INFLUENCE OF O-NITROBENZOIC ACID ON THE PROTOCATECHUATE OXYGENASE SYSTEM OF *PSEUDOMONAS FLUORESCENS*

By

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1961

Submitted to the faculty of the Graduate College of
the Oklahoma State University
in partial fulfillment of the requirements
for the degree of
MASTER OF SCIENCE
May, 1966

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ACKNOWLEDGMENTS

Thanks is extended to Dr. Norman Durham for his counsel and guidance throughout this investigation.

The author also expresses graditude for the encouragement and patience of her family and her husband.

TABLE OF CONTENTS

Chapter | Page

I. INTRODUCTION 1

Part A. Inducible enzymes 1
Part B. Oxidative phosphorylation 6
Part C. Protocatechuic acid metabolism. 9

II. MATERIALS AND METHODS. 12

Test organism. 12
Abbreviations used in the text 12
Media. 12
Growth of cells. 13
Cell extracts. 13
Substrates 14
Protocatechuic acid colorimetric assay 14
Manometric studies 14
Procedure for uptake of radioactive substrate. 16

III. RESULTS AND DISCUSSION 17

Inducible enzyme formation in Pseudomonas fluorescens cells. 17
Inhibition of the rate of enzyme synthesis by o-nitrobenzoic acid 17
Effect of 2,4-dinitrophenol on enzyme synthesis. 20
Effect of glucose on enzyme synthesis and inhibition . . 22
Effect of the inhibitor on cell viability. 22
Effect of o-nitrobenzoic acid on the utilization of protocatechuic acid by induced cells 27
Reversibility of the o-nitrobenzoic acid effect by excess substrate 27
Carbon dioxide production influenced by o-nitrobenzoic acid 31
Substrate uptake by cells in the presence and absence of o-nitrobenzoic acid. 34
Influence of exogenous adenosine triphosphate on the o-nitrobenzoic acid inhibition 35
Cell free extracts used in the inhibited system. 39
Reduction of an artificial acceptor by cell extracts in the presence and the absence of o-nitrobenzoic acid. . 39
Possible nonmetabolizable inducers tested. 41

IV. SUMMARY AND CONCLUSIONS. 46

LITERATURE CITED. 48

LIST OF TABLES

Table Page

I. Enzyme activity of nutrient agar-grown cells induced to protocatechuic acid in the presence and the absence of inhibitors 21

II. Viable cell count of noninduced and of induced cells incubated in the presence and the absence of o-nitrobenzoic acid and plated on their respective media. 28

III. Correlation of oxygen utilization and of carbon dioxide production by cells in the presence and the absence of o-nitrobenzoic acid. 33

IV. Uptake of succinic-2,3-^{14}C-acid in the presence and the absence of o-nitrobenzoic acid and of 2,4-dinitrophenol. . . 36

V. Protocatechuic acid depletion from medium by induced cells under the influence of o-nitrobenzoic acid and of 2,4-dinitrophenol. 37

VI. Uptake of p-aminosalicylic-^{14}C-carboxy-acid by nutrient agar-grown cells in the presence and the absence of o-nitrobenzoic acid. 43

LIST OF FIGURES

Figure		Page
1.	Regulation of enzyme formation as proposed by Monod, Jacob and Gros	5
2.	Proposed bacterial respiratory chains and points of oxidative phosphorylation (after Brodie and Adelson). . .	8
3.	Proposed oxidation of protocatechuic acid in bacteria. . .	10
4.	A standard curve for the colorimetric assay of protocatechuic acid	15
5.	Induction of protocatechuate oxygenase in *Ps. fluorescens* cells grown on nutrient agar	18
6.	Inhibition of protocatechuate oxygenase by o-nitrobenzoic acid	19
7.	Inhibition of protocatechuate oxygenase by 2,4-dinitrophenol.	23
8.	The influence of o-nitrobenzoic acid on the induction of *Ps. fluorescens* cells in the presence and the absence of glucose	24
9.	The influence of 2,4-dinitrophenol on the induction of *Ps. fluorescens* cells in the presence and the absence of glucose	25
10.	Effect of o-nitrobenzoic acid on the oxidation of succinic acid by nutrient agar-grown *Ps. fluorescens* cells. . . .	26
11.	Effect of o-nitrobenzoic acid on the utilization of protocatechuic acid by protocatechuic acid-grown cells.	29
12.	Effect of 2,4-dinitrophenol on the utilization of protocatechuic acid by protocatechuic acid-grown cells .	30
13.	Reversal of the o-nitrobenzoic acid inhibition by the addition of excess substrate	32
14.	Influence of adenosine triphosphate on the inhibition of enzyme synthesis by o-nitrobenzoic acid.	38

LIST OF FIGURES (Continued)

Figure | Page

15. Utilization of oxygen by cells and by cell extracts from nutrient agar-grown and from succinic acid-grown cells with protocatechuic acid and with succinic acid as respective substrates in the presence and the absence of o-nitrobenzoic acid 40

16. Reduction of methylene blue by cell extracts of induced <u>Ps. fluorescens</u> cells in the presence and the absence of o-nitrobenzoic acid 42

17. The utilization of protocatechuic acid by nutrient agar-grown cells in the presence of chloramphenicol after incubation of the cells in protocatechuic acid, in p-aminobenzoic acid, in p-aminosalicylic acid, or in 0.01 M Tris buffer for 2 hours 45

CHAPTER I

INTRODUCTION

Part A. Inducible enzymes

Cohn *et al*. (1953) proposed the term "enzyme induction" to define that increase of enzyme synthesis resulting from exposure to a specific chemical or "inducer". Enzyme formed in the absence of exogenous inducer was said to be "constitutive". First credit for distinguishing between the two types of enzyme synthesis goes to Karström (1937) who noted that *Betacoccus arabinosa* would ferment glucose and sucrose without prior exposure, but that the cells needed to be grown in the presence of galactose, arabinose, or lactose in order for their fermentive enzymes to be present in the cell.

Several instances of inducible enzymes had been observed earlier. In 1882, Wortman (cited in Pollock, 1959; p. 620) observed that without starch in the medium, cultures of "*Bacterium termio*" did not produce starch destroying ferments. Duclaux's *Traité de Microbiologie* (1899) contained one of the first distinct discussions of "adaptive" enzymes. He noted saccharase production by certain *Aspergilli* only in the presence of saccharose and two proteases if milk is incorporated into the medium. In 1901 Went (cited in Monod, 1947; p. 227) described the formation of proteases in the presence of casein or peptone by *Monilia* (*Neurospora*) *sitophila* while amylase was constitutive. Dienert (1901) showed that fermentation of galactose by *Saccharomyces cerevisiae* occurred only after several hours of induction but that glucose fermentation occurred

immediately. Stanier (1947) proposed sequential induction. If one enzyme in a catabolic series is induced then those enzymes which follow, but not those which precede, that step will also be induced.

In 1938, Yudkin proposed his theory of "mass action". The enzyme to be induced was already present in small quantities in the cell and in equilibrium with its precursor. When the inducer joined with the precursor to form more enzyme, the equilibrium was disturbed so that more enzyme continued to be formed. Halvorson and Spiegelman (1953) reported the first evidence that induced enzymes were synthesized de novo. They found that depletion of the amino acid pool, caused by glucose metabolism in a nitrogen-free medium, resulted in the decreased ability of yeast cells to produce enzyme. Replenishment of the pool restored the synthetic capacities. Radioactive studies employing ^{14}C and ^{35}S labeling with the B-galactosidase system of Escherichia coli (Rotman and Spiegelman, 1954; Hogness, Cohn, and Monod, 1955) and with the penicillinase system of Bacillus (Pollock and Kramer, 1958) confirmed the "new synthesis" theory. Cell protein was labeled and induction followed in an unlabeled medium. Analysis showed that less than 1.0 per cent of the induced enzyme could have come from existing cell material (Rotman and Spiegelman, 1954). Cohn and Monod (1953) proposed the unitary hypothesis of enzyme synthesis, which states there is no difference in the mechanism of synthesis for an inducible and a constitutive enzyme. Induced and constitutive penicillinases show no difference in properties (Kogut, Pollock, and Tridgell, 1956).

Adaptation can take place in non-growing cell cultures (Stephenson and Stickland, 1933; Stephenson and Yudkin, 1936) ruling out the idea that "natural selection" can account for the increased enzyme synthesis. Induction must be a phenotypic expression of genes already present and

not a genetic change (Pardee, 1962). In bacterial populations grown on low concentrations of inducer the individual cell is either induced or uninduced establishing that induction is an all-or-none phenomenon. Induction will occur in the cell as long as the inducer is present in the medium (Novick and Weiner, 1957). However, in the case of penicillinase, the inducer is adsorbed to the cell wall within one minute; inducible enzyme synthesis continues even after removal of the inducer from the medium (Pollock, 1950).

Induction is a stereospecific process (Halvorson, 1960) and the specificity bears some resemblance to that between enzyme and substrate. Various compounds, usually closely related in structure (Halvorson, 1960), may serve as inducers. Inducers are generally substrates or competitive inhibitors of the substrate (Pollock, 1957). In the case of penicillinase, induction is restricted to a few compounds which resemble benzylpenicillin, and the ability to induce appears to depend on the affinity of the compound for a specific penicillin binding component present in the cell (Pollock, 1957).

The kinetics of induction reflects on the number of bacteria induced rather than the degree of induction per cell (Novick and Weiner, 1957). Kinetic studies have been somewhat clouded because the overall kinetics depends on three factors - permeability, gratuity, and conditions of growth (Halvorson, 1960). Enzyme formation begins within a few minutes after exposure to the inducer (Pardee and Prestidge, 1961) and it is generally agreed that induction occurs at a constant rate (Novick and Weiner, 1957; Halvorson, 1960; Herzenberg, 1959) indicating a specific number of enzyme-forming centers in the cell (Halvorson, 1960).

Pardee and Prestidge (1961) state that an unstable ribonucleic acid (RNA) must be made before enzyme can be synthesized and that the RNA is

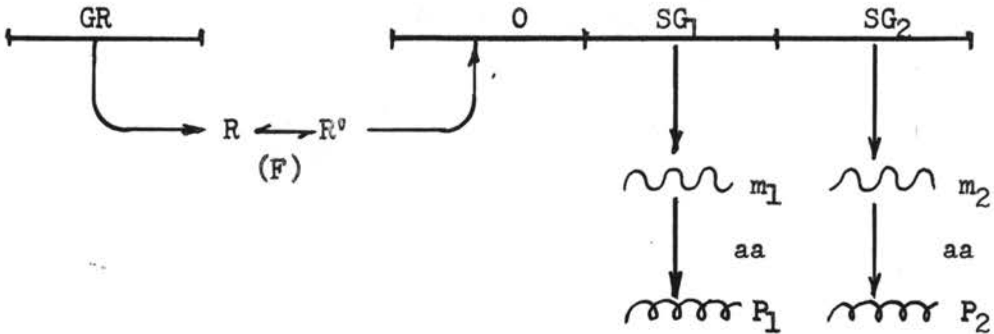
manufactured only after the genetic material is derepressed by the inducer. Herzenberg (1959) maintains that there is no increase in the enzyme forming system but that it is already present and is merely activated by the inducer.

Pardee (1962) states that since a set of enzymes found in a strain of bacteria remains constant through many generations, the controlling factor must be hereditary. If an allele or gene is lost by mutation, then that particular enzyme cannot be made. This would suggest a one-gene, one-enzyme mode of control (Yanofsky, 1960). Two major theories of enzyme control exist. The first, illustrated in Figure 1, originated with Monod, Jacob, and Gros (1962). A set of genes designated as the operon and under the regulation of the regulator gene control the synthesis of enzymes. The regulator gene causes production of a repressor which by itself or in combination with a smaller molecule, the effector, combines with the operator gene of the operon unit. When the repressor and the operator gene are joined, the structural genes of the operon do not manufacture the messenger RNA (mRNA) necessary for enzyme synthesis. In an inducible system the repressor alone combines with the operator gene and prohibits synthesis. When the effector (inducer) attaches to the repressor it is released from the operator gene and enzyme synthesis is initiated. This is sometimes referred to as derepression. A constitutive system is ineffective only when the repressor and the effector, usually a metabolic end product of the enzymatic pathway, are attached to the operator gene. If the effector is removed the repressor is released and synthesis proceeds. The effector, whether substrate or end product, controls the amount of enzyme produced (Yates and Pardee, 1957).

Lindegren (1963) maintains that a reaction occurs at the gene site (operon) between a protein receptor attached to the gene surface and the

Figure 1.

Regulation of enzyme formation as proposed by Monod, Jacob, and Gros (1962). GR, regulator gene; O, operator gene; SG_1, SG_2, structural genes; m_1, m_2, messengers made by SG_1, SG_2; P_1, P_2, proteins made by m_1, m_2; aa, amino acids; R. repressor converted to R' in the presence of the effector, F.


GR
O
SG1
SG2
R
R'
(F)
m1
m2
aa
aa
P1
P2

inducer. This reaction produces an excitation which initiates enzymatic synthesis by releasing mRNA.

Pardee and Prestidge (1959) concluded that the repressor is a gene product, possibly RNA. Further evidence that RNA may be the repressor was presented by Borek, Rockenbach, and Ryan (1956) and Yanagisawa (1962). Both used methionineless mutants of Escherichia coli and found that methionine starvation resulted in an accumulation of RNA and a delayed ability of the organism to synthesize both adaptive and constitutive enzymes. Yanagisawa (1962) included glycerol in his starvation medium and found that a second substance accumulated. He speculated that this substance might be a repressor or precursor of a repressor.

Part B. Oxidative phosphorylation

Energy which results from intracellular respiration is used predominantly for the synthesis of adenosine triphosphate (ATP) through oxidative phosphorylation (Slater, 1963). The stepwise downhill process of electron transport is coupled to the production of "high-energy" phosphate bonds (Brodie and Adelson, 1965). Kalchar (1939) was probably the first to realize that phosphorylation was a mechanism for trapping energy from aerobic oxidation. Ochoa (1953) established the phosphorylation product as adenosine polyphosphate and estimated the P:O ratio as three for a heart extract system.

There are two types of oxidative phosphorylation (both of which proceed via the following pathway: $AH_2 + B + ADP + P_i = A + BH_2 + ATP$). In substrate-linked phosphorylation AH_2, a substrate, may be phosphoglyceraldehyde, pyruvate, or a-ketoglutarate. In the second type of oxidative-phosphorylation, the symbols AH_2 and B are both members of the respiratory chain. The energy required for synthesis of ATP ($\Delta G = -8{,}000$

calories per mole) is supplied by hydrogen or by electron transfer (Slater, 1963).

Brodie and Adelson (1965) recently reported that the separation of a crude extract of Mycobacterium phlei cells into a supernatant solution and a particulate fraction prevented oxidation and phosphorylation. The supernatant solution contained proteinaceous components necessary for both oxidation and phosphorylation.

The respiratory enzymes of M. phlei closely resemble those in mammalian cells and have been identified as nicotinamide adenine dinucleotide (NAD^+), flavins, a naphthoquinone (as opposed to the benzoquinone), and cytochromes b, c_1, c, a, and a_3. Destruction of the naphthoquinone (vitamin K_9H) by irradiation resulted in the loss of both oxidation and phosphorylation with all substrates. The bacterial system contains additional respiratory enzymes which oxidize malate through FAD to naphthoquinone (Figure 2).

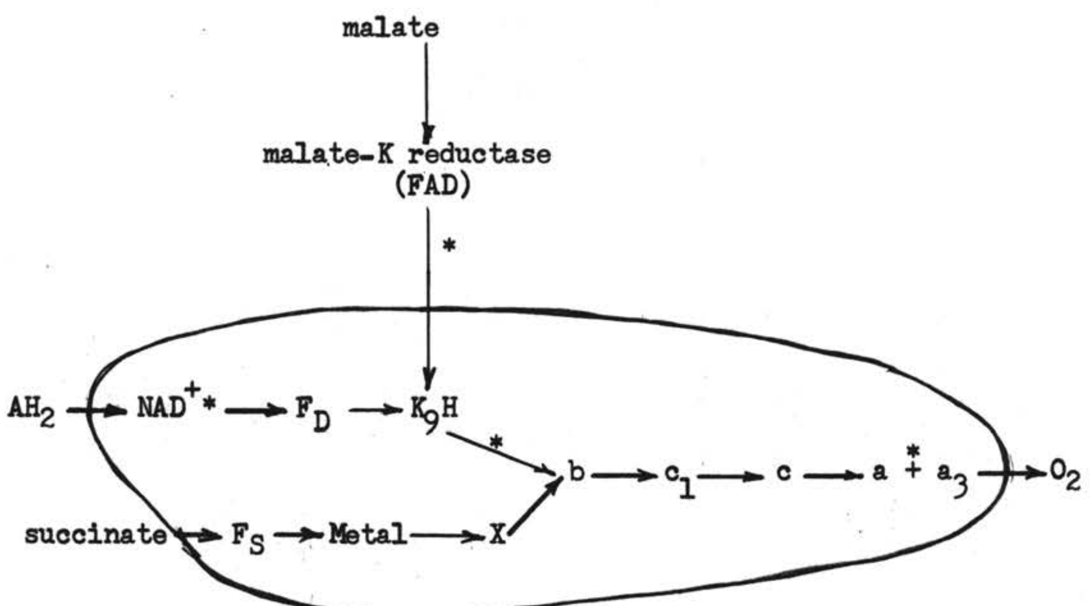
Phosphorylation sites were found in each of the three chains (succinic acid chain, malate-vitamin K reductase chain, and the NAD^+-linked chain). Brodie and Adelson (1965) find some evidence that naphthoquinone participates directly in phosphorylation and conclude that histidine phosphate is not an intermediate.

Compounds that uncouple oxidative phosphorylation permit oxidation to proceed without phosphorylation, while an oxidative phosphorylation inhibitor acts on the link between the oxidation reaction and the phosphorylation so that the overall respiration is completely inhibited. Pickett and Clifton (1943) noted that 2,4-dinitrophenol (DNP) blocks the oxidative utilization of glucose. Dodds and Greville (1934) showed that nitrophenols caused a stimulation of respiration in several tissues and that 4,6-dinitro-o-cresol in particular, increased aerobic glycolysis.

Figure 2.

Proposed bacterial respiratory chains and points of oxidative phosphorylation (after Brodie and Adelson, 1965).

The soluble components are located outside of the line.
*proposed sites of oxidative phosphorylation.
FAD (flavin adenine dinucleotide), NAD (nicotinamide adenine dinucleotide).


malate
malate-K reductase
(FAD)
*
AH2
NAD+ *
FD
K9H
*
b
c1
c
a
*
+
a3
O2
succinate
FS
Metal
X

It is generally accepted that nitrophenols stimulate intracellular respiration while inhibiting energy-requiring functions of the cell. The mode of action of an uncoupling agent is thought to be either prevention of phosphorylation while allowing oxidation, or dephosphorylation (hydrolysis) of a high energy phosphate intermediate (Lardy and Elvehjem, 1945).

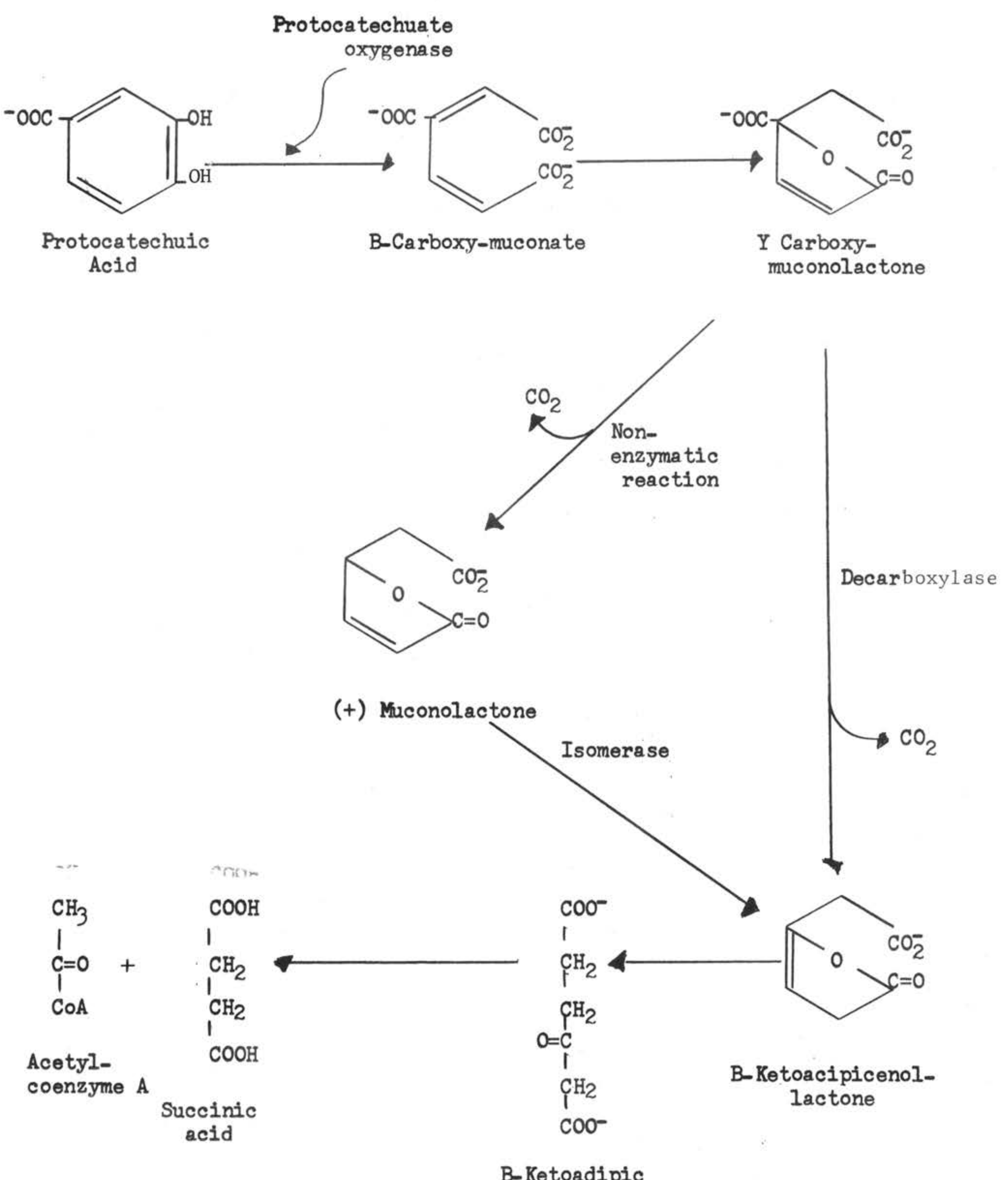
Part C. Protocatechuic acid metabolism

Pseudomonas fluorescens is able to utilize several aromatic compounds for growth (Durham, 1956; Durham, 1958) including protocatechuic acid (PA). The enzymes for the oxidation of PA are inducible and have been purified (Stanier, 1950). The bacterial degradation of PA proceeds through the pathway given in Figure 3 (Ornston and Stanier, 1964; Katagiri and Hayaishi, 1956). The addition of glucose to the medium shortens the lag time for induction to PA by Ps. fluorescens (Durham and McPherson, 1960).

Kirkland and Durham (1963) reported that o-nitrobenzoic acid (ONB) inhibits growth of a species of Flavobacterium using p-nitrobenzoic acid or succinic acid as the sole carbon and energy source. Protocatechuic acid is an intermediate in the degradation of p-nitrobenzoic acid (Durham, 1958). ONB did not kill the cells nor influence substrate uptake. The degree of growth inhibition was related to the concentration of ONB and not to the inhibitor-substrate ratio. Reversal of the inhibition could be effected by the addition of certain amino acids. This was taken to indicate that one mechanism of ONB inhibition might be interference with the synthesis or interconversion of specific amino acids. Hubbard and Durham (1961) found that PA oxidation by the

Figure 3.

Proposed oxidation of protocatechuic acid in bacteria. Ornston and Stanier, 1964; Katagiri and Hayaishi, 1956.



Protocatechuate oxygenase
^{-}OOC
OH
OH
Protocatechuic Acid
^{-}OOC
CO_2^-
CO_2^-
B-Carboxy-muconate
^{-}OOC
O
CO_2^-
C=O
Y Carboxy-muconolactone
CO_2
Non-enzymatic reaction
Decarboxylase
O
CO_2^-
C=O
(+) Muconolactone
Isomerase
CO_2
CH_3
C=O
CoA
+
COOH
CH_2
CH_2
COOH
Acetyl-coenzyme A
Succinic acid
COO^-
CH_2
CH_2
O=C
CH_2
COO^-
B-Ketoadipic acid
O
CO_2^-
C=O
B-Ketoacipicenol-lactone

Flavobacterium species could be inhibited by p-aminosalicylic acid (PASA). This inhibition appeared to be a competition for specific membrane transport sites.

ONB appeared to function as a metabolic growth inhibitor of the *Flavobacterium* species while PASA apparently inhibited PA oxidation by competing for membrane transport sites. The present work was undertaken to study the effect of ONB on the induction to PA by *Ps. fluorescens* cells and to elucidate the mechanism of inhibition.

CHAPTER II

MATERIALS AND METHODS

Test organism.

The microorganism used for this study is a laboratory strain of *Pseudomonas* demonstrating the following characteristics: Gram-negative, motile rod; raised, smooth, entire colonies on nutrient agar which are darker and rougher in the center; no pigment in a 24 hour nutrient agar culture; good growth at 25 C and 37 C, poor growth at 42 C; acid but no gas in glucose; produces slight alkalinity in litmus milk which is peptonized in 4 days. The organism was tested on Bacto-Psuedomonas Agar F which enhances the production of fluorescin and on Bacto-Psuedomonas Agar P which enhances production of pyocyanin. This bacterium gave a reaction identical with the reaction given by an organism previously described as *Pseudomonas fluorescens*. On the basis of the above tests the organism used was tentatively identified as *Ps. fluorescens*. Stock cultures were maintained on nutrient agar, succinate acid agar and protocatechuic acid agar slants stored at 4 C.

Abbreviations used in the text.

Abbreviations used in the text are as follows: adenosine triphosphate (ATP), 2,4-dinitrophenol (DNP), chloramphenicol (CAP), o-nitrobenzoic acid (ONB), p-aminobenzoic acid (PABA), p-aminosalicylic acid (PASA), protocatechuic acid (PA), and 2-amino-2-(hydroxymethyl)-3-propanediol (Tris).

Media.

A synthetic salts medium was used in various parts of this study.

The medium was composed of 0.2 per cent NaCl, 0.32 per cent KH_2PO_4, 0.42 per cent K_2HPO_4, 0.2 per cent NH_4Cl, and 0.2 per cent of the desired carbon source (succinic acid or PA). The pH of the medium was adjusted to 7.0 and agar (Difco) was added to give a final concentration of 2.0 per cent. The medium was sterilized by autoclaving at 121 C for 15 minutes and cooled to 52 C before 0.1 ml of a sterile mineral salts solution was added to each 100 ml of the medium. The mineral salts solution had the following composition: 5.0 g of $MgSO_4 \cdot 7\ H_2O$; 0.1 g of $MnSO_4$; 1.0 g of $FeCl_3$; 0.5 g of $CaCl_2$; 100 ml of distilled water.

Nutrient agar was prepared from the dehydrated medium (Difco) and agar (Difco) was added to give a final concentration of 2.0 per cent agar.

Growth of cells.

Nutrient agar, succinate agar, or PA agar slants were inoculated from the appropriate stock cultures and incubated for 16-18 hours at 37 C. The cells were suspended in sterile 0.01 M 2-amino-2-(hydroxymethyl)-1,3-propanediol buffer (Tris) (pH 7.0), agar plates of the appropriate medium were inoculated with 0.1 ml of the cell suspension, and the plates spread with a sterile glass rod. The plates were incubated at 37 C for 16-18 hours. The cells were harvested, washed twice, and suspended in 0.01 M Tris buffer. All cell suspensions were standardized to the same absorbance so that a 1:24 dilution gave a desired reading, usually 0.5 at 540 mμ in a Bausch and Lomb "Spectronic 20" spectrophotometer.

Cell extracts.

The cells were washed three times, suspended in 100 ml of 0.01 M Tris buffer, and incubated with constant shaking for 30 minutes at 37 C.

The cells were centrifuged, suspended in 0.01 M Tris buffer, and ruptured by passage through a French pressure cell at a pressure of 15,000 psi. Cell debris was removed by centrifugation in a Sorvall RC-2 refrigerated centrifuge at 29,000 X g for 20 minutes at 4 C.

Substrates.

Succinic acid, PA, and ONB were prepared fresh for each experiment. The compound used was dissolved in 0.01 M Tris buffer and the pH adjusted to 7.0 with KOH. DNP was kept frozen from one experiment to the next.

Protocatechuic acid colorimetric assay.

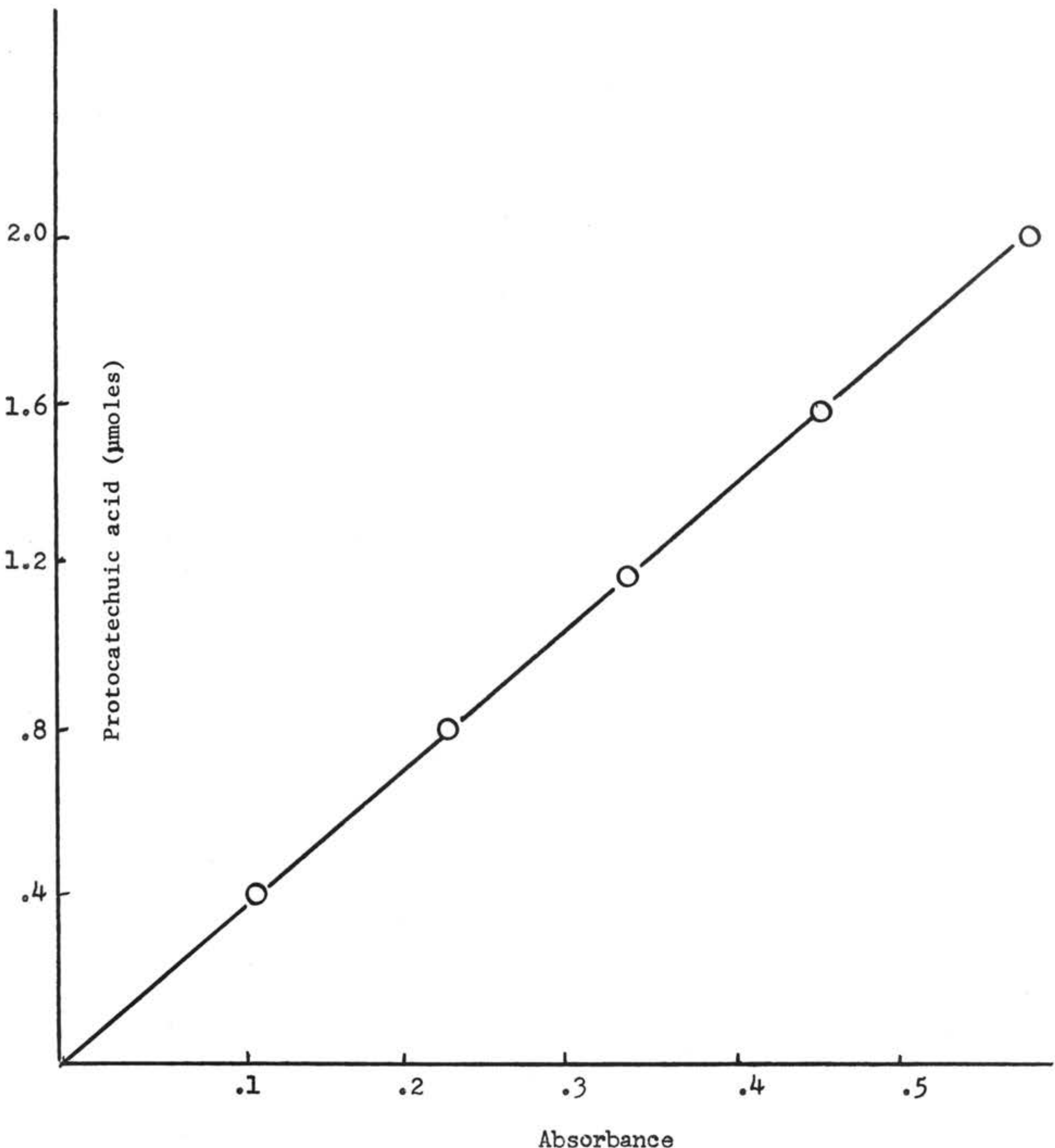
The colorimetric assay for PA was adapted from the procedure of Snell and Snell (1953). The sample (0.5 ml) to be tested was placed in a tube and 3.5 ml of distilled water was added. One ml of a solution containing 0.2 per cent ferrous sulfate and 1.0 per cent sodium potassium tartrate was added and the tubes were allowed to stand for 10 minutes at room temperature. Five ml of 20 per cent ammonium acetate was then added and the tubes read immediately at 540 mμ using the "Spectronic 20" spectrophotometer. A unit of enzyme activity is defined as that amount of enzyme required to transform 0.1 μmole of PA per hour. A standard curve for the absorbance of PA is given in Figure 4.

Manometric studies.

Oxygen uptake was measured using a Warburg apparatus at 37 C with air as the gas phase (Umbreit, Burris, and Stauffer, 1957). The center well contained 0.2 ml of 20 per cent potassium hydroxide to absorb carbon dioxide. The substrate and the inhibitor were placed in side arms, and the cell suspension was placed in the main chamber. The liquid volume was brought to a total of 3.0 ml by the addition of 0.01 M Tris buffer

Figure 4.

A standard curve for the colorimetric assay of protocatechuic acid.


2.0
1.6
1.2
.8
.4
Protocatechuic acid (μmoles)
.1
.2
.3
.4
.5
Absorbance

(pH 7.0). The flasks were equilibrated to temperature for 10 minutes before closing the manometers. Readings were taken for 30 minutes to determine the endogenous oxidation rate before the side arm contents were added into the flasks.

Procedure for uptake of radioactive substrate.

Ps. fluorescens cells grown on succinate agar were used for measuring the uptake of succinic-2,3-^{14}C-acid (Nuclear Chicago, specific activity of 4.32 μc/mmole). Five-tenths ml of the cell suspension, (a 1:10 dilution would give an absorbance of 0.25 at 540 mμ) was incubated for 75 seconds at 37 C with 0.1 ml of 0.01 M succinate; 0.02 ml of succinate-^{14}C (0.1 μc); 0.1 ml of the inhibitor; 0.1 ml of chloramphenicol (CAP) (750 μg/ml); and 0.01 M Tris buffer to bring the liquid volume to 1.0 ml. After 75 seconds incubation the cells were immediately filtered on a Millipore membrane filter (10 mm diameter; 0.45μ pore size) and washed four times with 0.5 ml of distilled water. The membrane filter was placed in a counting vial and dissolved with 0.5 ml of 3,4-dioxane. Counting fluid was prepared by dissolving 4 g of 2,5-diphenyloxazole and 0.05 g of p-bis-[2-(5-phenyloxazolyl)]-benzene in one liter of 3,4-dioxane. Counting fluid (9.5 ml) was added to each vial and the radioactivity was determined by counting in a Nuclear Chicago liquid scintillation spectrometer.

CHAPTER III

RESULTS AND DISCUSSION

Inducible enzyme formation in *Pseudomonas fluorescens* cells.

Protocatechuic acid can be used by this strain of *Ps. fluorescens* as a sole source of carbon and energy. The enzyme, protocatechuate oxygenase, responsible for the oxidation of this substrate, is inducible. In a typical experiment, washed nutrient agar-grown cells would adapt to protocatechuic acid in approximately 60-90 minutes (Figure 5). The per cent utilization of oxygen as compared with the theoretical was approximately 64 per cent (Table III).

Inhibition of the rate of enzyme synthesis by o-nitrobenzoic acid.

The addition of ONB simultaneously with the inducer in an inhibitor/substrate ratio of 10/1 resulted in a decrease in the rate of synthesis of protocatechuate oxygenase (Figure 6). Controls established that the autooxidation of PA and of ONB was negligible and the cells did not oxidize ONB. If the ONB was added to the cells 30 minutes prior to the PA, the rate of induction was reduced even more than when the two were added simultaneously. When ONB was added 40 minutes after induction was evident (80 minutes), the inhibition was more pronounced than when the substrate and inhibitor were added simultaneously. This may have resulted from the higher inhibitor/substrate ratio that prevails following utilization of some of the substrate by the cells.

The results obtained from the manometric experiment were augmented by an experiment run concommitantly in which the substrate was assayed

Figure 5.

Induction of protocatechuate oxygenase in Ps. fluorescens cells grown on nutrient agar. The protocatechuic acid concentration was 4.0 μmoles per flask. ○ , PA; ▲ , endogenous.

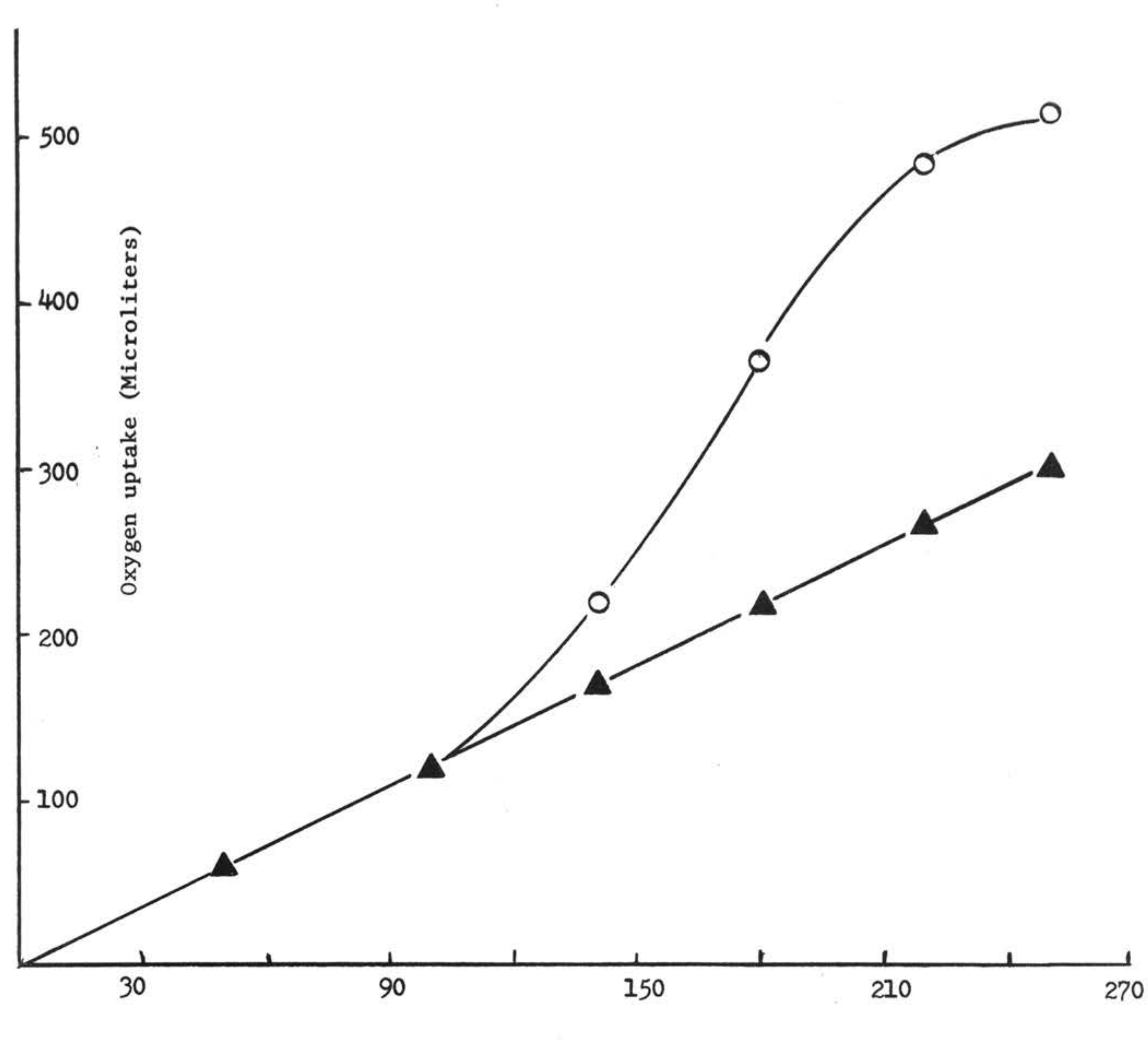
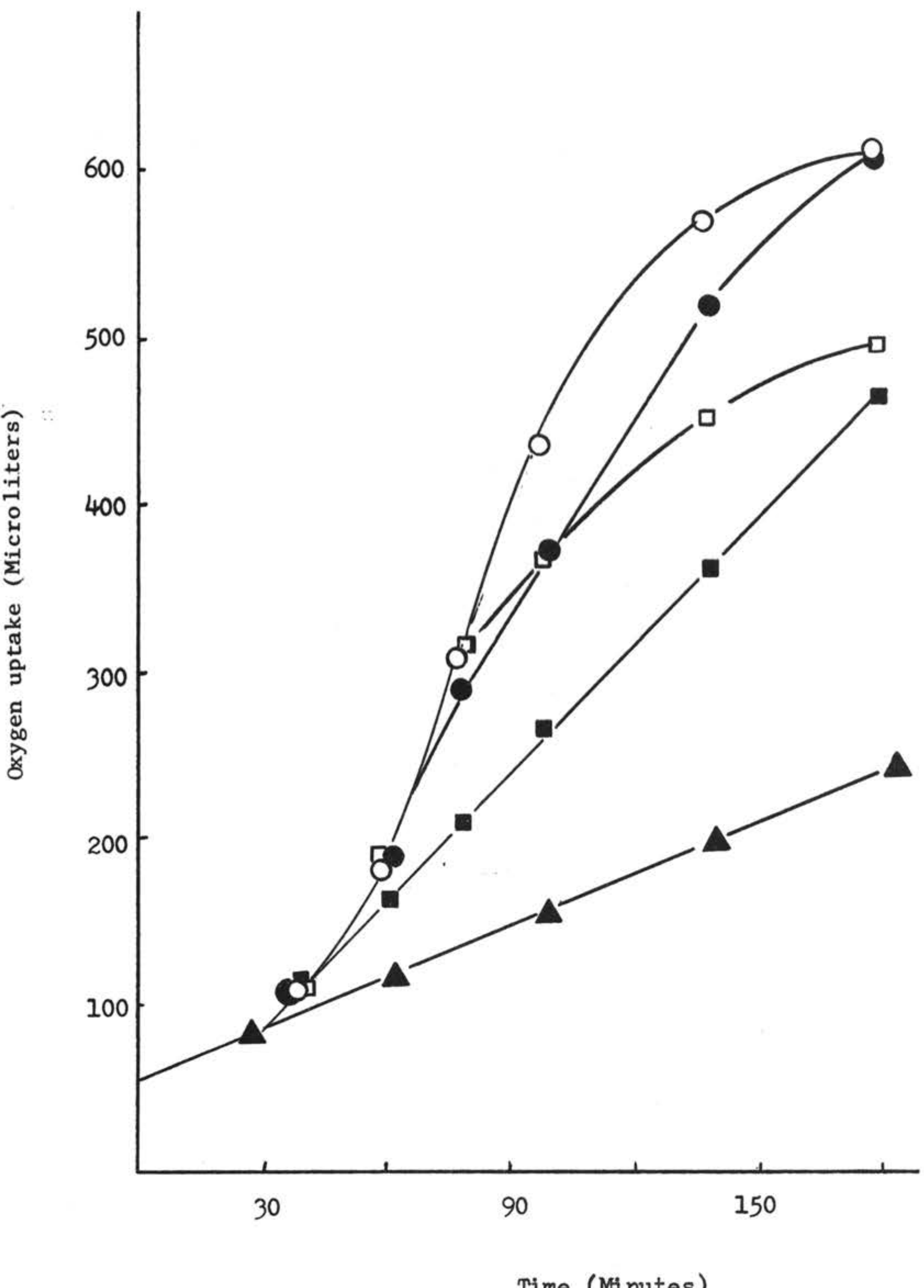

500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
210
270
Time (Minutes)

Figure 6.

Inhibition of protocatechuate oxygenase by o-nitrobenzoic acid. The concentration of protocatechuic acid was 4.0 µmoles per flask and of o-nitrobenzoic acid was 40 µmoles per flask. ○, PA (control); ●, PA + ONB (added simultaneously); ■, ONB added 30 minutes prior to PA; □, ONB added at 80 minutes; ▲, endogenous.

*PA added at 60 minutes.


600
500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
Time (Minutes)

colorimetrically. Two flasks containing 13 ml of a washed nutrient agar-grown cell suspension (1:20 dilution giving an absorbance of about 0.9 at 540 mμ) and 22.8 μmoles of PA were incubated at 37 C in an Eberbach shaking water bath. ONB (230 μmoles) was added to one of the flasks and 0.01 M Tris buffer (pH 7.0) was added to the flasks to bring the total liquid volume to 20 ml. At 0, 45, and 90 minutes, a 5.0 ml sample was removed from each flask and centrifuged in a Servall table model centrifuge at 4,000 x g at room temperature for 10 minutes. The cell pellet was suspended in 4.5 ml of 0.01 M Tris buffer and incubated with 0.2 ml toluene at 37 C with constant shaking. After 30 minutes, 0.5 ml of PA (0.04 M) was added to each tube and the tubes incubated for an additional 120 minutes. The cell suspensions were placed in a boiling water bath for 3 minutes to terminate enzymic activity, filtered, and the filtrates assayed for PA. The results (Table I) augment the findings from the manometric studies which show that ONB inhibits the synthesis of protocathechuate oxygenase and the presence of glucose does not influence the inhibition.

Effect of 2,4-dinitrophenol on enzyme synthesis.

Studies were conducted to compare the effect of ONB and of DNP, which uncouples oxidative phosphorylation, to determine if ONB might be acting by a similar mechanism. The presence of DNP in the induction system decreases the synthesis of protocatechuate oxygenase (Table I). The influence of DNP on induction was also evaluated in respirometric studies in which DNP (2.0 μmoles per flask) was substituted for ONB in the Warburg vessel. The results (Figure 7) show that DNP inhibits the synthesis of enzyme. However, the effect is not as great as with ONB but this may be due to the differences in concentration. Thus, it may

TABLE I. Enzymic activity of nutrient agar-grown cells induced to protocatechuic acid in the presence and the absence of inhibitors.

flask contents*	Enzymic activity (units/hr) Time in minutes 0	45	90
PA	0	4.0	8.5
PA + ONB	0	0	4.0
PA + DNP	0	1.5	2.0
PA + glucose	0	8.5	12.5
PA + glucose + ONB	0	2.0	2.5
PA + glucose + DNP	0	0	3.5

be that the two inhibitors - ONB and DNP - have a similar mechanism of action.

Effect of glucose on enzyme synthesis and inhibition.

Durham and McPherson (1960) reported that the addition of glucose shortened the lag period required for protocatechuate oxygenase synthesis. Therefore, studies were made to determine what effect glucose might have on a system inhibited by ONB. The noninduced system previously described was used. Glucose (1.5 µmoles per flask) shortened the lag period and ONB did not delay the time of induction but did decrease the rate of enzyme synthesis (Figure 8). DNP was also used as an inhibitor in the glucose-containing system. At a concentration of 1.0 µmoles per flask, DNP showed the same effect as ONB, that is, the rate of oxygen utilization was decreased (Figure 9).

Glucose was also used in experiments in which the enzymic activity was measured by the colorimetric assay of PA. Results are given in Table I and supplement the manometric data.

Effect of the inhibitor on cell viability.

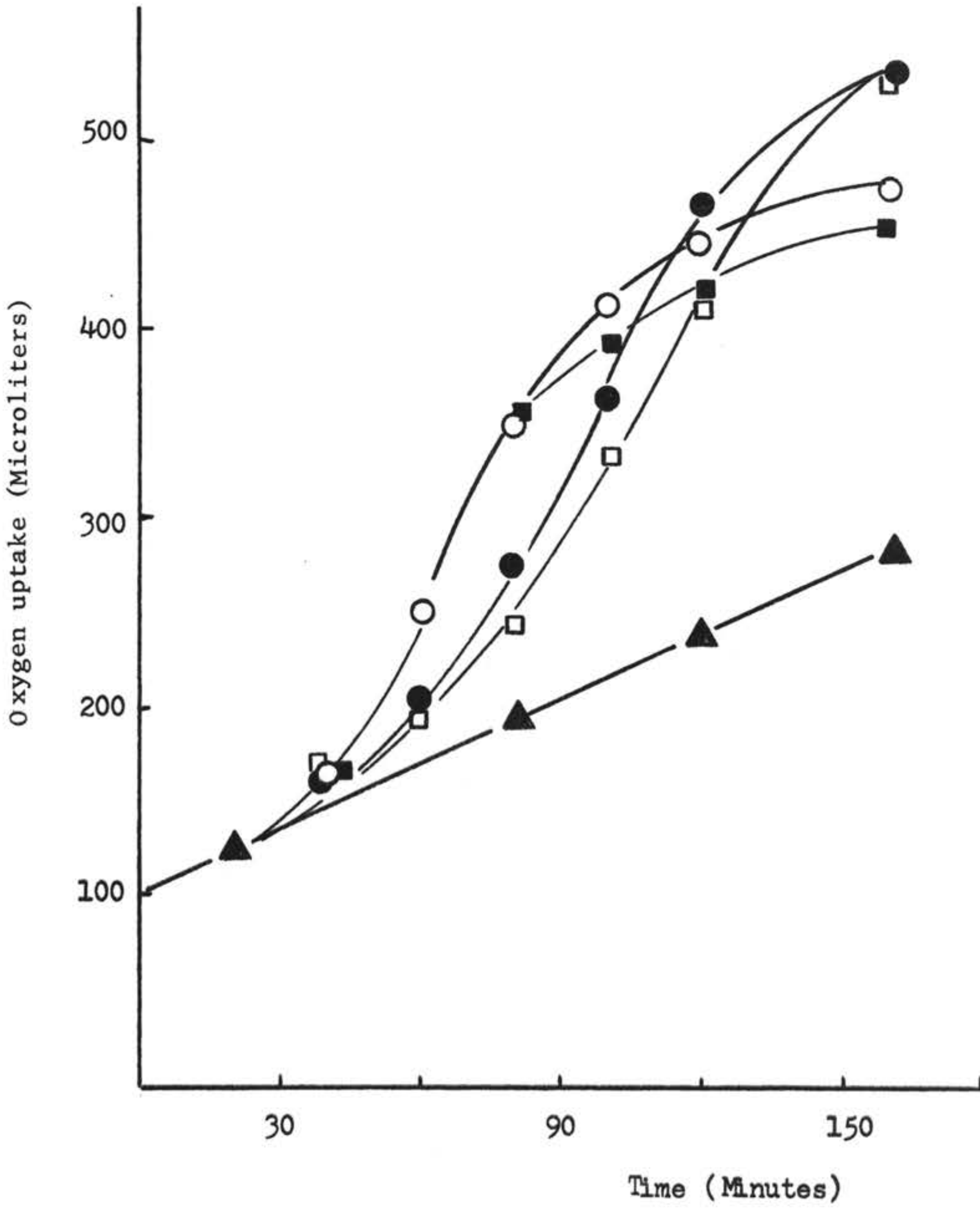
To determine whether or not the inhibitor was killing the cells, two types of experiments were conducted. The oxidation of succinic acid by succinic acid-grown cells was measured in the presence and absence of the inhibitor. Since succinic acid is oxidized by a constitutive enzyme, the killing of *Ps. fluorescens* cells by ONB might be reflected in the rate of substrate utilization. Manometric studies showed that the presence of ONB caused a slight increase in the total oxygen uptake, suggesting that the cells were probably not killed (Figure 10).

These results were confirmed by studies in which viable cell counts were made of samples taken from a system identical to that used for the

Figure 7.

Inhibition of protocatechuate oxygenase by 2,4-dinitrophenol. The concentration of protocatechuic acid was 4.0 μmoles per flask and of 2,4-dinitrophenol was 2.0 μmoles per flask. ○ , PA (control); ● , PA + DNP (added simultaneously); □ , DNP added 30 minutes prior to PA; ■ , DNP added at 60 minutes; ▲ , endogenous.

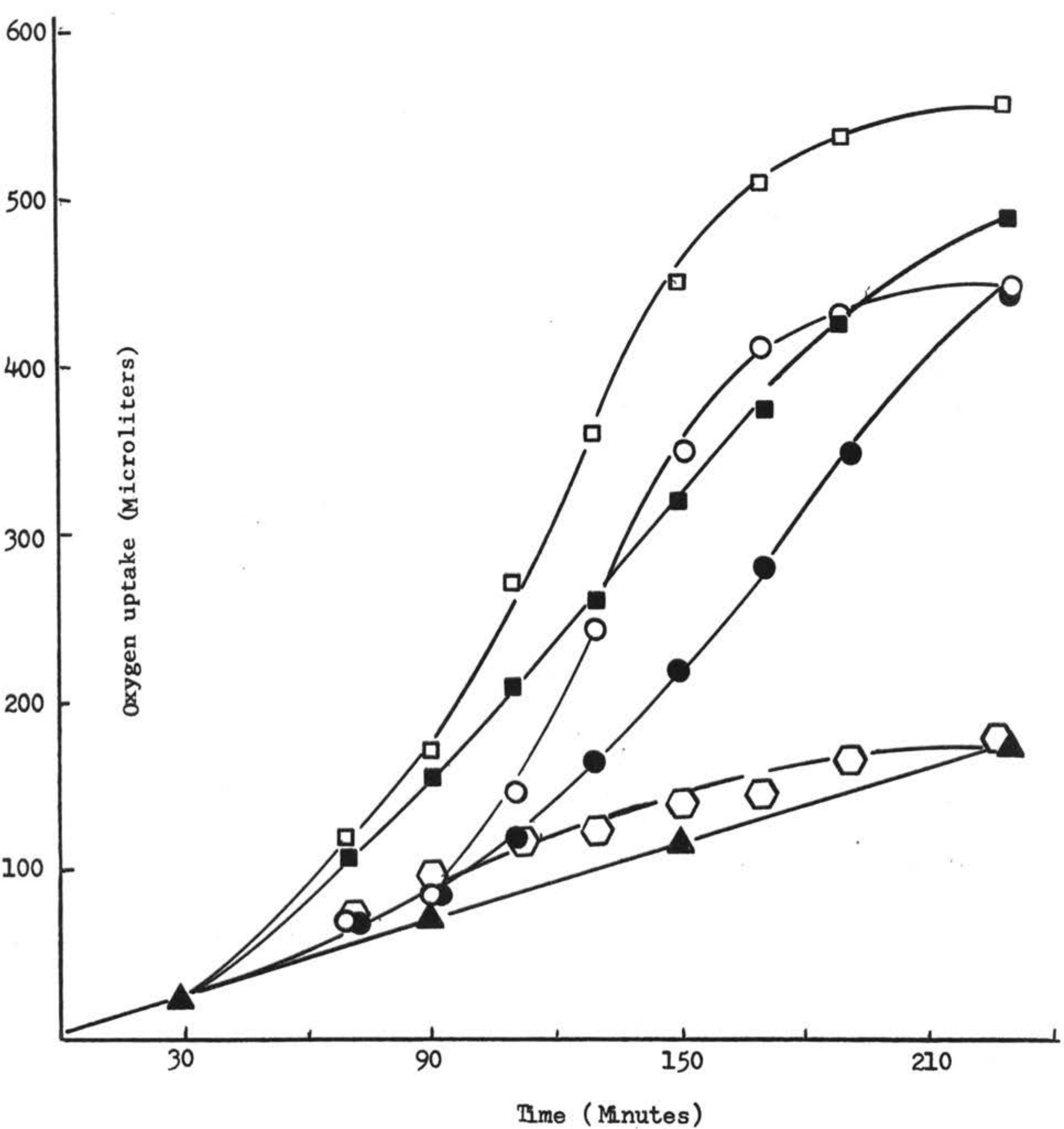
* PA added at 50 minutes.



500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
Time (Minutes)

Figure 8.

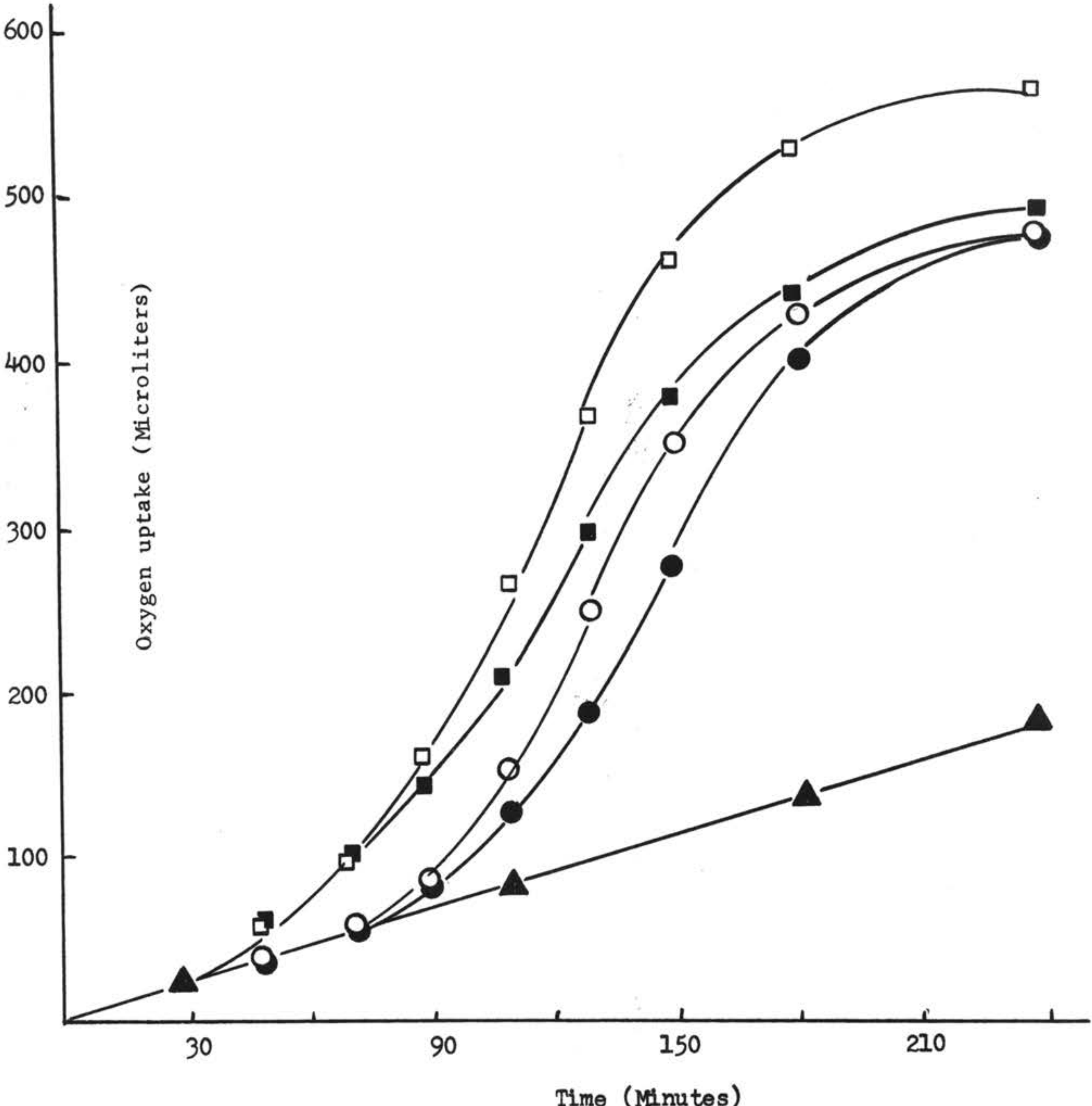
The influence of o-nitrobenzoic acid on the induction of Ps. fluorescens cells in the presence and the absence of glucose. The concentration of protocatechuic acid was 4.0 μmoles per flask, of o-nitrobenzoic acid was 40 μmoles per flask, and of glucose was 1.5 μmoles per flask. ○, PA (control); □, PA + glucose; ●, PA + ONB; ⬡, glucose (control); ■, PA + glucose + ONB; ▲, endogenous.



600
500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
210
Time (Minutes)

Figure 9.

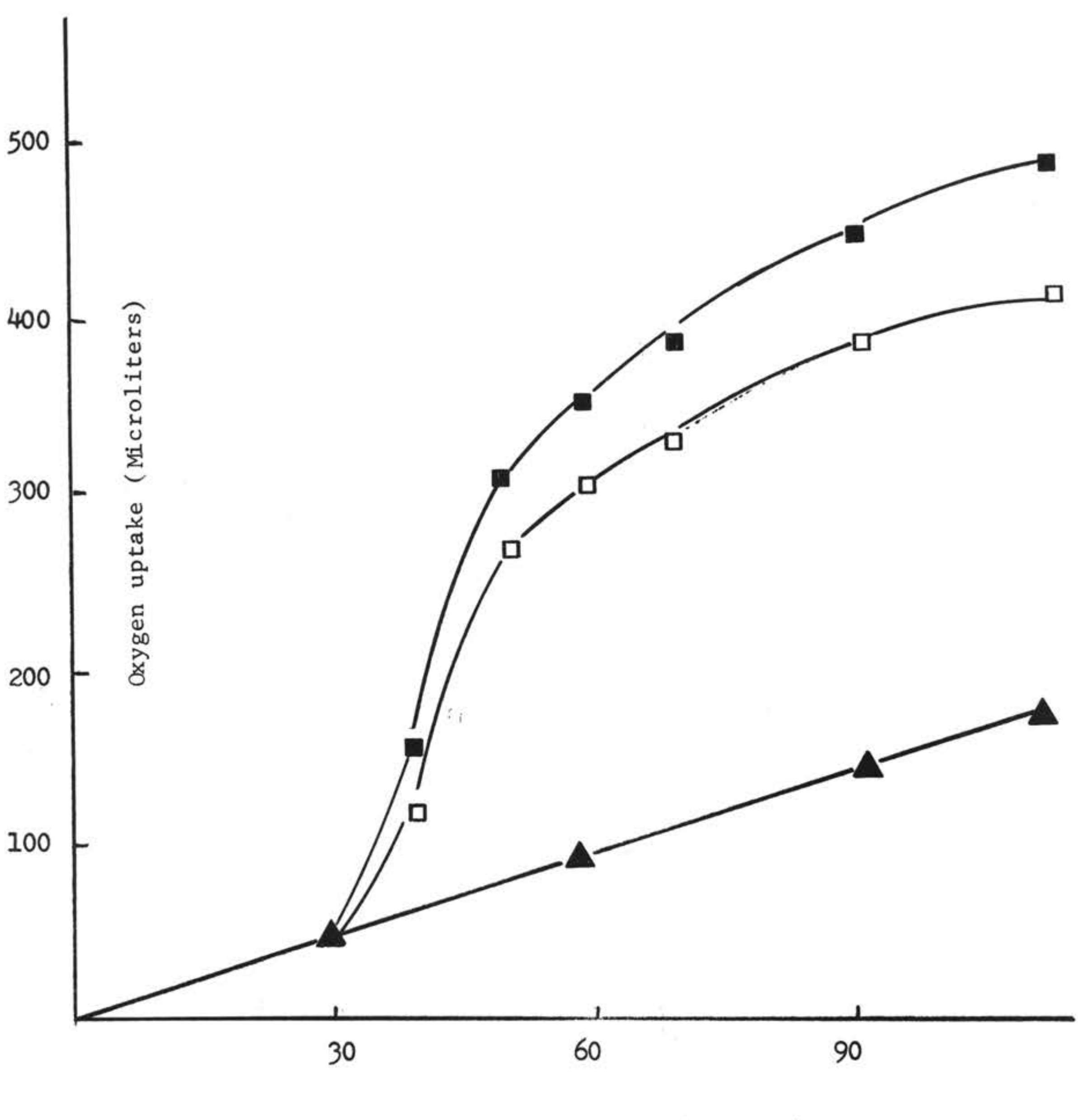
The influence of 2,4-dinitrophenol on the induction of Ps. fluorescens cells in the presence and the absence of glucose. The concentration of protocatechuic acid was 4.0 µmoles per flask, of 2,4-dinitrophenol was 1.0 µmole per flask, and of glucose was 1.5 µmoles per flask. ○ , PA (control); □ , PA + glucose; ●, PA + DNP; ■ , PA + glucose + DNP; ▲ , endogenous.



600
500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
210
Time (Minutes)

Figure 10.

Effect of o-nitrobenzoic acid on the oxidation of succinic acid by nutrient agar-grown Ps. fluorescens cells. The concentration of succinic acid was 4.0 µmoles per flask and of o-nitrobenzoic acid was 40 µmoles per flask. □ , succinic acid (control); ■ , succinic acid + ONB; ▲ , endogenous.


500
400
300
200
100
Oxygen uptake (Microliters)
30
60
90
Time (Minutes)

Warburg studies. Nutrient agar-grown cells in the presence and absence of ONB were incubated at 37 C in an Eberbach shaking water bath. One ml samples were removed at 0, 45, 60, 90, 120, 180, 240, and 300 minutes, diluted, and plated on nutrient agar using the seed-plate technique. The same experiment was repeated using PA-grown cells and the counts made on PA agar. A decrease in viable numbers was not observed in either the nutrient agar or PA-agar plate counts (Table II).

Effect of o-nitrobenzoic acid on the utilization of protocatechuic acid by induced cells.

To determine if the inhibitor was affecting the entrance of the substrate into the cell or the activity of existing enzyme, ONB was tested in a system using induced cells. A washed suspension of PA-grown cells was used in a series of investigations. The presence of ONB during respirometric studies resulted in an overall increase in oxygen uptake accompanied by a slight increase in the rate of utilization (Figure 11). The increase in oxygen utilization in the presence of ONB would indicate that neither the entrance of the substrate into the cell nor the activity of existing enzyme is affected by the inhibitor. DNP (2.0 µmoles per flask) produced similar results (Figure 12) again indicating that the two compounds may have a similar mode of action.

Reversibility of the o-nitrobenzoic acid effect by excess substrate.

Several explanations for the inhibition of PA utilization are possible and would include competition of ONB for a substrate or co-factor binding site on the protocatechuate oxygenase enzyme and interference with entrance of the substrate into the cell either by physical means - as attachment to the wall or membrane - or by some action on the permease-transfer mechanism. The following experiments to determine the

TABLE II. Viable cell count of noninduced and of induced cells incubated in the presence and the absence of o-nitrobenzoic acid and plated on their respective media.

Viable cells Number of colonies X 10^8									
Plated on	Incubated with	Time in minutes							
		0	45	60	90	120	180	240	300
Exp 1	PA	331	325	287	298	319	290	327	326
Nutrient agar	PA + ONB	329	309	360	330	279	295	319	269
Exp 2	PA	150	86	102	118	94	213	182	101
PA-agar	PA + ONB	75	147	133	131	94	207	168	94

Figure 11.

Effect of o-nitrobenzoic acid on the utilization of protocatechuic acid by protocatechuic acid-grown cells. The concentration of protocatechuic acid was 4.0 µmoles per flask and of o-nitrobenzoic acid was 40 µmoles per flask. ○ , PA (control) added at 30 minutes; ● , PA + ONB added simultaneously at 30 minutes; ▲, endogenous.

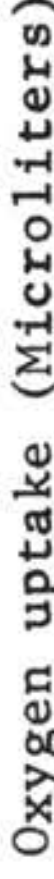
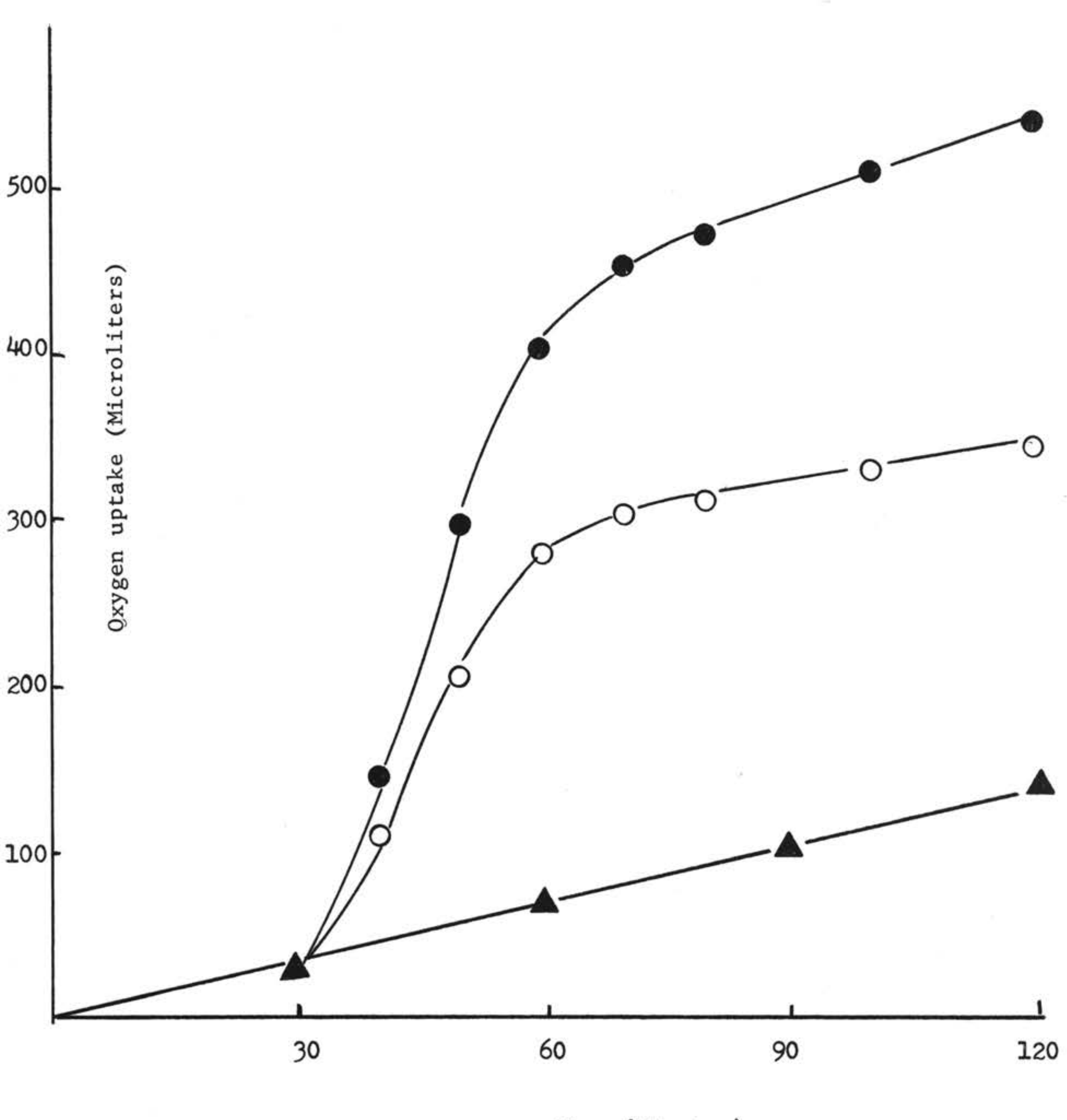

Oxygen uptake (Microliters)
500
400
300
200
100
30
60
90
Time (Minutes)

Figure 12.

Effect of 2,4-dinitropheol on the utilization of protocatechuic acid by protocatechuic acid-grown cells. The concentration of protocatechuic acid was 4.0 µmoles per flask and of 2,4-dinitrophenol was 2.0 µmoles per flask. ○ , PA (control) added at 30 minutes; ● , PA + DNP added simultaneously at 30 minutes; ▲ , endogenous.


500
400
300
200
100
Oxygen uptake (Microliters)
30
60
90
120
Time (Minutes)

reversibility of the inhibition in the presence of excess substrate were undertaken in an attempt to elucidate the mode of inhibitor action of ONB in the protocatechuate oxygenase system.

Nutrient agar-grown cells were washed three times and suspended in 0.1 M Tris buffer (pH 7.0) so that a 1:24 dilution gave a reading of 0.85 at 540 mμ. One side arm of the Warburg vessel contained 4 μmoles of PA and various concentrations of ONB. The second side arm contained additional PA (80 μmoles per flask). The cell suspension was placed in the main compartment along with sufficient 0.01 M Tris buffer to give a final liquid volume in the flask of 3.0 ml. Thirty minutes after induction was evident in the uninhibited control (130 minutes) the excess substrate was added (Figure 13). The inhibition by ONB was reversed by the addition of excess substrate since the rate in the presence of the inhibitor approached the rate evident in the control. These findings suggest that a competitive situation exists between the substrate and inhibitor.

Carbon dioxide production influenced by o-nitrobenzoic acid.

The increased oxygen uptake shown by the Warburg experiments with both the PA-grown and the succinic acid-grown cells is characteristic of inhibitors that act by uncoupling oxidative phosphorylation. An increase in carbon dioxide production often accompanies an uncoupling action of an inhibitor by virtue of the increased glycolysis (Dodds and Greville, 1934). Studies were made to determine the amount of carbon dioxide produced in the presence and absence of ONB (Umbreit et al., 1957). The contents of the flasks and the results are summarized in Table III. The results show that carbon dioxide production did increase in the presence of ONB concommitantly with the increased oxygen uptake indicating that ONB may be functioning as an uncoupling agent.

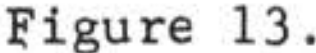
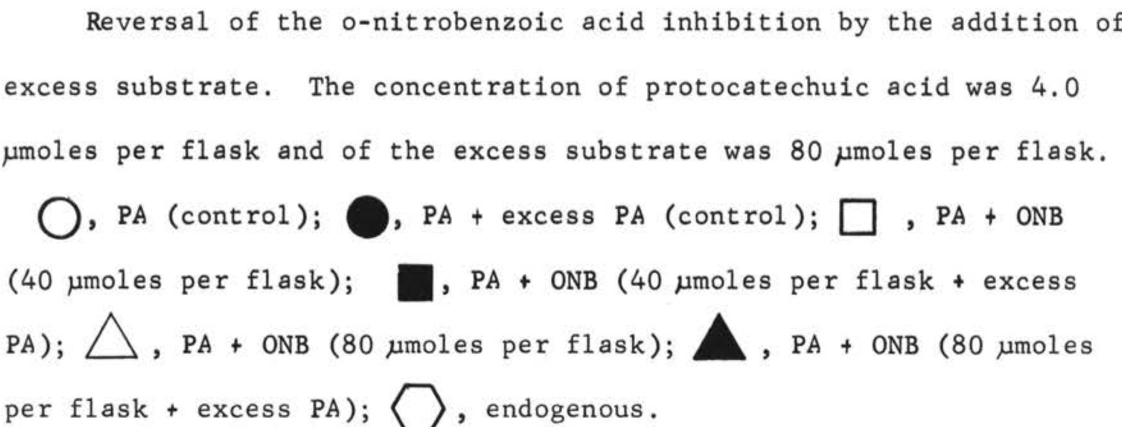
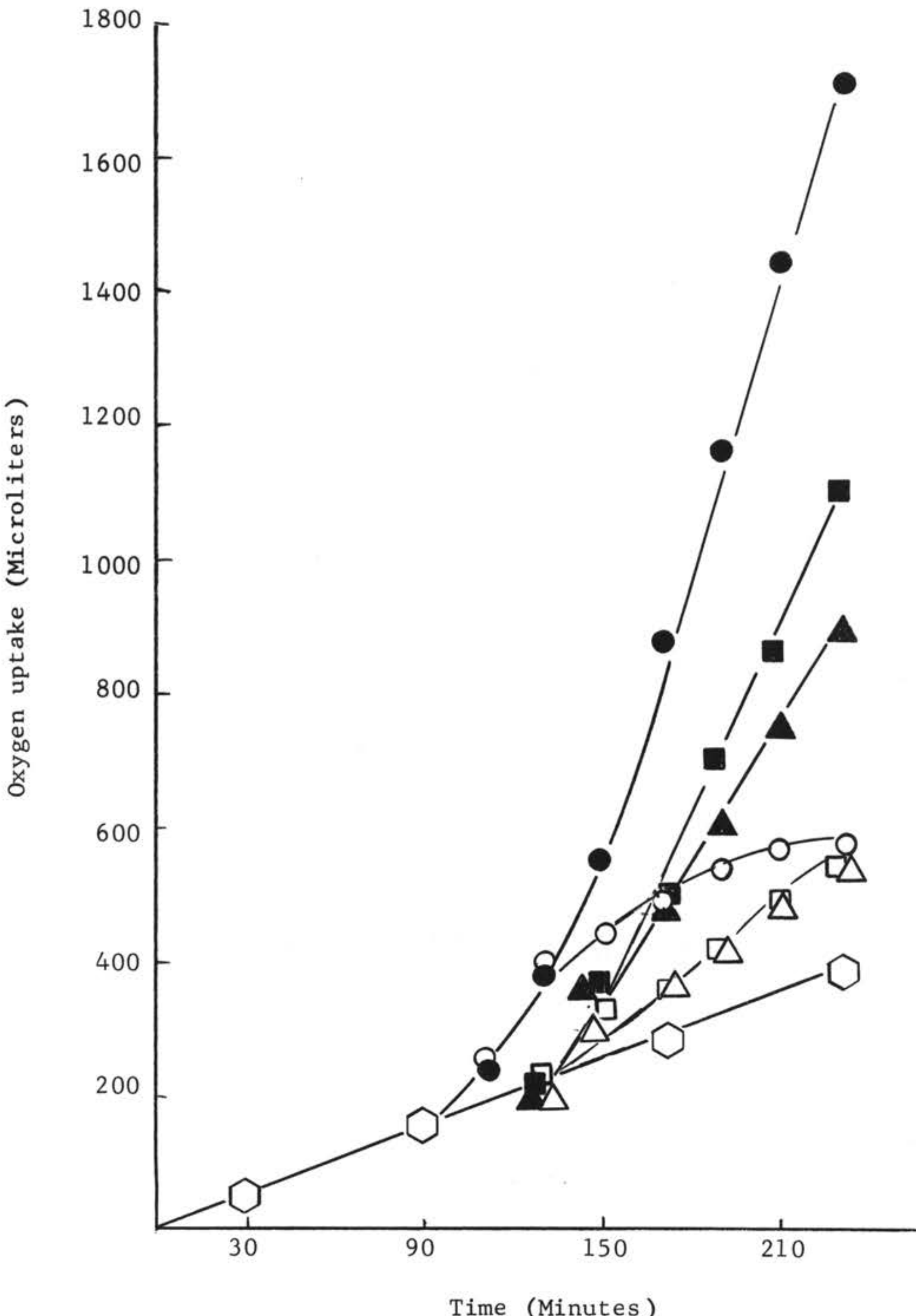


Figure 13.



Reversal of the o-nitrobenzoic acid inhibition by the addition of excess substrate. The concentration of protocatechuic acid was 4.0 µmoles per flask and of the excess substrate was 80 µmoles per flask. ○, PA (control); ●, PA + excess PA (control); □ , PA + ONB (40 µmoles per flask); ■, PA + ONB (40 µmoles per flask + excess PA); △ , PA + ONB (80 µmoles per flask); ▲ , PA + ONB (80 µmoles per flask + excess PA); ⬡, endogenous.



1800
1600
1400
1200
1000
800
600
400
200
Oxygen uptake (Microliters)
30
90
150
210
Time (Minutes)

TABLE III. Correlation of oxygen utilization and of carbon dioxide production by cells in the presence and the absence of o-nitrobenzoic acid.

	µmoles of O_2*	µmoles of CO_2 produced*
Succinate-grown cells		
1. 4 µmoles Succinate	1.05	1.20
2. 4 µmoles Succinate + 40 µmoles ONB	1.37	2.72
difference	0.32	1.52
PA-grown cells		
1. 4 µmoles PA	4.20	4.12
2. 4 µmoles PA + 40 µmoles ONB	4.73	5.62
difference	0.53	1.50

* values given in µmoles of gas per µmole of substrate

$$\text{(protocatechuic acid)} = C_7H_6O_4 \xrightarrow{6\frac{1}{2}\ O_2} 7\ CO_2 + 3\ H_2O$$

protocatechuic acid

The final products if complete oxidation occurs, contain 17 atoms of oxygen, four of which are supplied by the substrate, leaving 13 atoms or $6\frac{1}{2}$ µmoles of oxygen to be used from the atmosphere. Our data show that only 4.2 µmoles were taken from the air thus the oxidation of the protocatechuic acid was approximately 64 per cent complete.

Substrate uptake by cells in the presence and absence of o-nitrobenzoic acid.

If ONB is serving as an uncoupling agent, this would mean less ATP available for energy-requiring mechanisms and thus the active transport of substrate into the cell might be affected. The uptake of radioactive substrate was measured to determine if there was a difference in the substrate accumulation in the presence or absence of ONB.

Succinic acid agar-grown cells were incubated with succinic-2,3-^{14}C-acid as the substrate in one phase of this study (Table IV). ONB (10 µmoles per ml) inhibited the accumulation of succinic acid by the cells but a lower concentration (1.0 µmoles per ml) was without effect. Incubating the cells with the inhibitor for 20 minutes prior to adding the succinic acid did not prove to be more effective than when ONB and succinic acid were added simultaneously.

Similar studies were conducted with DNP and the results (Table IV) show that DNP in a concentration of 0.5 µmole per ml had little if any effect on the uptake of succinic acid but at an increased concentration of 1.0 µmole per ml substrate accumulation was inhibited. Exposure of the cells to the inhibitor for 20 minutes prior to adding the substrate may have enhanced the inhibition.

Radioactive PA was not available for similar studies, therefore, the depletion of PA using the colorimetric assay was used in an attempt to determine how the inhibitors influenced accumulation of PA by the cells. Cells grown on PA agar were harvested and washed three times in 0.01 M Tris buffer (pH 7.0). Two sets of tubes containing 2.0 ml of a heavy cell suspension (1:20 dilution giving an absorbance of 0.85 at 540 mµ) were incubated with either 3.0 µmoles ONB, 30 µmoles ONB, 1.5 µmoles DNP, or 3.0 µmoles DNP. Tris buffer (0.01 M) was added to bring the final

liquid volume to 3.0 ml. One set of tubes contained cells which were incubated with the inhibitor for 20 minutes prior to addition of the substrate. The cell-inhibitor mixture was exposed to the substrate for 6 minutes, placed in a boiling water bath for 3 minutes to stop the reaction, and centrifuged at approximately 4,600 rpm in a Servall table model centrifuge. The supernatant solution (0.5 ml) was used for the colorimetric assay. The results (Table V) indicate that neither ONB or DNP influence the uptake of PA by induced cells from the medium. Exposing the cells to the inhibitor for 20 minutes prior to adding the substrate did not affect the uptake. However, this uptake system is not as sensitive as those using labeled substrates so it may be that the effect is present but could not be detected under the condition of the experimentation.

Influence of exogenous adenosine triphosphate on the o-nitrobenzoic acid inhibition.

If the production of energy (ATP) by the cell is being decreased by ONB, it would seem feasible that reversal of the inhibition might be accomplished by adding exogenous ATP to the medium. The incubation of 3.0 µmoles of ATP with nutrient agar-grown cells in the typical respirometric induction system for 30 minutes prior to adding the substrate or the substrate plus inhibitor did not overcome the ONB effect and, in fact, this concentration of ATP lowered the total oxygen uptake both in the presence and absence of ONB (Figure 14). Higher concentration of ATP (9.0 µmoles per flask) inhibited to about the same degree, while lower quantities (1.5 µmoles per flask and 0.75 µmoles per flask) had no affect on the system either in the presence of absence or ONB. Failure to reverse the inhibitory effect of ONB by ATP may have been a consequence of the inability of ATP to permeate the cell barrier.

TABLE IV. Uptake of succinic-2,3-^{14}C-acid in the presence and the absence of o-nitrobenzoic acid and of 2,4-dinitrophenol.

Flask contents	Specific activity*
1. 1.0 µmoles/ml Succinic acid (control)	16,143
2. + 1.0 µmoles/ml ONB (20 minutes**)	16,914
3. + 1.0 µmoles/ml ONB	15,614
4. + 10 µmoles/ml ONB (20 minutes**)	9,237
5. + 10 µmoles/ml ONB	8,468
1. 1.0 µmoles/ml succinic acid (control)	3,916
2. + 0.5 µmoles/ml DNP (20 minutes**)	4,320
3. + 0.5 µmoles/ml DNP	3,202
4. + 1.0 µmoles/ml DNP (20 minutes**)	1,025
5. + 1.0 µmoles/ml DNP	1,735

* Specific activity was determined as the cpm per mg dry cell weight. The dry cell weight was 0.13 mg/ml.

** The inhibitor and the cells were incubated together for 20 minutes prior to the addition of the substrate.

TABLE V. Protocatechuic acid depletion from medium by induced cells under the influence of o-nitrobenzoic acid and of 2,4-dinitrophenol.

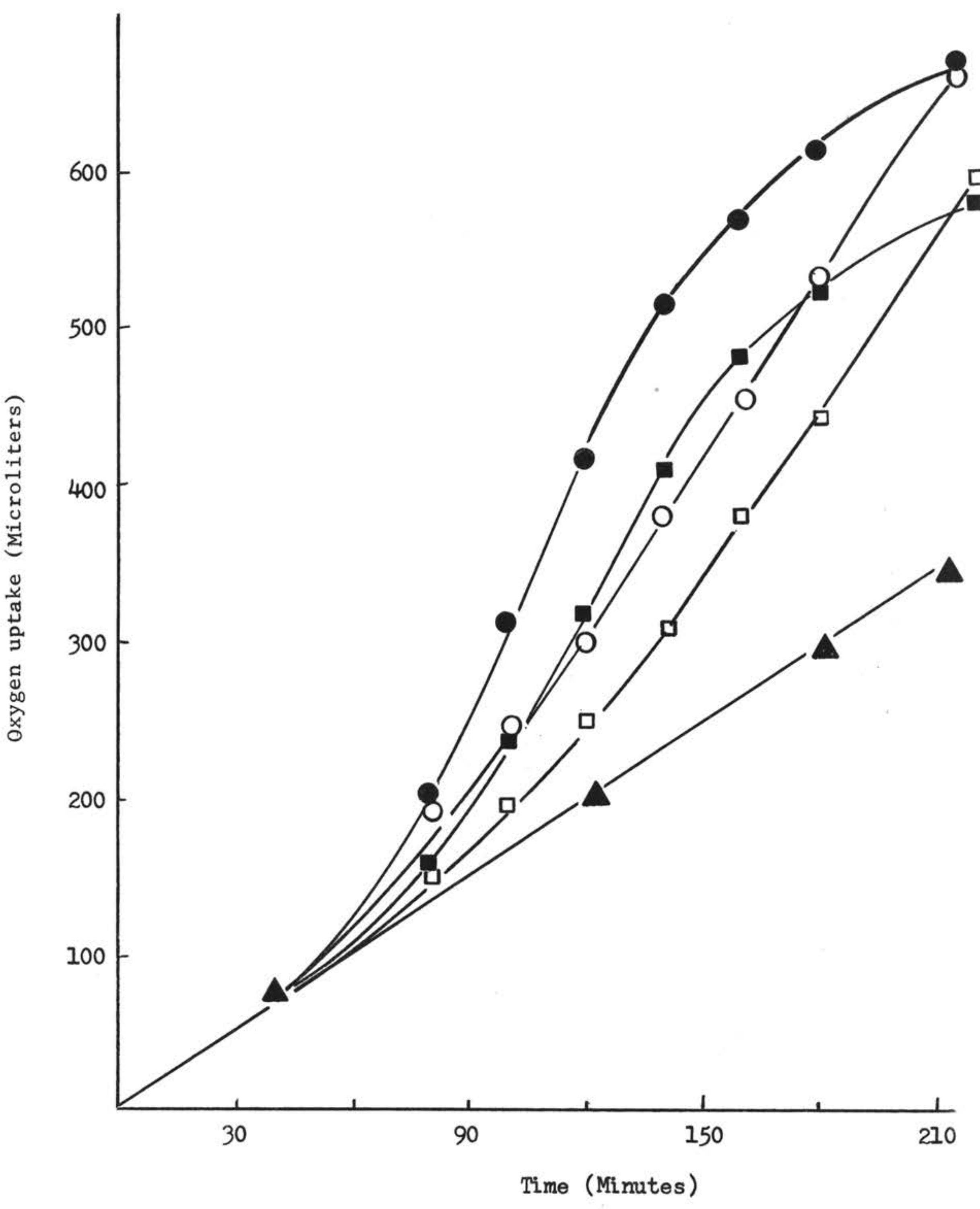
Flask contents	PA depleted,* µmoles/ml
1. PA (3.0 µmoles)	.015
2. + ONB (3.0 µmoles, 20 minutes)#	.020
3. + ONB (3.0 µmoles)	.027
4. + ONB (30 µmoles, 20 minutes)	.020
5. + ONB (30 µmoles)	.020
6. + DNP (1.5 µmoles, 20 minutes)	.020
7. + DNP (1.5 µmoles)	.020
8. + DNP (3.0 µmoles, 20 minutes)	.015
9. + DNP (3.0 µmoles)	.015

* µmoles PA/ml taken up from medium; original concentration of PA was 1 µmole/ml.

This time indicates that the inhibitor and the cell were incubated together for 20 minutes before addition of the substrate.

Figure 14.

Influence of adenosine triphosphate on the inhibition of enzyme synthesis by o-nitrobenzoic acid. The concentration of protocatechuic acid was 4.0 µmoles per flask, of o-nitrobenzoic acid was 40 µmoles per flask, and of adenosine triphosphate was 3.0 µmoles per flask. ●, PA (control); ○, PA + ONB; ■, PA + ATP; □, PA + ATP + ONB; ▲, endogenous.


600
500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
210
Time (Minutes)

Cell-free extracts used in the inhibited system.

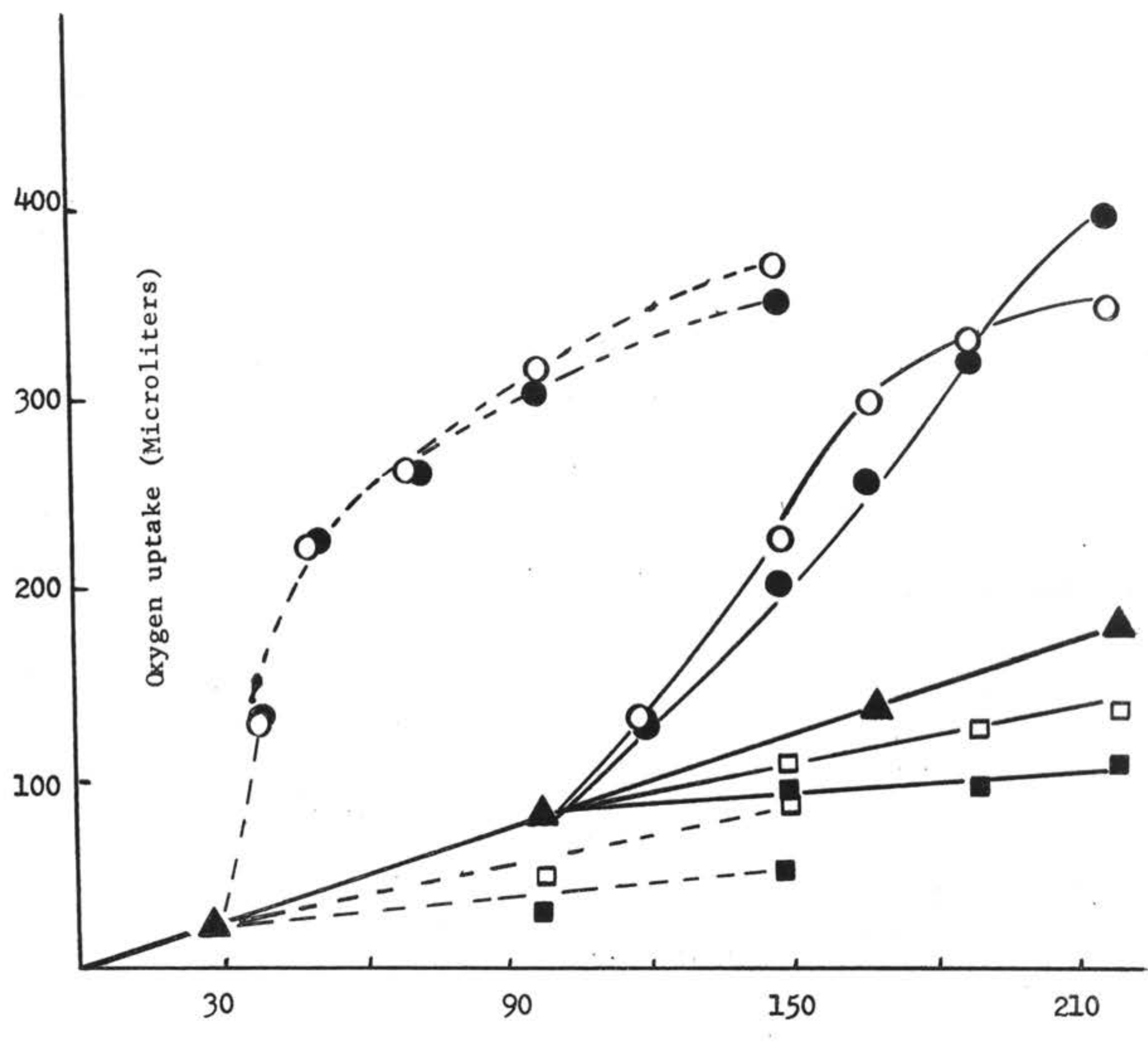
Hubbard and Durham (1961) reported that p-aminosalicylic acid (PASA) inhibited PA oxidation by *Ps. fluorescens* cells by competing with the substrate for the uptake mechanism. Therefore, it was of interest in this study to examine the role of permeability, if any, in the ONB inhibition. Cell extracts were prepared and examined manometrically. Extracts prepared from nutrient agar-grown cells did not induce to PA (Figure 15). Extracts prepared from cells grown on PA or succinic acid were unable to utilize their respective substrates. Since no oxygen utilization occurred in the constitutive succinic acid system it would appear that the cell-free system was lacking in a component of the electron transport chain.

Reduction of an artificial acceptor by cell extracts in the presence and the absence of o-nitrobenzoic acid.

To compensate for the above apparent loss of part of the election transport chain, methylene blue was used as an artificial acceptor. Two ml of extract of induced cells was placed in the side arm of Thunberg tubes. The tubes contained 40 μmoles PA as substrate plus, in a second tube, 400 μmoles ONB. As controls, a third tube contained neither PA or ONB and a fourth contained only ONB. To this was added 0.1 ml of a 0.1 per cent aqueous methylene blue solution and adequate 0.01 M Tris buffer (pH 7.0) to make a total liquid volume of 15 ml. Since only the two tubes which contained the substrate and the substrate plus inhibitor could be read spectrophotometrically due to the tube size, the other tubes were judged visually. The addition of ONB imparted some color to the mixture so the two tubes could not be standardized to the same initial reading. Therefore, only the rate of dye reduction and the shape of the curves

Figure 15.

Utilization of oxygen by cells and by cell extracts from nutrient agar-grown and from succinic acid-grown cells with protocatechuic acid and with succinic acid as respective substrates in the presence and the absence of o-nitrobenzoic acid. The concentration of protocatechuic acid was 4.0 µmoles per flask, of succinic acid was 4.0 µmoles per flask, and of o-nitrobenzoic acid was 40 µmoles per flask. The solid lines represent the nutrient agar-protocatechuic acid system and the broken lines represent the succinic acid system. ○, substrate (control) (cells); ●, substrate + ONB (cells); □, substrate (control) (extract); ■, substrate + ONB (extract); ▲, endogenous.



400
300
200
100
Oxygen uptake (Microliters)
30
90
150
210
Time (Minutes)

could be compared. These results are given in Figure 16. Since the curves appear to be similar in slope and shape, it is assumed that ONB does not influence the oxidation of PA by the enzyme. Therefore, ONB affects either the attachment of PA to the membrane or the transport therefrom. The control tubes showed no reduction.

Possible nonmetabolizable inducers tested.

Since labeled PA was not available, a nonmetabolizable inducer was sought in hope that we might come to a clearer understanding of the role of the cell membrane as associated with the transport system in the ONB inhibition. Techniques similar to those used with sugar and amino acid transport studies (Circillo, 1961; Marquis and Gerhardt, 1964; Noall *et al.*, 1957) were used in this study. Since PASA has been observed to compete with PA for the transport mechanism (Hubbard and Durham, 1961), this compound seemed to be a good possibility. Studies were conducted to determine if nutrient agar-grown *Ps. fluorescens* cells would take up ^{14}C-carboxy PASA. A nutrient agar-grown cell suspension (9.5 ml) was incubated with 0.1 ml of 0.01 M PASA as carrier and 0.03 ml ^{14}C-carboxy labeled PASA (0.09 μcuries/ml). The uptake was measured for 75 seconds in the presence and absence of ONB. Results (Table VI) show that, although PASA is taken up by the cell, neither concentration of ONB seems to have any inhibitory effect.

Studies were then made to ascertain whether PASA or p-aminobenzoic acid (PABA) would serve to induce protocatechuate oxygenase. Nutrient agar-grown cells were harvested, washed three times, and suspended in 0.01 M Tris buffer (pH 7.0) so that a 1:24 dilution gave a reading of 0.95 at 540 mμ. Nine ml of this suspension was placed in each of three 25 ml Erlenmeyer flasks containing respectively, 120 μmoles PA, 120 μmoles PABA,

Figure 16.

Reduction of methylene blue by cell extracts of induced *Ps. fluorescens* cells in the presence and the absence of o-nitrobenzoic acid. The concentration of protocatechuic acid was 4.0 μmoles per tube and of o-nitrobenzoic acid was 400 μmoles per tube. ◯, PA (control); ☐ , PA + ONB.

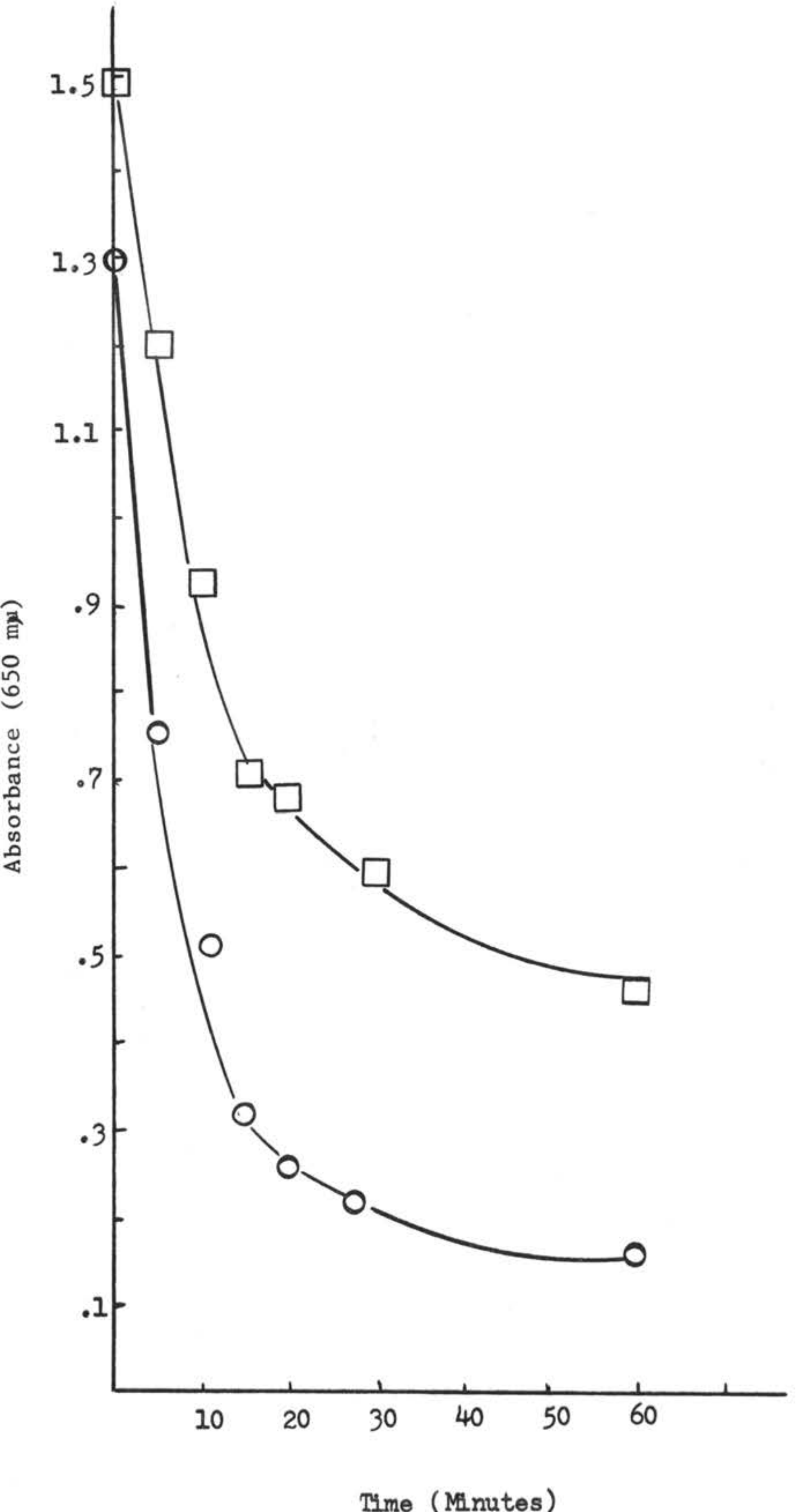

1.5
1.3
1.1
.9
.7
.5
.3
.1
Absorbance (650 mμ)
10
20
30
40
50
60
Time (Minutes)

TABLE VI. Uptake of p-aminosalicylic-^{14}C-carboxy-acid by nutrient agar-grown cells in the presence and the absence of o-nitrobenzoic acid.

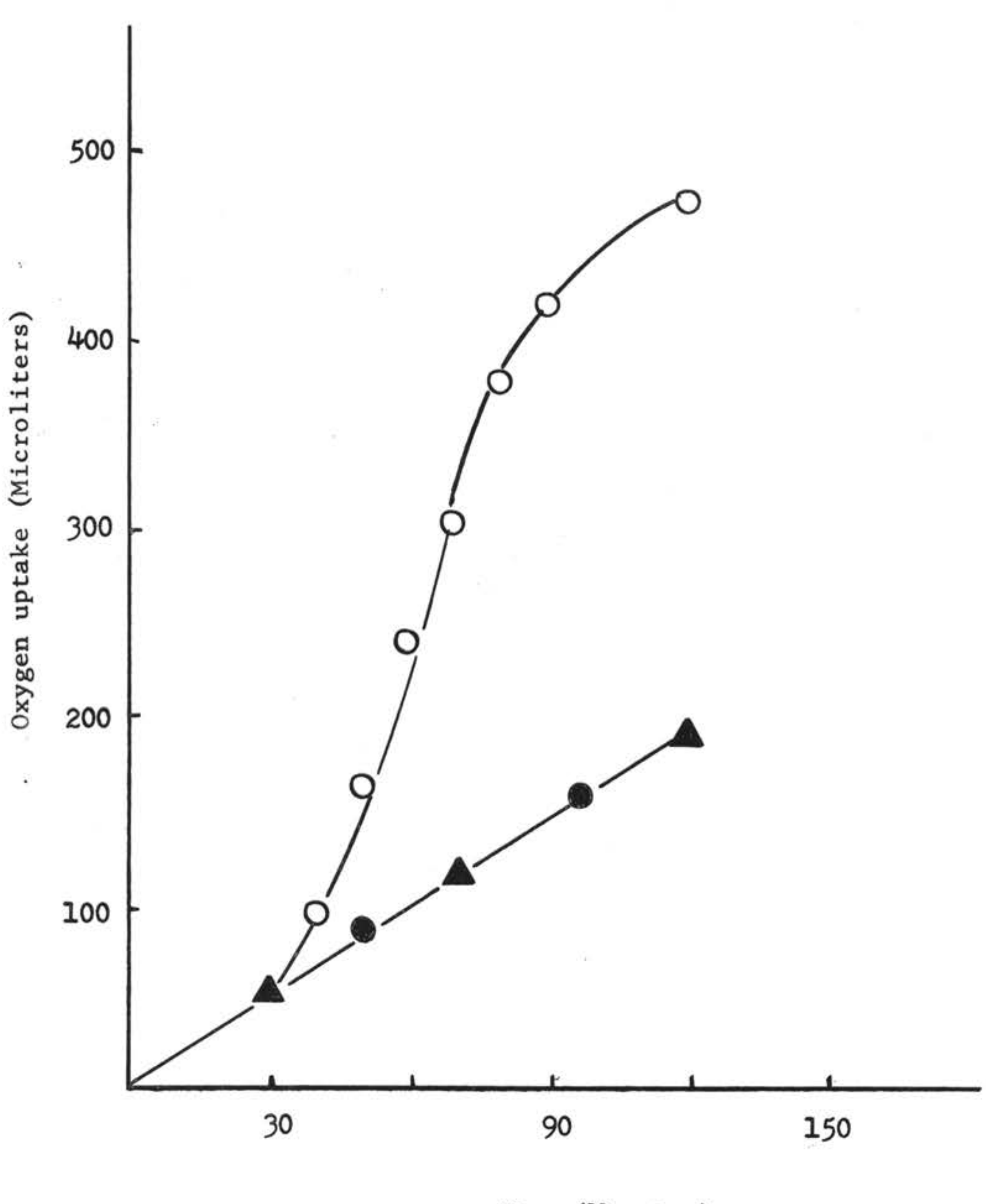
	Flask contents	Specific activity
1.	no PASA to control	431
2.	PASA (1.0 µmoles/ml)	3,438
3.	PASA + ONB (1.0 µmoles/ml, 20 minutes*)	2,806
4.	PASA + ONB (1.0 µmoles/ml)	2,949
5.	PASA + ONB (10 µmoles/ml, 20 minutes*)	3,466
6.	PASA + ONB (10 µmoles/ml)	3,062

* The inhibitor and the cells were incubated together for 20 minutes prior to the addition of the substrate.

or 120 µmoles PASA. A fourth flask contained only the cell and buffer suspension. These flasks were incubated at 37 C in an Eberbach shaking water bath for 2 hours at which time they were washed three times and suspended in 10 ml of 0.01 M Tris buffer. Two ml of each of the four suspensions were used in Warburg flasks containing PA (4.0 µmoles/per flask), 375 µg chloramphenicol (CAP) to prevent further enzyme synthesis, and sufficient 0.01 M Tris buffer to give a final liquid volume of 3.0 ml. Figure 17 shows that only those cells incubated in PA had synthesized protocatechuate oxygenase during the two hour time interval.

Figure 17.

The utilization of protocatechuic acid by nutrient agar-grown cells in the presence of chloramphenicol after incubation of the cells in protocatechuic acid, in p-aminobenzoic acid, in p-aminosalicylic acid, or in 0.01 M Tris buffer for 2 hours. The concentrations during the incubation were 120 μmoles of protocatechuic acid, p-aminobenzoic acid, or p-aminosalicylic acid per flask. The concentrations in the Warburg vessel were 4.0 μmoles of protocatechuic acid per flask and 375 μg of chloramphenicol per flask. ○, PA incubated cells; ▲—●, PASA, PABA, and Tris incubated cells and endogenous.



500
400
300
200
100
Oxygen uptake (Microliters)
30
90
150
Time (Minutes)

CHAPTER IV

SUMMARY AND CONCLUSIONS

The enzymes which oxidize protocatechuic acid in *Ps. fluorescens* cells are inducible. The rate of induction can be decreased by the addition of ONB to the induction system. The reduced rate of induction caused by ONB was also observed in a system containing glucose which reduced the lag time required for induction.

Cell-free extracts were used to determine whether ONB affected the activity of existing enzymes or the transport system. Preparation of the cell extracts apparently destroyed one or more components of the electron transport chain necessitating the use of an artificial acceptor. Methylene blue appeared to be reduced at the same rate in the presence and absence of ONB; therefore, it is concluded that the existing enzyme system is not directly affected by the inhibitor. Since the reversal of inhibition of oxygen uptake can be accomplished by the addition of excess substrate, the transport system is implicated as a site of competitive inhibition by ONB. Studies to find a nonmetabolizable inducer which could be obtained in radioactive form and thus elucidate the effect of ONB on the membrane transport site were unsuccessful.

The addition of ONB to a PA induced system or to the constitutive succinic acid system did not lower the rate of oxygen utilization, but resulted in an increased rate and total uptake of oxygen. This would implicate an uncoupling of oxidative phosphorylation. This was supported by results which showed a corresponding increase in carbon dioxide

production and a decrease in the accumulation of labeled substrate (succinic acid). Addition of ATP failed to reverse the action of ONB, possibly because of impermeability.

These results would suggest that the effect of ONB on the PA enzyme system in Ps. fluorescens cells may be a result of several sites of inhibition. There appears to be both an uncoupling of oxidative phosphorylation and a competition for transport sites. These effects alone or in combination could cause the inhibition in the rate of synthesis of protocatechuate oxygenase.

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