THE MICROBIAL DEGRADATION OF SOME DERIVATIVES OF ACETANILIDE

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A thesis
submitted to
the Faculty of Graduate Studies and Research
University of Manitoba

In partial fulfillment
of the requirements for the degree
Master of Science in Pharmacy

Faculty of Pharmacy
1973

AKNOWLEDGEMENTS

The author expresses her sincere gratitude to Dr. J.A.M. Shaw, who initiated this project, for his guidance and supervision throughout the course of this investigation.

The author thanks Miss Ellen Ho for her assistance in GLC analysis.

The facilities and assistance extended to the author by other members of Faculty of Pharmacy are gratefully aknowledged. The author also thanks the Faculty of Pharmacy, University of Manitoba for the teaching fellowship.

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ABSTRACT

An acetanilide utilizing microorganism which was isolated from the garden soil and classified as a Pseudomonas sp. was used in this study to investigate the degradation of some ring-substituted acetanilides (o-acetotoluidide, m-acetotoluidide, p-acetotoluidide, 2,6-dimethylacetanilide, 2'-hydroxyacetanilide, 3'-hydroxyacetanilide and 4'-hydroxyacetanilide).

The results indicate the deacylation of the substituted acetanilides to anilines which are further hydroxylated, and prepared for ring fission. o-Toluidine and catechol; m-toluidine and protocatechuic acid; p-toluidine and protocatechuic acid; o-aminophenol and catechol were found to be the metabolites in the bacterial cultures grown on o-acetotoluidide, m-acetotoluidide, p-acetotoluidide and 2'-hydroxyacetanilide, respectively. The enzymes involved in the microbial degradation of the substituted acetanilides studied have been shown to be inductive rather than constitutive.

The growth of the organism has been shown to be dependent upon two energy providing steps:

(1) Deacylation of the acetanilides ---- This reaction provides energy and carbon source for the growth of the organism in the form of acetate.

(2) Fission of the aromatic nucleus ---- This follows hydroxylation of anilines and produces simple aliphatic compounds which are readily utilizable by the organism.

INTRODUCTION

1. General

Our environment is constantly being polluted with many synthetic chemicals in the form of pesticides, herbicides and industrial effluents. Many of these chemicals are the derivatives of the aromatic compound, benzene. If these pollutents cannot be degraded by the microbial flora in soil, they will accumulate in soil and cause serious ecological changes. There is also a strong possibility that these chemicals will accumulate in animal tissues and cause serious problems. It is, therefore, important to understand the mechanisms by which natural and synthetic chemicals are metabolized by microorganisms, so that measures may be taken to prevent the destruction of our environment.

Although aromatic compounds are generally quite stable, microorganisms with versatile abilities do exist which can completely degrade a number of such compounds. The metabolism and utilization of aromatic compounds by certain bacterial species is now very well understood. Their isolation from various sources has been reported by numerous workers. The metabolic pathways leading to the microbial degradation of these compounds have also been studied and proposed, and some of these pathways are now well established.

There are two ways by which herbicides and pesticides in the soil may be decomposed: (1) chemical reactions; (2) biodegra-

dation (by plants or by microorganisms present in soil, the latter are especially important for the degradation and disappearance of such compounds).

2. Microbial Degradation of Aromatic Compounds

The wide use of aromatic compounds and the current concern of the ecologists over the pollution of our environment by the over use of such compounds has stimulated considerable interest and extensive studies in the mechanism of their degradation and their disappearance.

Stormer (1908) tested the effects of a number of organic compounds, including toluene, xylene, phenol and cresol, on soil and claimed to have isolated organisms that were capable of decomposing these compounds. Fowler, Arden and Lockett (1911) suspected the existence of phenol utilizing bacteria on sewage beds. The disappearance of aromatic compounds as a result of bacterial activities was studied quantitatively by Sen-Gupta (1921).

Mechanism of oxidative metabolism of aromatic compounds by bacteria was studied first by Grey and Thornton (1928) and Tattersfield (1928). They successfully isolated and identified several types of soil bacteria capable of degrading phenol, o-, m-, and p-cresol, naphthalene, phloroglucinol, resorcinol and toluene. These compounds are commonly used as soil sterilizing agents. Pure cultures of organisms were capable of utilizing these compounds as sole source of carbon, when incorporated into

a simple mineral salts medium containing an inorganic nitrogen supply. The strains were classified morphologically and from their growth characteristics, into six families: Coccaceae, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirillaceae and Bacillaceae. Happold (1930) has studied the capacity of the bacterial suspensions to catalyze the oxidation of catechol, guaiacol, resorcinol, phenol, p-cresol and tyrosine.

The interest in the studies of the microbial degradation of aromatic compounds involves: (a) The study of intermediates involved in the metabolic degradation of different aromatic substrates; (b) the elucidation of the mechanism of hydroxylation and ring fission; (c) the control of the enzymes participating in the metabolism of these aromatic compounds by microorganisms.

Microorganisms display a notorious versatility in releasing organic carbon, locked up in aromatic structures. The various steps in the metabolic pathways leading to the degradation of aromatic compounds can be clearly divided into two classess: (1) Pathways in the oxidative metabolism of aromatic compounds prior to ring fission; (2) reaction sequences associated with fission of aromatic nucleus.

It appeared, until recently, that the main problems of interest in the catabolism of aromatic compounds by microorganisms were those concerned with the unexplored reaction sequences leading to the already known modes of ring fission.

(a) Preparation for Ring Fission

It is generally accepted that dihydroxylation is a pre-requisite for enzymatic fission of the benzene ring. The hydroxyl groups may be ortho- to each other, as in the case of catechol and

protocatechuic acid, or para- to each other, as in gentisic acid and homogentisic acid. Catechol and protocatechuic acid have been shown to be substrates for ring fission in microbial degradation of many aromatic compounds (see Table 1, Gibson, D.T., 1968).

A substitution in the aromatic nucleus presents microorganisms with a choice, as to their mode of attack. Results obtained in the studies of microbial degradation of phenyl-substituted acids by Nocardia species, indicated that the acid side chains are metabolized by a beta-oxidation process; that is, the microorganism removes two-carbon atoms at one time. Thus side chains with an odd number of carbon atoms are metabolized to benzoic acid, which is further converted to catechol prior to ring fission. Phenyl-substituted acids that contain an even number of carbon atoms are metabolized through either homogentisic acid (2,5-dihydroxy-phenylacetic acid), or homoprotocatechuic acid (3,4-dihydroxy-phenylacetic acid), depending on the species of microorganisms (Dagley, S. et al., 1953; Kunita, N., 1955).

Gibson, D.T. (1968) studied the metabolism of toluene and isopropyl benzene by Pseudomonas putida and showed that these compounds are converted to o-dihydroxy-compounds in which the side chains are left intact. The enzymes, which catalyze the hydroxylation of the aromatic ring, have been termed "mixed function oxidases" by Mason, H.S., et al. (1955). In such reactions one atom of oxygen is incorporated into the substrate molecule.

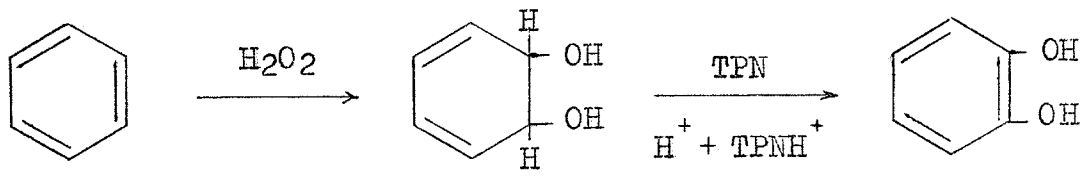
Marr and Stone (1961), using Pseudomonas species that utilized benzene as sole carbon source for growth, detected the

TABLE 1

Catechol and protocatechuic acid as central intermediates in microbial degradation of many aromatic compounds (Gibson, D.T., 1968)

Compounds metabolized via	
Catechol	Protocatechuic acid
Benzoic acid	m-Cresol
Salicylic acid	p-Cresol
Phenol	p-Hydroxybenzoic acid
Naphthalene	p-Hydroxymandelic acid
Phenanthrene	p-Aminobenzoic acid
Anthracene	Phthalic acid
Mandelic acid	
o-Cresol	
Benzene	

presence of catechol chromatographically. They excluded phenol as an intermediate in benzene metabolism, due to the inability of benzene grown cells to metabolize this compound; from this study, they thus proposed the following pathway for benzene oxidation to catechol:



Benzene — 3,5-Cyclo-hexadiene-1,2-diol — Catechol

Marr and Stone suggested that the first steps in the oxidation of benzene and other aromatic compounds are apparently unique, and can be carried out only by a limited number of strains. In the case of benzene, the enzymes were inductive, as removal of the organism from the benzene medium resulted in the loss of ability to oxidize benzene. Hypothetical pathways, given in this study, for the formation of catechol from benzene, suggested epoxidation of the aromatic nucleus, followed by hydrolysis to form trans-benzene-glycol (1,2-dihydroxy-3,5-cyclohexadiene) as one of the possible sequences in the formation of catechol; Fig.1. Enzymatic dehydration of trans-benzene-glycol resulted in the catechol formation (Gibson, D.T., 1968).

Taniuchi, H. et al. (1964) isolated an enzyme from a Pseudomonas sp., which was capable of converting anthranilic acid

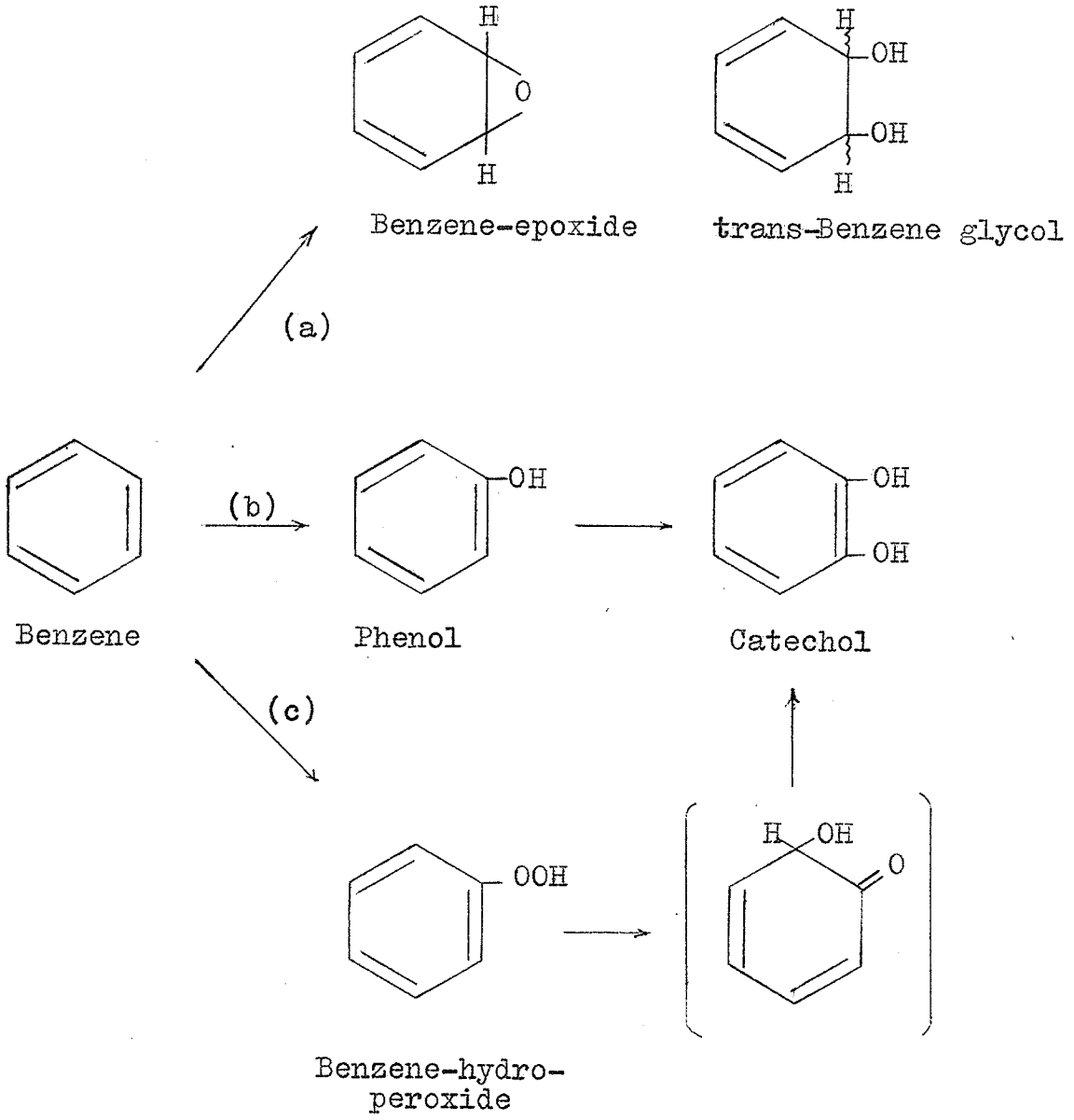
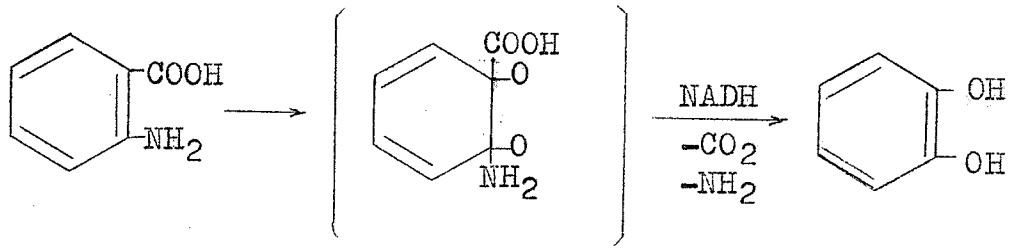


Fig. 1. Hypothetical pathways for the formation of catechol from benzene (Marr and Stone, 1961).

to catechol involving an epoxide intermediate; but isotopic studies by Kobayshi, S. et al (1964) showed that both atoms of the oxygen molecule were incorporated into the aromatic substrate. Thus a cyclic peroxide intermediate has been proposed as a product of the anthranilic acid oxidation:



Anthranilic acid — Hypothetical peroxide intermediate — Catechol

Diversity is found commonly in pathways leading to the oxidation of aromatic compounds prior to ring fission. Hayaishi and Stanier (1951) reported that there were two distinct pathways of tryptophan metabolism among the Pseudomonas. Kunita, N. (1955) investigated the microbial dissimilation of phenylacetic acid. The results indicated that one strain of Pseudomonas fluorescens produced homoprotocatechuic acid from phenylacetic acid, while another strain appeared to take the homogentisate route.

There is a remarkable diversity in the pathways employed for the utilization of benzoate among microbial species and strains prior to ring fission, Fig. 2. Formation of aniline was

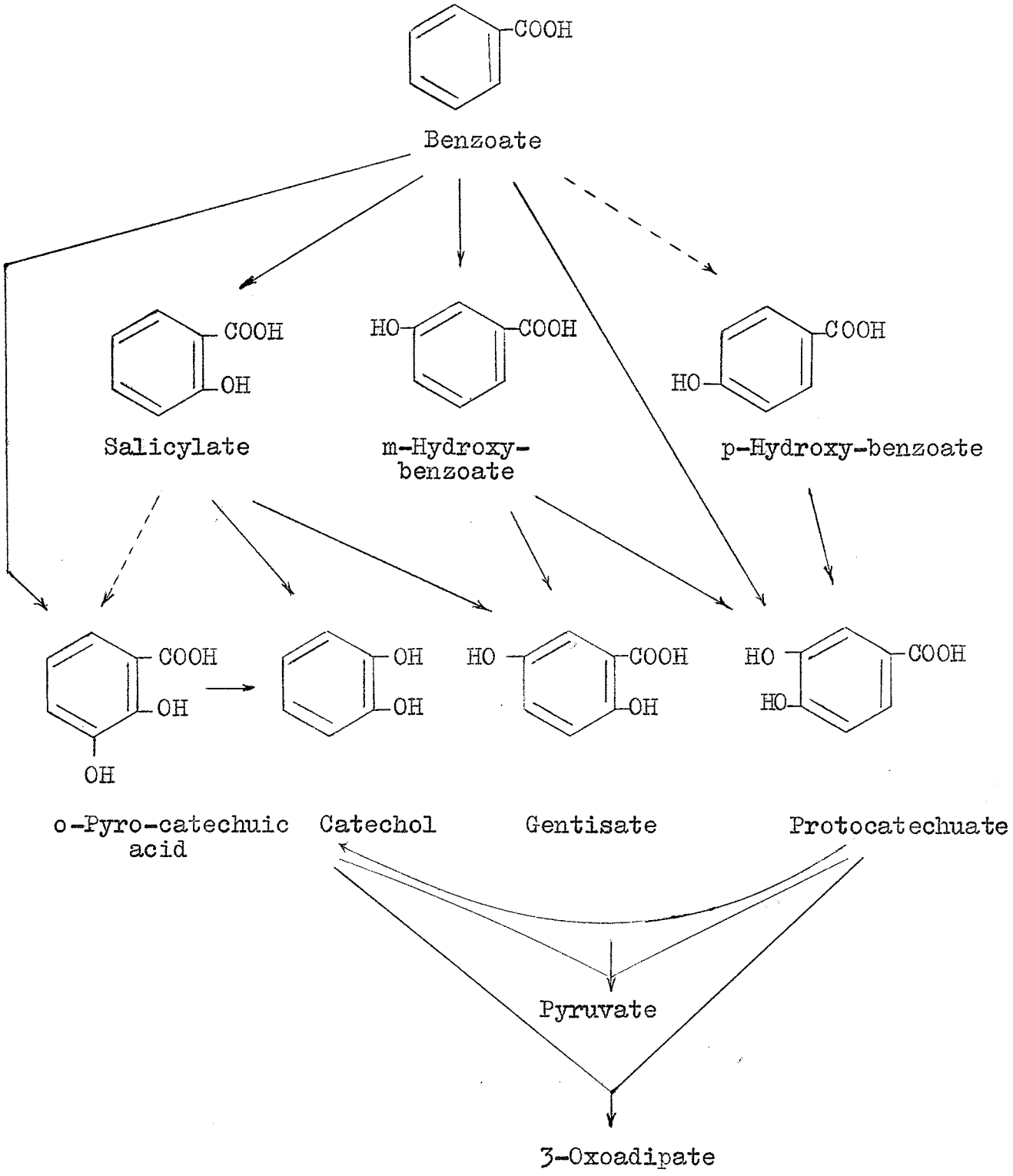


Fig. 2. Diversity in the microbial degradation of benzoate (Dagley, S. et al., 1960).

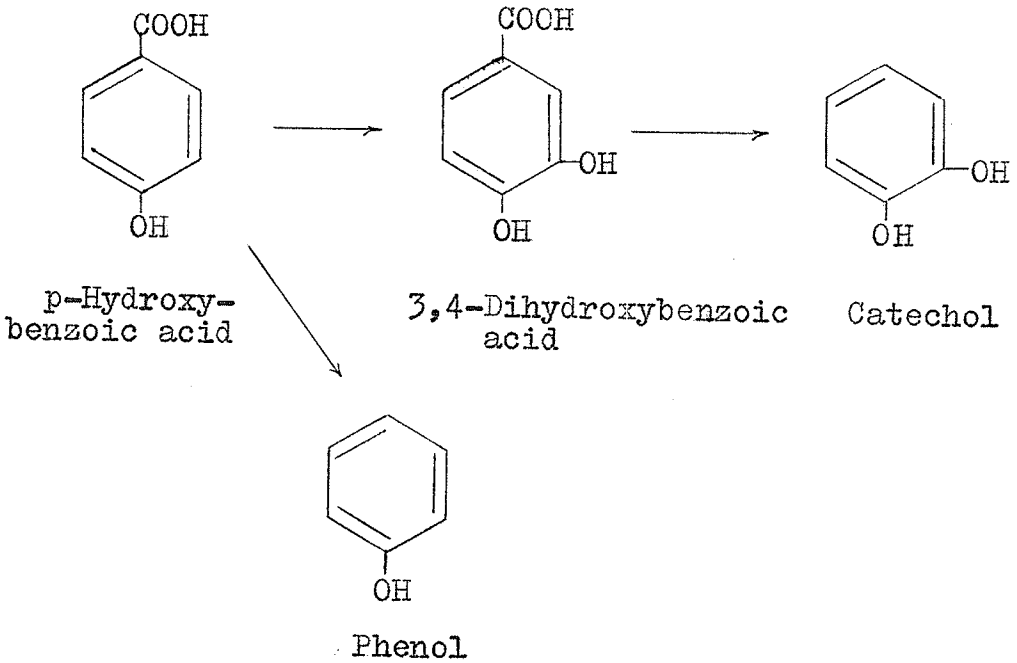
reported from p-aminobenzoic acid by Mycobacterium smegmatis, which was further oxidized to p-aminophenol. Further study with acid fast bacteria revealed the following reaction sequence:

p-Aminobenzoic acid ⟶ p-Aminobenzyl alcohol

↓

p-Hydroxyaniline

The metabolic pathways used by members of the genus Azotobacter for the oxidation of benzoic acid and p-hydroxybenzoic acid were studied by Hardisson, C. et al. (1969). They showed that several Azotobacter species oxidized benzoic acid via catechol, and p-hydroxybenzoic acid through protocatechuic acid. Patel and Grant (1969) demonstrated the presence of phenol in the culture fluid of Klebsiella aerogenes after growth in a chemically defined medium consisting of mineral salts and p-hydroxybenzoic acid. They thus proposed a metabolic pathway for the degradation of p-hydroxybenzoic acid through catechol, and the formation of phenol was attributed to a side reaction:



p-Hydroxybenzoic acid

3,4-Dihydroxybenzoic acid

Catechol

Phenol

Dagley, S. et al (1953) reported that phenylalanine was oxidized to homogentisic acid by Vibrio via two different pathways: one through phenylpyruvic acid, and the other via tyrosine and p-hydroxyphenylpyruvic acid. Walker and Wiltshire (1953) found the presence of salicylic acid and catechol as intermediates during the metabolism of naphthalene by a soil bacterium, using "simultaneous adaptation techniques" (Stanier, R.Y., 1947).

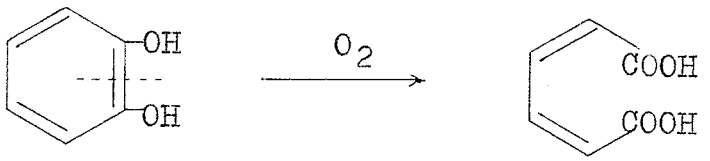
The enzymatic transformations involved in these reactions are hydroxylations and decarboxylations. The particular route taken by a microorganism, capable of utilizing an aromatic compound, will presumably depend on its genetic potential coupled with the environmental influences for the synthesis of the relevant enzymes.

Hayaishi, O.(1964) proposed the term "mono-oxygenases" for enzymes that catalyze the incorporation of one atom of oxygen molecule into a substrate molecule, and the term "di-oxygenases" for enzymes incorporating both atoms of the oxygen molecule. Di-oxygenases function principally in the enzymatic fission of the aromatic nucleus.

(b) Fission of the Aromatic Nucleus

The dihydroxylated benzene derivatives, catechol and protocatechuic acid, have been studied quite intensively, due to the fact that they are demonstrated to be metabolic intermediates of many aromatic compounds. An enzyme, which was capable of cleaving the dihydroxylated derivatives of benzene between the two hydroxyl groups, was isolated by Hayaishi and Hashimoto (1950).

The reaction products, in the case of catechol and protocatechuic acid, were identified as cis, cis-muconic acid and β-carboxy-cis-cis-muconic acid, respectively.



Catechol — cis-cis-Muconic acid



Protocatechuic acid — β-Carboxy-cis-cis-muconic acid

Dagley and Stopher (1959) reported an alternate enzymatic cleavage of catechol by a Pseudomonas sp., which utilized o-cresol as sole carbon source for growth. Catechol was oxidized to α-hydroxy-muconic semialdehyde by a reaction catalyzed by the enzyme catechol-2,3-dioxygenase, Fig. 3. Dagley and Evans (1960) demonstrated the induction of another enzyme, protocatechuic acid-4,5-dioxygenase, by growing another Pseudomonas sp. on p-cresol. In this case, protocatechuic acid was oxidized to α-hydroxy-γ-carboxy-muconic semialdehyde, Fig. 4.

The metabolism of these ring fission products were reinvestigated by Ornston and Stanier (1966). They used Pseudomonas

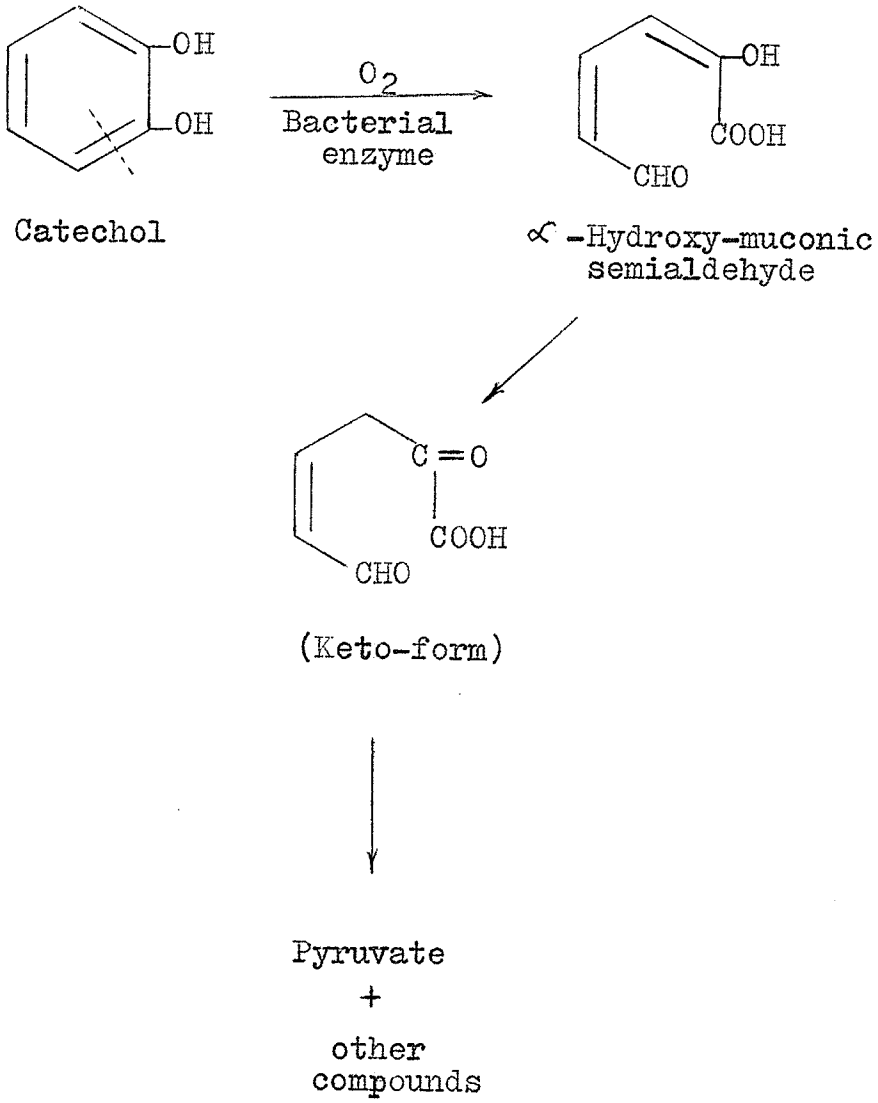


Fig. 3. Suggested pathway for the oxidation of catechol by microorganisms possessing the catechol-2,3-dioxygenase system. (Dagley, S. and Stopher, D.A., 1959)

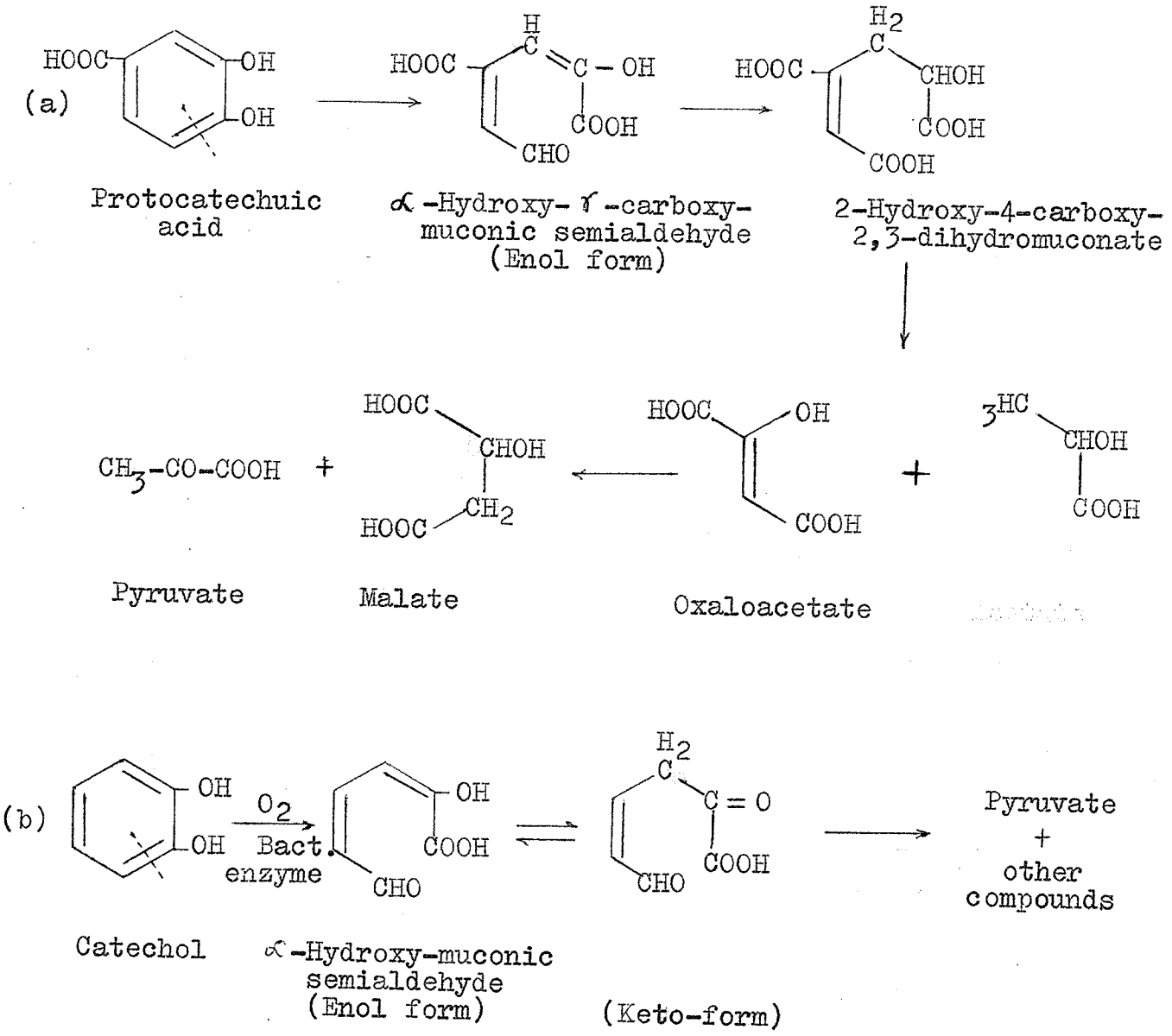


Fig. 4. Proposed pathways for oxidative metabolism of protocatechuic acid and catechol by Pseudomonas sp., possessing protocatechuic acid-4,5-dioxygenase enzyme system (Dagley, S. and Evans, C.W., 1960).

putida and showed that β-oxoadipic acid enol lactone (γ-carboxymethyl-Δ^{β}-butenolide) as a common intermediate in the sequence of reactions initiated by catechol-1,2-dioxygenase, Fig. 5. Table 2 shows the ring fission substrates corresponding to some aromatic compounds (Gibson, D.T., 1968).

Diversity in pathways in the enzymatic fission of aromatic nucleus, during oxidative metabolism of aromatic compounds, is found quite commonly in organisms utilizing aromatic compounds as sole carbon source for growth. The metabolism of benzoate and phenol was studied by Feist and Hegeman (1969) by using strains of Pseudomonas putida. In the case of growth at the expense of benzoate, catechol was shown to cleave ortho- and metabolize via β-keto-adipate pathway; in the case of cresol and phenol, the catechol or substituted catechol formed were metabolized by a separate pathway, following meta-cleavage of aromatic ring of catechol. Due to this diversity in pathways of ring fission, Dagley, S. et al. (1964) proposed a general scheme for the degradation of catechol, Fig. 6. This has been successfully applied to microbial degradation of several aromatic compounds. However, microorganisms do not live by a rigid set of rules; it is therefore, not surprising to find exceptions to the proposed scheme, Table 3.

Many alternate routes of metabolism have been found. It is thus clear that catechol, protocatechuic acid, gentisic acid, and homogentisic acid are not the only substances susceptible to ring fission. Further, that the oxygenative cleavage of the

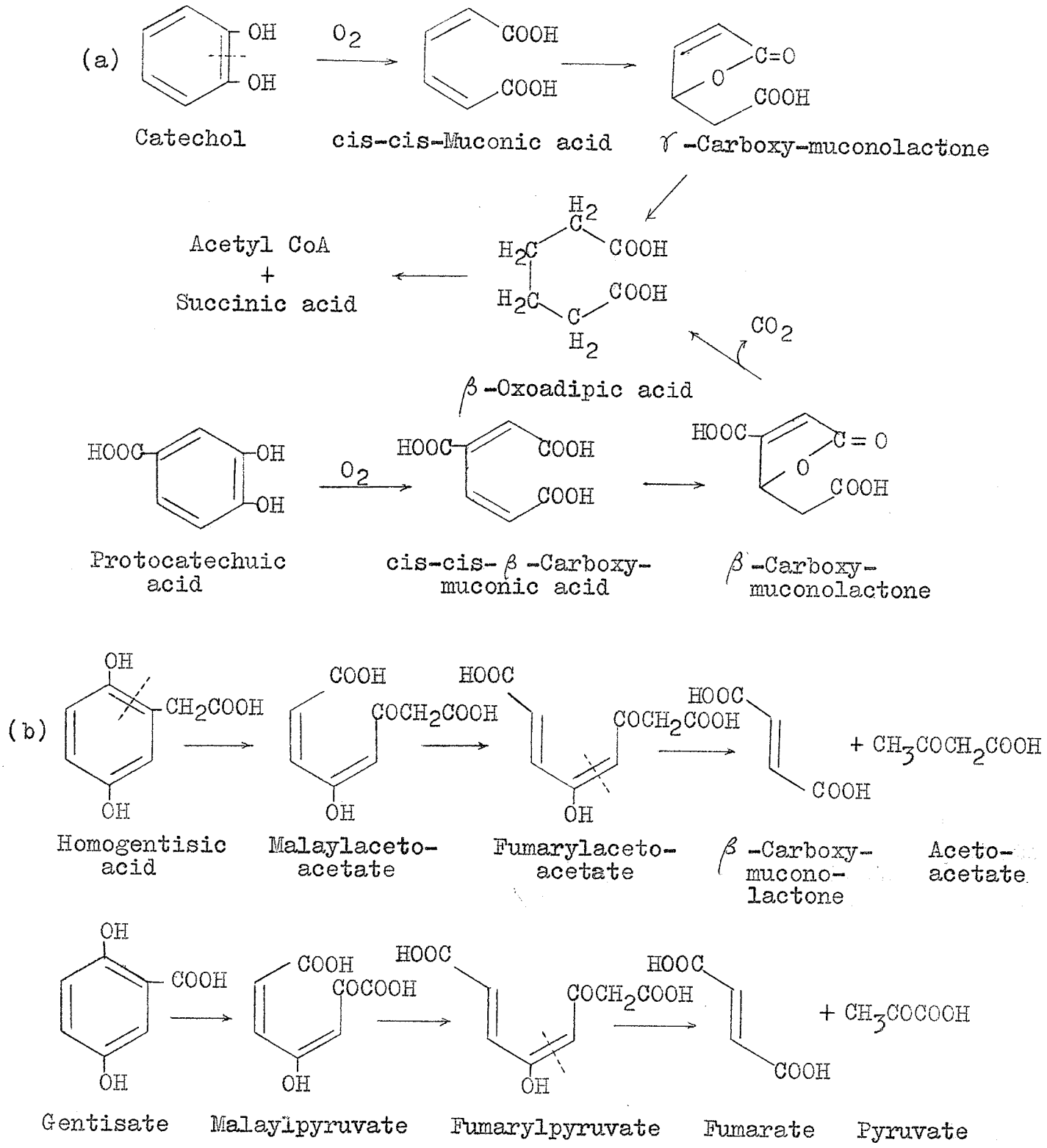


Fig. 5. Oxidative pathways for fission of aromatic nucleus by micro-organisms: (a) Oxidative fission of the bond between carbon atoms bearing the hydroxyl groups of an o-dihydroxyphenol; (b) rupture of the bond between carbon atoms bearing a hydroxyl group and an adjacent carbon atom carrying a carbon side chain or a carboxyl group (Ornston and Stanier, 1966).

TABLE 2

Some aromatic compounds and their ring fission substrates (Gibson, D.T., 1968)

Aromatic compounds	Ring fission substrates
Toluene or o-cresol	2,3-Dihydroxytoluene
β-Phenylpropionic acid	2,3-Dihydroxy-β-phenylpropionic acid
Phenylacetic acid	3,4- or 2,5-Dihydroxyphenylacetic acid
Naphthalene	1,2-Dihydroxynaphthalene
Anthracene	1,2-Dihydroxyanthracene
Phenanthrene	3,4-Dihydroxyphenanthrene
Estrone	4-Hydroxyestrone

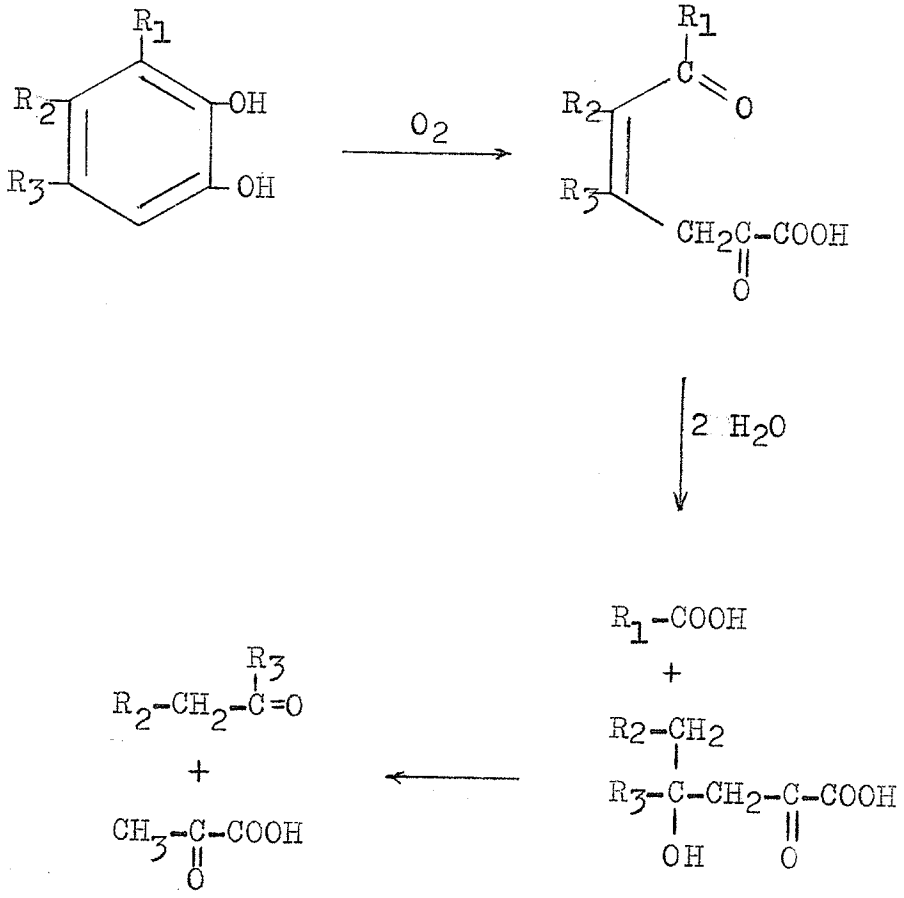


Fig. 6. General scheme for the microbial degradation of catechol and substituted catechols (Dagley, S. et al., 1964).

TABLE 3

A general scheme for the degradation of catechols (Dagley, S. et al., 1964)

Substrate	Nuclear substituent	Products
Catechol	$R_1 = R_2 = R_3 = H$	Formate, acetaldehyde pyruvate
3-Methylcatechol	$R_2 = R_3 = H$; $R_1 = CH_3$	Acetate, acetaldehyde pyruvate
4-Methylcatechol	$R_1 = R_3 = H$; $R_2 = CH$	Formate, propionaldehyde, pyruvate
2,3-Dihydroxy-β-phenylpropionic acid	$R_1 = HOOC-CH_2-CH_2$ $R_2 = R_3 = H$	Succinate, acetaldehyde, pyruvate
Protocatechuic acid	$R_1 = R_2 = H$; $R_3 = COOH$	Formate, pyruvate (2 moles)

former two compounds does not always yield a muconic acid.

3. Microbial Degradation of Aromatic Amino Compounds

Although microbial degradation of aromatic compounds in general has been studied quite intensively, very little attention has been paid to the metabolic pathways leading to the degradation of aromatic amino compounds which are essential constituents of several herbicides and pesticides. The studies of the metabolic pathways leading to their disappearance in soil is very important from an ecological point of view. Until such investigations are carried out, it is impossible to assess meaningfully their toxicity to human body.

Von Plotho (1948) isolated two species of Proactinomyces which were capable of decomposing aniline. It was stated that both species grew in the presence of phenol. Moore (1949) obtained organisms of the genus Proactinomyces from soil enrichment cultures which were reported able to utilize pyridine, aniline, nicotinic acid, nitrobenzene, or phenol + ammonium ion as sole source of carbon, nitrogen and energy.

Sloane, Crane and Mayer (1951) studied the degradation of p-aminobenzoic acid by Mycobacterium smegmatis. Their results implied that the substrate was metabolized firstly to aniline and that further bacterial activity resulted in the formation of p-aminophenol. The metabolic pathway involved was reported later to proceed via p-aminobenzoic acid $\longrightarrow$ aniline $\longrightarrow$ p-aminobenzyl alcohol $\longrightarrow$ p-aminophenol (Sloane and Untch, 1964).

The decarboxylation of p-aminobenzoic acid and anthranilic

acid to aniline was demonstrated by McCullough et al. (1957), using cell-free enzyme preparations from E. coli. The aniline formed was further metabolized via p-aminophenol.

Bartha, R. et al (1968) studied the metabolic transformations of aniline and eleven chloroanilines in soil. They found that all mono-chloro- and some dichloro-anilines were transformed to their corresponding dichloro- and tetrachloro-azo compounds, but no azo compound was formed from aniline. The formation of 3,3',4,4'-tetrachloro-azo-benzene from 3,4-dichloro-aniline in soil was reported by Sprott and Corke (1971). Tweedy, B.G. et al. (1970) reported the acetylation of p-bromoaniline by a soil microorganism as a detoxification mechanism. They suggested that acetylation competed with azobenzene formation in utilizing the aniline formed by metabolism of substituted urea herbicides.

Walker and Harris (1969) studied the utilization of aniline as sole carbon source for growth by microorganisms. They suggested that aniline was converted to catechol with the liberation of ammonia.

Acetanilide forms the basic structure of a wide variety of herbicides and analgesics, but very little is known about the microbial degradation of acetanilide. The conversion of acetanilide to 4'-hydroxyacetanilide in the mammalian systems has been reported by Bernheim and Bernheim (1937). Lester and Greenberg (1947) as well as Brodie and Axelrod (1948) showed that in human body, the major fraction of acetanilide administered is converted to 4'-hydroxyacetanilide, followed by conjugation with sulfuric acid and glucuronic acid at the hydroxyl group; a minor fraction

is deacylated to form aniline. Brodie et al (1953) successfully hydroxylated acetanilide in the 2'- and 4'-positions with their model system, consisting of ascorbic acid, ferrous ion (Fe^{++}), ethylenediamine-tetra-acetate (EDTA) and oxygen.

Dagley, J. (1970) studied the metabolism of various substituted acetanilides with 3-methyl-cholane-anthrene induced rat liver microsomes. Metabolism of acetanilide, which occured primarily at para-position, was markedly influenced by the presence of other substituents in the ortho-, meta-, or para-position. Metabolism of acetanilide herbicides by soil microorganisms was studied by Bartha and Pramer (1970). They reported that the side chain, once released, was metabolized via conventional pathways to carbon dioxide, water and cell materials; and the aromatic nucleus was possibly metabolized by established mechanisms. These included ring hydroxylation, and cleavage to an aliphatic product which would be further oxidized.

Hydroxylation of acetanilide by several microorganisms was studied by Theriault and Longfield (1967). 2'-Hydroxyacetanilide was found to be the major product, a small amount of 4'-hydroxyacetanilide was also formed.

Work has been carried out on the degradation of acetanilide by a soil Pseudomonas sp. (Shaw and Aziz, 1972). It was shown that deacetylation of acetanilide resulted in the formation of aniline. The aniline was then found to be further metabolized through o-aminophenol. Both of the metabolites of acetanilide were isolated and identified by chromatographic techniques. Aniline, o-aminophenol and 4-aminocatechol were found to be the

major metabolites of acetanilide.

This project presents an attempt to study the metabolic pathways for the degradation of some ring substitutes of acetanilide, o-acetotoluidide, m-acetotoluidide, p-acetotoluidide, 2,6-dimethylacetanilide, 2'-hydroxyacetanilide, 3'-hydroxyacetanilide and 4'-hydroxyacetanilide by a soil Pseudomonas sp. which utilizes them as sole carbon and energy source. Specifically, the study is concerned with:

(a) Elucidation of the preliminary steps in the metabolism of these substituted acetanilides, such as deacetylation of the substituted acetanilides studied, hydroxylation of the aromatic nucleus and preparation for ring fission, assuming that the pathways for ring fission are common to those which have already been reported for other aromatic compounds.

(b) Study of the influence on the microbial degradation of acetanilide by the presence of various substituents in the ortho-, meta-, or para-position of acetanilide.

MATERIALS AND METHODS

MATERIALS

I. Reagents

(a) Origin and Purity

All inorganic chemicals were Analar grade. Organic chemicals were redistilled or recrystallized before use, and were shown to be chromatographically pure. 2'-Hydroxyacetanilide, 4'-hydroxyacetanilide, o-toluidine and m-toluidine were obtained from J.T. Baker Chemical Co., Phillipsburg, New Jersey; 3'-hydroxyacetanilide was supplied by Eastman Kodak Co., Rochester, N.Y.; p-toluidine was purchased from BDH Chemicals Ltd., Poole, England; catechol, protocatechuic acid and o-aminophenol were obtained from Matheson, Coleman and Bell, Norwood, Ohio. o-Acetotoluidide, m-acetotoluidide, p-acetotoluidide and 2,6-dimethylacetanilide were synthesized, and were shown to be pure by both melting point determination and TLC analysis. Solvents used for extraction and other purposes were redistilled.

(b) Preparation of Chemicals

2,6-dimethylacetanilide , o-, m- and p-acetotoluidide were prepared by means of the following procedures:

(1) 6 ml of acetyl chloride was added slowly and with stirring to 5 ml of toluidine (o-, m-, and p-) and 2,6-dimethylaniline (this was an exothermic reaction).

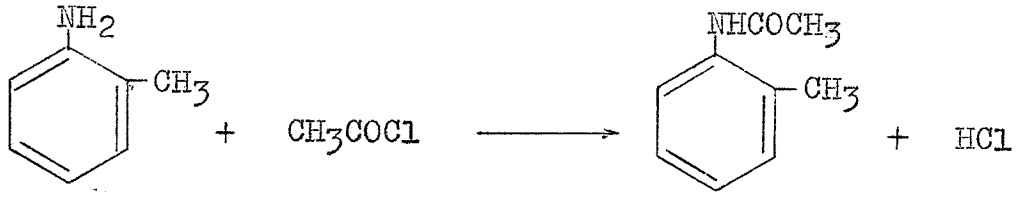
(2) The crude product was washed several times with small quantities of cold distilled water to remove excess acetyl chloride.

(3) The product was then dried at 30 to 40°C.

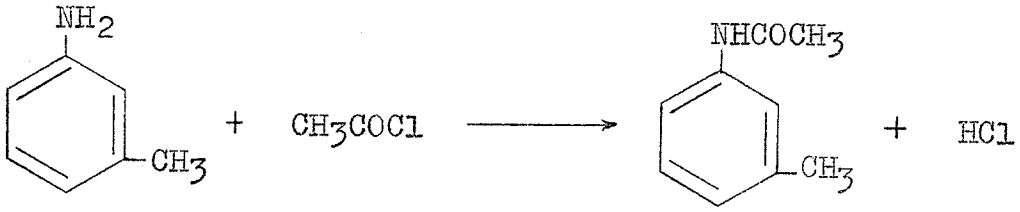
(4) The dried product was purified and recrystallized with 50 % ethyl alcohol.

(5) Crystals were collected and air-dried.

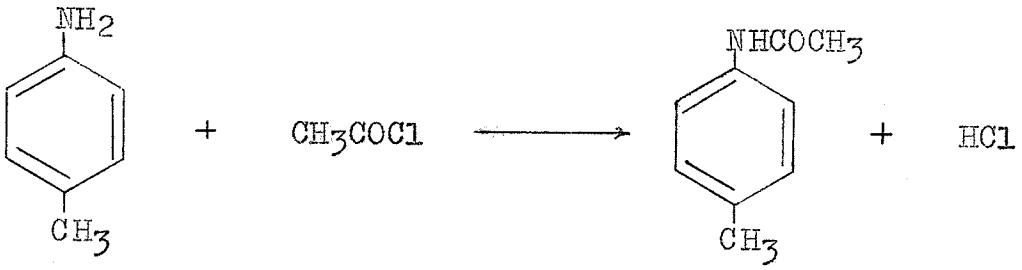
Reactions involved:



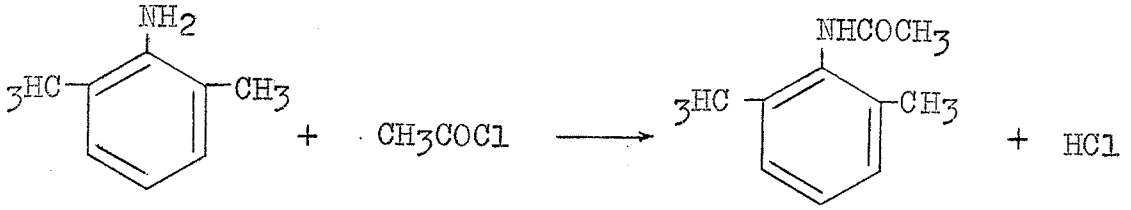
o-Toluidine Acetyl chloride o-Acetotoluidide (o-Methylacetanilide)



m-Toluidine Acetyl chloride m-Acetotoluidide (m-Methylacetanilide)



p-Toluidine Acetyl chloride p-Acetotoluidide (p-Methylacetanilide)



2,6-Dimethyl-aniline Acetyl chloride 2,6-Dimethylacetanilide

II. Organism

(a) Isolation

A microorganism, capable of utilizing acetanilide as sole carbon and energy source for growth, was isolated by screening several aniline utilizing organisms obtained from garden soil (Shaw and Aziz, 1972). The organism was identified as Pseudomonas sp., and was used in these experiments for studying the microbial degradation of some ring substituted acetanilides.

(b) Cultivation

Stock cultures and inocula were grown in 125 ml-Erlenmeyer flasks containing 100 ml of a mineral salts medium (Halvorson, 1970) with 0.1% (W/V) substituted acetanilides (o-, m-, and p-acetotoluidide, 2,6-dimethylacetanilide, 2'-, 3'- and 4'-hydroxy-acetanilide) as sole carbon and energy source for growth. The simple mineral salts medium used had the following composition:

NH_4Cl	0.3	Gm
K_2HPO_4	0.1	Gm
$CaCl_2$ (anhydrous)	0.2	Gm
$MgSO_4 \cdot 7\ H_2O$	0.2	Gm
$FeSO_4 \cdot 7\ H_2O$	0.005	Gm
Double distilled water to	1	L.

Substituted acetanilides studied were added at the final concentration of 0.1%. pH of the medium was adjusted to 6.8 with 1N-HCl to prevent the precipitation of mineral salts on autoclaving. The medium was autoclaved at 15 lbs pressure for 30 minutes. After cooling, each of these flasks, was then inoculated with the acetanilide utilizing organism and incubated at 30°C for a week. After

the incubation period, 1 ml of cell suspensions were transferred from the flasks showing growth to fresh medium and again incubated for one week at 30°C. Before the study of metabolites, subculturing was carried out several times to make sure that growth was really due to the utilization of substituted acetanilides but not due to the presence of trace of carbon and energy source transferred from the stock cultures.

METHODS

1. Growth Studies

(1) Growth Response

Presence and absence of growth was observed visually. This studies was carried out together with controls.

(2) Optimum Temperature for Growth

Growth was observed at 30 and 37°C, in 125 ml-Erlenmeyer flasks of nutrient broth and in 125 ml-Erlenmeyer flasks of simple mineral salts medium containing 0.1% (W/V) of substituted acetanilides used as sole carbon and energy source. Growth was measured turbidimetrically at regular time intervals in Reflectance Spectrophotometer, Unigalvo type 20, Evans Electroselanium Ltd., Essex, using appropriate reference standards.

(3) Optimum Concentration for Growth

The course of growth on o- and p-acetotoluidide and 2'-hydroxyacetanilide was followed turbidimetrically by using three concentrations, 0.1, 0.2 and 0.3%. For m-acetotoluidide, four concentrations were tried, namely, 0.05, 0.1, 0.2 and 0.3 %. The organism was grown in 100 ml of simple mineral salts medium con-

taining different concentrations of substituted acetanilides at 30°C. 5 ml of aliquots were taken at regular time intervals, and each aliquot was measured for turbidity in Reflectance Spectrophotometer.

2. Quantitative Studies on the Release of Aromatic Amino Compounds

The production of aromatic amino compounds in cultures during growth of the organism on various substituted acetanilides studied was followed at regular time intervals by means of Daniels quantitative method (Daniel, 1961) with reference to a standard calibration curve.

Various procedures have been described for determining aromatic amino compounds. When the amine is present in microgram amounts, the favoured procedure involves diazotisation with nitrous acid and then reaction with a suitable coupling reagent to give a highly coloured azo compound. The intensity of the colour can then be related to the concentration of the amine by using a photoelectric colorimeter.

The amine (0.100 Gm) was dissolved in a little dilute hydrochloric acid, and the solution was diluted to 100 ml with water; further dilution was made from this solution as required. The formation of the coloured substance was based on the following two reactions: (1) Diazotisation --- A 1 ml-portion of solution, containing 10 μg of amine, was placed in each of a series of glass-stoppered volumetric flasks, and to the contents of each flask were then added 1 ml of 2N-HCl and 1 ml of 0.25% solution of sodium nitrite. Stoppers were inserted in the flasks, and the

contents were mixed by inversion. A reagent blank was prepared simultaneously, and the flasks were set aside at room temperature for 30 minutes before proceeding with the coupling stage. (2) Coupling --- To each portion of diazotised amine was added 1 ml of 2.5 % (W/V) ammonium sulphamate solution, and 1 minute later, 2 ml of 3 M-sodium acetate solution and 1 ml of 1 % solution of N-1-naphthylenediamine dihydrochloride were added. The azo colour was developed by adding 0.5 ml of concentrated hydrochloric acid, and the solution was diluted to 10 ml with water.

Colorimetric measurements were carried out with a Spectronic 20 Colorimeter, at a wave length of 545 mμ. A standard calibration curve was drawn up.

For measuring the aromatic amines released in the cultures during growth of the organism, cells were grown at 30°C in a series of 125 ml-Erlenmeyer flasks containing 100 ml of mineral salts medium with 0.1, 0.2 and 0.3 % of substituted acetanilides. For m-acetotoluidide, only two concentrations, 0.05 and 0.1 %, were used. One flask of culture was taken at regular time intervals, and the cells were removed by centrifugation at 2500 r.p.m. for 30 minutes. The clear supernatant was used for colorimetric measurement of the aromatic amines. This study was carried out together with controls.

3. Qualitative Studies of Metabolites

A) Extraction of Metabolites

The organism was grown on 500 ml of liquid simple mineral salts medium containing 0.1 % (W/V) substituted acetanilides in Erlenmeyer flasks at 30°C. Cells were removed at the end of the

logarithmic phase of growth (12 days) by centrifugation at 2500 r.p.m. for 30 minutes. The supernatant was cleared of any soluble proteins by adding few drops of 20 % trichloroacetic acid and centrifuging to remove any precipitate.

The clear supernatant thus obtained was then adjusted to pH 8 with 1N-NaOH and extracted in a separatory funnel with five portions of 200 ml of ether. The ethereal extracts were combined, dried over anhydrous sodium sulfate, and evaporated to a small volume in a water bath at 40°C. This alkaline ethereal extract was collected and kept for analysis of metabolites. The supernatant, remaining after the first extraction, was adjusted to pH 3 with 1N-HCl and extracted with five protions of 200 ml of ether. This acidic ethereal extract was then dried and evaporated to a small volume, and was preserved for studies of metabolites.

Control flasks, containing 500 ml of mineral salts medium with 0.1 % of substituted acetanilides were incubated at 30°C for 12 days; and extracted with ether by a similar procedure used for extracting metabolites. The acidic and alkaline ethereal extracts of bacterial cultures and the ethereal extracts of controls were then subjected to analytical procedures for identification of metabolites.

B) Identification of Metabolites

Analysis of metabolites was done by means of three methods, namely, thin layer chromatography, gas-liquid chromatography, and oxygen uptake experiments.

(1) Thin Layer Chromatography

Ethereal extracts of bacterial cultures and controls were

chromatographed on glass plates (20 × 20 cms), coated with 0.25 mm layers of silica gel G 254 (E. Merck, according to Stahl, 1969), activated at 105°C for 30 minutes. The chromatograms were developed at room temperature by the following two solvent systems:

(a) Dibutyl ether-Ethyl acetate-Acetic acid (10:10:1)
(M. Gillio-Tos, S.A. Previtera and A. Vimercati, 1964)

(b) Chloroform-Methanol-Ammonium hydroxide (85:15:1)
(Theriault and Longfield, 1967)

After the chromatograms had been developed, the plates were air-dried, and sprayed with the following spraying reagents:

(a) Spray reagent I: 5 % sodium nitrite solution in 3N-HCl
Spray reagent II: 0.1 % N-1-naphthylenediamine dihydrochloride solution (aqueous)
The chromatographic plates were sprayed with reagent I, allowed to stand for 5 minutes and then sprayed with reagent II. The plates were dried at 60°C for 30 minutes to intensify the colours.
This is a diazotisation and coupling reaction. The reagent is specific for compounds having free amino-group (Stahl, 1969).

(b) Potassium Ferricyanide-Ferric Chloride Solution
Solution a: 1 % aqueous solution of potassium ferricyanide
Solution b: 2 % aqueous ferric chloride solution
Equal amounts of solution a and b were mixed just before use.
Treatment after spraying: The colours were intensified

by subsequent spraying with 2N-HCl.

(M. Gillio-Tos, S.A. Previtera and Vimercati, 1964; H. Wagner, L. Hoerhammer and U.H. Nufer, 1965)

This reagent mixture reveals phenolic compounds as intense blue spots on a pale yellow background, changing to pale blue with age. Although the reagent is not completely specific under the conditions used, it is sensitive and positive for many phenolic compounds (Hathway, D.E., 1960).

(2) Preparative-Plate Chromatography

(Separation of metabolites for gas-liquid chromatographic studies)

The ethereal extracts of metabolites were applied on glass plates (20 20 cms) coated with 1 mm layer of silica gel G 254. The chromatograms were developed with the two solvent systems listed in the previous section. After being developed, the plates were air-dried and scanned under ultra-violet light. The areas on silica gel layer where the bands appeared were marked and scraped from the plates separatively. The organic components of the bands thus collected were then extracted with three 20 ml portions of absolute ethanol. The extracts were filtered and the solvent was removed by evaporation. The dried residues thus obtained were then redissolved in ether and used for gas-liquid chromatographic analysis.

(3) Gas-Liquid Chromatography

The metabolites isolated by preparative-plate chromatography were detected with GC-5 gas chromatograph (Beckman), using the

following column system. Ethereal solutions of pure reference standards were used in this study. Recording was done at a chart speed of 0.1 in/minute.

Column system:

Column type: 0.5 per cent Amine 220 on 100-120 mesh chromosorb G, HpDmcs, 6' teflon column

Injection type: Glass-lined

Detector type: Hydrogen flame

Flask inlet temperature: 180°C

Column temperature: 150°C

Detector temperature: 260°C

Detector line temperature: 205°C

The carrier gas was helium with a flow rate of 50 mls/min. The inlet pressure was 50 lbs/sq.in.

(4) Manometric Studies

i) Preparation of Bacterial Cell Suspension

The manometric studies were performed on two types of bacterial cell suspensions:

(a) Cells grown on nutrient agar

The bacterial cells were grown on sterilized nutrient agar at 30°C, using standard inoculum and harvested after 7 days of growth, by shaking with 0.02 M-phosphate buffer (pH 7). The cells were scraped from the nutrient agar surface with the help of a glass rod. The lumps of agar were removed by filtering through muslin cloth. Cells were harvested by centrifuging at 5°C for 30 minutes, resuspended in phosphate buffer and aerated for 24 hrs., by passing filtered air, to exhaust any metabolites

which may have been present in the cells. The cells were then washed twice with phosphate buffer, and adjusted to the required dry cell weight.

(b) Cells grown on substituted acetanilides

The cells were grown on liquid mineral salts medium containing 0.1 % (W/V) of substituted acetanilides at 30°C in 2 litre-Erlenmeyer flasks, and harvested at the end of the logarithmic phase of growth (12 days), by centrifuging at 2500 r.p.m. for 30 min. at 5°C. The cells thus obtained were then resuspended in phosphate buffer and aerated by passing filtered air for 24 hrs. After aeration, the cells were washed twice with phosphate buffer and adjusted to the required dry cell weight.

ii) Oxygen Uptake Experiments

Oxygen uptake by the organism in the presence of various substrates was measured in a Warburg constant respirometer, according to the technique of manometry of Umbreit, Burris and Stauffer (1964), using a 13 ml-single arm flask at 30° ± 0.5, with air as the gas phase. The flasks were calibrated by the Ferricyanide-Hydrazine method. This method has the marked advantage of being able to employ a weighed or a chemically standardized primary standard. The reaction involved is shown below, which occurs under alkaline conditions:

$$4\ Fe^{+++}(CN)_6 \ +\ N_2H_4 \longrightarrow 4\ Fe^{++}(CN)_6 + 4\ H^+ + N_2$$

The potassium ferricyanide was used as 0.1 M solution, which was stable if preserved in the dark for several days. Each ml of 0.1 M potassium ferricyanide liberated 2240/4 = 560 μl N_2, thus normally 0.4 ml (= 224 μl) of 0.1 M solution was added. The other

reagents required were 4N-NaOH and hydrazine. Hydrazine solution was prepared by dissolving 5 Gm of hydrazine sulfate in 100 ml of hot water; the excess separated on cooling, and the clear saturated solution was used.

A typical system employed the following:

Main compartment:

0.4 ml 0.1 M potassium ferricyanide (= 224 μl N_2)

0.4 ml 4N-NaOH

1.2 ml water

Side arm:

0.5 ml hydrazine sulfate solution

0.5 ml 4N-NaOH

After equilibration, the contents of the side arm were tipped in and h was determined (h is the manometer reading in mm; the difference in height of the manometer liquid in the limbs of the manometer). The reaction was completed in about 30 min.

The flask constant (K) was calculated for each flask by using the following equation:

$K = x/h = 224/h$ (this is $K_{N_2} = K_{O_2}$)

x is the volume of gas evoled at NTP (see Manometric Techniques and Tissue Metabolism, Umbreit, Burris and Stauffer, 1964).

For oxygen uptake experiments, the main compartment of each flask contained 0.5 ml of 0.02 M phosphate buffer (pH 7), and 1 ml of bacterial cell suspension. The volume of the cell suspension was adjusted with phosphate buffer, using a standardized turbidity vs. dry weight scale. The side arm contained 0.5 ml of

aqueous substrate solution, which was neutralized to pH 7 with 1N-NaOH where necessary. The centre well contained 0.2 ml of 20 % (W/V) KOH. The surface area of the KOH solution was increased by adding a small folded filter paper to absorb all CO_2 which was liberated by the bacterial cells in flask atmosphere. The flasks were incubated in the water bath at 30°C for 30 min. to equilibrate the temperature of their contents and surroundings. The absorption of oxygen by the living cells depended almost entirely on the oxygen in the solution and the flask atmosphere; in order to keep this at a maximum, the flasks were shaken. The reaction was started by tipping the contents of the side arm into the main compartment.

RESULTS

1. Some Characteristics of the Organism Used

The organism which utilized acetanilide as sole carbon source for growth was Gram negative, rod-shaped, measuring $3 \times 1 \mu$, and usually with flagella, Plate I (Shaw and Aziz, 1972). Colonies on nutrient agar were smooth and translucent with spreading margins. No pigmentation was observed during growth. Fluorescence was not observed under ultra-violet light and it gave positive oxidase test. It showed growth on glucose and fructose which was oxidative and aerobic. It gave positive indole nitrite test, but it did not grow on urea broth; it showed positive Methyl Red-Voges Proskauer test. The organism also grew on Koser's citrate slant, TSL slant, starch agar and gelatine without liquification. It was capable of nitrogen fixation (atmospheric nitrogen) and degradation of oxalate. The optimum temperature for growth ranged between 28 to 32° C. No growth at 42°C was observed. pH range of 6.5 to 7.5 was found suitable for optimum growth.

These results suggested that the organism belongs to the genus Pseudomonas and resembles greatly Pseudomonas azotogenesis (Shewan and Hendrie, 1966).

2. Growth Response

The organism showed very good growth on o-, m-, p-acetotoluidide and 2'-hydroxyacetanilide. Both 3'- and 4'-hydroxyacetanilide were very unstable and even turned dark in colour at room temperature, and were not used in these studies. The organism did not grow on 2,6-dimethylacetanilide contained in the simple mineral

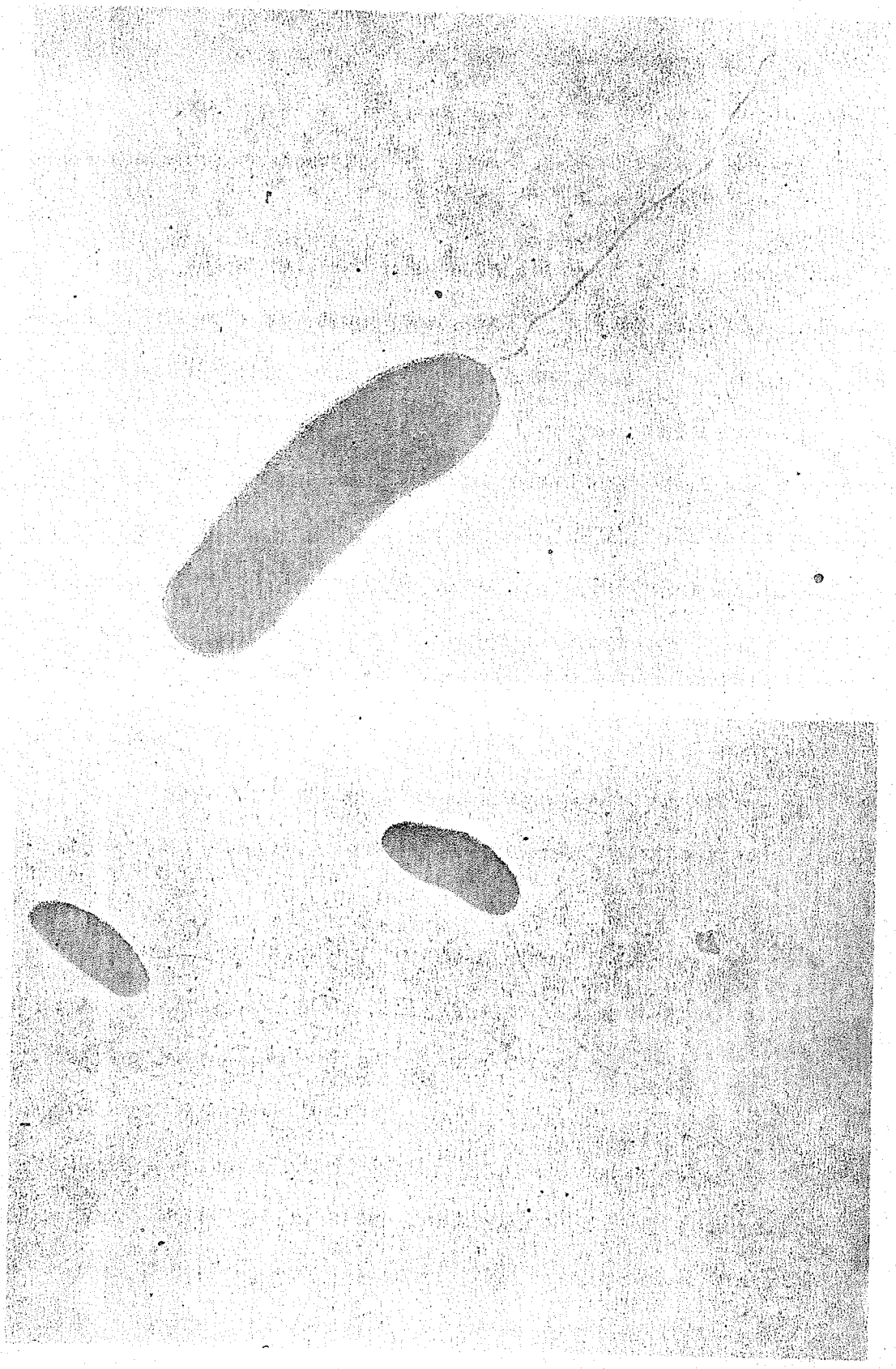


Plate I. Photomicrograph of acetanilide-decomposing Pseudomonas sp., Top: x10,000 magnification; Bottom: x5,000 magnification.

salts medium, but it did show very good growth on the same compound when nutrient broth instead of simple mineral salts medium was used as the culture medium. This may be due to the fact that the organism is not capable of utilizing 2,6-dimethylacetanilide as sole carbon and energy source.

3. Optimum Temperature for Growth

Growth was observed to be better at 30°C than at 37°C, this is shown in Fig. 7. However, less growth at the higher temperature does not simply mean that this relates only to the temperature. It is possible that less growth may be due to the lower oxygen tension in the liquid culture medium at the higher temperature. The reason for doing a temperature growth curve was to determine the best temperature for use under our conditions of static cultures. If aeration was used, the results might be different.

4. Optimum Concentration for Growth

By measuring the growth turbidimetrically at regular time intervals, the optimum concentration for growth of the organism on o-acetotoluidide, m-acetotoluidide and p-acetotoluidide was found to be 0.1%. It was 0.3 % in the case of 2'-hydroxyacetanilide. For m-acetotoluidide, the higher concentrations, 0.2 and 0.3 %, gave no growth even after an incubation period of 4 weeks; it is possible that m-acetotoluidide, or one of its metabolites, is toxic to the organism at higher concentrations.

The relationship between concentration of the substrate and growth of the organism is shown in Fig. 8, 9, 10 and 11.

5. Quantitative Studies on the Release of Aromatic Amino Compounds

The release of aromatic amino compounds in the cultures during growth of the organism was followed at regular time inter-

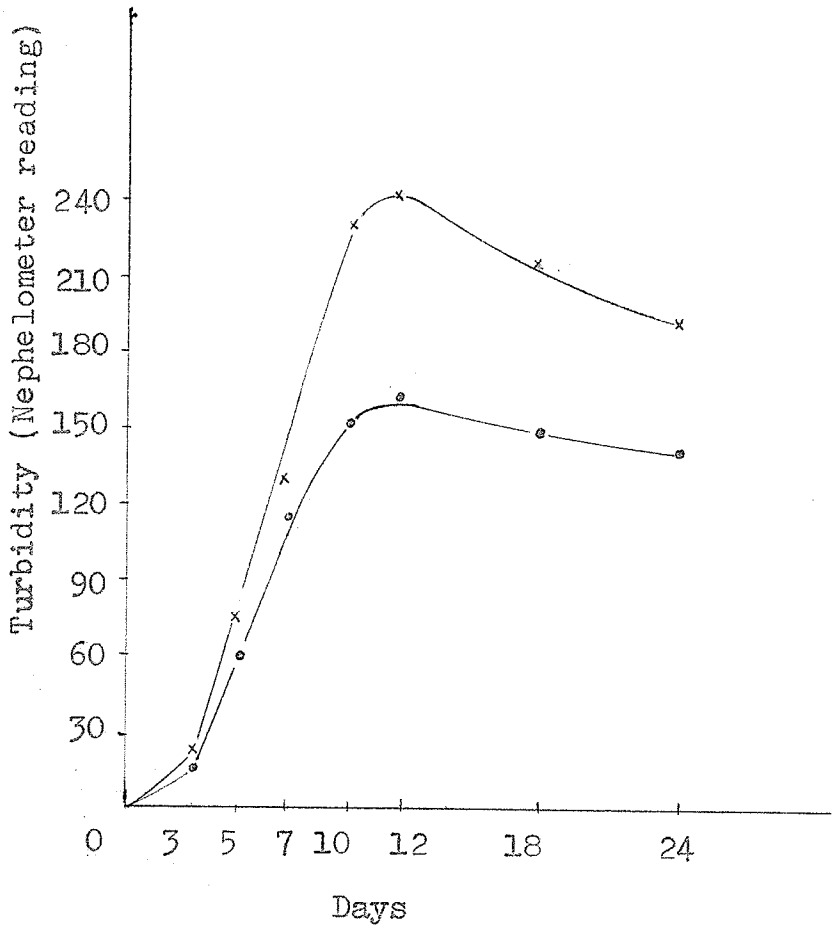


Fig. 7. Temperature growth curve. The Pseudomonas sp. was grown in nutrient broth at two different temperature, 30°C (---- x) and 37°C (---- .).

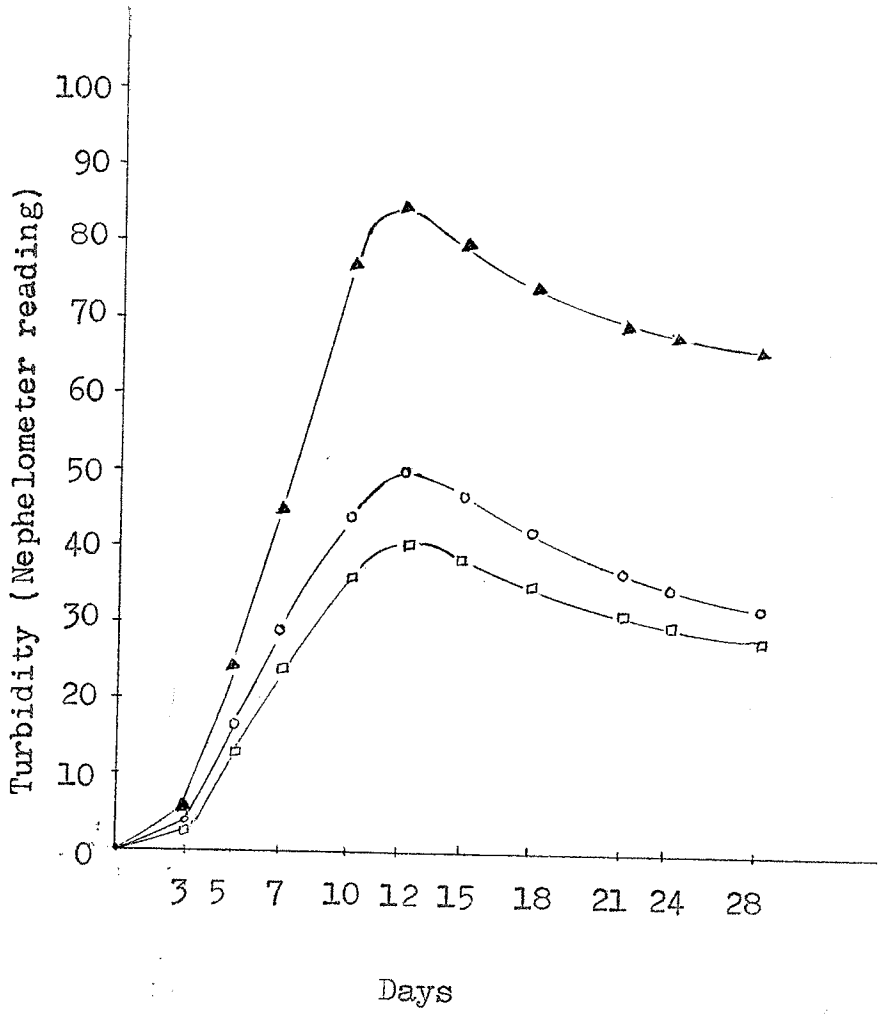


Fig. 8. Growth curves of the Pseudomonas sp. on o-acetotoluidide at three different concentrations: ▲ ---- 0.1%; o ---- 0.2%; □ ---- 0.3%.

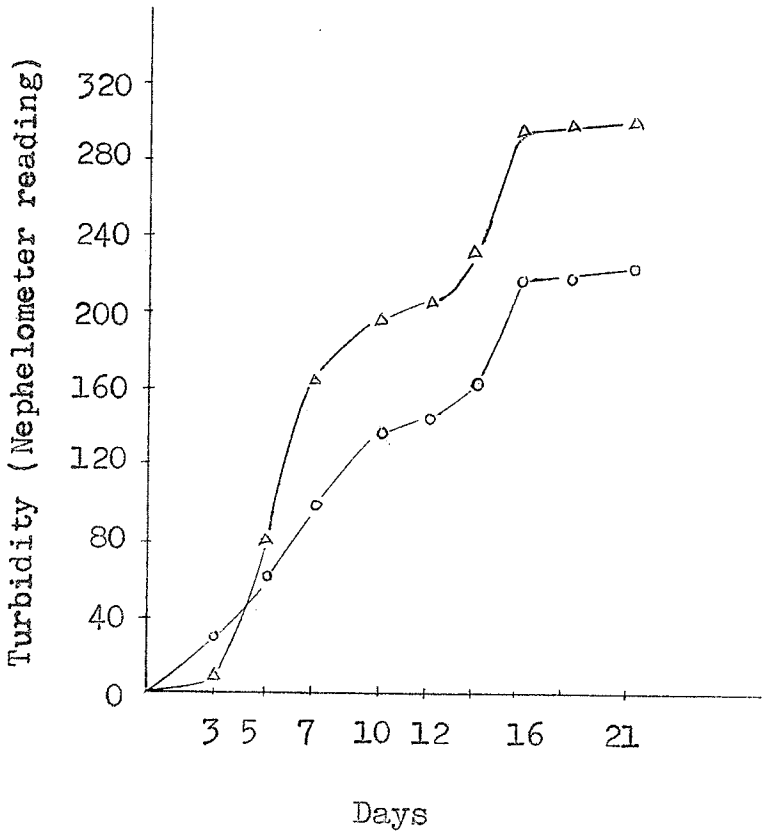


Fig. 9. Growth curves of the Pseudomonas sp. on m-acetotoluidide at two different concentrations: o ---- 0.05%; Δ ---- 0.1%.

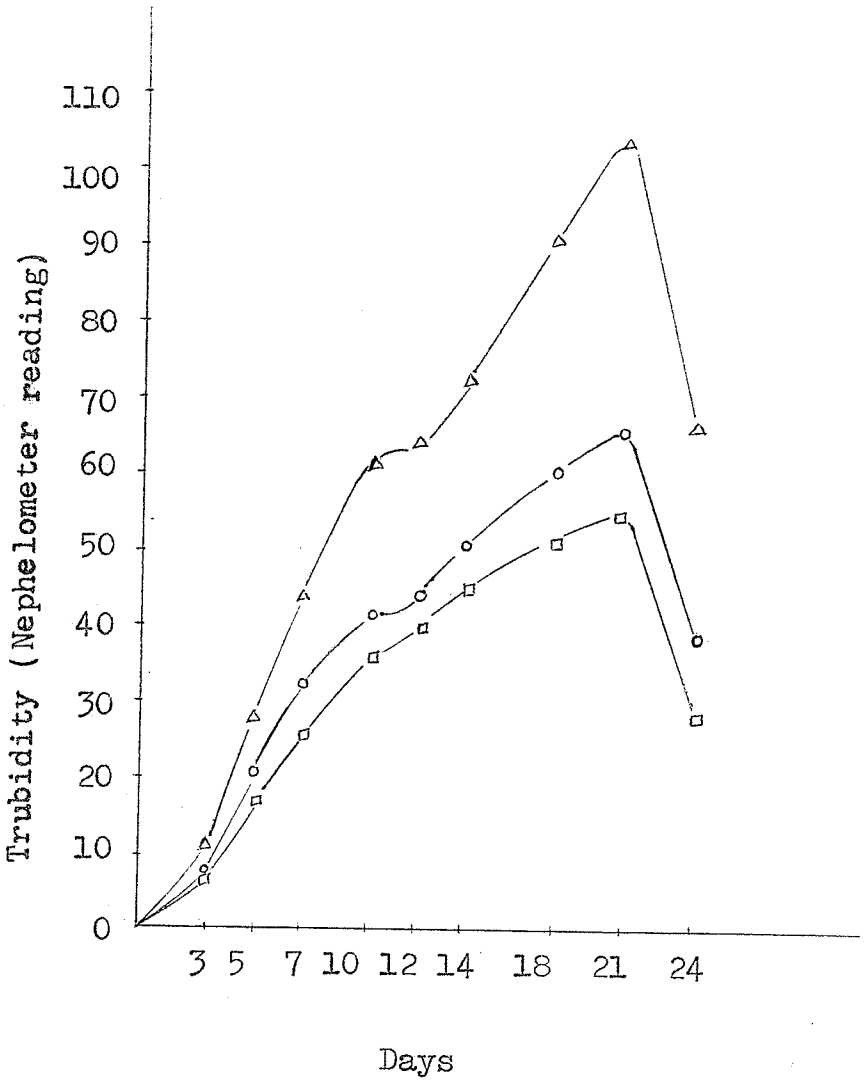


Fig. 10. Growth curves of the Pseudomonas sp. on p-acetotoluidide at three different concentrations: △ ---- 0.1%; o ---- 0.2%; □ ---- 0.3%.

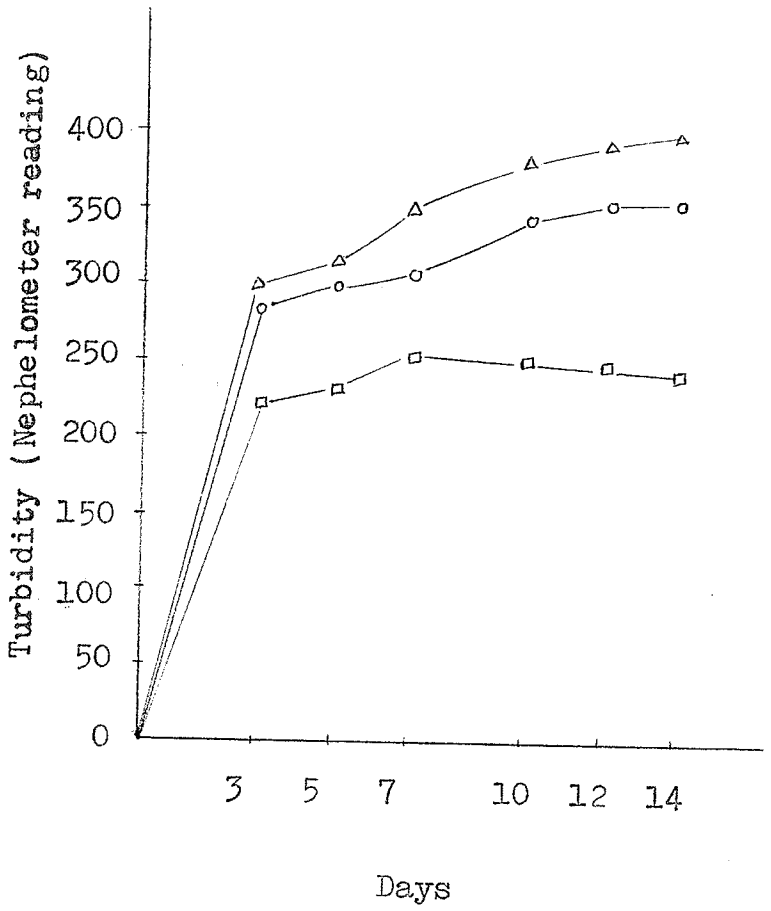


Fig. 11. Growth curves of the Pseudomonas sp. on 2'-hydroxyacetanilide at three different concentrations: □ ---- 0.1%; o ---- 0.2%; Δ ---- 0.3%.

vals and is shown in Tables 4, 5, 6 and 7 and Fig. 12, 13, 14 and 15. No amine was measurable in control flasks.

6. Thin Layer Chromatography

(1) o-Acetotoluidide

TLC analysis of ethereal extracts of metabolites from cultures of the organism grown on o-acetotoluidide (0.1 %, W/V) revealed three distinct spots with the alkaline ethereal extracts (obtained at pH 8.0), and two spots with the acidic ethereal extracts (obtained at pH 3.0). Ethereal extracts from control flasks did not show any spots of metabolites except the original compound, o-acetotoluidide (with reference to the standard o-acetotoluidide spot) when chromatographed under identical conditions.

Spot A, B and D appeared blue when the plates were sprayed with potassium ferricyanide-ferric chloride reagent. Spot D appeared with both alkaline and acidic ethereal extracts. The Rf values for these spots in solvent system (1): Dibutyl ether-Ethyl acetate-Acetic acid (10:10:1) are given in Table 8 .

Spot C had a Rf value of 0.59, corresponding to standard o-toluidine spot, which had a Rf value of 0.59 under identical conditions, and both appeared pink after the plate was sprayed with the diazotising reagent. When this spot C was co-chromatographed with o-toluidine, only a single spot with a Rf value of 0.59 appeared.

Spot A and B did not show any colour after the plates were sprayed with the diazotising reagent. Spot D, which appeared when both alkaline and acidic ethereal extracts of the metabolites

TABLE 4

Release of aromatic amino compounds during growth of Pseudomonas sp. on o-acetotoluidide

Conc. / Days	Aromatic amines (µg/ml)		
	0.1%	0.2%	0.3%
3	74	82	163
5	158	185	197.5
7	77	385	545
10	68	565	855
14	60	630	655
18	53	410	535
21	43	180	460

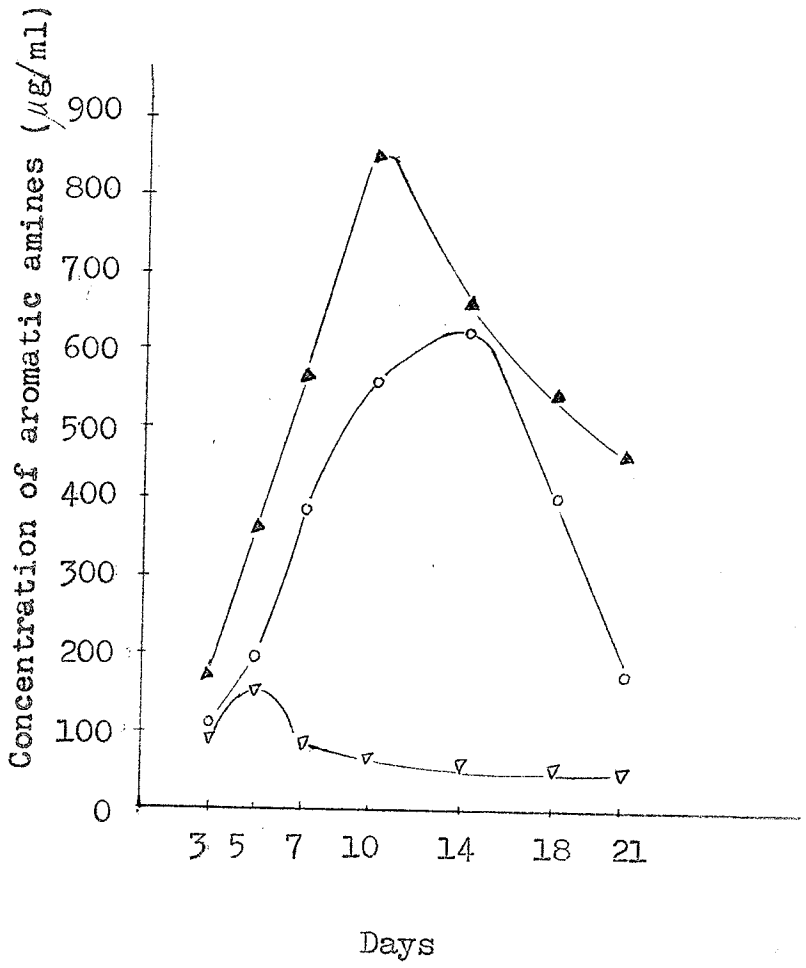


Fig. 12. Release of aromatic amines during growth of the Pseudomonas sp. on o-acetotoluidide at three different concentrations: ▽ ---- 0.1%; o ---- 0.2%; ▲ ---- 0.3%.

TABLE 5

Release of aromatic amino compounds during growth of Pseudomonas sp. on m-acetotoluidide

Conc. / Days	Aromatic amines (µg/ml)	
	0.05%	0.1%
3	5.8	13.40
5	0	20.60
7	0	10.60
10	0	3.84
12	0	1.58
14	0	1.30
17	0	1.20
21	0	1.20

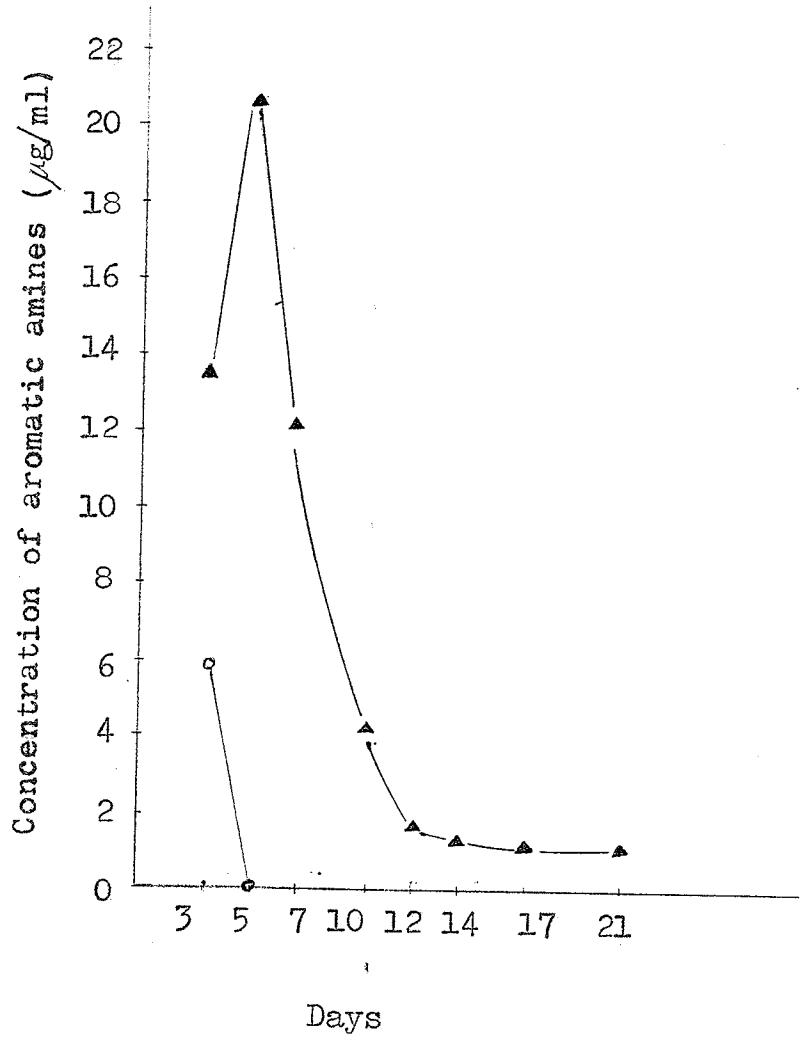


Fig. 13. Release of aromatic amines during growth of the Pseudomonas sp. on m-acetotoluidide at two different concentrations: o ---- 0.05%; ▲ ---- 0.1%.

TABLE 6

Release of aromatic amino compounds during growth of Pseudomonas sp. on p-acetotoluidide

Conc. / Days	Aromatic amines (μg/ml)		
	0.1%	0.2%	0.3%
3	197	201	236
5	330	1060	1186
7	305	860	1140
10	3.80	810	1050
12	0.97	785	980
14	0.90	750	820
18	0.80	690	810
21	0.78	690	775

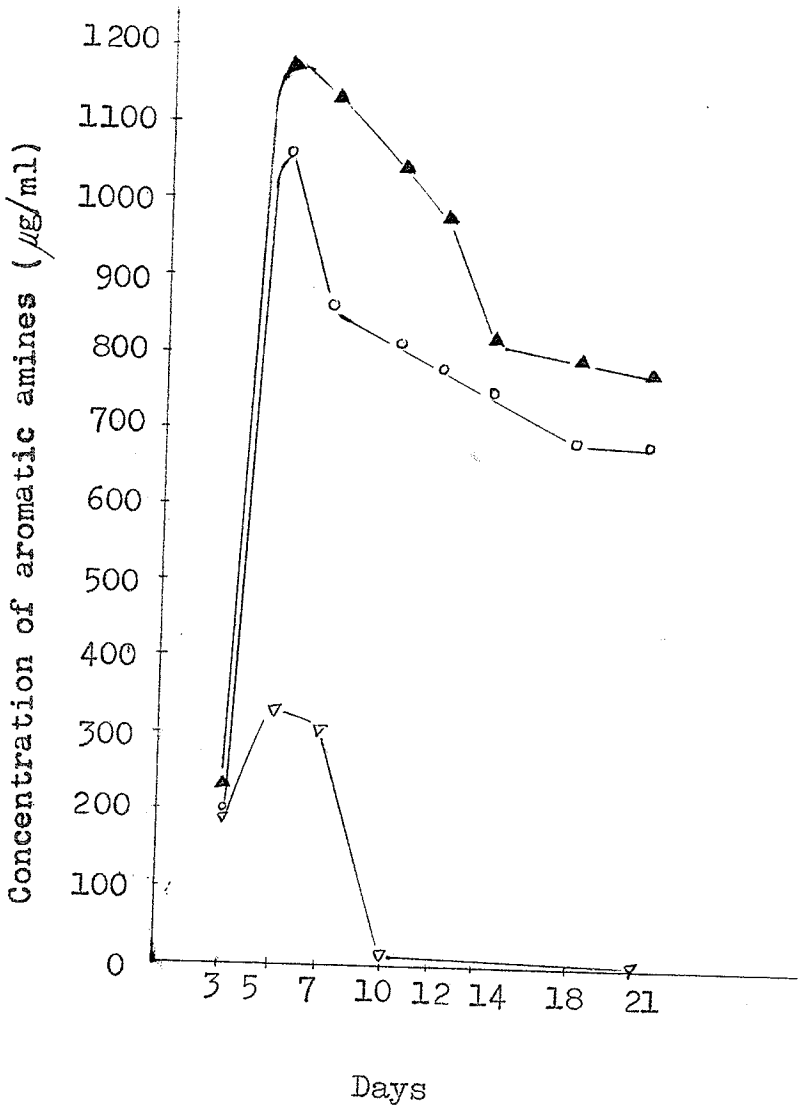


Fig. 14. Release of aromatic amines during growth of the Pseudomonas sp. on p-acetotoluidide at three different concentrations: ▽ ---- 0.1%; o ---- 0.2%; ▲ ---- 0.3%.

TABLE 7

Release of aromatic amino compounds during growth of *Pseudomonas* sp. on 2'-hydroxyacetanilide

Conc. / Days	Aromatic amines (μg/ml)		
	0.1%	0.2%	0.3%
3	3.40	9.40	9.80
5	2.30	6.01	8.10
7	1.57	2.87	7.85
10	1.47	2.63	5.15
14	1.37	2.40	2.97
18	0.80	1.67	2.20

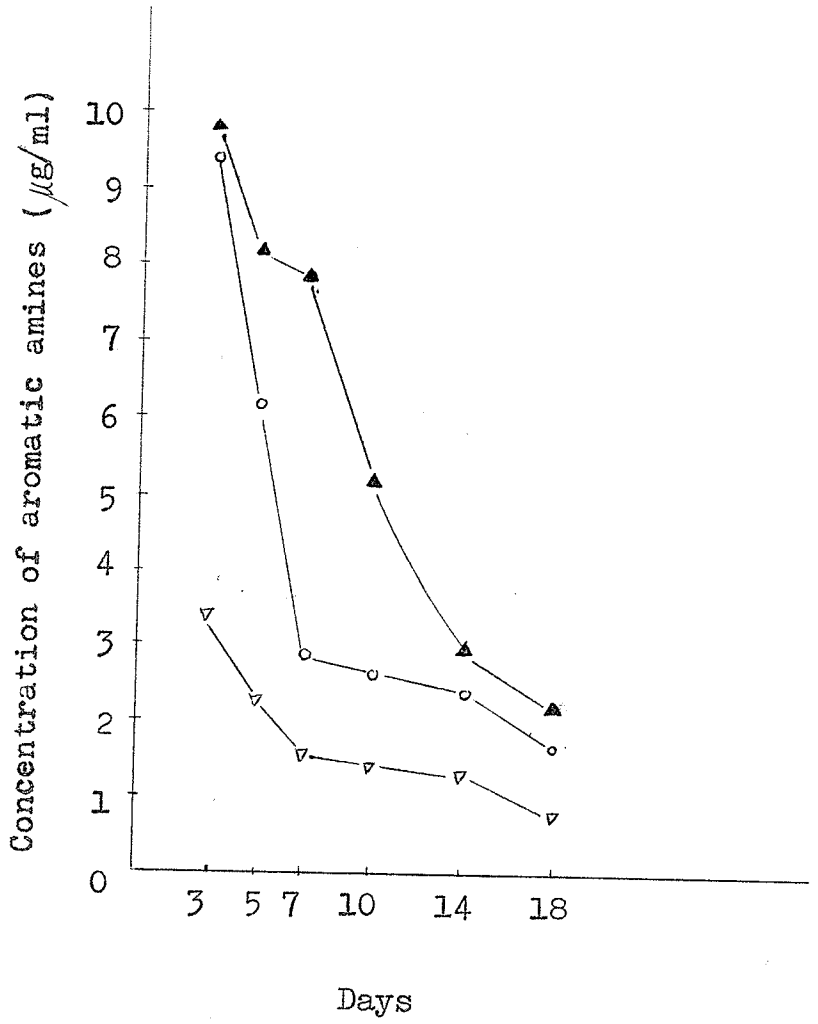


Fig. 15. Release of aromatic amines during growth of the Pseudomonas sp. on 2'-hydroxy-acetanilide at three different concentrations: ▽ ---- 0.1%; o ---- 0.2%; ▲ ---- 0.3%.

were chromatographed, had a value of 0.84. It appeared as a yellow spot when the diazotising reagent was sprayed.

TABLE 8

Rf values for metabolites of o-acetotoluidide and reference standard (TLC analysis)
Solvent system: Dibutyl ether-Ethyl acetate-Acetic acid (10:10:1)

Metabolites and reference standard	Rf value (Mean of 8 trials)	
	Basic ethereal extract (pH 8.0)	Acidic ethereal extract (pH 3.0)
Spot A		0.12
Spot B	0.29	
Spot C	0.59	
o-Toluidine	0.59	
Spot D	0.84	0.84

(2) m-Acetotoluidide

Table 9 shows the Rf values for the metabolites of m-acetotoluidide in both solvent systems (1) and (2).

TLC analysis of ethereal extracts of metabolites from cultures of the organism grown on m-acetotoluidide (0.1 %, W/V) revealed three distinct spots with the alkaline ethereal extract, and two spots with the acidic ethereal extracts. Ethereal extracts from control experiments showed only single spot which was identified as the original compound, m-acetotoluidide (with reference to the standard m-acetotoluidide spot) when chromatographed under identical conditions.

Spot A, B and D appeared blue when the plates were sprayed with potassium ferricyanide-ferric chloride reagent. Spot C had a Rf value of 0.46 and 0.75 in solvent system (1) and (2), respectively, corresponding to the standard m-toluidine spot, which had a Rf value of 0.46 and 0.75 in solvent system (1) and (2), respectively under the same conditions; and both appeared pink after the plates were sprayed with the diazotising reagent.

Spot D appeared with both the alkaline and acidic ethereal extracts. It had a Rf value of 0.76 and 0.86 in solvent systems (1) and (2), respectively, and it was seen as yellow spot after the plates were sprayed with the diazotising reagent.

TABLE 9

Rf values for metabolites of m-acetotoluidide and reference standard (TLC analysis)
Solvent system (1): Dibutyl ether-Ethyl acetate-Acetic acid (10:10:1)
Solvent system (2): Chloroform-Methanol-Ammonium hydroxide (85:15:1)

Metabolites and reference standard	Rf value (Mean of 12 trials)			
	Alkaline ethereal extract (pH 8.0)		Acidic ethereal extract (pH3.0)	
	Solvent sys. (1)	Solvent sys. (2)	Solvent sys. (1)	Solvent sys. (2)
Spot A			0.12	0.21
Spot B	0.26	0.40		
Spot C	0.46	0.75		
m-Toluidine	0.46	0.75		
Spot D	0.76	0.86	0.76	0.86

(3) p-Acetotoluidide

TLC analysis of ethereal extracts of metabolites from cultures of the organism grown on p-acetotoluidide (0.1 %, W/V) revealed two spots with the alkaline ethereal extract, and two spots with the acidic ethereal extract. Ethereal extracts from control flasks did not show any spot of the metabolites except the original compound, p-acetotoluidide (with reference to the standard p-acetotoluidide spot) under identical conditions. The Rf values of the spots detected in solvent system (1) are shown in Table 10.

TABLE 10

Rf values for metabolites of p-acetotoluidide and reference standard (TLC analysis)
Solvent system (1): Dibutyl ether-Ethyl acetate-Acetic acid (10:10:1)

Metabolites and reference standard	Rf value (Mean of 10 trials)	
	Alkaline ethereal extract (pH 8.0)	Acidic ethereal extract (pH3.0)
Spot A		0.11
Spot B	0.59	
p-Toluidine	0.59	
Spot C	0.59	0.95

Spot A and C appeared blue when the plates were sprayed with potassium ferricyanide-ferric chloride reagent. Spot B had a Rf value of 0.59, corresponding to the standard p-toluidine spot which had a Rf value of 0.59 under identical conditions, and both appeared as a pink spot after the plates were sprayed with the diazotising reagent.

Spot C had a Rf value of 0.95, and appeared with both the

alkaline and acidic ethereal extracts were chromatographed. It was seen as a yellow spot when the plate was sprayed with the diazotising reagent.

(4) 2'-Hydroxyacetanilide

TLC analysis of ethereal extracts of metabolites from cultures of the organism grown on 2'-hydroxyacetanilide (0.3 %, W/V) revealed three distinct spots with the alkaline ethereal extract, and four spots with the acidic ethereal extract. Ethereal extracts obtained from control experiments did not show any spot of metabolites except the original compound, 2'-hydroxyacetanilide (with reference to the standard 2'-hydroxyacetanilide spot when chromatographed under identical conditions.

Spots A, B, C, D and E all appeared blue when the plates were sprayed with potassium ferricyanide-ferric chloride reagent.

The Rf value for these spots in both solvent systems (1) and (2) are given in Table 11. Spot B had Rf values of 0.72 and 0.37, in solvent systems (1) and (2), respectively, corresponding to the standard catechol spot which had Rf values of 0.72 and 0.37 in solvent systems (1) and (2), respectively under identical conditions; and both appeared yellowish brown after the plates were sprayed with the diazotising reagent.

Spot C, which appeared when both the alkaline and acidic ethereal extracts were chromatographed, had Rf values of 0.43 and 0.51 in solvent systems (1) and (2), respectively, corresponding to the standard o-aminophenol spot which had the same Rf values under identical conditions. It was seen as a yellow spot when the plates were sprayed with the diazotising reagent and thus resem-

bles o-aminophenol in these respects. Co-chromatography of this spot C and o-aminophenol showed single spot with identical Rf value.

Spots D and E appeared pink and yellow, respectively after the plates were sprayed with the diazotising reagent.

TABLE 11

Rf values for metabolites of 2'- hydroxyacetanilide and reference standards (TLC analysis)
Solvent system (1): Dibutyl ether-Ethyl acetate-Acetic acid (10:10:1)
Solvent system (2): Chloroform-Methanol-Ammonium hydroxide (85:15:1)

Metabolites and reference standards	Rf value (Mean of 8 trials)			
	Alkaline ethereal extract (pH 8.0)		Acidic ethereal extract (pH 3.0)	
	Solvent sys. (1)	Solvent sys. (2)	Solvent sys. (1)	Solvent sys. (2)
Spot A			0.25	0.04
Spot B			0.72	0.37
Catechol			0.72	0.37
Spot C	0.43	0.51	0.43	0.51
o-Aminophenol	0.43	0.51	0.43	0.51
Spot D	0.81	0.75		
Spot E	0.93	0.83	0.93	0.83

The possible metabolic intermediates in the bacterial cultures grown on substituted acetanilides are given in Table 12. (based on the results of TLC analysis)

TABLE 12

Possible metabolic intermediates in bacterial cultures grown on substituted acetanilides (based on the results of TLC analysis)

Substituted acetanilides	Possible metabolic intermediates
1. o-Acetotoluidide	o-Toluidine
2. m-Acetotoluidide	m-Toluidine
3. p-Acetotoluidide	p-Toluidine
4. 2'-Hydroxyacetanilide	o-Aminophenol Catechol

7. Gas-Liquid Chromatography

GLC analysis of ethereal extracts of metabolites and controls gave results which are in agreement with those obtained from TLC studies. The results shown in Table 13 suggest the presence of o-toluidine, m-toluidine, p-toluidine, and o-aminophenol as the major metabolite in the bacterial cultures grown on o-acetotoluidide, m-acetotoluidide, p-acetotoluidide and 2'-hydroxyacetanilide, respectively.

8. Oxygen Uptake Experiments

(a) With nutrient agar grown organism

This organism was used to test whether the oxidation of substituted acetanilides used depended on an induced enzyme system. The results (Fig. 16, 17, 18 and 19) indicate that the organism failed to show rapid uptake of oxygen on the substrates tested; however, adaptation, followed by active oxidation, could be ob-

TABLE 13

Results of gas-liquid chromatographic analysis of ethereal extracts of metabolites

Samples and reference standard	Retention time
1. Spot C from o-acetotoluidide	9'30"
o-Toluidine	9'38"
Co-chromatography-Spot C and o-toluidine	9'35", single peak
2. Spot C from m-acetotoluidide	13'55"
m-Toluidine	13'45"
Co-chromatography-Spot C and m-toluidine	13'48", single peak
3. Spot B from p-acetotoluidide	12'10"
p-Toluidine	12'04"
Co-chromatography-Spot B and p-toluidine	12'05", single peak
4. Spot C from 2'-hydroxyacetanilide	7'25"
o-Aminophenol	7'12"
C o-chromatography-Spot C and o-aminophenol	7'20", single peak

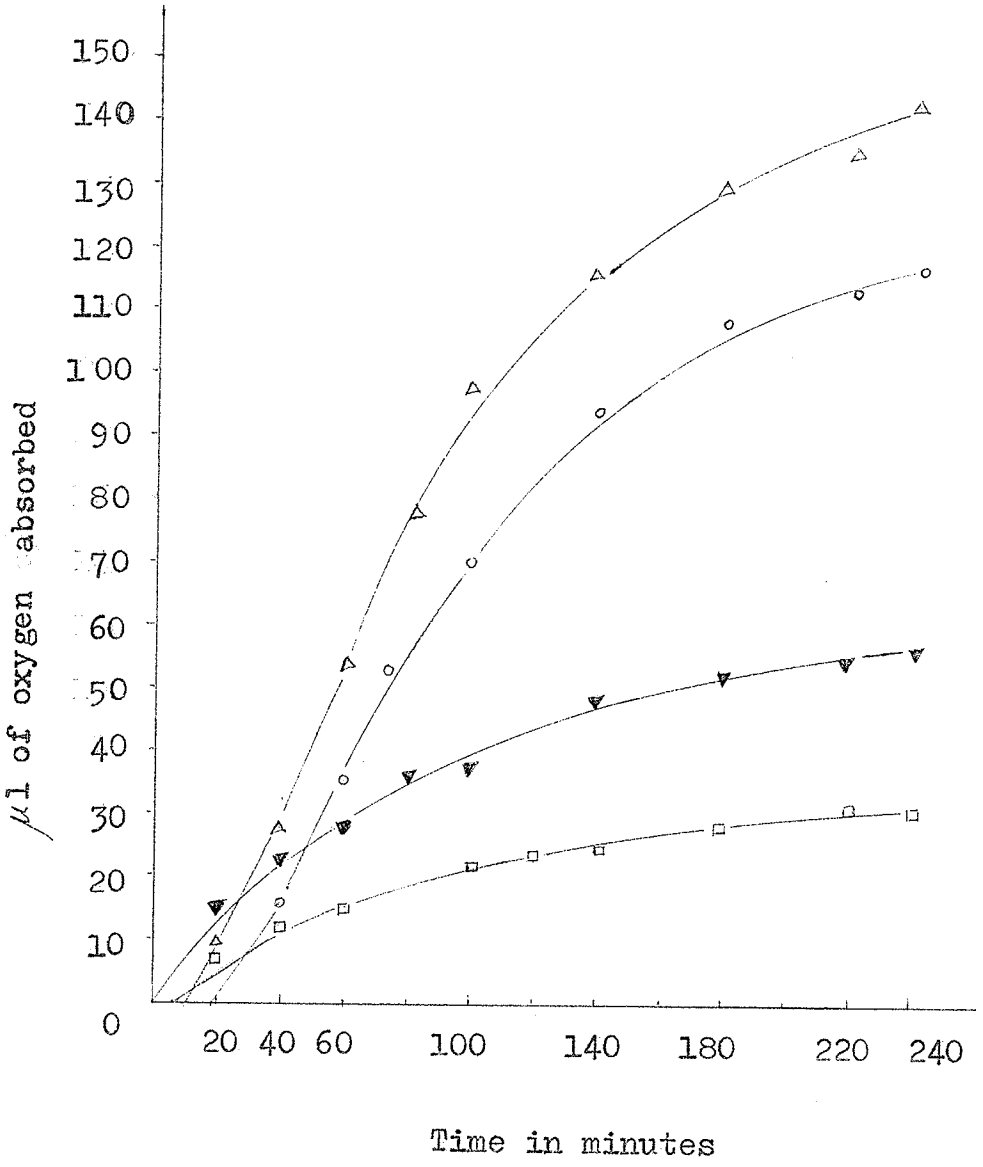


Fig. 16. Rate of oxygen uptake by washed nutrient agar grown Pseudomonas sp. 20 mgs dry weight of cells per ml. o ---- o-acetotoluidide; □ ---- catechol; △ ---- o-toluidine; ▼ ---- acetate (Plots are an average of 5 trials, exogenous).

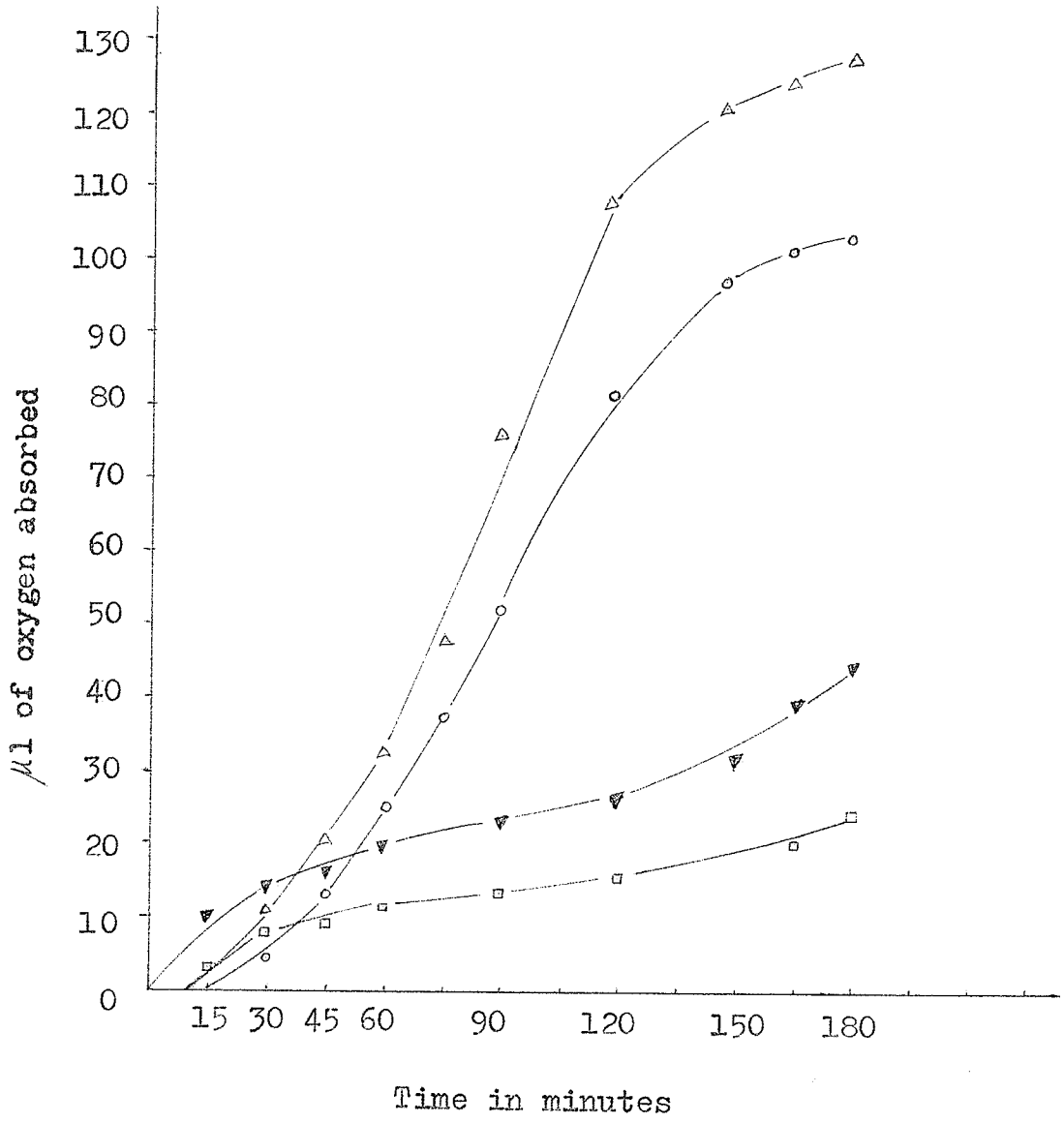


Fig. 17. Rate of oxygen uptake by washed nutrient agar grown Pseudomonas sp. 16 mgs dry weight of cells per ml. o ---- m-acetotoluidide; △ ---- m-toluidine; □ ---- protocatechuic acid; ▼ ---- acetate (Plots are an average of 4 trials; exogenous)

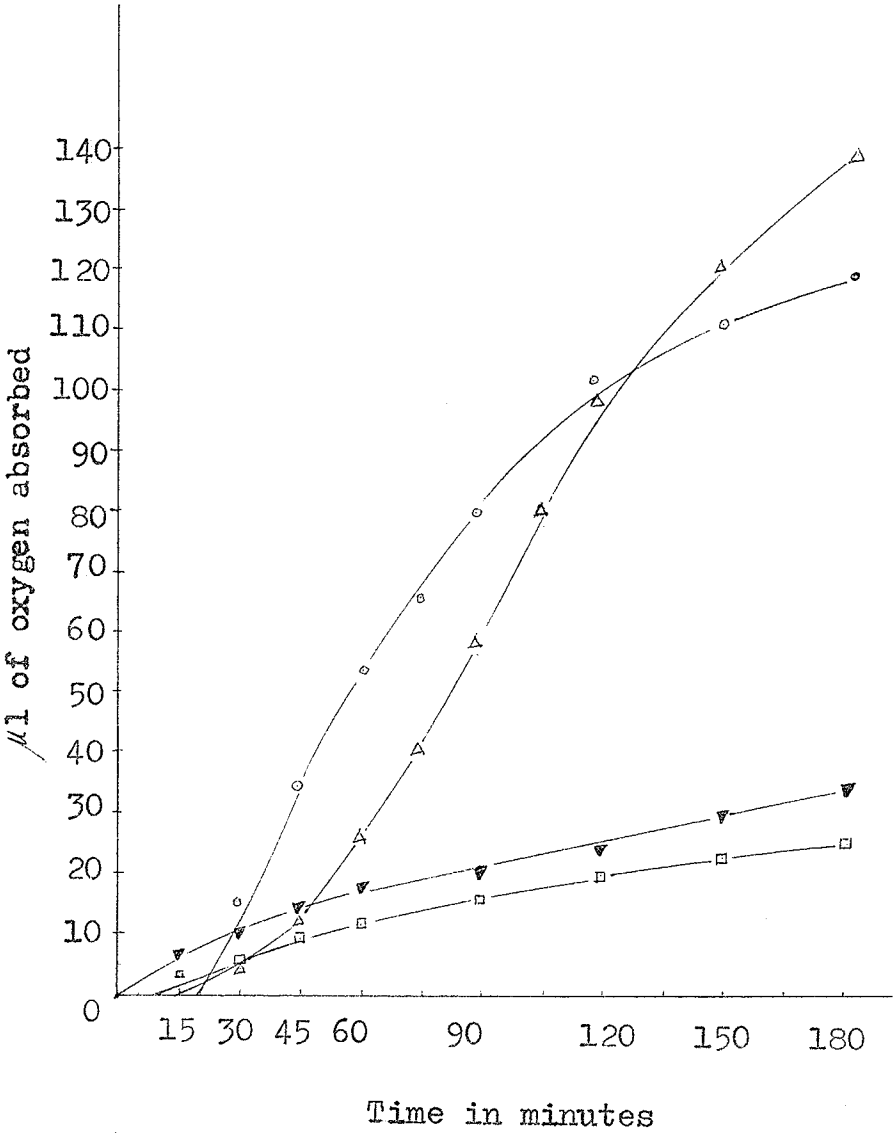


Fig. 18. Rate of oxygen uptake by washed nutrient agar grown Pseudomonas sp. 18 mgs dry weight of cells per ml. o ---- p-aceto-toluidide; △ ---- p-toluidine; □ ---- protocatechuic acid; ▼ ---- acetate (Plots are an average of 3 trials; exo-genous).

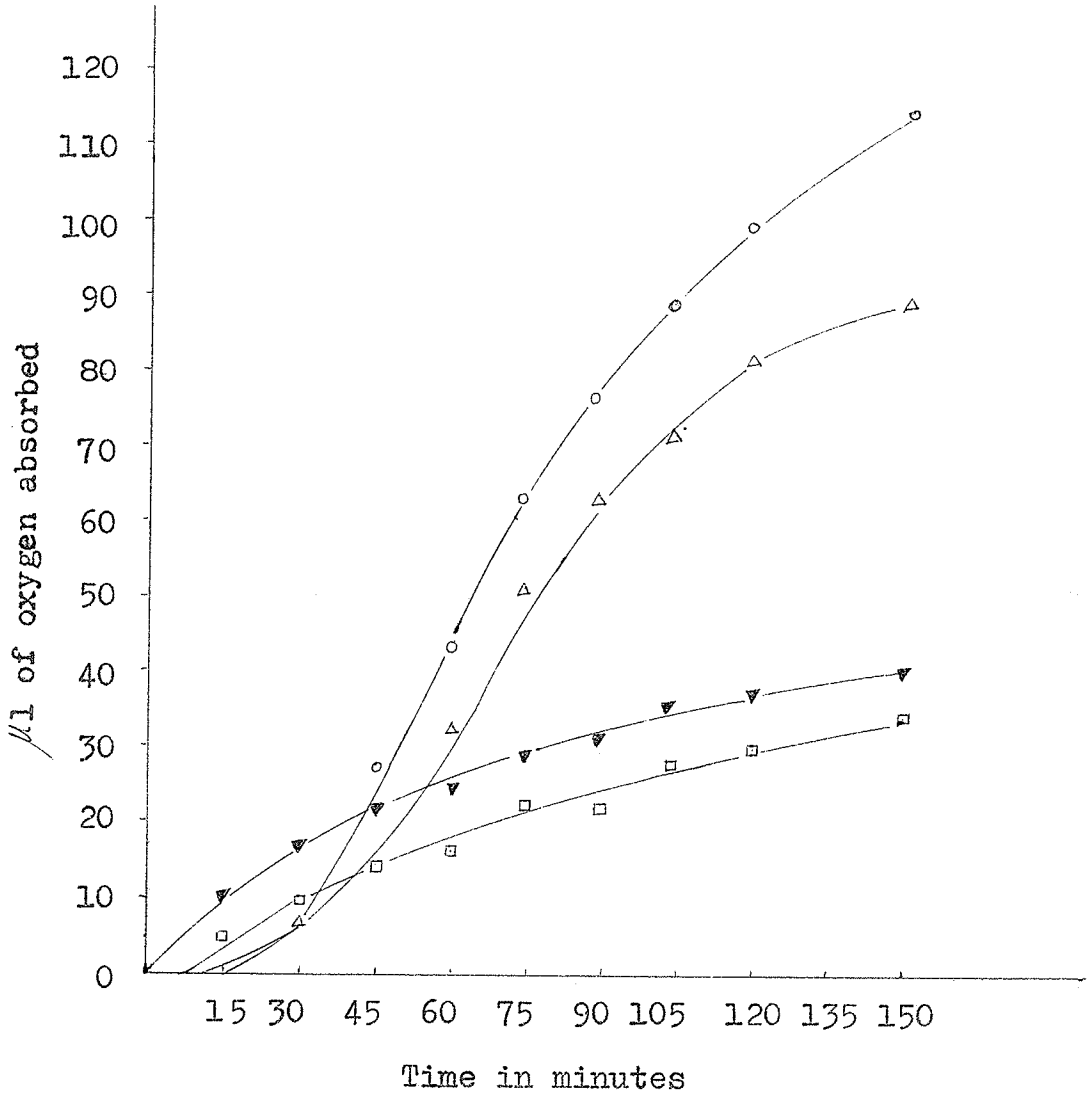


Fig. 19. Rate of oxygen uptake by nutrient agar grown Pseudomonas sp. 16 mgs dry weight of cells per ml. o ---- 2'-hydroxyacetanilide; △ ---- o-aminophenol; □ ---- catechol; ▼ ---- acetate (Plots are an average of 3 trials; exogenous).

served. The substrates were oxidized after a lag period and the total amount of oxygen consumed was low. Significant rapid oxygen uptake was observed in the case of o-acetotoluidide after a lag period of 18 minutes, whereas o-toluidine and catechol showed a lag period of 10 and 7 minutes, respectively. However, acetate showed a rapid uptake of oxygen, although the total volume of oxygen consumed was small. These results thus indicate that the nutrient agar grown organism does not possess o-acetotoluidide oxidizing enzymes. In other words, the oxidation of o-acetotoluidide is accomplished by an induced enzyme system.

From the results given in Fig. 17, 18 and 19, we know that m-acetotoluidide, p-acetotoluidide and 2'-hydroxyacetanilide are also oxidized by the action of an induced enzyme system rather than by the action of the constitutive enzymes which already present in the organism.

(b) With substituted acetanilides grown organism

The oxygen consumption by the Pseudomonas sp., grown aerobically on the substituted acetanilides studied are given in Fig. 20, 21, 22 and 23. In the case of o-acetotoluidide grown cells, o-acetotoluidide, o-toluidine, catechol and acetate were immediately oxidized without any induction periods, indicating that o-acetotoluidide acts as an enzyme inducer, and induces the formation of an enzyme system specifically capable of oxidizing itself. From the results of oxygen uptake experiments, it indicates that m-acetotoluidide, p-acetotoluidide and 2'-hydroxyacetanilide are also enzyme inducers, they induce the formation of enzyme systems specifically capable of catalyzing their own oxidation.

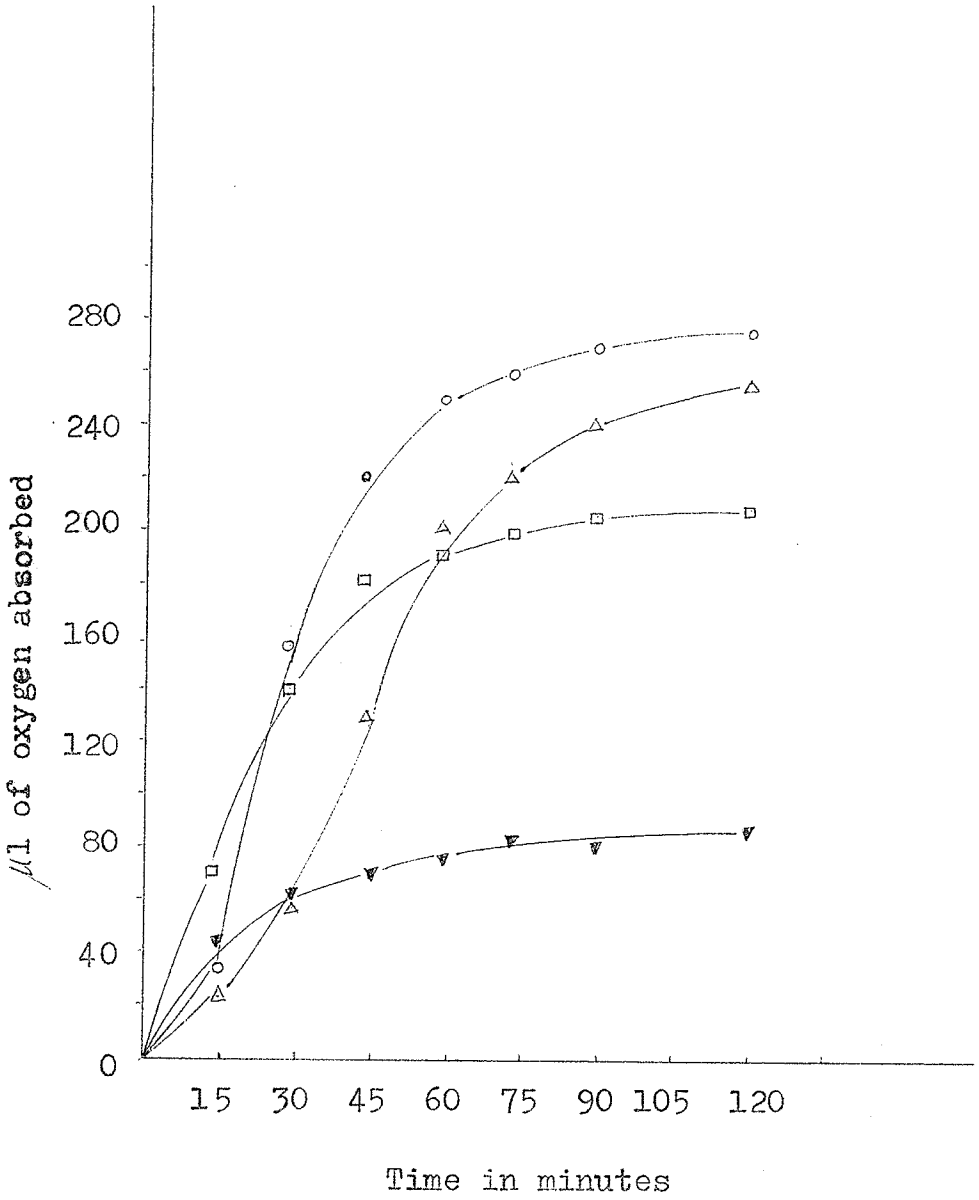


Fig. 20. Rate of oxygen uptake by washed o-acetotoluidide grown cell suspension of Pseudomonas sp. 20 mgs dry weight of cells per ml. o ——— o-acetotoluidide; △ ----- o-toluidine; □ ——— catechol; ▼ ——— acetate (Plots are an average of 5 trials; exogenous).

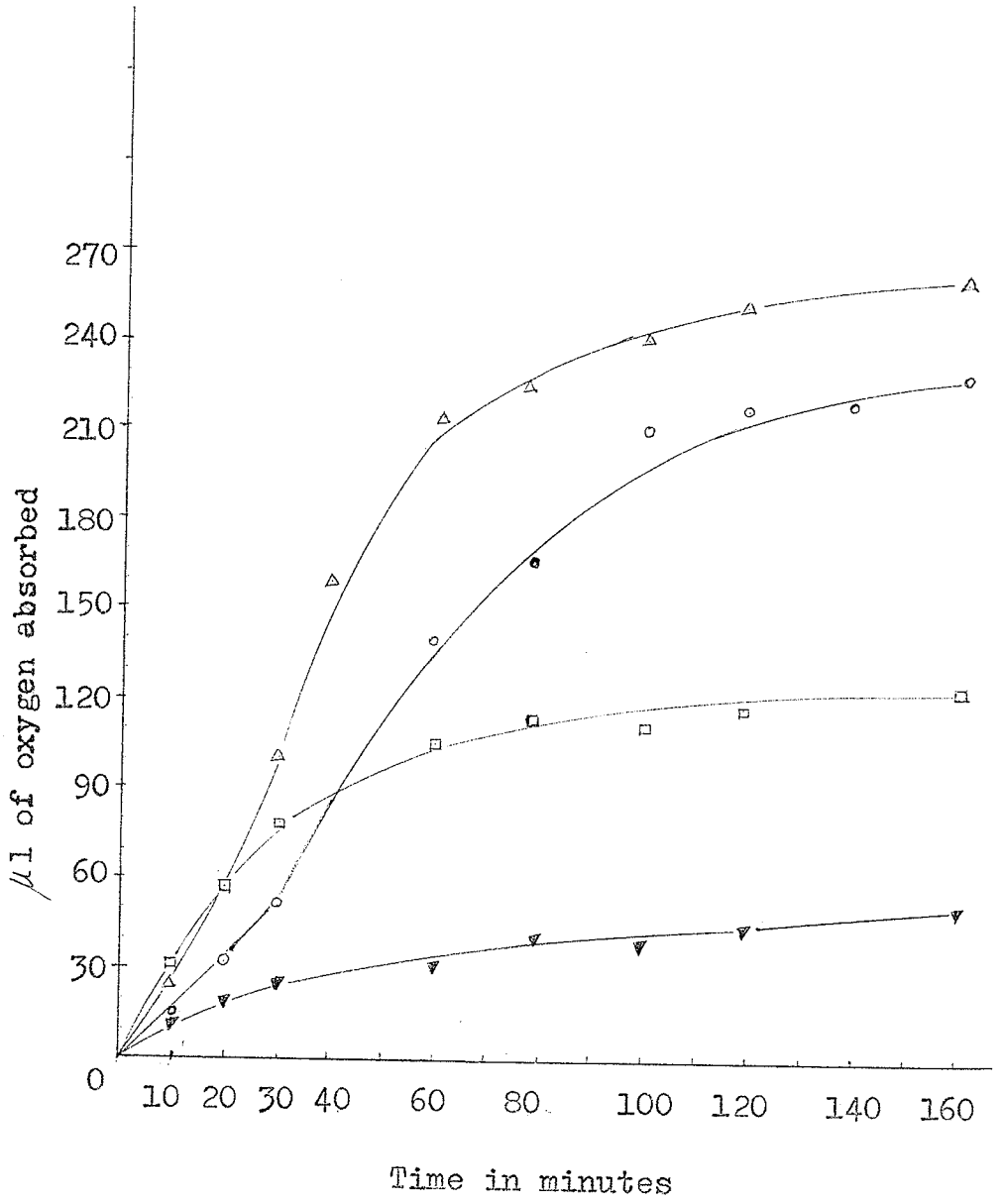


Fig. 21. Rate of oxygen uptake by washed m-acetotoluidide grown cell suspension of the Pseudomonas sp. 18 mgs dry weight of cells per ml.
o ---- m-acetotoluidide; △ ---- m-toluidine;
□ ---- protocatechuic acid; ▼ ---- acetate
(Plots are an average of 3 trials; exogenous).

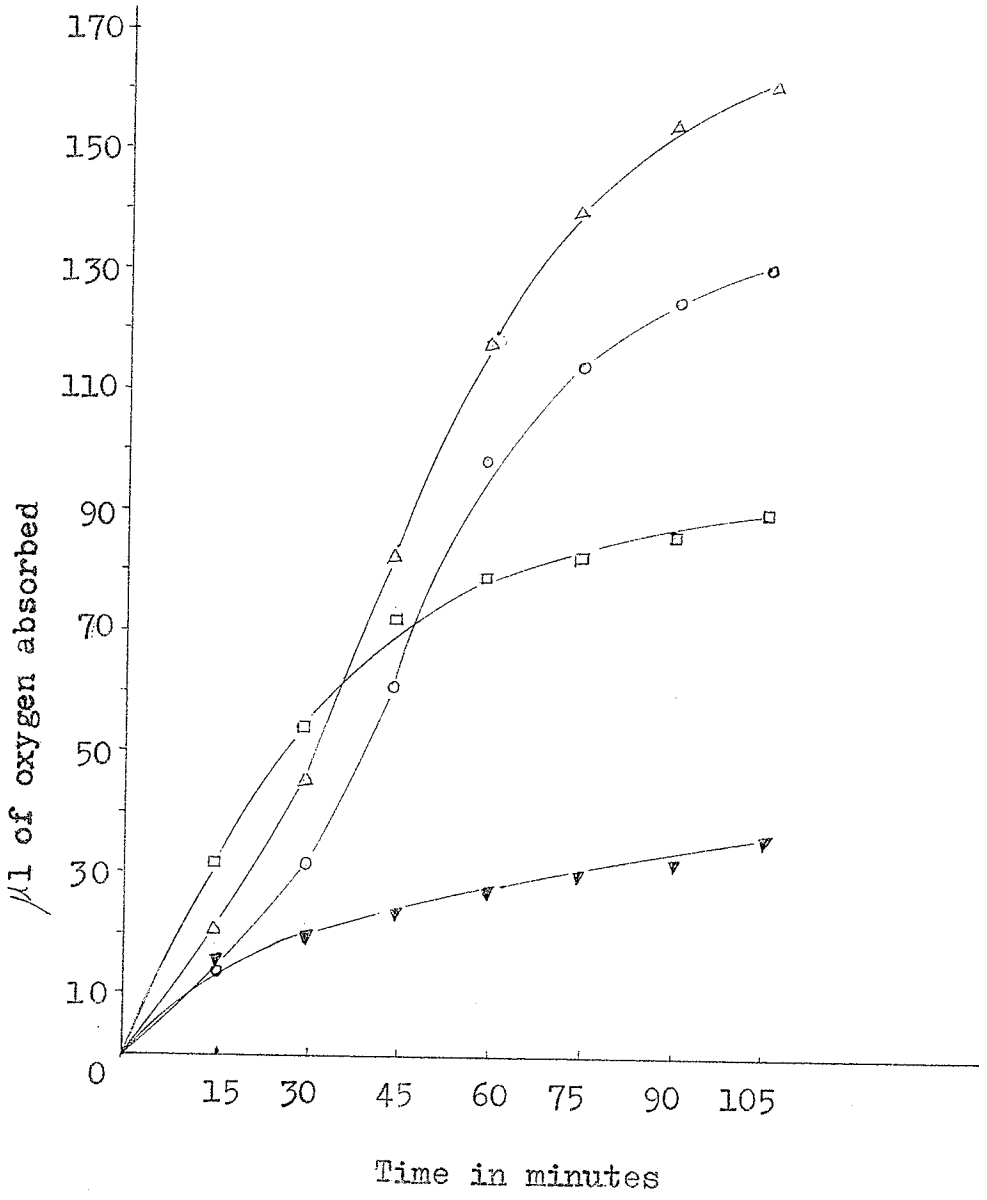


Fig. 22. Rate of oxygen uptake by washed p-acetotoluidide grown cell suspension of Pseudomonas sp. 16 mgs dry weight of cells per ml. o ——— p-acetotoluidide; △ ---- p-toluidine; □ ——— protocatechuic acid; ▼ ---- acetate (Plots are an average of 4 trials; exogenous).

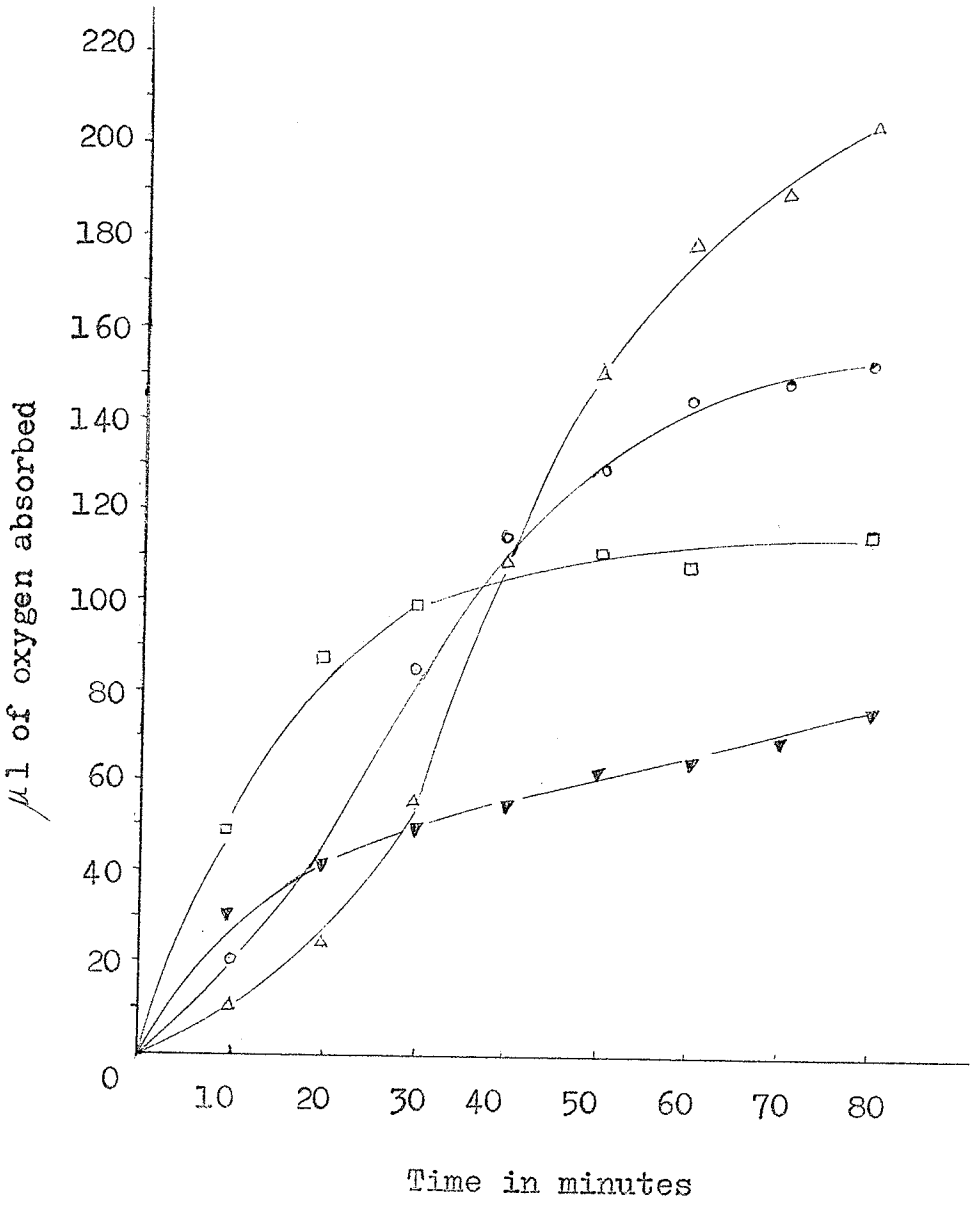


Fig. 23. Rate of oxygen uptake by 2'-hydroxyacetanilide grown cell suspension of Pseudomonas sp. 20 mgs dry weight of cells per ml. o ---- 2'-hydroxy-acetanilide; △ ---- o-aminophenol; ▼ ---- acetate; □ ---- catechol (Plots are an average of 5 trials; exogenous).

These results suggest that the substrates which show rapid oxygen uptake and consume a large amount of oxygen may be the metabolic intermediates of the substituted acetanilides studied. The possible metabolites of these aromatic compounds indicated by the oxygen uptake experiments are given in Table 14.

TABLE 14

Possible metabolites of the substituted acetanilides indicated by the oxygen uptake experiments

Substituted acetanilides	Possible metabolites
1. o-Acetotoluidide	o-Toluidine, catechol, acetate
2. m-Acetotoluidide	m-Toluidine, protocatechuic acid
3. p-Acetotoluidide	p-Toluidine, protocatechuic acid
4. 2'-Hydroxyacetanilide	o-Aminophenol, catechol, acetate

The rate of oxygen uptake by nutrient agar grown cells as well as washed cell suspensions of the Pseudomonas sp. grown on the substituted acetanilides for oxidizing various substrates are given in Tables 15, 16, 17 and 18.

The rate of oxygen uptake by washed cell suspensions of the Pseudomonas sp. grown on o-toluidine, m-toluidine and p-toluidine are shown in Table 19, 20, 21 and Fig. 24, 25 and 26.

The results shown in Fig. 24 indicate that catechol and acetate are possible metabolic intermediates of o-toluidine. Fig. 25 and 26 suggest that protocatechuic acid is the possible meta-

TABLE 15

Oxidation of aromatic compounds and acetate by washed cell suspension of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# o-Acetotoluidide	
	Oxidation of subs. after lag period (minutes)	QO_2E	Oxidation of subs. after lag period(min.)	QO_2E
1. o-Acetotoluidide	18	1.70	0	12.35
2. o-Toluidine	10	2.55	0	10.00
3. Catechol	7	0.80	0	9.55
4. Acetate	0	1.30	0	3.50

* ---- Substrates used at a concentration of 5 μmole/ml, except o-toluidine, which was used at a concentration of 10 μmole/ml.

\# ---- Substrates used at a concentration of 5 μmole/ml.

QO_2E ---- Represents rate of oxygen uptake in the presence of exogenous substrate after subtraction of the endogenous uptake. The units being microlitres (μl) of uptake STP/mg dry weight of cells/hour.

TABLE 16

Oxidation of aromatic compounds and acetate by washed cell suspension of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# m-Acetotoluidide	
	Oxidation of subs. after lag period (min.)	QO_2E	Oxidation of subs. after lag period (min.)	QO_2E
1. m-Aceto-toluidide	14	1.56	0	8.22
2. m-Tolui-dine	10	1.94	0	12.11
3. Protoca-techuic acid	9	0.63	0	7.67
4. Acetate	0	1.13	0	1.56

* ---- substrates used at a concentration of 5 μmole/ml, except m-toluidine, which was used at a concentration of 10 μmole/ml.

\# ---- substrates used at a concentration of 5 μmole/ml, except m-toluidine and protocatechuic acid, which were used at a concentration of 10 and 2.5 μmole /ml, respectively.

TABLE 17

Oxidation of aromatic compounds and acetate by washed cell suspensions of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# p-Acetotoluidide	
	Oxidation of subs. after lag period (min.)	QO_2E	Oxidation of subs. after lag period (min.)	QO_2E
1. p-Acetotoluidide	19	3.00	0	6.12
2. p-Toluidine	14	1.39	0	7.56
3. Protocatechuic acid	10	0.50	0	4.88
4. Acetate	0	0.89	0	1.50

* ---- substrates used at a concentration of 5 μmole/ml, except p-acetotoluidide and p-toluidine, which were used at a concentration of 10 μ mole/ml.

---- substrates used at a concentration of 5 μmole/ml, except p-acetotoluidide and protocatechuic acid, which were used at a concentration of 2.5 μmole/ml.

TABLE 18

Oxidation of aromatic compounds and acetate by washed cell suspensions of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# 2'-Hydroxyacetanilide	
	Oxidation of subs. after lag period (min.)	QO_2E	Oxidation of subs. after lag period (min.)	QO_2E
1. 2'-Hydroxy-acetanilide	15	2.56	0	7.40
2. o-Aminophenol	12	2.00	0	9.35
3. Catechol	8	0.88	0	5.65
4. Acetate	0	1.38	0	3.20

* ---- substrates used at a concentration of 5 μmole/ml, except 2'-hydroxyacetanilide and o-aminophenol, which were used at a concentration of 10 μmole/ml.

\# ----substrates used at a concentration of 5 μmole/ml, except 2'-hydroxyacetanilide and catechol, which were used at a concentration of 2.5 and 1.0 μ mole /ml, respectively.

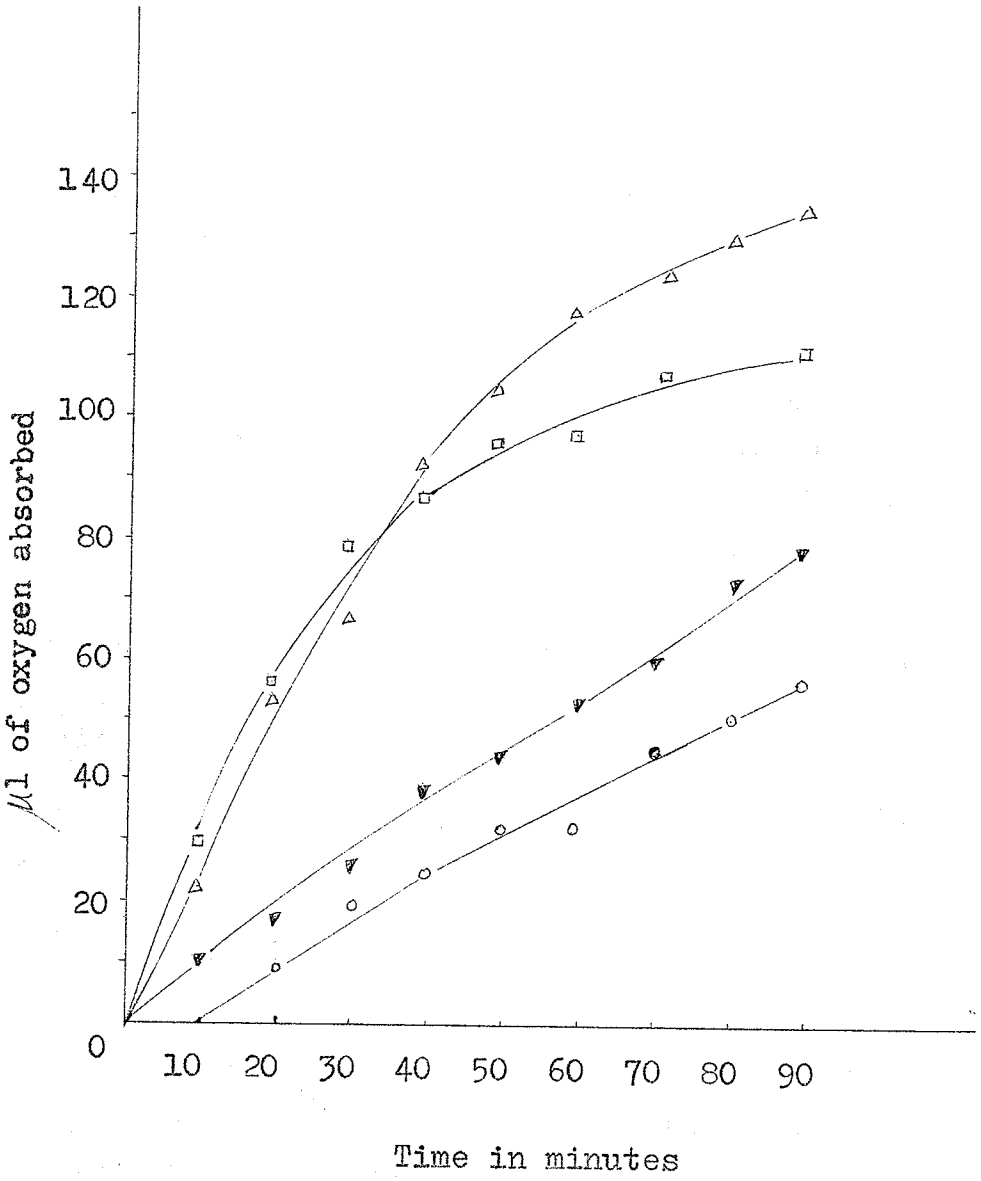


Fig. 24. Rate of oxygen uptake by washed o-toluidine grown cell suspension of Pseudomonas sp. 16 mgs dry weight of cells per ml. o ---- o-acetotoluidide; △ ---- o-toluidine; □ ---- catechol; ▼ ---- acetate (Plots are an average of 4 trials; exogenous).

TABLE 19

Oxidation of aromatic compounds and acetate by washed cell suspensions of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# o-Toluidine	
	Oxidation of subs. after lag period	QO_2E	Oxidation of subs. after lag period	QO_2E
1. o-Toluidine	10	2.55	0	7.63
2. o-Acetotoluidide	18	1.70	10	1.75
3. Catechol	7	0.80	0	6.06
4. Acetate	0	1.30	0	3.31

* ---- substrates used at a concentration of 5 μmole/ml, except o-toluidine, which was used at a concentration of 10 μmole/ml.

\# ---- substrates used at a concentration of 5 μmole/ml.

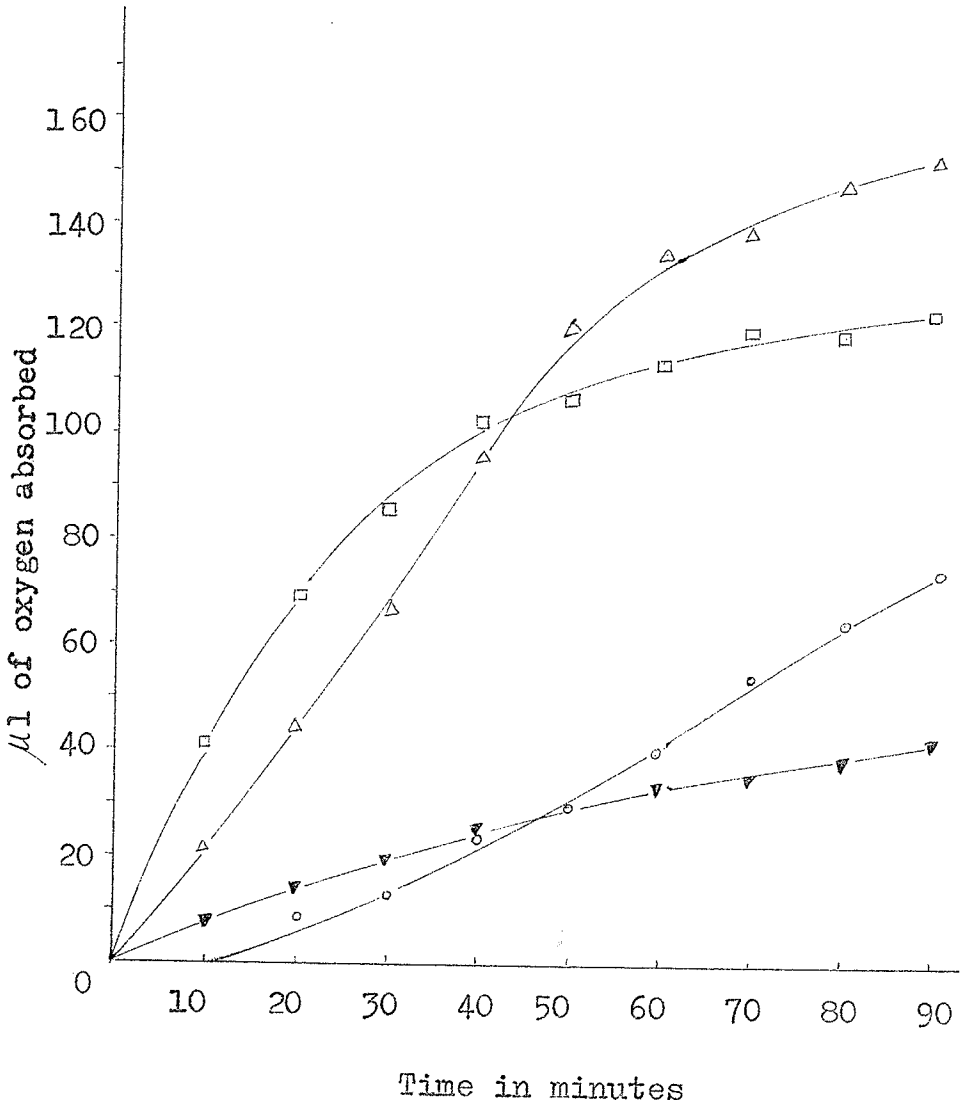


Fig. 25. Rate of oxygen uptake by washed m-toluidine grown cell suspension of Pseudomonas sp. 16 mgs dry weight of cells per ml. o ---- m-acetotoluidide; Δ ---- m-toluidine; □ ---- protocatechuic acid; ▼ ---- acetate (Plots are an average of 3 trials; exogenous).

TABLE 20

Oxidation of aromatic compounds and acetate by washed cell suspensions of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# m-Toluidine	
	Oxidation of subs. after lag period (min.)	QO_2E	Oxidation of subs. after lag period (min.)	QO_2E
1. m-Toluidine	10	1.95	0	8.00
2. m-Acetoto-luidide	14	1.56	12	1.81
3. Protocate-chuic acid	9	0.63	0	6.75
4. Acetate	0	1.13	0	1.31

* ---- substrates used at a concentration of 10 μmole/ml, except protocatechuic acid and acetate, which were used at a concentration of 5 μmole/ml.

\# ---- substrates used at a concentration of 5 μmole/ml, except m-acetotoluidide, which was used at a concentration of 10 μ mole/ml.

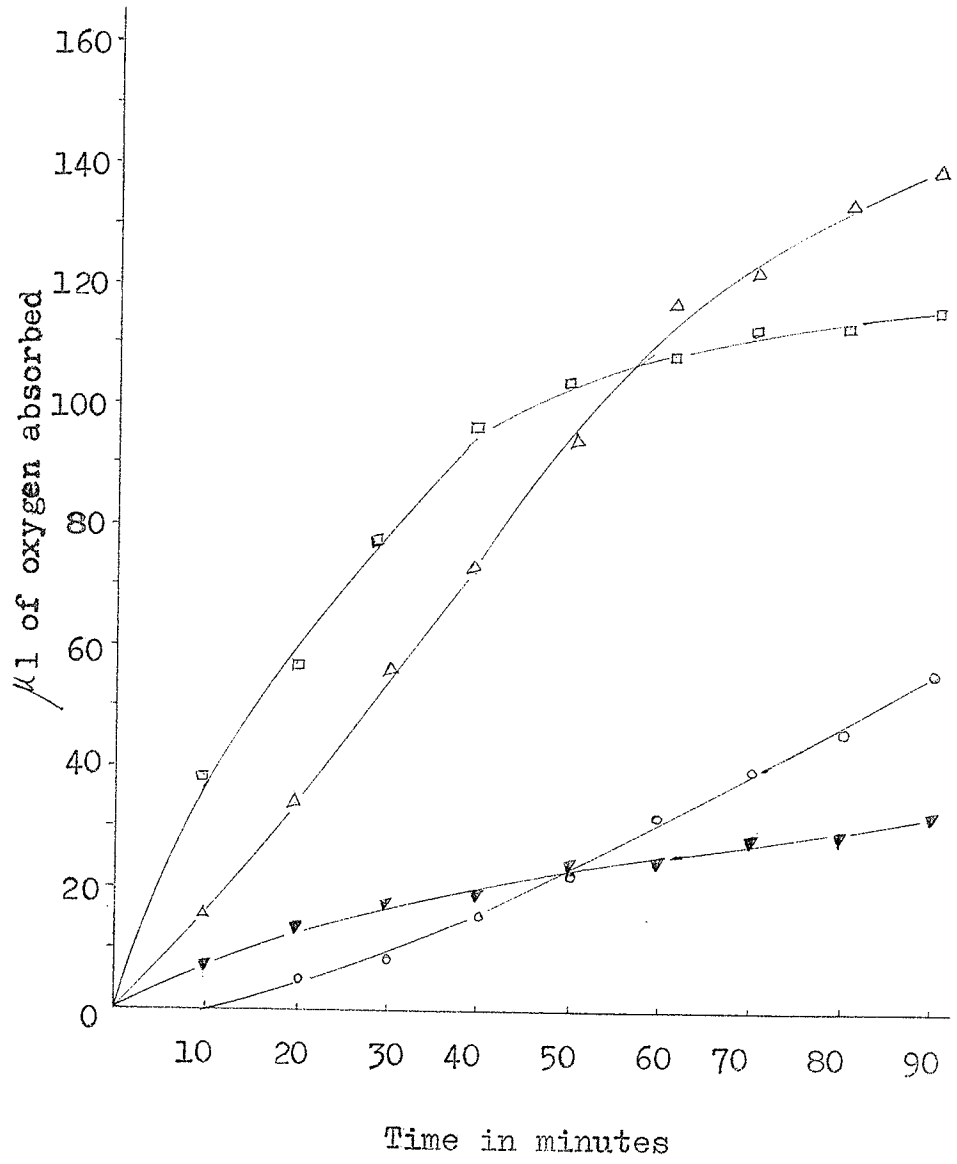


Fig. 26. Rate of oxygen uptake by washed p-toluidine grown cell suspension of Pseudomonas sp. 16 mgs dry weight of cells per ml. o ---- p-acetotoluidide; △ ---- p-toluidine; □ ---- protocatechuic acid; ▼ ---- acetate (Plots are an average of 5 trials).

TABLE 21

Oxidation of aromatic compounds and acetate by washed cell suspensions of Pseudomonas sp.

Substrate	Cells grown in presence of			
	* Nutrient agar		# p-Toluidine	
	Oxidation of subs. after lag period (min.)	QO_2E	Oxidation of subs. after lag period (min.)	QO_2E
1. p-Toluidine	14	1.39	0	7.00
2. p-Acetotoluidide	19	3.00	0	1.25
3. Protocatechuic acid	10	0.50	11	6.19
4. Acetate	0	0.89	0	1.06

* ---- substrates used at a concentration of 10 μ mole/ml, except protocatechuic acid and acetate, which were used at a concentration of 5 μ mole/ml.

\# ---- substrates used at a concentration of 5 μ mole/ml, except p-acetotoluidide, which was used at a concentration of 2.5 μ mole/ml.

bolite of m- and p-toluidine. By using "simultaneous adaptation" technique, we can thus determine part of the reaction sequences for the metabolism of the substituted acetanilides studied (Stanier, R.Y., 1947). These reaction sequences for the oxidative metabolism of the substituted acetanilides are given in Fig. 27.

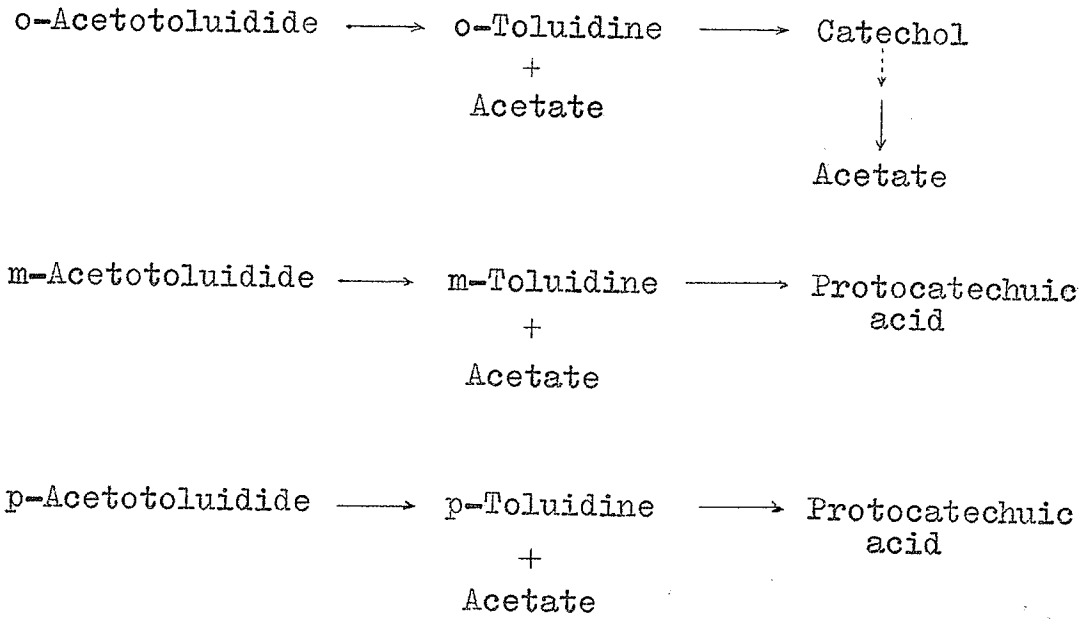


Fig. 27. Proposed reaction sequences for the oxidative metabolism of substituted acetanilide (by using simultnaeous adaptation technique).

DISCUSSION

The metabolism of acetanilide in the rabbit was studied by Smith and Williams (1948); who identified aniline, o-aminophenol and p-aminophenol as metabolites. An argument was presented by this study that if an animal can deacylate acetanilide to aniline, then it is to be expected that acetanilide will give rise to o-aminophenol and p-aminophenol in such an animal. Jaffe and Hilbert (1888) fed a dog with acetanilide and detected both o- and p-aminophenol in the urine. Their results suggested that the dog probably possesses an active deacylating enzyme for metabolizing aromatic acetamido compounds.

The findings presented in the study of the microbial degradation of acetanilide (Shaw and Aziz, 1972) are to some extent in agreement with Smith and Williams' hypothesis. However, a difference does exist between the microbial and mammalian oxidation of acetanilide. The results of Shaw and Aziz (1972) do not seem to agree with those presented by Theriault and Longfield (1967), who reported the conversion of acetanilide to 2'- and 4'-hydroxyacetanilide, by several bacteria. No indication of deacylation of acetanilide was given, which seems to be the major metabolic step in the pathway for degradation proposed by Shaw and Aziz (1972). This difference in the results presumably is due to the fact that in Theriault and Longfield's work, acetanilide was not used as sole carbon and energy source, but as a constituent of the peptone medium, which was the main energy providing

source. Under such conditions it may be possible for the organism to convert acetanilide to 2'- and 4'-hydroxyacetanilide as a detoxification mechanism, but not to deacylate acetanilide since its growth is not dependent on it. Our present results indicate that deacylation is the first step for degradation of substituted acetanilides by Pseudomonas sp. This is in agreement with the findings of Shaw and Aziz (1972) using the non-substituted acetanilide.

Thin layer chromatographic and gas-liquid chromatographic analysis have provided data which indicates the presence of the following metabolites: o-toluidine, m-toluidine, and p-toluidine for degradation of o-acetotoluidide, m-acetotoluidide and p-acetotoluidide, respectively. These studies also reveal that o-aminophenol and catechol are metabolites of 2'-hydroxyacetanilide. Manometric studies have shown rapid oxygen uptake by the substituted acetanilides grown Pseudomonas sp. in the presence of these substrates, which indicates that these compounds may be metabolic intermediates of the substituted acetanilides studied. In addition to these metabolites, manometric studies also suggest the presence of catechol as a metabolic intermediate of o-acetotoluidide and 2'-hydroxyacetanilide, and protocatechuic acid as the common metabolic intermediate of m- and p-acetotoluidide.

The results presented in our studies indicate the deacylation of substituted acetanilides to the related anilines and the presence of a deacylating enzyme system in the acetanilide utilizing Pseudomonas sp. This enzyme system is inductive rather than cons-

titutive; since nutrient agar grown organism oxidized the substrates after lag period and the total volume of oxygen consumed was low, whereas the substituted acetanilides grown organism consumed large amounts of oxygen rapidly, without any lag period. This clearly indicates that the enzymes involved are inductive, and the organism requires a induction before it can start oxidizing the substituted acetanilides.

The other metabolite of 2'-hydroxyacetanilide was identified by TLC analysis as catechol. Its presence in the culture was also supported by manometric studies. Even though catechol could not be isolated in sufficient amount to carry out gas-liquid chromatographic analysis and chemical assays to confirm its existence, strong evidence still has been presented in our results for the presence of catechol as a metabolic intermediate in the culture of 2'-hydroxyacetanilide grown Pseudomonas sp.

Catechol was suggested by manometric studies as another metabolite of o-acetotoluidide. In the case of m-acetotoluidide and p-acetotoluidide, protocatechuic acid rather than catechol was suggested as a metabolic intermediate by manometric studies. Unfortunately, since we failed to identify these compounds by chromatographic analysis and chemical methods, there is no strong direct evidence for their existence.

The presence of o-aminophenol and catechol in the culture of 2'-hydroxyacetanilide grown Pseudomonas sp. suggests that there are two major sets of reactions involved in the microbial degradation of 2'-hydroxyacetanilide, Fig. 28.

(1) Deacylation: Conversion of 2'-hydroxyacetanilide to o-aminophenol.

(2) Hydroxylation: Hydroxylation of o-aminophenol and formation of catechol.

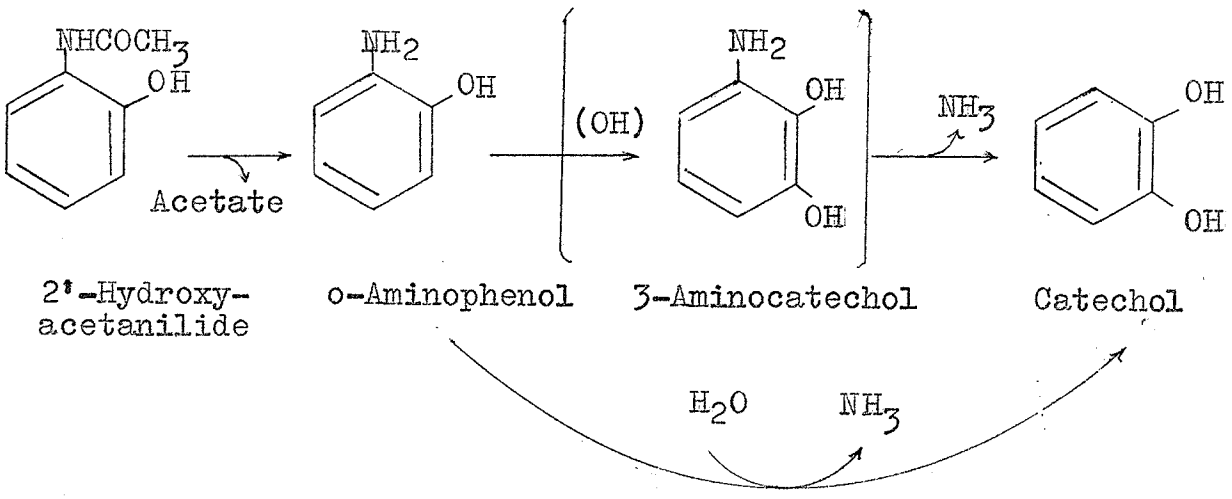


Fig. 28. Proposed pathways for degradation of 2'-hydroxyacetanilide by Pseudomonas sp.

Hypothetically, o-aminophenol could be converted to 3-aminocatechol, which is quickly converted to catechol. Therefore, 3-aminocatechol could not be accumulated in sufficient amount to be detected by the analytical techniques used in our experiments. Our studies do not provide concrete evidence to support this assumption.

From our experimental results a pathway for the degradation of 2'-hydroxyacetanilide through o-aminophenol, 3-aminocatechol and catechol was suggested. In the case of o-acetotoluidide, the pathway of degradation is proposed to be through o-toluidine and

catechol. m-Acetotoluidide and p-acetotoluidide are suggested to be metabolized via m-toluidine and p-toluidine, respectively, and protocatechuic acid. No rigid conclusion can justifiably be made from data obtained with intact cells until intermediates can be isolated, and shown to be the substrates of cell-free extracts.

No precedent could be found in the literature for the occurrence of o-aminophenol as an intermediate in microbial degradation of aromatic compounds. The workers, Walker and Harris (1969), who studied the degradation of aniline by a Pseudomonas sp. and suggested the presence of o-aminophenol as a metabolite, had based their entire presentation upon the findings resulting from manometric studies. However, the work of Shaw and Aziz (1972) and of Shaw and Fingerote (1972) as well as our present project have detected and identified the existence of o-aminophenol as an intermediate in the cultures of Pseudomonas sp., when acetanilide, aniline or 2'-hydroxyacetanilide was provided as sole carbon and energy source.

Walker and Harris (1969), studying the bacterial metabolism of aniline, suggested its conversion to catechol, which acts as the ring fission substrate, but they presented no direct evidence for its existence. Instead, they based their assumption on the results of manometric studies. Shaw and Aziz (1972) were also able to demonstrate a large oxygen uptake with their acetanilide grown Pseudomonas sp., when catechol was provided as a substrate, but they evidently could not show the presence of catechol in their culture. Our results suggest the existence of catechol in the

culture of 2'-hydroxyacetanilide grown Pseudomonas sp. by manometric studies, and also provide direct evidence for its presence by TLC analysis.

Concentrations of various substrates had to be varied to get appreciable amount of oxygen uptake in manometric studies; this could be explained assuming the correctness of the Jacob-Monad hypothesis for enzyme induction. In this case the efficiency with which the substrate binds to the repressor in order to promote enzyme synthesis may vary widely, so that for a poor inducer, high intracellular concentration of substrate would have to be reached before derepression occurs. As a corollary, if a substrate is itself non-inducing but its intermediate metabolic product fulfils its role, then induction would be dependent on the rate of substrate conversion by a low concentration of enzyme already present in the repressed cells.

From the results of growth studies, it became evident that the amount of growth obtained, as measured by the maximum turbidity displayed by the cultures, was dependent on the concentrations of acetanilides present. In the case of 2'-hydroxyacetanilide, for three concentrations used (0.1, 0.2 and 0.3%, W/V), growth increased with increasing concentration, Fig. 5. The organism grew very rapidly by utilizing 2'-hydroxyacetanilide as sole carbon and energy source (the growth was heavy after only three days of incubation); on the other hand, acetanilide gave only slight growth after an incubation period of three days under identical conditions. This may be due to the fact that monohydroxylated product (o-aminophenol) formed after the deacylation of 2'-hydroxyacetanilide is

more readily utilizable by the organism. This can be explained when we compare the following two proposed reaction sequences:

(1) Acetanilide ⟶ Aniline ⟶ o-Aminophenol
↓
Simple aliphatic compounds ⟵ Catechol

(2) 2'-Hydroxyacetanilide ⟶ o-Aminophenol
↓
Simple aliphatic compounds ⟵ Catechol

The organism needs three steps to convert acetanilide into the ring fission substrate, catechol; but it needs only two steps in the case of 2'-hydroxyacetanilide. Since the enzymes involved in these reactions are inductive rather than constitutive, it is time and energy consuming for the organism to carry out these reactions. From our results we reach a conclusion that mono-hydroxylation at the ortho-position of acetanilide provides a carbon and energy source which is more readily utilizable by the Pseudomonas sp. than the parent compound. The increase in water solubility after mono-hydroxylation at the ortho-position of acetanilide may be another factor for the increased rate and amount of growth. The effects of mono-hydroxylation at the meta- or para-position and dihydroxylation at both ortho-positions of acetanilide could not be investigated since we were unable to synthesize the dihydroxylated acetanilide, and both m- and p-hydroxyacetanilide could not be used due to their instability.

On the other hand, methylation on the ortho-, meta-, or para-position of the aromatic nucleus of acetanilide resulted in compounds with lower water solubilities. The rate of growth of the organism on these methyl derivatives was slower than on the parent compound. The growth was dependent upon the concentrations

of the methyl acetanilides present in the culture medium. With the three concentrations used (0.1, 0.2 and 0.3%, W/V), growth of the organism on both o- and p-acetotoluidide (o- and p-methylacetanilide) was observed to be best at 0.1%. The peak of growth with 0.1% substrate was two fold of that with 0.3% substrate. An inability to completely metabolize and an accumulation of the carbon source present as anilines when the concentrations of o- or p-acetotoluidide in the culture medium was increased up to 0.2% was evident. In other words, the decrease in the maximum growth of the organism with increased concentrations of o- or p-acetotoluidide may be due to the accumulation of a toxic metabolite, possibly o- or p-toluidine. Indeed toluidines were found to be increasing inhibitory, or bacteriostatic, with increasing concentrations.

Growth of the organism on m-methylacetanilide (m-acetotoluidide) was studied with four concentrations, 0.05, 0.1, 0.2 and 0.3% (W/V). The growth was observed to be best at 0.1%; the two higher concentrations, 0.2 and 0.3%, gave no growth at all. This may be due to the accumulation of a highly toxic metabolite, possibly m-toluidine, at the higher concentrations; or may be due the toxicity of m-toluidine, at higher concentrations, The organism did not grow on the dimethyl derivative of acetanilide (2,6-dimethylacetanilide). However, it grew very well in the presence of both 2,6-dimethylacetanilide and nutrient broth. This shows that 2,6-dimethylacetanilide is not toxic to the organism; the inability of showing growth is simply due to the fact that the

organism is unable to utilize this compound as sole carbon and energy source.

We also studied the growth of Pseudomonas sp. on o-, m-, and p-toluidine. The organism grew quite well when 0.01, and 0.05% (W/V) of toluidines were provided as sole carbon and energy source. However, higher concentrations (up to 0.1%) of toluidines gave no growth under identical conditions. This result shows that toluidines at higher concentrations are inhibitory to the growth of Pseudomonas sp. And it provides evidence that the failure of the organism to grow at higher concentrations of o-, m-, or p-acetotoluidide is due to its inability to completely metabolize and the accumulation of toluidines.

From these growth studies, we reach the conclusion that o-hydroxylation of the aromatic nucleus of acetanilide favors the utilization of the substrate and the growth of the Pseudomonas sp. On the other hand, mono-methylation of the aromatic nucleus of acetanilide results in the formation of compounds which are not so readily utilizable by the organism as the non-substituted acetanilide, and will give rise to more toxic metabolites. Dimethylation on both ortho-positions of the aromatic nucleus of acetanilide results in the formation of a compound which is not utilizable by the organism.

The growth profiles of the Pseudomonas sp. on m-acetotoluidide and p-acetotoluidide showed a distinct logarithmic phase, followed a lag phase of two days during which growth was very slow. After this period, the organism started to grow rapidly

again and attained maximum growth within 21 days. The appearance of this lag period suggests that the energy providing steps is shifted from one to another. A possible explanation for the existence of this lag phase in the growth profiles of the Pseudomonas sp. on m- or p-acetotoluidide exists, based upon the assumption that during the early stages of growth, the first step of deacylation of m- or p-acetotoluidide to m- or p-toluidine, is the main carbon and energy providing reaction, which releases a large amount of acetate, which is readily utilizable by the organism. Deacylation, on the other hand, causes the accumulation of m- or p-toluidine in the cultures, which cannot be metabolized by the organism at this phase of growth. The organism lacks the enzyme systems for the degradation of toluidines and the amounts of toluidines are not yet high enough to induce the synthesis of enzymes. As the m- or p-acetotoluidide in the culture medium is exhausted, the growth ceases as the deacylation is the main energy providing reaction (in the form of acetate) at this phase of growth. The concentrations of m- or p-toluidine in the culture medium is now high enough for inducing enzymes for their own degradation. This induction is a slow process and so, a lag period appears before the organism starts utilizing m- or p-toluidine. After this lag period, the organism starts growing rapidly again by utilizing m- or p-toluidine as energy and carbon source, which are broken down to simpler readily utilizable compounds.

The results obtained from these growth studies show that the adaptation to metabolize m- and p-acetotoluidide is not alone

responsible for simultaneous adaptation to the utilization of m- and p-toluidine. In other words, the presence of m- or p-acetotoluidide in the culture medium induces the formation of enzymes for its own degradation; the formation and accumulation of m- or p-toluidine again acts as an inducer which causes the synthesis of enzymes for its own degradation.

Our results indicate that there are two main energy providing steps in the degradation of m- and p-acetotoluidide by Pseudomonas sp. :

(1) Deacylation: This reaction releases acetate, which is readily utilizable as energy and carbon source for growth of the organism in the first phase.

(2) Fission of the aromatic nucleus after dihydroxylation and formation of simple aliphatic compounds, which provide energy for growth in the second phase.

The failure of the organism to exhibit a characteristic plateau during growth on o-acetotoluidide and 2'-hydroxyacetanilide present difficulty in deciding whether there is any or at which stage the shift in energy providing steps for growth of the organism exist.

Our study does not reveal the mode of cleavage of the aromatic nucleus by Pseudomonas sp. No rigid conclusion can be made on the influence of ring substitution on the mode of microbial degradation of acetanilide. Further studies of the microbial degradation of acetanilides are necessary for elucidating the mode of ring fission and the steric factors which are important in de-

termining the mode of attack on the acetanilides by microorganisms. Cell-free extracts and more sophisticated analytical procedures are needed for confirming some of our results.

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