STUDIES ON THE METABOLISM OF PHENYLACETIC ACID BY A *PSEUDOMONAS*: CHANGES IN INDUCED ENZYME ACTIVITY AS A FUNCTION OF GROWTH

by

Lawrence Van Caeseele

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

August, 1966

THE UNIVERSITY OF MANITOBA ELIZABETH DAFOE LIBRARY

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr. H. Halvorson, Department of Microbiology, University of Manitoba for his continued guidance throughout the course of this investigation and in the preparation of the manuscript.

Appreciation is also extended to Dr. I. Suzuki for his helpful discussions throughout this study.

ABSTRACT

The induced enzyme activity of a Pseudomonas sp. grown on phenylacetic acid as the sole carbon source was found to be a function of growth. Studies carried out at various cell densities over the entire range of the growth curve showed that activity toward phenylacetate and 3,4-dihydroxyphenylacetate was consistently high while activity toward the monohydroxylated derivatives of phenylacetate increased as the cell density increased.

The induced enzyme systems responsible for the oxidative degradation of phenylacetate and its hydroxylated derivatives were stable for at least 8 days when cells were stored in a packed state at 4 ^{o}C. After 16 days storage under the same conditions the cells showed no activity toward the monohydroxylated derivatives of phenylacetate but still retained the ability to form induced enzymes anew.

o-Hydroxyphenylacetic acid, m-hydroxyphenylacetic acid and p-hydroxyphenylacetic acid were detected in the fermentation liquor of cells grown on phenylacetate during an early stage of growth but only o-hydroxyphenylacetic acid was detectable in the fermentation liquor at a late stage of growth.

Interestingly, when the organism was grown with decreasing initial phenylacetate concentrations the time required for the organism to enter the logarithmic phase of growth was reduced. As expected, larger cell yields were obtained when the initial phenylacetate concentration was increased.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Cain studied the oxidation of o-nitrobenzoate in Nocardia opaca (7) and found that this substrate was oxidized through catechol. Anthranilate detected in fermentation liquor during early stages of growth proved to be a product of the metabolism of o-nitrobenzoate. Interestingly, accumulation of anthranilate reached a threshold level where it served as an inducer for the production of anthranilate oxidase. This phenomenon was termed "metabolic induction" by Cain. Differences in enzyme activity of o-nitrobenzoate grown cells toward anthranilate were observed and were found to be directly related to the anthranilate oxidase content of the cell.

Benzoate is also metabolized by induced enzymes and Donawa, while studying the oxidation of benzoate by Pseudomonas convexa (12), found that in cells grown on salicylate benzoate oxidase was more labile than salicylate oxidase. This suggested that in cells containing a series of induced enzymes, some are more labile than others.

While studying the oxidation of phenylacetic acid by a Pseudo monas sp., Blakely et al (3) observed differences in induced enzyme activity from one preparation to another toward the monohydroxylated derivatives of phenylacetate. A satisfactory explanation for this phenomenon was not offered. This study was undertaken in an attempt to determine whether the differences in activity were due to changes in the enzyme content of the cells as growth of the organism progressed or whether it was due to the stability of the enzyme systems involved.

HISTORICAL

Phenylacetic acid and its hydroxylated derivatives occur rarely in animal, plant and microbial systems and are usually found in association with other aromatic structures. In man, a number of clinical conditions cause an accumulation of the hydroxylated derivatives of phenylacetate where they would not occur under natural conditions. 2,5-Dihydroxyphenylacetate was found in the urine of humans suffering from alkaptonuria and phenylketonuria (18). Aromatic acids may also play a role in some mental illnesses. p-Hydroxyphenylacetic acid has been found in abnormal amounts in the urine of patients suffering from simple or paranoid schizophrenia; its source is probably from the abnormal metabolism of tyrosine caused by this condition (6). Though the role of phenylacetate and its hydroxylated derivatives in plants is unknown, o-hydroxyphenylacetate has been detected in the leaves of three species of Astible (22) and p-hydroxyphenylacetate has been found in the roots of the dandelion, Taraxacum officinale (17). Since phenylacetic acid is a moiety of penicillin G it is added to the growth medium of moulds during the commercial production of the antibiotic (27). o-Hydroxyphenylacetate has been detected in the mother liquor of some Penicillium sp. under natural conditions (1) while p-hydroxyphenylacetate is a cellular constituent of some yeasts (17). As an example of the diversity of situations under which this series of compounds is found, 3,4-dihydroxyphenylacetic acid along with other aromatic acids has been shown to serve as a substrate for cuticle hardening processes in some arthropods (15).

The breakdown of complex organic compounds produced by plants

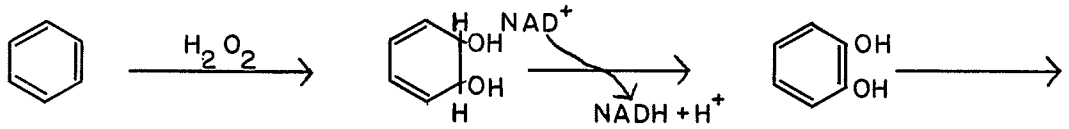
and animals is mandatory for the release of carbon, hydrogen, oxygen and nitrogen that would otherwise exist only in a bound form. When aromatic compounds are fed orally to animals, rarely are transformations of the benzenoid structure carried out to obtain energy. On the contrary, many aromatic compounds are extremely toxic to mammalian tissue and transformations exerted by animals are principly ones of detoxication. For example, when rabbits were fed phenol orally, catechol and hydroquinone were isolated from the urine (13). Mammals do possess enzymes to cleave the aromatic nucleus for a preparation of liver enzymes from a mammalian source was able to convert 3-hydroxyanthranilic acid to a straight chain semialdehyde (44, 26).

Plants are able to transform some simple aromatic compounds. Salicylate was detected as a product of benzoate metabolism by etiolated Helianthus hypocotyls (23) and other plants have been shown to convert benzoate not only to salicylic acid but also to gentisic acid, 2,3-dihydroxybenzoic acid and p-hydroxybenzoic acid (21).

The principle agents responsible for the breakdown of complex aromatic compounds are bacteria, yeasts and fungi. Some members of the families Coccaceae (39), Mycobacteriaceae (25), Pseudomonadaceae (25), Spirillaceae (16), Bacteriaceae (41) and Bacillaceae (41) are active metabolizers of aromatic compounds while yeasts such as Candida and Saccharomyces (18) are able to oxidize some simple aromatic compounds. Many fungi are able to attack a wide variety of simple and complex compounds (14).

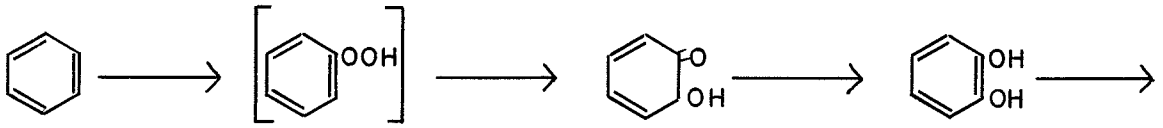
It has been known since 1913 that microorganisms are able to oxidize benzene (34) but the precise mechanisms by which the energy

associated with aromatic compounds is made available to microbial systems have been proposed only in recent years. Marr and Stone found that benzene could be used as a sole source of carbon by a Pseudomonas sp. and a Mycobacterium sp. (25) and they suggested that this compound is oxidized according to the following mechanism:



benzene — 3,5-cyclohexadiene-1,2-diol — catechol

This scheme proposes the simultaneous introduction of two hydroxyl groups into the benzene nucleus. A model involving the sequential introduction of single hydroxyl groups was proposed by Wieland et al for the oxidation of benzene by a Nocardia sp. (19). This scheme is shown below:

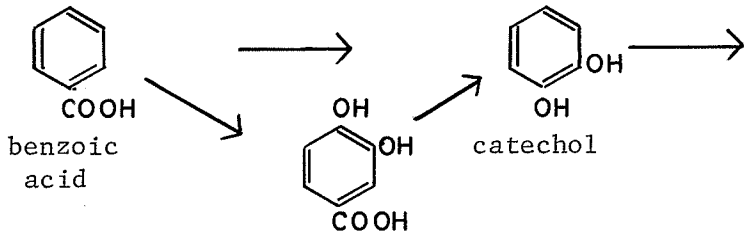


benzene — catechol

The mechanism proposed for catechol formation from benzene in different biological systems are at variance and require further study. While the fate of catechol is known an exhaustive review of aromatic ring cleavage is beyond the scope of this study and the reader is referred to the concise review by Evans (14) of the known mechanisms for the cleavage of aromatic compounds.

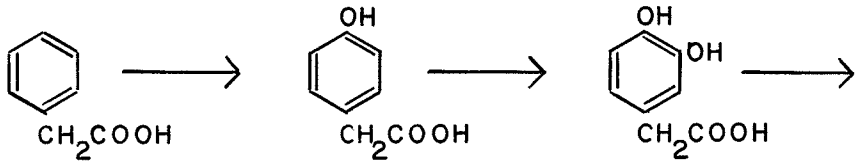
When one carboxyl group is introduced into the benzenoid ring

to form benzoic acid, the degradation of the aromatic moiety by bacteria may occur by one of several pathways. For example, benzoic acid may undergo either a simultaneous oxidative decarboxylation and hydroxylation to form catechol (12, 36) or it may be degraded via 3,4-dihydroxybenzoic acid (30) as shown below:

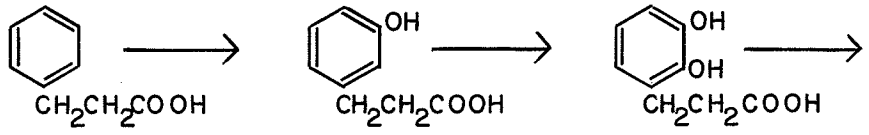


benzoic acid — catechol — 3,4-dihydroxybenzoic acid

There is evidence that side chain length plays an important role in determining the method by which the aromatic moiety of a molecule will be oxidized by microorganisms. Blakley et al (3) have suggested that a Pseudomonas strain 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 — 2,3-dihydroxyphenylpropionic acid

While advances have been made in resolving the degradation of aromatic compounds, there still exist many unsolved problems associated with the metabolic fate of these relatively simple structures.

In the majority of cases studied to date, the enzyme systems responsible for attacking the aromatic moiety are of the induced type. Enzyme induction or enzymic adaptation are equivalent terms used to describe the synthesis of an enzyme or series of enzymes required for the alteration of a specific inducing molecule. This occurs without a genotypic change in the organism as was proven by Marr and Stone who showed that a strain of Pseudomonas aeruginosa was able to utilize benzene as a substrate by induction rather than mutation when growth of equal numbers of organisms on solid medium with benzene as the sole carbon source and on nutrient agar demonstrated that essentially all the organisms were capable of oxidizing benzene (25). Also, Benzer (2) has shown that induced β-galactosidase was formed at approximately the same rate in most cells of a population of Escherichia coli demonstrating induction rather than mutation.

The synthesis and breakdown of enzymes is a continuous process in every living organism. Even proteins which are stable "in vitro" for lengthy periods of time exist in a condition of dynamic equilibrium "in vivo" and the catabolic removal of intracellular enzymes is reversed by the anabolic activities of the cell. This has been demonstrated by Schoenheimer et al (32) with studies on the incorporation of labelled amino acids into protein. Recent results of isotopic experiments (11) have shown that the half-life of liver proteins in animals is 2 to 4 days.

Mandelstam (24) has shown that in nongrowing cells of E. coli there is a turnover of protein through free amino acids in the cell at the substantial rate of 4 to 5% per hour. The proteins involved in this study were constituitive enzymes. Induced penicillinase from growing Bacillus cereus was broken down at a rate of approximately 1.5% per hour (43) indicating that induced enzyme protein, too, is subject to resynthesis. In most cases of enzyme induction the concentration of induced enzyme returns to the basal level immediately after the inducer is removed from the environment. In growing cells, the induced enzyme activity is lost because the protein is divided equally among daughter cells until dilution causes its apparent disappearance (31). Nongrowing cells exhibit a decrease in induced enzyme activity by one of several mechanisms ranging from simple protein turnover to complicated mechanisms such as enzyme interaction (29).

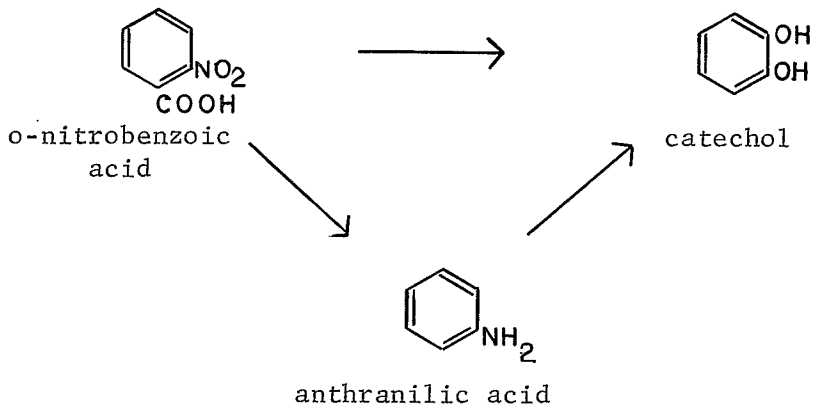
The stability of induced enzymes in the nongrowing state has received little attention. Donawa, while studying the oxidation of benzoate, found that cells grown on salicylate induced enzymes to degrade both benzoate and salicylate but upon storage, the activity toward benzoate disappeared at a higher rate than the activity toward salicylate (12).

Enzyme induction can occur in the presence of minute amounts on the inducer molecule and the quantity of induced enzyme synthesized bears no relation to the amount of inducer present (9).

If a substrate causes the induction of an enzyme, and the product of their interaction induces a second enzyme, a sequence of induced enzymes results. This was demonstrated by Hockenhull and

Herbert when Clostridium acetobutylicum grown on maltose induced maltase alone but when it was grown on starch, it induced both amylase and maltase (20). The significance of sequential induction was first realized by Stanier (35) and by Suda et al (39). Working independently, they showed that by demonstrating the presence of individual induced enzymes it was possible to elucidate metabolic pathways. This method is referred to as the "sequential induction" technique.

The sequential induction technique was used by Cain to study the oxidative degradation of o-nitrobenzoate by Nocardia opaca (7). The organism oxidized it directly to catechol but at the same time reduced small amounts of it to anthranilate as illustrated below:



The anthranilate was excreted into the medium and accumulated there until it reached a level where it became utilizable as a substrate. Therefore, cells that were grown on o-nitrobenzoate displayed activity toward anthranilate and application of the criteria of the sequential induction technique could not exclude anthranilate from the metabolic pathway for the degradation of o-nitrobenzoate. Cain termed this phenomenon "metabolite induction".

Of major significance was a marked variation in activity of whole cells toward anthranilate after they were grown on o-nitrobenzoate. This variation appeared to depend upon the time at which the organism was harvested with respect to growth.

Blakley et al, when studying the oxidation of phenyl-acetic acid by a Pseudomonas sp., found that cell free extracts prepared from cells grown on phenylacetate displayed variations in induced enzyme activity toward the monohydroxylated derivatives of that compound. This study was undertaken in an attempt to determine if the fluctuation was due to changes in the enzyme content of the cells as the growth of the organism progressed or whether the fluctuation was due to stability of the enzymes involved in the oxidative process.

MATERIALS AND METHODS

Culture

The organism used throughout this study was a *Pseudomonas* sp. isolated from soil by the enrichment culture technique. It was kindly provided by Dr. E.R. Blakley, Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan. This organism can utilize many aromatic compounds including phenylacetic acid as the sole source of carbon and energy.

Maintenance of Culture

The culture was maintained on Brain Heart Infusion (BBL) agar slants. From liquid suspension, the organism was streaked onto the surface of the agar, incubated for 24 hours at 28 ^{o}C and stored at 4 ^{o}C until required.

Growth Medium

An aqueous mineral salts solution of the following composition was used routinely:

K_2HPO_4	0.1%
KH_2PO_4	0.1%
NH_4NO_3	0.1%
$MgSO_4.7H_2O$	0.002%
$CaCl_2.2H_2O$	0.002%
$FeCl_3$	0.0003%

The salts were dissolved in distilled water and the solution was heated to 121 °C and then allowed to cool. A precipitate which formed was filtered from the solution and discarded. The mineral salts solution was supplemented with either 0.4% glucose or 0.4% phenylacetate as the carbon source and the pH was adjusted to 6.8 with NaOH. When phenylacetic acid was used as the carbon source it was added to the mineral salts solution before sterilization; when glucose was used as the carbon source it was sterilized in aqueous solution and added asceptically to sterile mineral salts solution. In the case of carboys containing mineral salts solution or mineral salts solution supplemented with phenylacetate, sterilization was achieved by autoclaving at 15 pounds pressure for 1 hour at 121 °C. The glucose solution was sterilized by autoclaving at 15 pounds pressure for 15 minutes at 121 °C.

Preparation of Inocula

Inocula were prepared by incubating the culture at 28 °C for 24 hours in 100 ml of growth medium in 300 ml 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 0.4% glucose.

Growth of Cells for Manometric Studies

To obtain a large amount of cells for manometric studies cells were grown in 15 litre carboys containing 10 litres of sterile growth medium and phenylacetate as substrate. After the addition of the total contents of an Erlenmeyer flask containing fresh inoculum the culture was

incubated at 28 ^{o}C with forced aeration. Air entering the carboy was sterilized by passage through a cotton filter and dispersed in the medium by means of fritted glass dispersion tubes. Growth was followed by measuring the cell density using a Klett-Summerson Photoelectric Colorimeter equiped with a KS-54 filter. As growth reached approximately the midpoint of the logarithmic phase, extensive foaming developed. A small amount of Silicone Antifoam (Canadian General Electric)was added to the medium at the start of the logarithmic phase to prevent this undesirable growth feature.

Manometric Experiments

Effect of Growth on Induced Enzyme Activity

In order to determine the effect of culture growth on induced enzyme activity, cells were grown with phenylacetate as the sole source of carbon and individual carboys were harvested when growth had reached a cell density of roughly 20, 40, 60, 80, 100, 120 and 140 Klett units. These values were selected arbitrarily as points distributed over the entire range of the growth curve. The organism was harvested by means of a steam driven Sharples Super Centrifuge at a speed of 20,000 revolutions per minute (20 pounds per square inch steam pressure), washed once in 0.1 M sodium phosphate buffer pH 6.8, resuspended in the same buffer and the cell density adjusted so that a 1:20 dilution of the suspension gave a reading of 340 Klett units using a KS-54 filter. Resting cell suspensions were prepared from the cells of each carboy and the activity of these preparations toward phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate

was measured immediately by manometric methods.

Manometric experiments were carried out in a Bronwill Warburg Respirometer at 30 ^{o}C with air as the gas phase using standard manometric techniques (42). Each flask contained: 0.2 ml 20% KOH and a small fluted filter paper in the center well; 4.0 μmoles substrate in the side arm; 1.0 ml standardized resting cell suspension in the main compartment of the flask. To the main compartment of the flask, 0.1 M sodium phosphate buffer pH 6.8 was added such that the total liquid volume in the flask was 3.0 ml.

Effect of Storage on Induced Enzyme Activity

To measure the effect of storage on the "in vivo" induced enzyme systems, cells were grown on phenylacetate and harvested when the cell density had reached 153 Klett units. They were washed once in 0.1 M sodium phosphate buffer pH 6.8 and centrifuged in a Servall Centrifuge model RC-2 at 20,000 x g for 20 minutes. At 2 day intervals for a period of 16 days standardized resting cell suspensions were made as described previously from portions of the cell mass and the activity of these suspensions toward phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate was measured manometrically. During the interim periods, the remaining cells were stored in a packed state at 4 ^{o}C.

Paper Chromatographic Experiments

Preparation of Samples for Chromatography

The identification of metabolites in fermentation liquor provides

a useful clue to resolving the mechanism of transformation of a utilizable substrate. Frequently, metabolites may appear but subsequently disappear as growth ensues. This means that rate controlling transformation mechanisms are a function of growth; intermediates are not excreted to detectable levels but are utilized as rapidly as they are formed.

Fermentation liquor of the Pseudomonas sp. grown on phenylacetate was examined for the presence of phenolic metabolites in an early and late stage of growth. Cells were grown on 1.0% phenylacetate and large samples were taken when the cell density had reached 20 and 153 Klett units. Each sample was treated as follows: The cells from a five litre sample were removed by centrifugation. The protein was removed from the supernatant by adding 10% by volume of 25% zinc sulfate and the pH was adjusted to 7.6 to 7.8 (28). The mixture was stirred and allowed to stand overnight. The supernatant was decantated, saturated with NaCl to salt out excess phenylacetate and filtered through Whatman No. 1 filter paper. The filtrate was placed in a separatory funnel with one-half volumes of petroleum ether and the mixture was shaken vigorously. The ether layer was collected and evaporated to dryness at room temperature. The residue was redissolved in 2.0 ml water and spotted on a chromatogram in amounts ranging from 100 to 400 µlitres.

Preparation of Reagents for Chromatography

The solvent used was n-butanol: ethanol: 2% NH_3 = 160:40:90 was prepared by mixing 160 parts n-butanol, 40 parts ethanol and 90 parts 2% NH_3 (17). The chromatogram was run in a descending manner for 16 hours. Whatman No. 1 filter paper and chromatography tanks of single

unit glass construction were used.

Diazotized sulfanilic acid (5) was used as the developing agent and was prepared as follows:

Solution 1. To 10 ml concentrated HCl was added 0.9 grams sulfanilic acid and the solution was diluted to 100 ml in water.

Solution 11. A 5% aqueous solution of sodium nitrite was prepared.

Both solutions were chilled in an ice bath. To 5.0 ml of Solution 1, 25 ml of Solution 11 was added. The mixture was kept on ice for two to four hours before use.

After being sprayed with diazotized sulfanilic acid, the chromatograms were oversprayed with a 20% aqueous solution of sodium carbonate while still moist.

RESULTS

Observations on Growth

The organism, in accordance with the results of Blakley et al (3), was able to grow with either phenylacetate or glucose as the sole source of carbon. When it was grown on glucose the liquid culture medium appeared creamy white; when it was grown on phenylacetate the liquid medium appeared pale green. The color probably resulted from the presence of pyocyanin, a green water soluble pigment often found with pseudomonads but no attempt was made to identify with confidence the pigment produced. When the culture reached maximum cell density in liquid medium the cells began to aggregate into large flocculant masses. Floccultation, which accompanied foam production, was probably caused by a high concentration of protein and nucleic acids released into the growth medium by cells undergoing autolysis. The organism was particularly susceptible to autolysis when grown on glucose but was stable when grown on phenylacetate under conditions where foaming was rigidly controlled. A characteristic odour was emitted from growth flasks when cells underwent lysis.

Effect of Initial Phenylacetate Concentration on Growth

When cells were grown with 0.4% phenylacetate as the sole source of carbon and were harvested at the maximum cell density, a large portion of the phenylacetate was not utilized. To determine if decreased initial concentrations of phenylacetate would yield cell densities of equivalent values, cells were grown in mineral salts medium containing phenylacetate from 0.4% to 0.05%. The culture was grown in 500 ml Erlenmeyer flasks

fitted with standardized culture tube side arms. Each flask contained 50 ml of medium and was inoculated with 1.0% by volume of a cell suspension grown on glucose. Growth was carried out at 28 ^{o}C on an Eberbach rotary shaker. At regular intervals, the contents of the flask were tipped into the side arm and the cell density measured in Klett units as described previously.

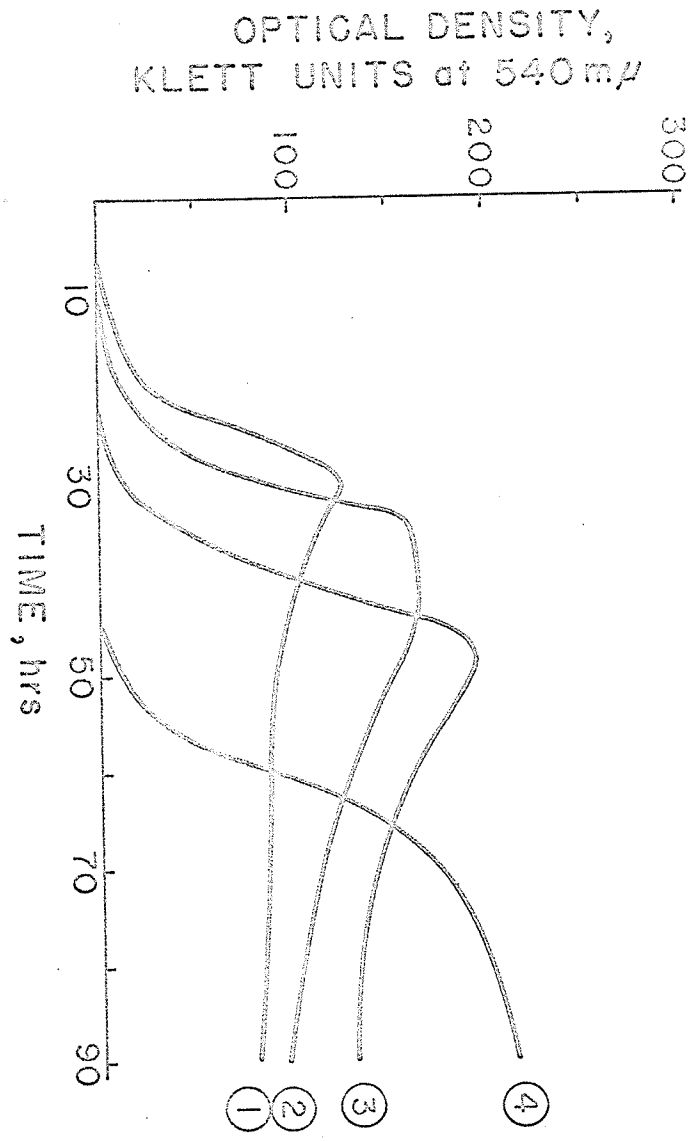
Figure 1 shows the response of the organism to a variation in the initial concentration of phenylacetate. Throughout the range of substrate concentrations studied the length of the lag period decreased as the initial phenylacetate concentration was decreased. In addition, maximum cell density increased as the initial concentration of phenylacetate was increased. The validity of the results obtained at the highest cell densities examined may be in doubt because flocculation of cells had begun to occur when these readings were made.

Manometric Experiments

Effect of Growth on Induced Enzyme Activity

Figure 2 shows the activity of a standardized resting cell suspension toward phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate. It shows the activity of phenylacetate grown cells harvested at a cell density of 20 Klett units. Curves are exogenous plots representative of several trials. Phenylacetate and 3,4-dihydroxyphenylacetate were both metabolized readily and at equal rates but activity toward all of the monohydroxylated compounds was considerably lower. o-Hydroxyphenylacetate was oxidized at the lowest rate. Figure 3 shows the activity of cells harvested at 42 Klett units. Again, consistently high activity

Fig. 1. Effect of phenylacetate concentration on the growth of a Pseudomonas sp., Substrate concentrations were as follows: 1 - 0.05%; 2 - 0.1%; 3 - 0.2%; 4 - 0.4%.


OPTICAL DENSITY,
KLETT UNITS at 540 mμ
100
200
300
10
30
50
70
90
TIME, hrs
1
2
3
4

toward phenylacetate and 3,4-dihydroxyphenylacetate was displayed. A strong response toward the monohydroxylated derivatives was not achieved. In fact, there was no activity toward o-hydroxyphenylacetate in the preparation used. When cells were harvested at 85 Klett units, the activity toward the monohydroxylated intermediates increased as shown in Figure 4. p-Hydroxyphenylacetate was metabolized equally as well as phenylacetate, the original growth substrate. Both o-hydroxyphenylacetate and m-hydroxyphenylacetate showed increased activity relative to earlier stages in the growth curve. Figure 5 shows the activity of cells harvested when the cell density had reached 140 Klett units. The activity toward both m-hydroxyphenylacetate and p-hydroxyphenylacetate had approached that of phenylacetate. The relative activity toward o-hydroxyphenylacetate had also increased appreciably, but it was still metabolized at a rate lower than the other compounds.

To determine if initial phenylacetate concentration affected the activity of cells grown to a specific cell density, the organism was grown in carboys under conditions identical to those described previously except that the initial phenylacetate concentration was made to 0.05%. Cells were harvested at 20 and 80 Klett units respectively and activity of suspensions prepared from these cells was determined against phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate under conditions as previously described. Activities were equivalent to values obtained from cells grown with 0.4% initial phenylacetate concentration and harvested at the same cell density. The results show conclusively that ensyme activity is a function of growth and not growth substrate

Fig. 2. Oxidation of phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate by resting cell suspensions of a Pseudomonas sp. grown on phenylacetate. Cells were harvested at a cell density of 20 Klett units. Curves are exogenous plots representative of several trials.

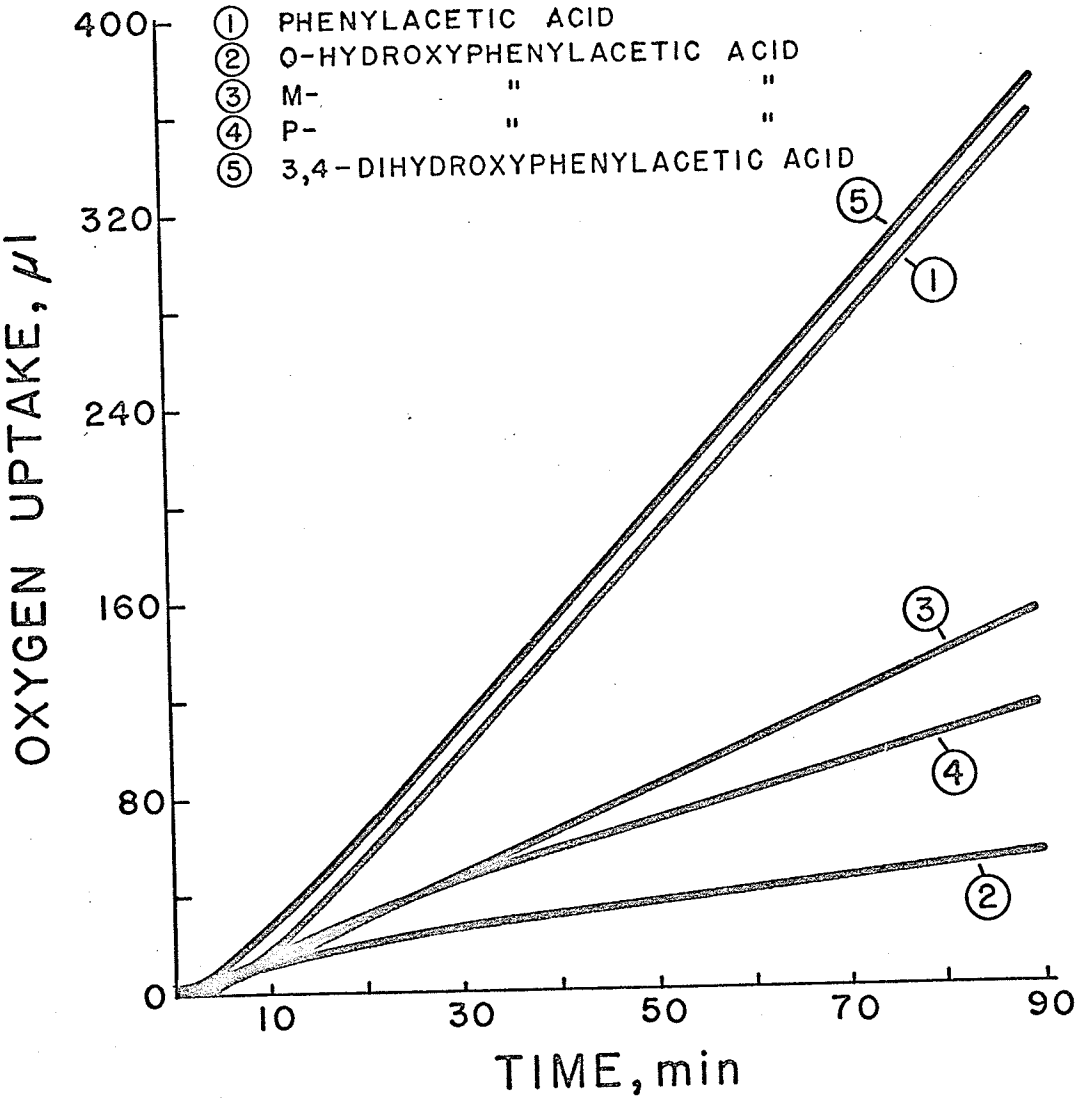

① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, μl
400
320
240
160
80
0
10
30
50
70
90
TIME, min

Fig. 3. Oxidation of phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate by resting cell suspensions of a Pseudomonas sp. grown on phenylacetate. Cells were harvested at a cell density of 42 Klett units. Curves are exogenous plots representative of several trials.

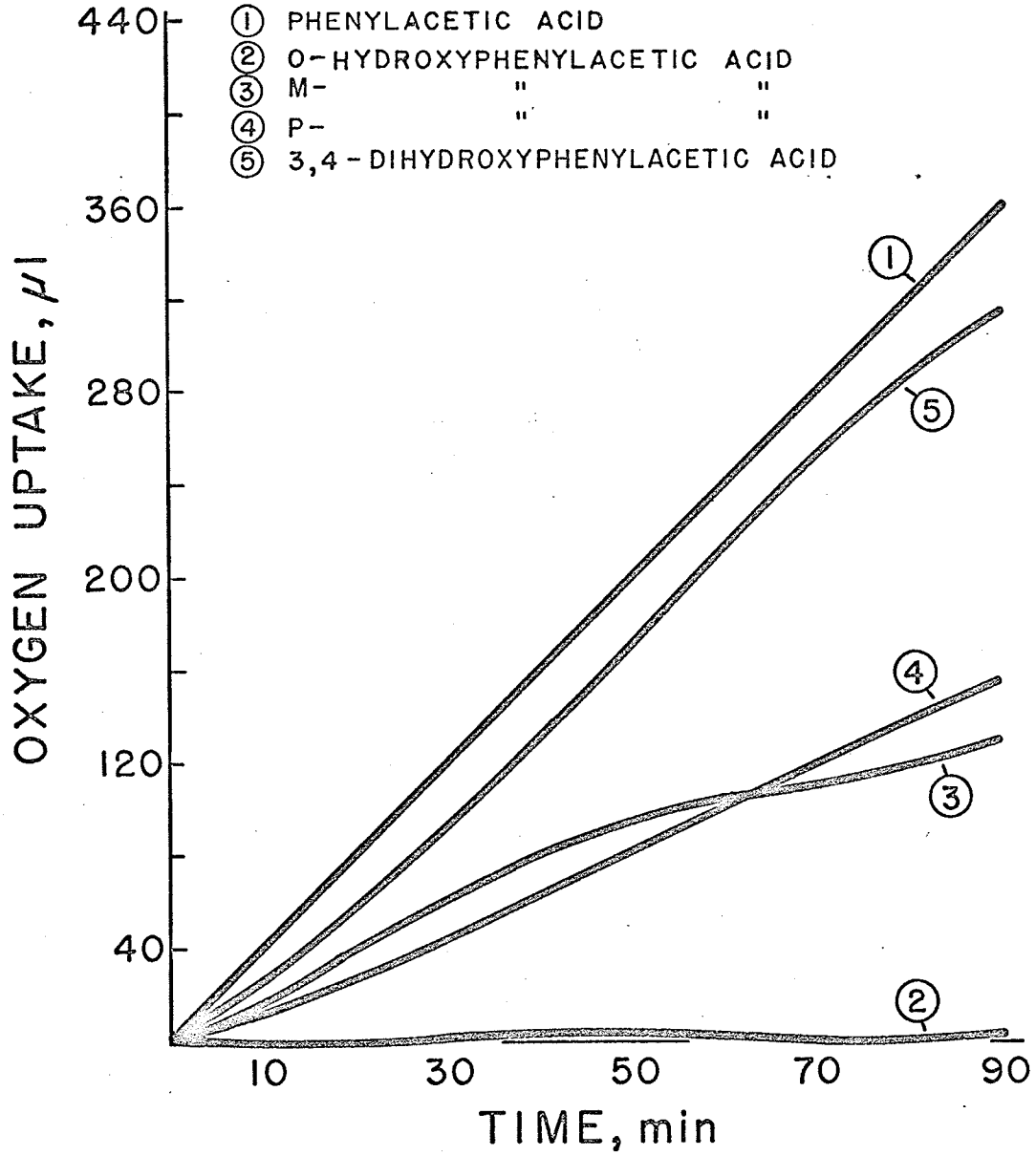

① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, μl
440
360
280
200
120
40
10
30
50
70
90
TIME, min

Fig. 4. Oxidation of phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate by resting cell suspensions of a Pseudomonas sp. grown on phenylacetate. Cells were harvested at a cell density of 85 Klett units. Curves are exogenous plots representative of several trials.

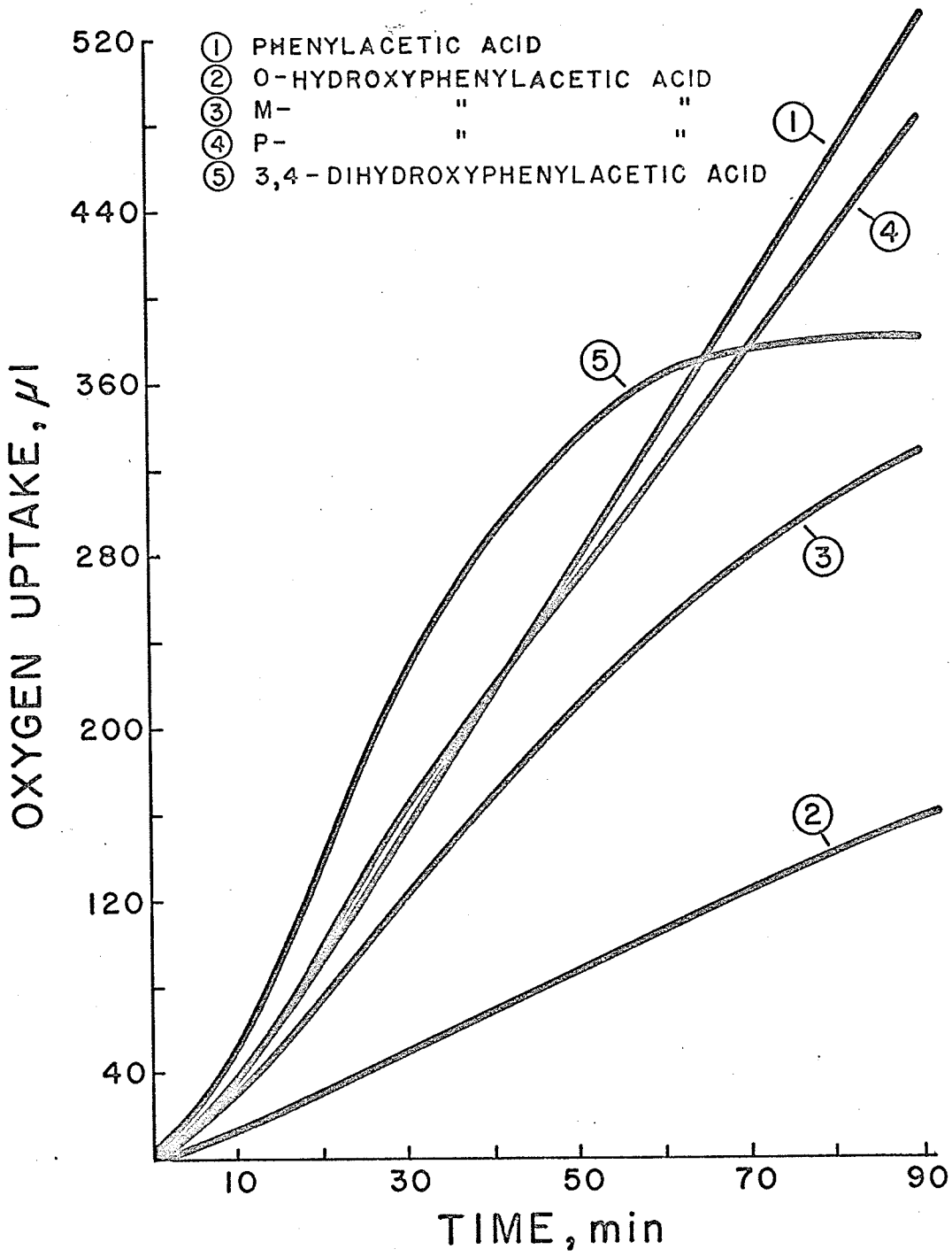
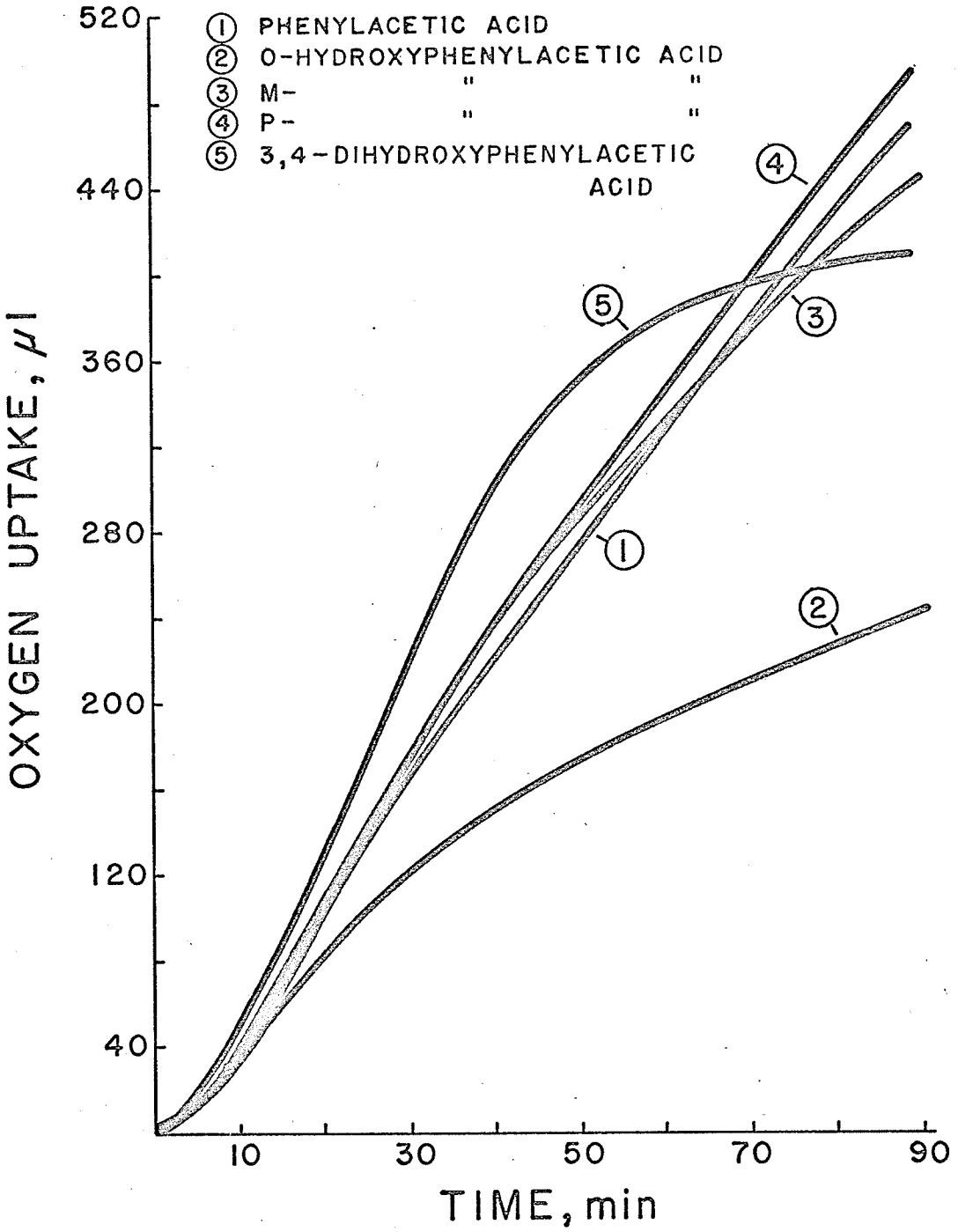

① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, μl
520
440
360
280
200
120
40
10
30
50
70
90
TIME, min

Fig. 5. Oxidation of phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate by resting cell suspensions of a Pseudomonas sp. grown on phenylacetate. Cells were harvested at a cell density of 140 Klett units. Curves are exogenous plots representative of several trials.


① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, μl
520
440
360
280
200
120
40
10
30
50
70
90
TIME, min

concentration.

Effect of Storage on Induced Enzyme Activity

Figures 6, 7, 8 and 9 are representative of the activity toward phenylacetate, 3,4-dihydroxyphenylacetate, o-hydroxyphenylacetate, m-hydroxyphenylacetate and p-hydroxyphenylacetate of standardized cell suspensions prepared from cells grown on phenylacetate and stored in a packed state at 4 ^{o}C for varying periods of time. The results are expressed as exogenous plots for all trials.

Figure 6 shows the activity of a suspension prepared after 4 days storage at 4 ^{o}C. The activity after 4 days storage was virtually identical to freshly harvested cells and subsequently a figure showing the results at zero time is not included. Even after 8 days storage there was no appreciable change in induced enzyme activity as shown in Figure 7. Figure 8 shows the activity of cells toward phenylacetate and its hydroxylated derivatives after 12 days storage. There was a marked drop in activity toward all compounds tested. No one compound showed a radical change in activity with respect to the other compounds suggesting that all the induced enzyme systems were of equal stability. Figure 9 shows the activity of cells after storage for 16 days. The induced enzyme activity had decreased markedly and a lag period was observed before oxidation of phenylacetate and its monohydroxylated derivatives occured.

Paper Chromatographic Results

Detection of Metabolites in Fermentation Liquor

Figure 10 shows a chromatogram prepared from fermentation liquor

Fig. 6. Effect of storage for 4 days at 4 ^{o}C on induced enzyme stability in a Pseudomonas sp..

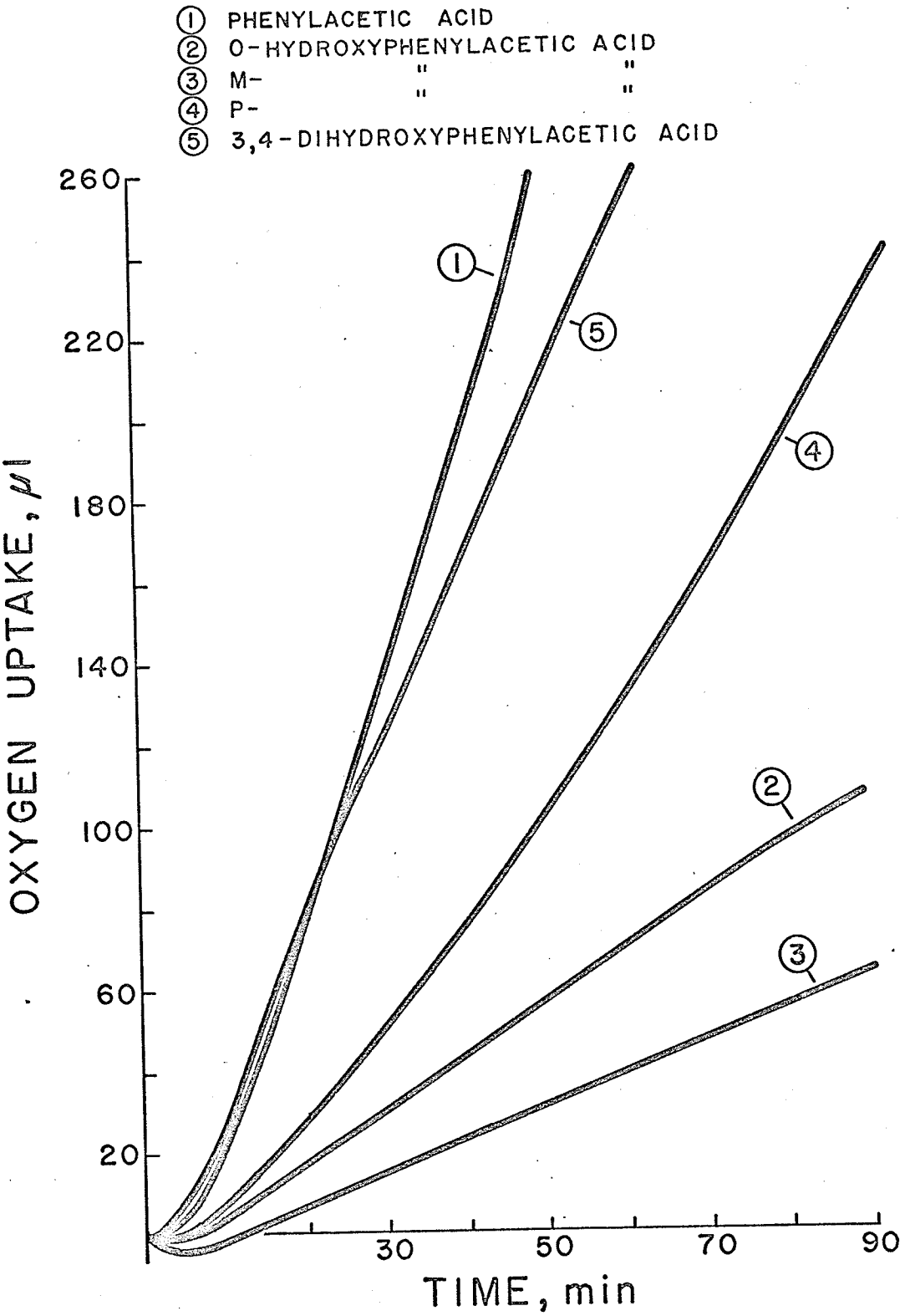

① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, μl
260
220
180
140
100
60
20
30
50
70
90
TIME, min

Fig. 7. Effect of storage for 8 days at 4 ^{o}C on induced enzyme stability in a Pseudomonas sp..

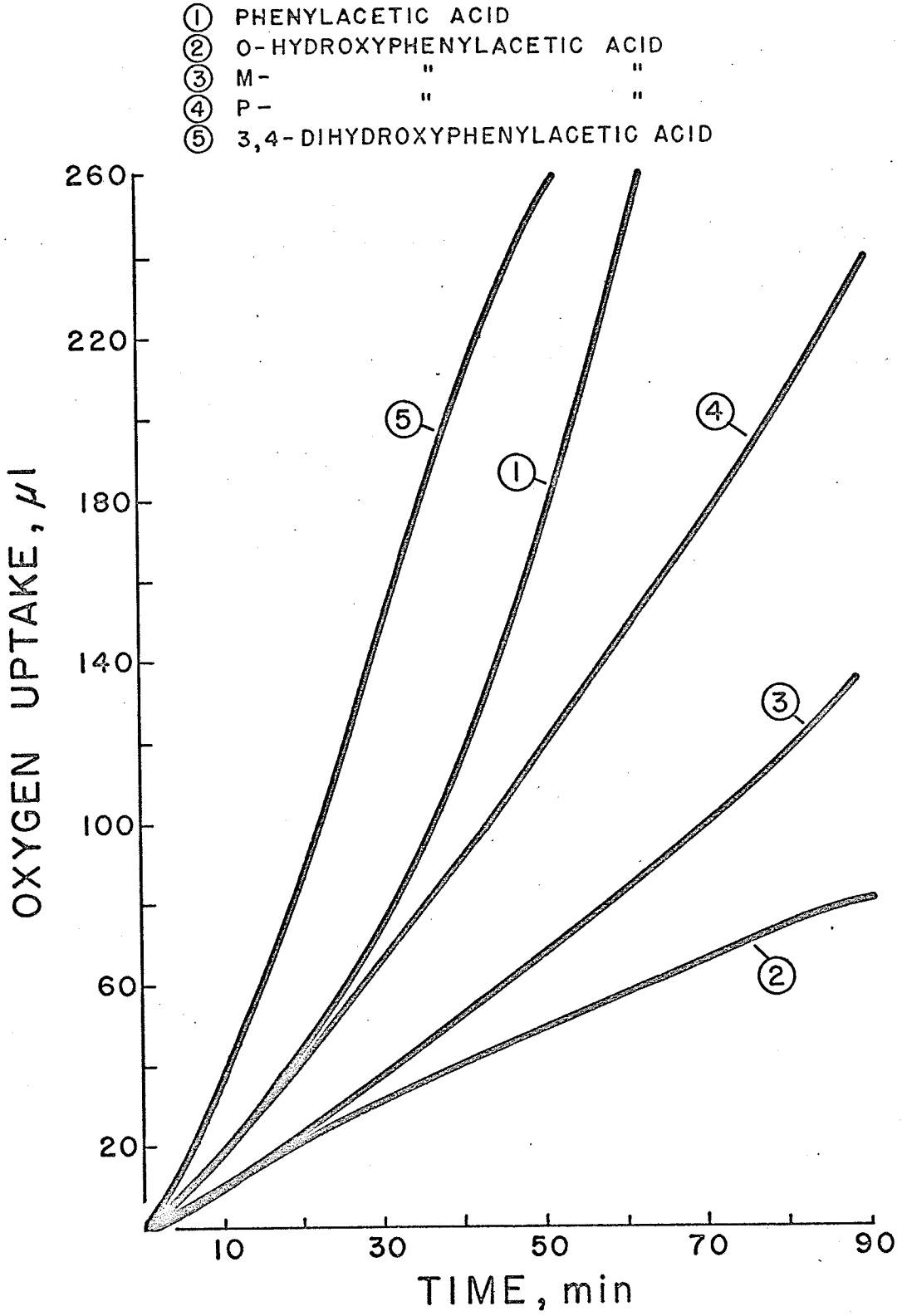

① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, μl
260
220
180
140
100
60
20
10
30
50
70
90
TIME, min

Fig. 8. Effect of storage for 12 days at 4 ^{o}C on induced enzyme stability in a Pseudomonas sp..

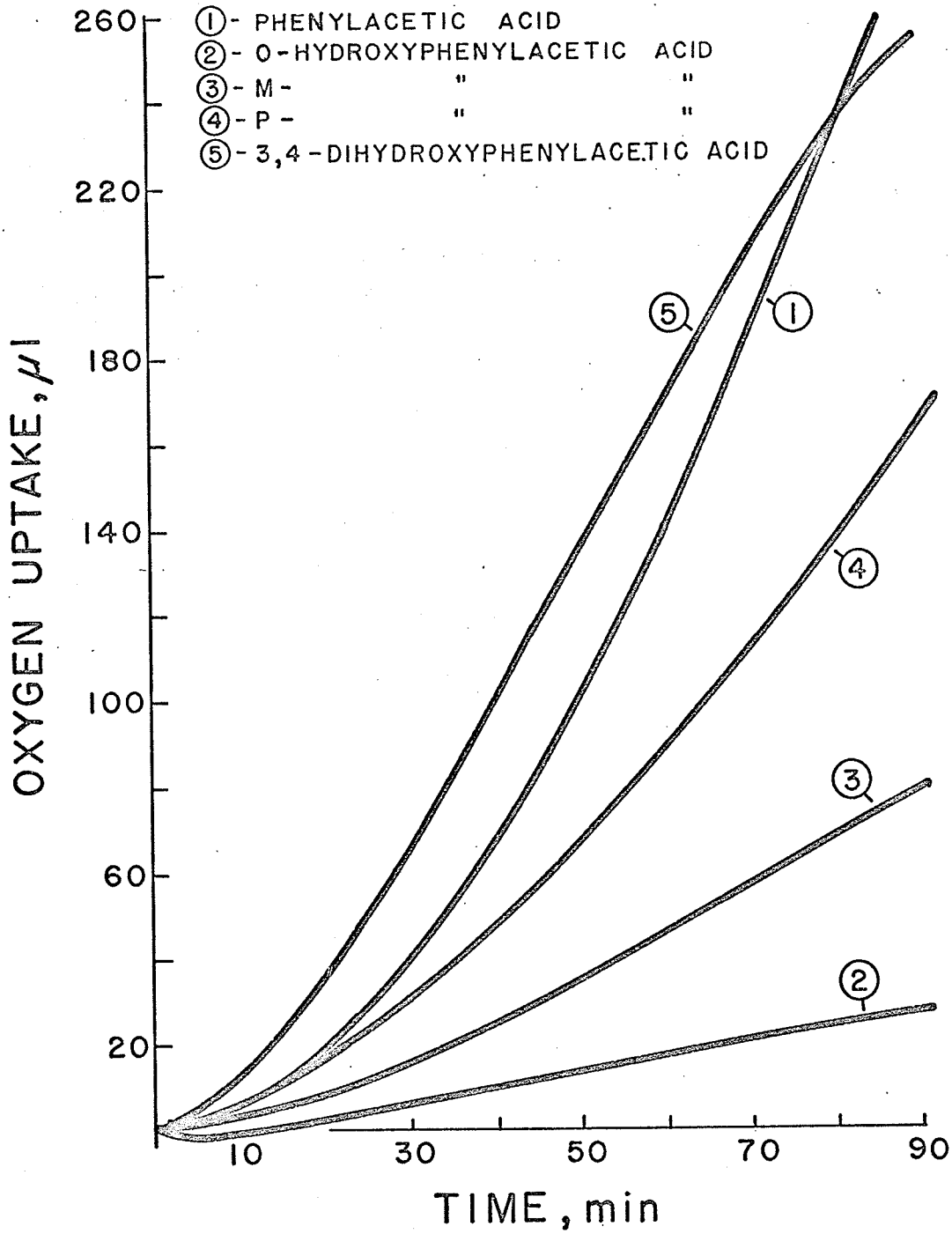
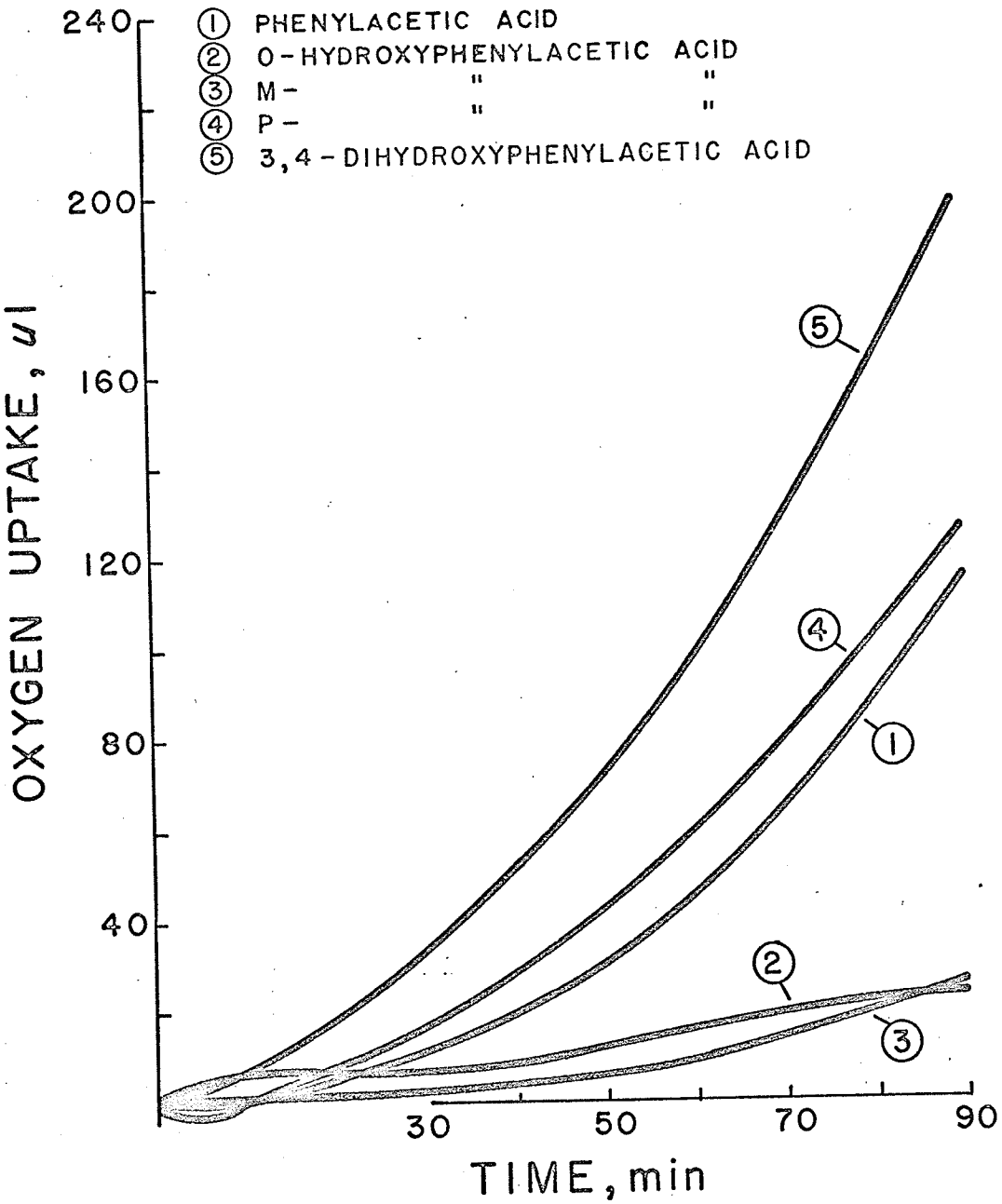
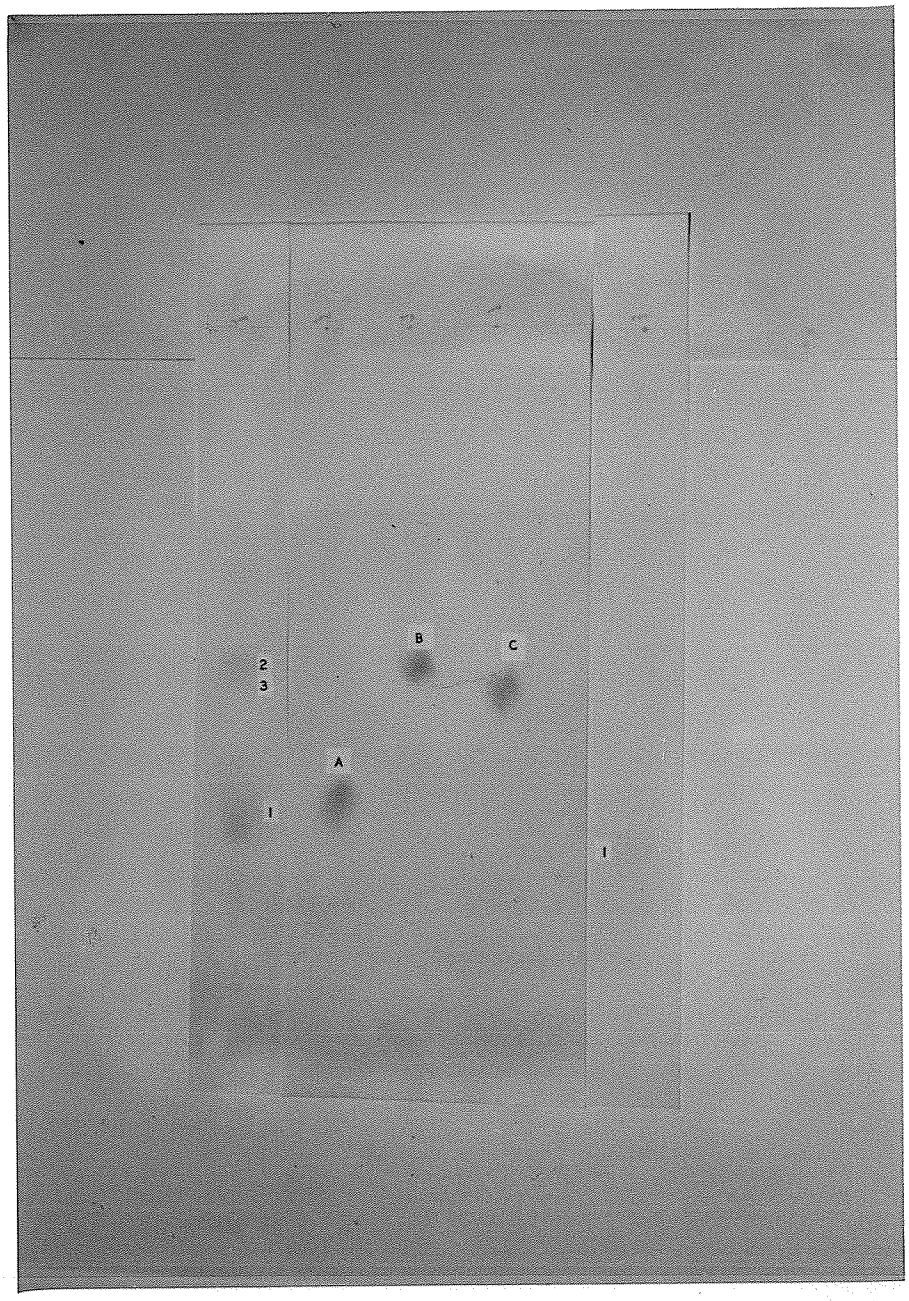

① - PHENYLACETIC ACID
② - O-HYDROXYPHENYLACETIC ACID
③ - M- " "
④ - P- " "
⑤ - 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, µl
260
220
180
140
100
60
20
10
30
50
70
90
TIME, min

Fig. 9. Effect of storage for 16 days at 4 ^{o}C on induced enzyme stability in a Pseudomonas sp..


① PHENYLACETIC ACID
② O-HYDROXYPHENYLACETIC ACID
③ M- " "
④ P- " "
⑤ 3,4-DIHYDROXYPHENYLACETIC ACID
OXYGEN UPTAKE, µl
240
200
160
120
80
40
30
50
70
90
TIME, min

of cells harvested at 20 and 153 Klett units. Authentic standards of o-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid and p-hydroxyphenylacetic acid, designated as A, B and C, were applied to the chromatogram along with the samples and were used for the purpose of identifying metabolites. All of the monohydroxylated derivatives of phenylacetic acid were found in the fermentation liquor of cells harvested at an early stage of growth as shown on the chromatogram; only o-hydroxyphenylacetic acid could be detected in the fermentation liquor of cells harvested at a late stage of growth.

Fig. 10. Production of metabolites of phenylacetic acid in fermentation liquor by a Pseudomonas sp. as a function of growth.


B
C
2
3
A
1
1

DISCUSSIONS

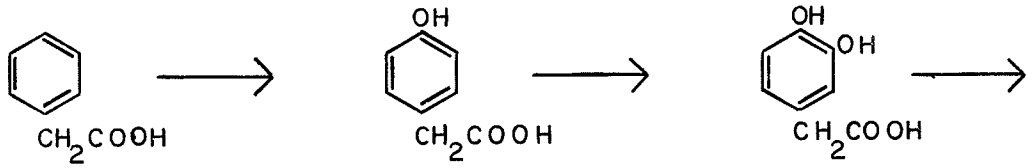
Studies with resting cell suspensions prepared from cells grown on phenylacetate and harvested at arbitrarily chosen points over the entire range of the growth curve showed that activity toward the monohydroxylated derivatives of phenylacetate increased as culture growth progressed. The absolute activity toward these compounds did not remain constant relative to cell density, for, cells that were harvested at a specific cell density from different growth preparations showed some minor variations in activity toward the monohydroxylated derivatives. It was necessary, therefore, to choose results representative of several trials at each cell density studied throughout the whole range of the growth curve.

It seems likely that the variation in activity with respect to growth is caused by different levels of intracellular enzyme rather than by different levels of specific permeases associated with the cell membrane. Blakley et al (3) have prepared cell free extracts active against 3,4-dihyroxyphenylacetate and p-hydroxyphenylacetate which varied in activity from one preparation to another. This shows that the enzymes responsible for differences in activity are contained within the cytoplasm of the cell.

The induced enzyme systems responsible for the oxidation of phenylacetate and its hydroxylated derivatives were stable for 8 days if cells were stored in a packed state at 4 ^{o}C. Fluctuation in induced enzyme activity with respect to growth was, then, not caused by enzyme lability and was assumed to be caused by a variation in the actual intracellular enzyme content of the cells.

o-Hydroxyphenylacetic acid, m-hydroxyphenylacetic acid and p-hydroxyphenylacetic acid were found in the fermentation liquor obtained from the cells grown to a density of 20 Klett units. When cells were grown to a density of 153 Klett units only o-hydroxyphenylacetic acid could be detected in the fermentation liquor. It was hypothesized that non-specific hydroxylation of phenylacetate occurred, and the mono-hydroxylated derivatives of phenylacetate accumulated in the fermentation liquor during early stages of growth. When accumulation reached a threshold level, the organism induced enzymes to degrade the hydroxylated compounds such that they were not detectable during latter stages of growth. This phenomena has been termed "metabolite induction" by Cain (7). o-Hydroxyphenylacetate was probably detectable at a late stage of growth because of its low rate of oxidation independent of the growth phase.

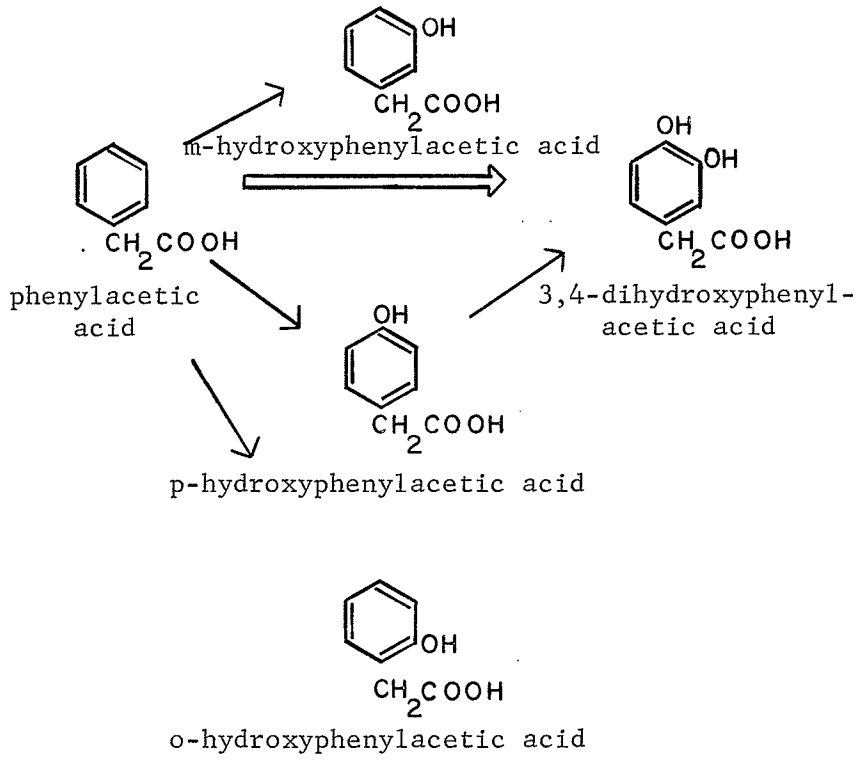
Blakley et al (3) have postulated that the organism used in this study oxidizes phenylacetic acid by the following mechanism:



phenylacetic acid — p-hydroxyphenylacetic acid — 3,4-dihydroxy-phenylacetic acid

This pathway involves the sequential introduction of individual hydroxyl groups and was proposed on the basis of results obtained using the sequential induction technique with cells obtained in the late stages of growth. The results obtained from this study suggest that the above pathway may be of only minor importance and that yet another pathway

involving the simultaneous introduction of two hydroxyl groups into the aromatic moiety of phenylacetate could be operating as the principle degradation mechanism. The simultaneous introduction of two hydroxyl groups into the benzenoid structure by microorganisms has been demonstrated previously in a number of instances. The oxidation of benzene (25), benzoic acid (12, 36) and anthranilic acid (7) has been shown to occur in this manner. The activity toward the monohydroxylated derivatives of phenylacetate present in late stages of growth can be accounted for because of metabolite induction. A suggested overall mechanism for the oxidation of phenylacetate by this Pseudomonas sp. is presented below:



Further work would be required, however, to confirm this series of transformations.

REFERENCES

1. Bate-Smith, E. C. Ferulic, sinapic and related acids in leaves. Chem. and Ind. (Rev.) 1457-1458 (1954).
2. Benzer, S. Induced synthesis of enzymes in bacteria analysed at the cellular level. Biochem. Biophys. Acta 11, 383-395 (1953).
3. Blakley, E. R., Kurz, W., Halvorson, H., and Simpson, F. J. The metabolism of phenylacetic acid by a Pseudomonas. Can. J. Microbiol. In press.
4. Blakley, E. R. and Simpson, F. J. The microbial metabolism of cinnamic acid. Can. J. Microbiol. 10, 175-185 (1964).
5. Bray, H. G., Thorpe, W. V. and White, K. The fate of certain organic acids and amides in the rabbit. 10. The application of paper chromatography to metabolic studies of hydroxybenzoic acids and amides. Biochem. J. 46, 271-275 (1950).
6. Briggs, M. H. and Harvey, N. Urinary metabolites of aromatic amino acids in schizophrenia. Life Sciences 2, 61-64 (1962).
7. Cain, R. B. Induction of anthranilate oxidation system during the metabolism of o-nitrobenzoate by certain bacteria. J. Gen. Microbiol. 42, 197-217 (1966).
8. Cohen, G. N. and Monod, J. Bacterial permeases. Bacteriol. Rev. 21, 169-194 (1957).
9. Cohn, M. Contributions of studies on the β-galactosidase of E. coli to our understanding of enzyme synthesis. Bacteriol. Rev. 21, 140-169 (1957).

10. Davies, R. and Gale, E. F. in "Adaptation in Microorganisms". ed. by Davies, R. and Gale, E. F., Cambridge University Press, Cambridge, England.

11. Dixon, M. and Webb, E. C. in "Enzymes" pp. 477 ed. by Dixon, M. and Webb, E. C., Academic Press Inc., New York. 1964.

12. Donawa, A. On the role of salicylate in the intermediate metabolism of benzoate by some Pseudomonas spp. M. Sc. Thesis, University of Manitoba, Winnipeg. 1965.

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

14. Evans, W. C. The microbial degradation of aromatic compounds. J. Gen. Microbiol. 32, 177-184 (1963).

15. Gilmour, D. in "Biochemistry of Insects". ed. by Gilmour, D., Academic Press Inc., New York. 1960.

16. Happold, F. C. and Key, A. The bacterial purification of gasworks liquors. The action of the liquors on the bacterial flora of the sewage. J. Hyg., Cambridge 32, 573 (1932).

17. Harborne, J. B. and Simmonds, N. W. in "Biochemistry of Phenolic Compounds". pp. 83, 496 ed. by Harborne, J. B. and Simmonds, N. W., Academic Press Inc., New York. 1964.

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

19. Hayaishi, O. In "Oxygenases". ed. by Hayaishi, O. Academic Press Inc., New York. 1964.

20. Hockenhull, D. J. and Herbert, D. The amylase and maltase of Clostridium acetobutylicum. Biochem. J. 39, 102-106 (1945).

21. Ibrahim, R. K. and Towers, G. H. N. Conversion of salicylic acid to gentisic acid and o-pyrocatechuic acid with C^{14} in plants. Nature 184, 1803 (1959).

22. Kindl, H. and Billek, G. 2-Hydroxyphenylacetic acid, a new constituent of higher plants. Nature, London 194, 579 (1962).

23. Klambt, H. D. Conversion in plants of benzoic acid to salicylic acid and its β-d-glucoside. Nature 196, 491 (1962).

24. Mandelstam, J. Induced biosynthesis of lysine decarboxylase in Bacterium cadaveris. J. Gen. Microbiol. 11, 426-437 (1954).

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

26. Mehler, A. H. Proc. Fourth Int. Cong. Biochem. Colloquia 13, 164 (1958) Cited from Dagley, S., Evans, W.C. and Ribbons, D. W. New pathways in the oxidative metabolism of aromatic compounds by microorganisms. Nature 188, 560-566 (1960).

27. Moyer, A. J. and Coghill, R. D. The effect of phenylacetic acid on penicillin production. J. Bacteriol. 53, 329-341 (1947).

28. Neish, A. C. Analytical methods for bacterial fermentations. Report No. 46-8-3 (Second Revision). Prairie Regional Laboratory, Saskatoon, Sask. 1952.

29. Pollock, M. R. In "The Enzymes" Vol. I, pp. 629 ed. by Boyer, P. D., Lardy, H. and Myrback, K., Academic Press Inc., New York. 1959.

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

31. Rickenberg, H. V., Yanofsky, C. and Bonner, D. M. Enzymatic deadaptation. J. Bacteriol. 66, 683-687 (1953).

32. Schoenheimer, R. in "The Dynamic State of Body Constituents" ed. by Schoenheimer, R., Harvard University Press. 1946.

33. Sleeper, B. P., Tsuchida, M. and Stanier, R. Y. The bacterial oxidation of aromatic compounds. J. Bacteriol. 59, 129-133 (1950).

34. Sohngen, N. L. Benzin, Petroleum, Parafinol und Parafin als Kohlenstoff und Energiequelle fur Mikroben. Centr. Bakteriol. Parasitenk. Abt. II, 37, 339-348 (1913).

35. Stanier, R. Y. Simultaneous adaptation; a new technique for the study of metabolic pathways. J. Bacteriol. 54, 339-348 (1947).

36. Stanier, R. Y. The oxidation of aromatics by fluorescent pseudomonads. J. Bacteriol. 55, 477-493 (1948).

37. Stanier, R. Y. Enzymatic adaption in bacteria. Ann. Rev. Microbiol. 5, 35-56 (1951).

38. Stanier, R. Y. in "Aspects of Synthesis and Order in Growth" pp. 43. Princeton University Press, Princeton, New Jersey. 1955.

39. Suda, M., Naono, S., Kato, A., Inoue, S. and Yugari, Y. Adaptive formation of a specific series of enzymes in bacterial cells. Proc. Int. Symp. on Enz. Chem., Tokyo and Kyoto (1957).

40. Sumner, J. B. and Myrback, K. Modern aspects of enzymatic adaptation. In "The Enzymes", Vol. I, Pt. 1, pp. 167 ed. Sumner, J. B. and Myrback, K., New York Academic Press Inc. 1959.

41. Tausson, V. O. In "Basic Principles of Plant Bioenergetics" (Collected Works) pp. 73 ed. by Maximov, N. A. Moscow: Academy of Sciences of the U. S. S. R. 1954.

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

43. Urba, R. C. Protein breakdown in Bacillus cereus. Biochem. J. 71, 513-518 (1959).

44. Wiss, O., Simmer, H., and Peters, H. Uber die Umwandlung der 3-hydroxyanthranilsaure und Nicotinsaure im tierischen Organismus. Z. physiol. Chem. 304, 221-231 (1956).