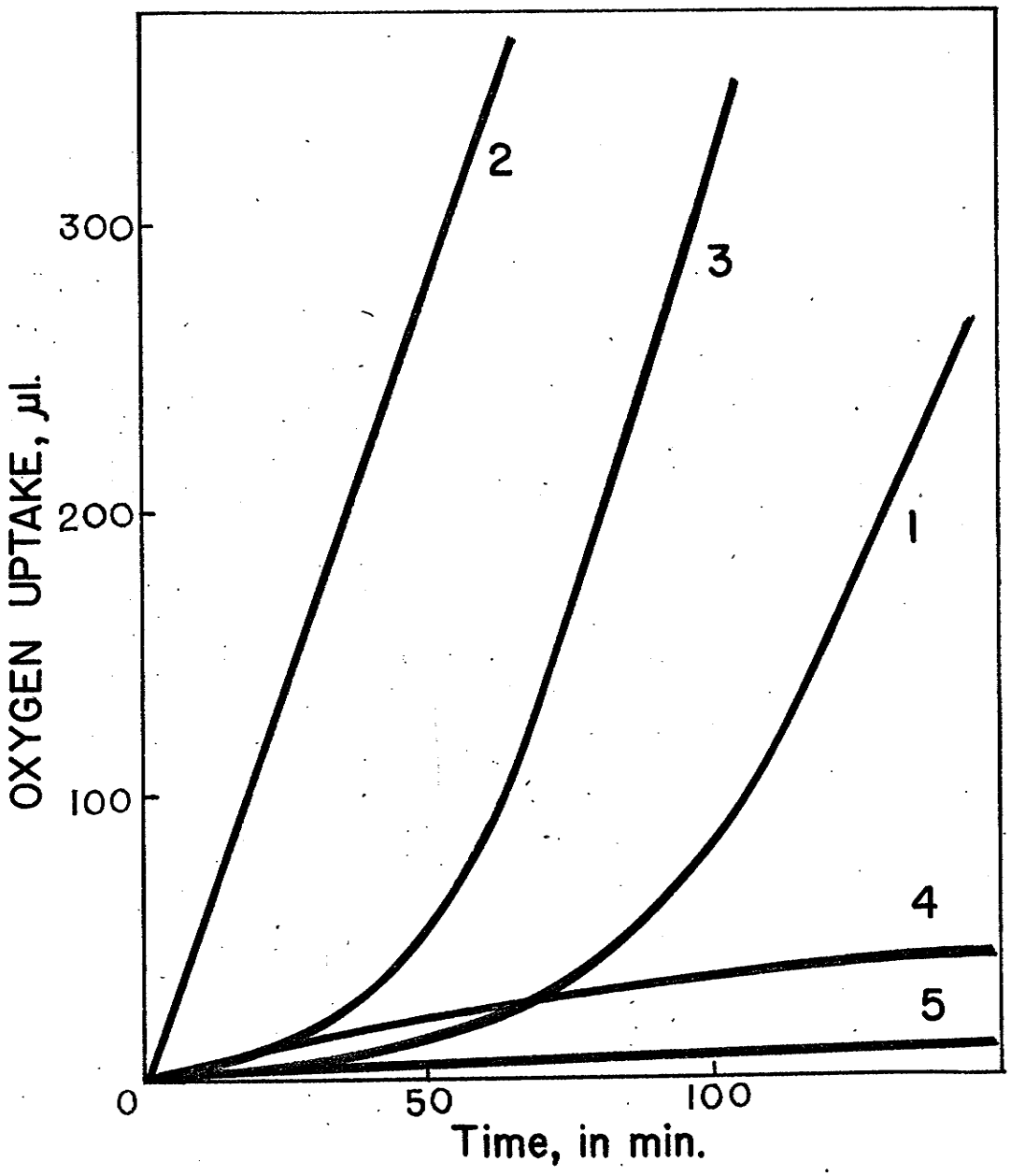

OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

Fig. 11. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown 3,4-dihydroxyphenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 50 minutes. Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. o-hydroxyphenylacetic acid
5. m-hydroxyphenylacetic acid

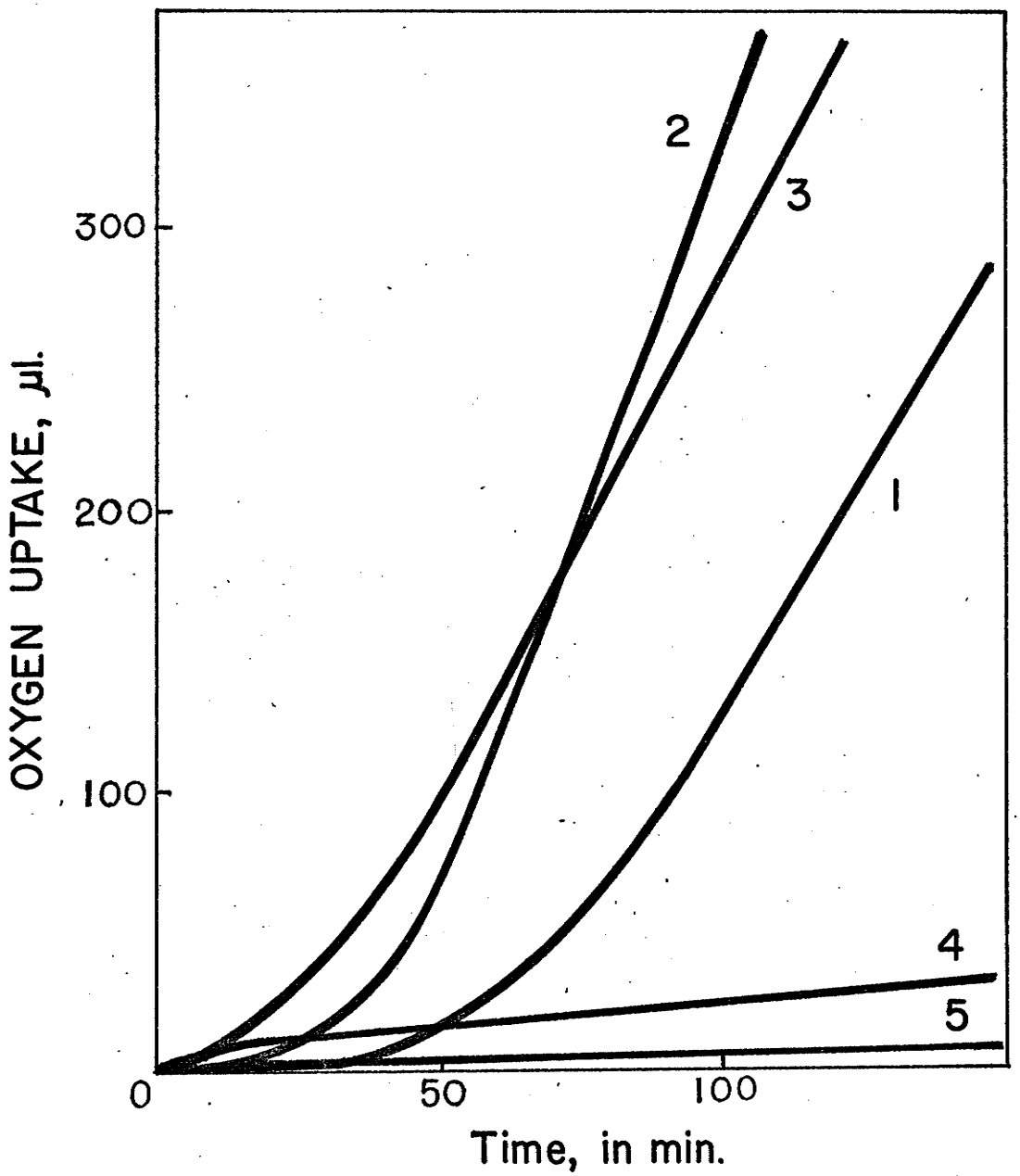

OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min.
1
2
3
4
5

Fig. 12. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown 3,4-dihydroxyphenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 100 minutes.

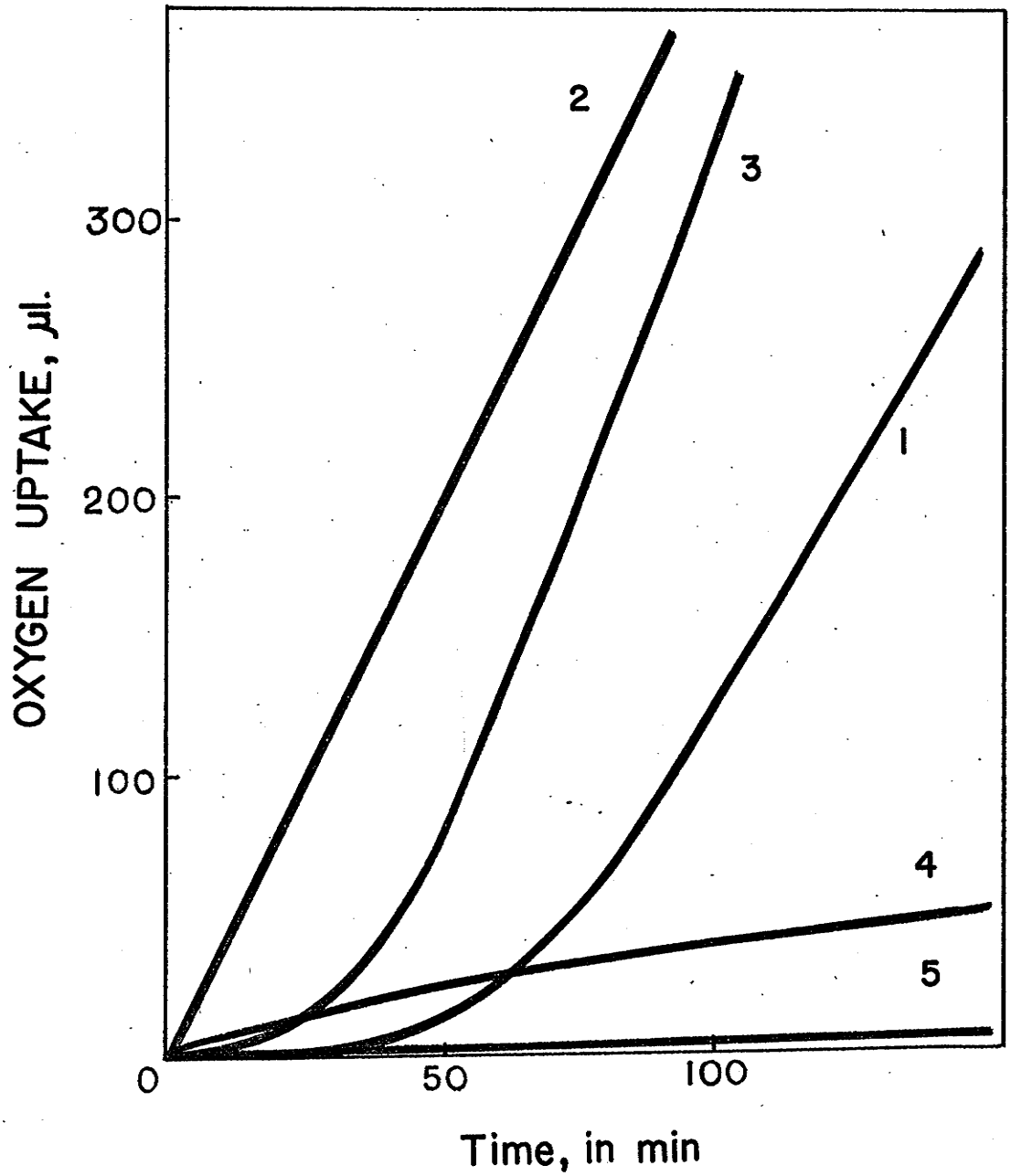
Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. o-hydroxyphenylacetic acid
5. m-hydroxyphenylacetic acid

Fig. 13. Oxidation of phenylacetic acid, p-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, o-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid by a resting cell suspension of a glucose-grown 3,4-dihydroxyphenylacetic acid-induced Pseudomonas sp. Cells were harvested after an induction period of 200 minutes.

Curves are exogenous plots representative of:

1. phenylacetic acid
2. 3,4-dihydroxyphenylacetic acid
3. p-hydroxyphenylacetic acid
4. o-hydroxyphenylacetic acid
5. m-hydroxyphenylacetic acid


OXYGEN UPTAKE, µl.
300
200
100
0
50
100
Time, in min
1
2
3
4
5

p-hydroxy- and 3,4-dihydroxyphenylacetic acid. These experiments were done to determine whether exposure time to the inducer affected the presence or formation of induced enzyme. Further, they were done to test whether 'metabolite induction' contributes to enzyme activity as a function of growth; short exposure to an inducer would be expected to preclude the production of enzymes that were dependent for formation upon the accumulation of metabolites.

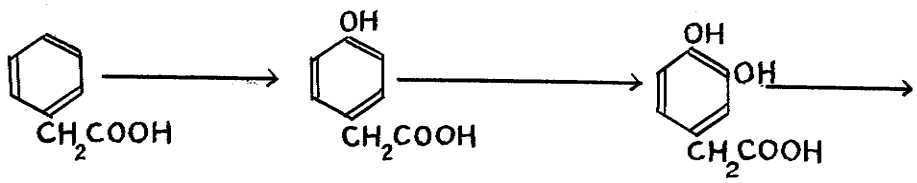
Figs. 5, 6, and 7 show the results of glucose grown cells induced toward phenylacetic acid for 50, 100, and 200 minutes respectively. The figures show that phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid were metabolized but only after a lengthy lag period. The length of the lag period was shortened as the induction increased but a close sigmoidal relationship between induction and lag reduction was not evident but this is probably a technical problem of removing the cells from the influence of the inducer after the induction period had been completed. The method is not sufficiently sensitive to compare the rates of substrate oxidation after induction has been initiated. These results and those of

subsequent figures show that o-hydroxyphenylacetic acid and m-hydroxyphenylacetic acid are not important intermediates in the oxidation of phenylacetic acid.

Figs. 8, 9, and 10, show the results when p-hydroxyphenylacetic acid was used as the inducer. It proved to be a better inducer than did phenylacetic acid after 50 minutes enzyme induction had been completed; increased exposure time did not increase the amount of activity. Phenylacetic acid was oxidized after a lag period and according to the principles of 'sequential induction' it must preceed p-hydroxyphenylacetic acid in a metabolic pathway.

Figs. 11, 12, and 13 show that 3,4-dihydroxyphenylacetic acid was not as good an inducer as p-hydroxyphenylacetic acid but better than phenylacetic acid; the induction period was between 100 and 200 minutes. Again, no significant difference was found in the amount of enzyme activity as the induction period increased.

The results of Figs. 5-13 support the metabolic pathway proposed by Blakley et al (23):



Phenylacetic acid | p-hydroxyphenylacetic acid | 3,4-dihydroxyphenylacetic acid

Further, there is no evidence to suggest that 'metabolite induction' plays a role in regulating the mechanism of phenylacetic acid decomposition; activity against p-hydroxyphenylacetic acid occurred in a time period too short to allow the accumulation of a metabolite inducer.

Partial Purification of 3,4-Dihydroxyphenylacetic Acid-2,3-Dioxygenase

A summary of the partial purification of 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase is given in Table I. The yield was 60% of the original activity of the cell free extract and a 16 fold purification was achieved.

Effect of pH on Enzyme Activity

Effect of pH on the activity of crude p-hydroxyphenylacetic acid monooxygenase is shown in Fig. 14. The crude preparation was found to have an optimum activity at

TABLE I

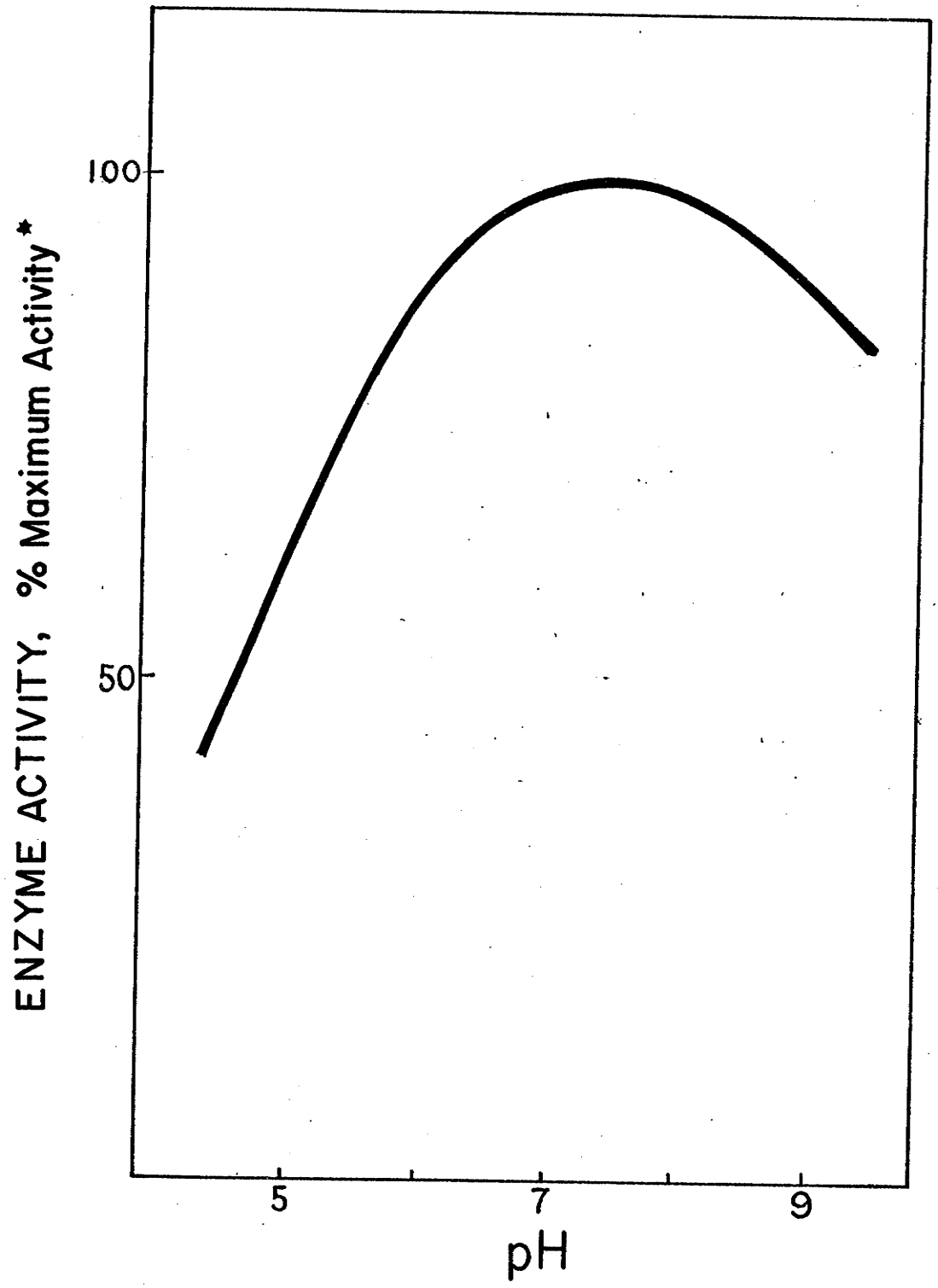
Summary of partial purification of 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase from a Pseudomonas sp.

Fractionation Step	Total Volume (ml)	Total Protein (mg)	Total Enzyme Units*	Specific Activity (units/mg protein)	Recovery %
Crude extract	20	2410	5300	2.2	100
First ammonium sulfate	18	466	4845	10.4	82
Second ammonium sulfate	16	310	4773	16.4	80
Third ammonium sulfate	10	107	3286	30.5	62
Heat treatment	9	90	3168	35.2	60

*One unit of enzyme activity was defined as the amount of enzyme which caused an O.D. increase of 0.01 in 1 min. at 380 mμ.

Fig. 14. Effect of pH on the activity of p-hydroxy-phenylacetic acid monooxygenase.

*100% activity is defined as 10 μl/min/12 mg protein.


ENZYME ACTIVITY, % Maximum Activity*
100
50
5
7
9
pH

pH 8.4. The activity decreased when the pH was altered; at a pH of 5.4 only approximately 50% of the optimum enzyme activity was retained. No attempt was made to purify the enzyme due to its instability.

Effect of pH on the activity of 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase is shown in Fig. 15. The optimum pH was 7.9; at neutral pH only 50% of the enzyme activity was retained.

Effect of o-Hydroxyphenylacetic Acid on 3,4-Dihydroxyphenylacetic Acid-2,3-Dioxygenase at Different pH Values

As shown in Fig. 16, the inhibition of o-hydroxyphenylacetic acid on 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase varied as the pH changed. At maximum velocity and at optimum pH (7.9), 4 mM of o-hydroxyphenylacetic acid gave approximately 50% inhibition; when the pH deviated from the optimum pH, the inhibition by o-hydroxyphenylacetic acid on the function of the enzyme was repressed.

Effect of o-Hydroxyphenylacetic Acid on Growth

The growth of the organism in a medium with

Fig. 15. Effect of pH on the activity of 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase. The assay contained: 0.185 mM $FeSO_5 \cdot 5H_2O$, 4 mM 3,4-dihydroxyphenylacetic acid, and 0.1 M phosphate buffer at the indicated pH.

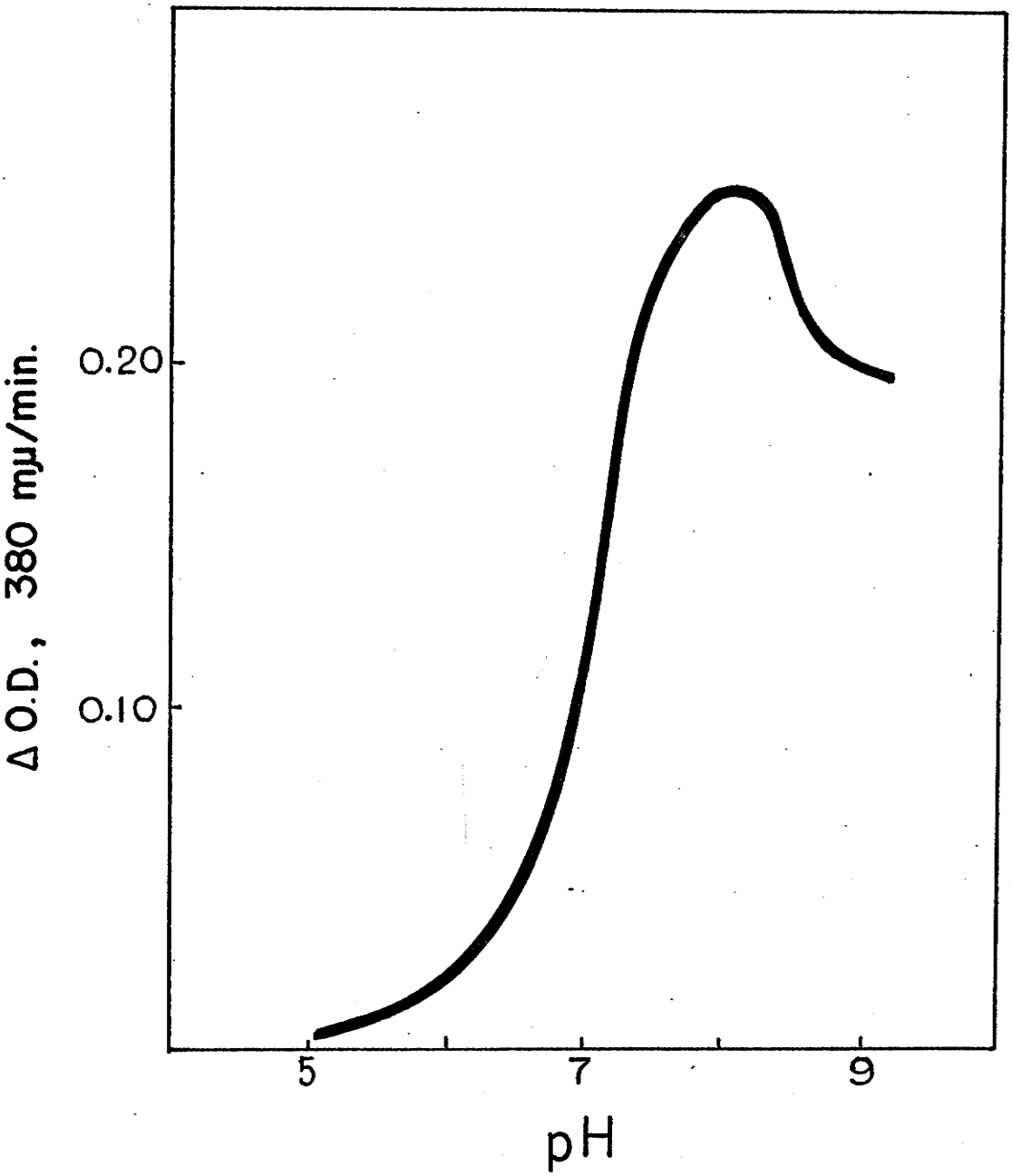

Δ O.D., 380 mμ/min.
0.20
0.10
5
7
9
pH

Fig. 16. Effect of o-hydroxyphenylacetic acid on 3,4-dihydroxyphenylacetic acid-2,3-dioxygenase at different pH values. The assays contained: 0.185 mM $FeSO_4 \cdot 5H_2O$, 4 mM o-hydroxyphenylacetic acid, 0.1 M phosphate buffer and variable amounts of substrate at three different pH values.

pH 7.9: A without inhibitor

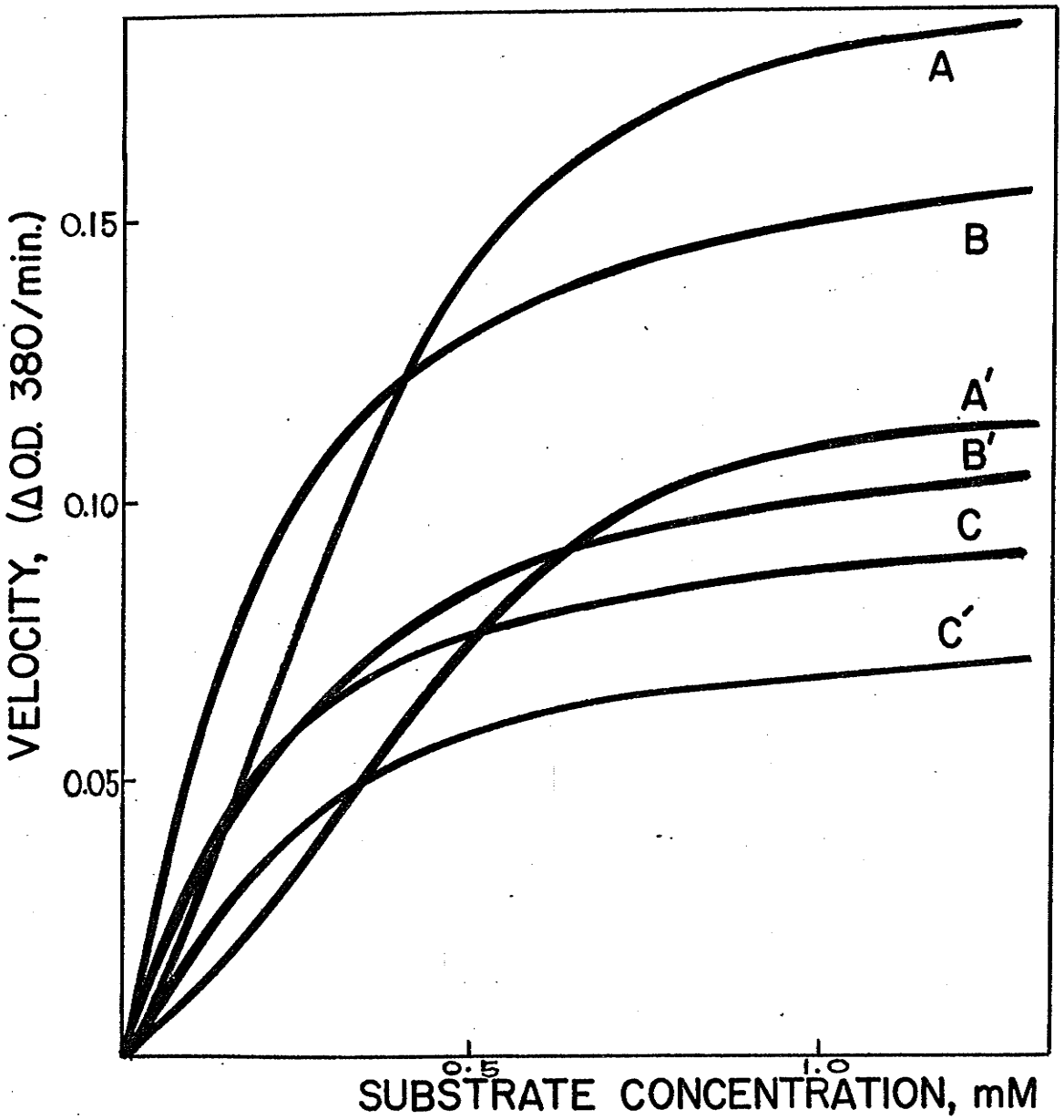
A′ with inhibitor

B without inhibitor

B′ with inhibitor

C without inhibitor

C′ with inhibitor


A
B
A'
B'
C
C'
0.15
0.10
0.05
VELOCITY, (ΔO.D. 380/min.)
0.5
1.0
SUBSTRATE CONCENTRATION, mM

phenylacetic acid as the major carbon source in the presence of o-hydroxyphenylacetic acid showed several interesting changes in activity of cells compared with cells grown in a medium without o-hydroxyphenylacetic acid. As shown in Table II, 0.01% of o-hydroxyphenylacetic acid incorporated into the medium did not affect either the cell density or the ability of resting cell suspensions to utilize phenylacetic acid, p-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid as determined by manometry. As the concentration of o-hydroxyphenylacetic acid in the medium was increased from 0.04% to 0.2%, the growth rate decreased somewhat and the activity of cells toward phenylacetic acid, p-hydroxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid was considerably repressed, particularly at the late stage of growth.

Formation of o-Hydroxyphenylacetic Acid and Phenylacetaldehyde

A gas chromatographic profile of the Tri-Sil derivatives of phenylacetic acid, o-hydroxyphenylacetic acid, phenylacetic acid, and phenylacetaldehyde is shown in Fig. 17. Both o-hydroxyphenylacetic acid and o-hydroxyphenylacetic acid and phenylacetaldehyde were

TABLE II

Inhibition of growth and oxidation of phenylacetic acid, p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid by whole cells of a Pseudomonas sp. grown on phenylacetic acid in the presence of various concentrations of o-hydroxyphenylacetic acid*

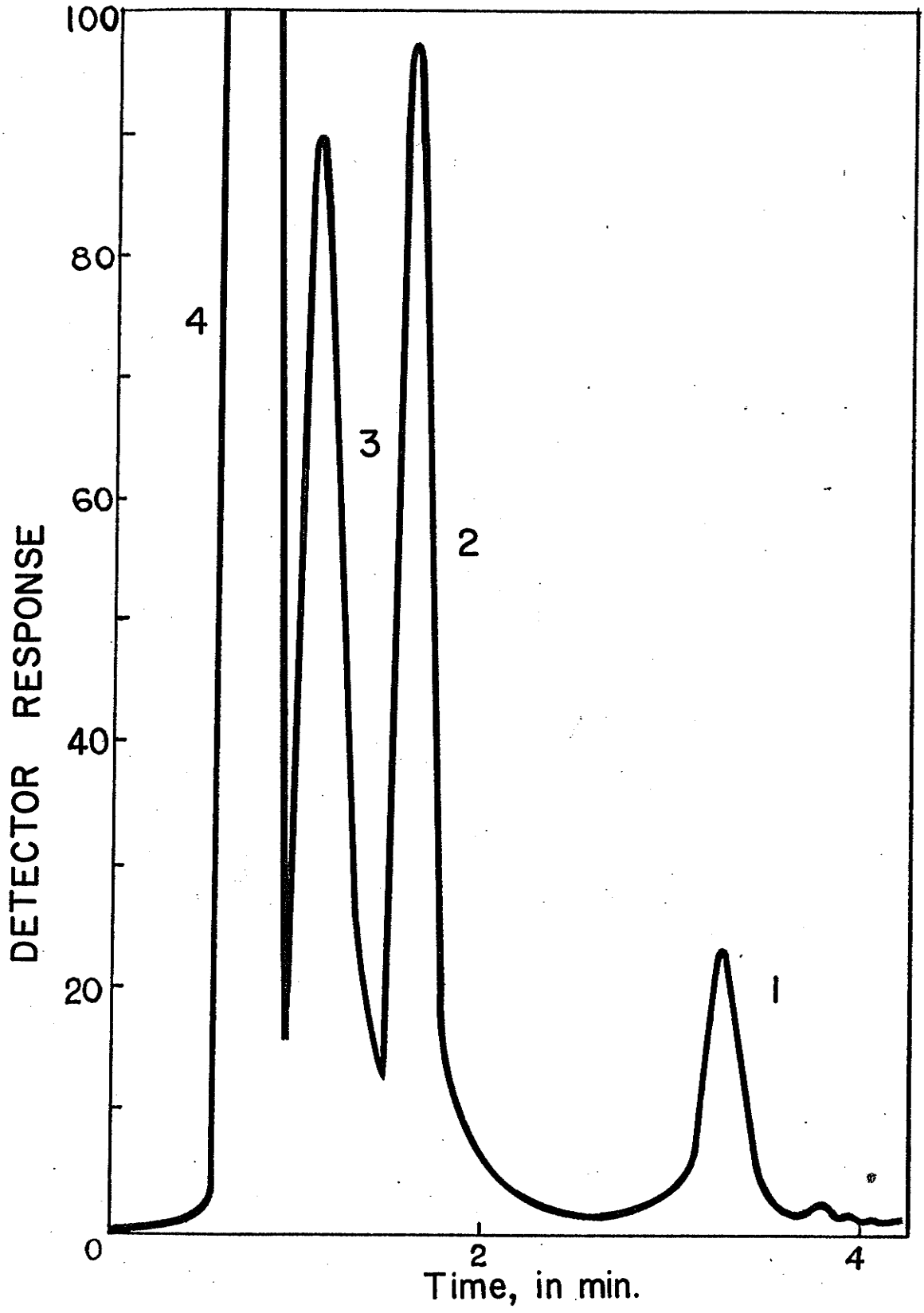
Concentration of o-hydroxyphenylacetic acid added to growth media	Age of Culture (hours)	Optical Density (Klett units)	Relative percent O_2 uptake/hr			Degree of Inhibition
			Phenylacetate	p-Hydroxyphenylacetate	3.4	
0.01%	6	48	100.0**	99.5	99.2	
	12	98	99.8	100.2	101.3	None
	21	101	99.2	97.8	98.2	
	28	108	97.3	87.8	95.2	
0.04%	6	42	101.2	83.2	59.9	
	12	68	78.3	63.9	70.3	Slight
	21	82	55.4	41.1	32.2	inhibition
	28	101	45.2	38.7	41.5	
0.12%	6	35	92.5	78.3	43.2	
	12	60	70.1	45.0	50.1	Inhibition
	21	75	41.7	38.2	48.0	
	28	101	33.4	30.1	51.9	
0.20%	6	19	97.2	78.8	58.9	
	12	31	68.4	43.5	48.6	Severe
	21	67	41.6	28.9	41.9	inhibition
	28	82	30.2	26.7	41.0	

*Initial concentration of phenylacetic acid was 0.2%.

**The oxygen uptake in 60 minutes by whole cells of a Pseudomonas sp. grown in a medium without o-hydroxyphenylacetic acid as 100%.

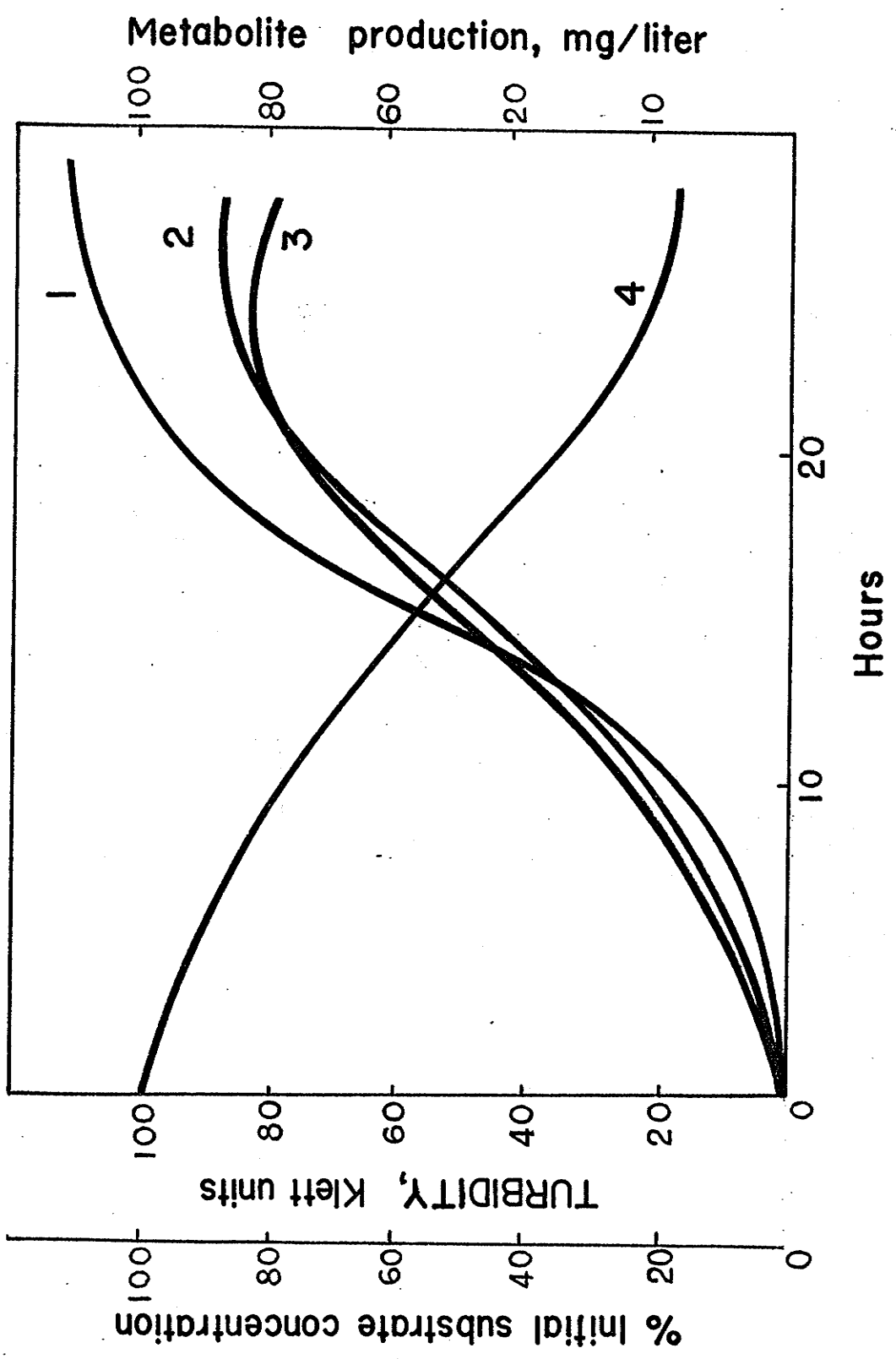
Fig. 17. Gas chromatographic profile of:

1. o-hydroxyphenylacetic acid
2. phenylacetic acid
3. phenylacetaldehyde
4. solvent


100
80
60
40
20
0
DETECTOR RESPONSE
4
3
2
1
2
4
Time, in min.

produced by the Pseudomonas sp. and were detected in fermentation liquor. The products increased in concentration as the cell density increased. The accumulation reached a maximum (approximately 85 mgm per liter for both compounds) as the culture approached the stationary phase of growth as shown in Fig. 18. m-Hydroxyphenylacetic acid and p-hydroxyphenylacetic acid were not found in any samples analyzed.

Fig. 18. Production of o-hydroxyphenylacetic acid and phenylacetaldehyde from phenylacetic acid by a Pseudomonas sp. 1. cell density, 2. o-hydroxyphenylacetic acid, 3. phenylacetaldehyde, 4. phenylacetic acid.


Metabolite production, mg/liter
100
80
60
20
10
1
2
3
4
20
10
Hours
0
20
40
60
80
100
TURBIDITY, Klett units
0
20
40
60
80
100
% Initial substrate concentration

DISCUSSION

DISCUSSION

Studies with resting cell suspensions and cell free extracts prepared from cells grown on phenylacetic acid as sole carbon source and harvested at chosen points over the entire range of the growth curve showed that enzyme activity against p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid increased as growth progressed. But the levels of enzyme activity for the degradation of p-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid did not remain constant relative to activity against phenylacetate, especially the enzyme which catalyzed the oxidation of p-hydroxyphenylacetic acid.

According to these results, variation in enzyme activity with respect to growth is caused by changes in the level of intracellular enzymes, rather than to changes in permease activity.

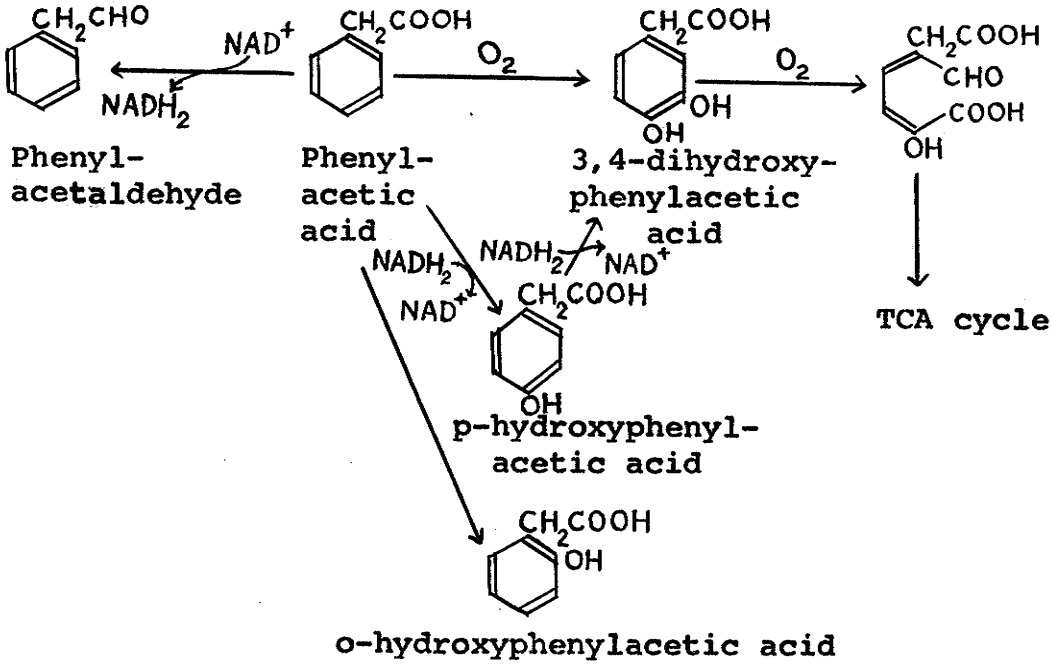
The accumulation of o-hydroxyphenylacetic acid and phenylacetaldehyde is notable. Both the cell density and the enzyme systems responsible for the degradation of phenylacetic acid are affected by the presence of o-hydroxy-phenylacetic acid on 3,4-dihydroxyphenylacetic acid-2,3-

dioxygenase can be quite pronounced. Regrettably, attempts to obtain cell free extracts active against phenylacetic acid were unsuccessful. The effect of the inhibitor upon the enzyme(s) leading to the formation of 3,4-dihydroxyphenylacetic acid would be useful in assigning a metabolic role for o-hydroxyphenylacetic acid in phenylacetate metabolism. A regulatory role is anticipated because growth was also impaired. It might play an important role in coordinate control (5, 28, 29) because inhibition failed to allow the accumulation of 3,4-dihydroxyphenylacetic acid as a metabolite which suggests that inhibition is also occurring at a site(s) leading to the production of the dihydroxy compound.

Since short exposure time to phenylacetate as an inducer did not lead to reduced activity against p-hydroxyphenylacetic acid, and since p-hydroxyphenylacetic acid never could be detected in fermentation liquor, it is certain that 'metabolite induction' does not play a regulatory role in the metabolism of phenylacetic acid by this organism. Van Caeseele reported that o-, m-, and p-hydroxyphenylacetic acids could be detected in fermentation liquor. However, the method used to identify these

'metabolites' was by paper chromatography. The spray used was a diazotized sulfanilic acid spray that is not particularly specific. It was not possible to detect m-, and p-hydroxyphenylacetic acid by the much more sensitive and specific gas chromatographic techniques employed in this study; undoubtedly they are artifacts.

A plausible mechanism to account for the results of the present study is presented below:



This scheme suggests that phenylacetic acid, at an early stage of growth, is principally converted directly to 3,4-dihydroxyphenylacetic acid without involving a

monohydroxylated intermediate. This mechanism is operative independent upon the presence of NADH + H^+ and is because of the absence (or limited availability) of NADH + H^+. The mechanism, therefore, acts to conserve energy during the demanding energy requirements of the early logarithmic phase of growth. At the exponential or late logarithmic phase of growth, an active TCA cycle relieves the conservation of energy mechanism and the metabolic pathway proceeds via the branch containing p-hydroxyphenylacetic acid which does involve NADH + H^+ no longer present in limiting supply. The regeneration of NAD^+ can occur at the expense of phenylacetate to yield phenylacetaldehyde (detected as a metabolite) or it can be regenerated by alternate mechanisms involving the TCA cycle. In addition, it is tempting to speculate that o-hydroxyphenylacetic acid acts as a catabolite repressor which plays a role in economizing the energy demands of the cell by controlling the supply of intermediates that enter the TCA cycle.

REFERENCES

REFERENCES

1. Adachi, K., Takeda, and Senoh, S., Oxidation of p-hydroxyphenylacetic acid by Pseudomonas sp. Biophys. Acta, 93, 438-442 (1964).

2. Blakley, E. R., Halvorson, H., and Kurz, W., The microbial production and some characteristics of δ-carboxymethyl-α-semialdehyde. Can. J. Microbiol. 13, 157-165 (1967).

3. Blakley, E. R., Kurz, W., Halvorson, H., and Simpson, F. J., The metabolism of phenylacetic acid by a Pseudomonas. Can. J. Microbiol. 13, 147-157 (1967).

4. Cain, R. B., Induction of anthranilate oxidation system during the metabolism of o-nitrobenzoate by certain bacteria. J. Gen. Microbiol. 42, 197-217 (1966).

5. Canóvas, J. L., Ornston, L. N., and Stanier, R. Y., Evolutionary significance of metabolic control systems. Science, 150, 1697-1702 (1967).

6. Dagley, S. and Wood, J. M., Oxidation of phenylacetic acid by Pseudomonas. Biochim. Biophys. Acta, 99, 383-385 (1965).

7. Dagley, S. and Stopher, D. A., A new mode of fission of the benzene nucleus by bacteria. Biochem. J. 73, 169-173 (1959).

8. Dagley, S., Evans, W. C., and Ribbons, D. W., New pathways in the oxidative metabolism of aromatic compounds by microorganism. Nature, 188, 560-566 (1960).

9. Dawson, P.S.S., The theory of simultaneous adaptation as a method for the analysis of metabolic pathways. Can. J. Microbiol. 11, 893 (1965).

10. Dawson, P.S.S., Continuous phased growth with a modified chemostat. Can. J. Microbiol. 9, 671 (1963).

11. Dawson, P.S.S., The intracellular amino acid pool of Canidia utilis during growth in batch and continuous flow culture. Biochim. Biophys. Acta, 111, 51 (1965).

12. Evans, W. C., The microbiological degradation of aromatic compounds by yeast. Nature, 195, 473-474 (1962).

13. Evans, W. C., Oxidation of phenol and benzoic acid by some soil bacteria. Biochem. J. 41, 373-382 (1947).

14. Gibson, D. T., J. R. Koch, C. L. Schuld, R. E. Kallio, Oxidative degradation of aromatic hydroxycarbons by microorganisms. I. Enzymatic formation of catechol

from benzene. Biochem. J. 7, 2653-2661 (1968).

15. Harris, G. and Ricketts, R. W., Metabolism of phenolic compounds by yeast. Nature, 195, 473-474 (1962).

16. Hayaishi, O. and Stanier, R. Y., The oxidation of tryptophan. III. Enzymatic activities of cell free extracts of bacteria employing the aromatic pathway. J. Bacteriol. 62, 690-709 (1951).

17. Hayaishi, O., In "Oxygenase". Edited by Hayaishi, O., Academic Press, New York, pp. 180-195 (1962).

18. Hiroshi, Kita., The hydroxylation of p-hydroxyphenyl-acetic acid by a Pseudomonas sp. J. Biochem. 58 116-123 (1965).

19. Kornberg, H. L., The role of acetic acid in isocitrate lyase induction. Biochim. Biophys. Acta, 73, 517-519 (1963).

20. Kornberg, H. L. and Elsden, S. R., The metabolism of 2-carbon compounds by microorganisms. Advan. Enzymol. 23, 401-470 (1961).

21. Kornberg, H. L., The role and control of the glycoxylate cycle in E. coli. Biochem. J. 99, 1-11 (1966).

22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the folin

phenol reagent. J. Biol. Chem. 193, 265-275 (1951).

23. Lack, L., Enzymatic oxidation of gentisic acid. Biochim. Biophys. Acta, 34, 117-124 (1959).

24. Marr, E. K. and Stone, R. W., Bacterial oxidation of benzene. J. Bacteriol. 3, 425-430 (1961).

25. Macleod, R. A., Personal communication.

26. Nishizukam, Y., Ichiyama, A., Nakamura, S., and Hayaishi, O., A new metabolic pathway of catechol. J. Biol. Chem. 237, 268-274 (1962).

27. Ornston, L. N. and Stanier, R. Y., The conversion of catechol and protocatechuate to β-ketoadipate by Pseudomonas putida. I. Biochemistry of the protocatechuate pathway. J. Biol. Chem. 241, 3776-3784 (1966).

28. Ornston, L. N., The conversion of catechol and protocatechuate to β-ketoadipate by Pseudomonas putida. II. Enzymes of the protocatechuate pathway. J. Biol. Chem. 241, 3787-3795 (1966).

29. Proctor, M. H. and Scher, S., Decomposition of benzoate by photosynthetic bacteria. Biochem. J. 76, 33 (1960).

30. Sleeper, B. P. and Stanier, R. Y., The bacterial oxidation of aromatic compounds. J. Bacteriol. 59, 117-127 (1950).

31. Stanier, R. Y. and Ingraham, J. L., Protocatechuic acid oxidase. J. Biol. Chem. 210, 799-808 (1957).

32. Stanier, R. Y., Sleeper, B. P., Tsuchida, M. and Macdonald, D. L., The bacterial oxidation of aromatic compounds. III. The enzymatic oxidation of catechol and protocatechuic acid to β-ketoadipic acid. J. Bacteriol. 59, 137-151 (1950).

33. Stanier, R. Y., The oxidation of aromatic compounds by fluorescent Pseudomonas. J. Bacteriol. 55, 477-493 (1948).

34. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric Techniques, Burgess Publishing Co., Minneapolis (1957).

35. Van Caeseele, L. A., M. Sc. Thesis, Studies on the metabolism of phenylacetic acid by a Pseudomonas. Changes in induced enzyme activity as a function of growth. Univ. of Manitoba (1966).

36. Wegener, W. S. and Romano, A. H., Control of malate synthetase formation in Rhizopus nigricans. J. Bacteriol. 87, 156-161 (1964).

37. Warner, S. W. and Schell, J. E., Control of malate synthetase formation in Rhizopus nigricans. J.

Bacteriol. 94, 1851-1956 (1967).

38. Wheelis, M. L., Palleroni, N. J., and Stanier, R. Y., The metabolism of aromatic acids by Pseudomonas testosteroni and P. acidovorans. Archiv. für Mikrobiologie, 59, 302-314 (1967).