BACTERIAL METABOLISM

OF

ACETANILIDE

A THESIS

submitted by

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Some men see things as they are, and say why?

I dream of things which never were, and say why not?

Bernard Shaw.

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Aleem uddin Aziz

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Chapter 1

INTRODUCTION

1.1 General.

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

Although aromatic compounds are very stable, microorganisms do exist which can completely degrade a number of such compounds. The utilization of aromatic compounds by certain bacterial species is now very well understood, and numerous workers have described their isolation from various sources; some species are known to be capable of metabolising phenolic compounds. The metabolic pathways leading to the degradation of these compounds have also been studied. The interest in 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; and (c) The control of the enzymes participating in the metabolism of these aromatic compounds.

1.2 Microbial metabolism of aromatic compounds.

Stormer (1908) tested the effect of a number of organic compounds including toluene, xylene, phenol, and p-cresol, on soil and claimed to have isolated organisms that could destroy these compounds. Sen Gupta (1921) has studied quantitatively, the disappearance of phenols and cresols from the soil. Occurrence of phenol utilizing bacteria was also reported by Fowler, Adren and Lockett (1911).

Mechanism of oxidative metabolism of aromatic compounds was studied first by Grey & Thornton (1928) and Tattersfield (1928). They isolated many types of soil bacteria capable of destroying the following aromatic compounds: phenols, ortho-, meta-, and para-cresol, naphthalene, phloroglucinol, resorcinol and toluene. These compounds are commonly used as soil sterilizing agents. Pure cultures of the organisms were capable of utilizing the compounds as sole carbon source, when incorporated into a mineral salt medium containing an inorganic nitrogen supply. The strains were classified, morphologically and from their growth characteristics, into six families: Coccacea, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirillaceae and Bacillaceae.

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 classes.

(a) Pathways in oxidative metabolism of aromatic compounds prior to ring fission.

(b) 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 con-

cerned with 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 catechol and protocatechuic acid, or para- to each other, as in gentisic and homogentisic acids. Catechol and protocatechuic acid have been shown to be substrates for ring fission in the microbial degradation of many different aromatic compounds; see Table I; 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 a Nocardia species, indicated that the acid side chain is metabolised by a beta-oxidation process; that is, the microorganism removes two-carbon chains at one time. Thus side chains with an odd number of carbon atoms are metabolised to benzoic acid, which is converted to catechol prior to ring fission. Phenyl substituted acids that contain an even number of carbon atoms are metabolised through phenyl-acetic acid. The further metabolism of phenyl-acetic acid may proceed through either homogentisic acid (2,5-dihydroxy-phenyl-acetic acid) or homoprotocatechuic acid (3,4-dihydroxy-phenyl-acetic acid), depending on the species of microorganism; Dagley, S. et al. (1953); Kunita, N. (1955).

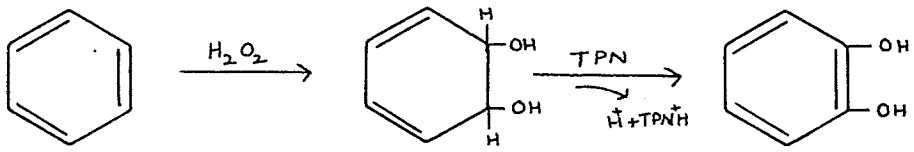
Studies of the metabolism of toluene and isopropyl benzene by Pseudomonas putida have shown that these compounds are converted to ortho-dihydroxy compounds in which the side chain is left intact; Gibson, D. T. (1968). The enzymes, catalyzing the hydroxylation of the aromatic ring,

TABLE I

Compounds metabolised via	
Catechol	Protocatechuic acid
Benzoic acid Salicylic acid Phenol Naphthalenes Phenanthrene Anthracene Mandelic acid ortho-Cresol Benzene	m-Cresol p-Cresol p-Hydroxy-benzoic acid p-Hydroxy-mandelic acid p-Amino-benzoic acid Phthalic acid

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.

In view of the considerable interest in the degradation of aromatic compounds there is surprisingly very little work done on benzene metabolism. Marr and Stone (1961), working with Pseudomonas organism that utilized benzene as sole carbon source for growth, detected catechol chromatographically. Phenol was excluded as an intermediate in benzene metabolism, due to the inability of benzene grown cells to metabolise this compound. This study 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 by only a limited number of strains. In the case of benzene, the enzymes are not constitutive, as removal of the organism from benzene medium results in loss of ability to oxidize benzene. Hypothetical pathways, given in this study, for the formation of catechol from benzene, suggested epoxidation of the aromatic ring, 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 dehydrogenation of trans-benzene glycol results in catechol for-

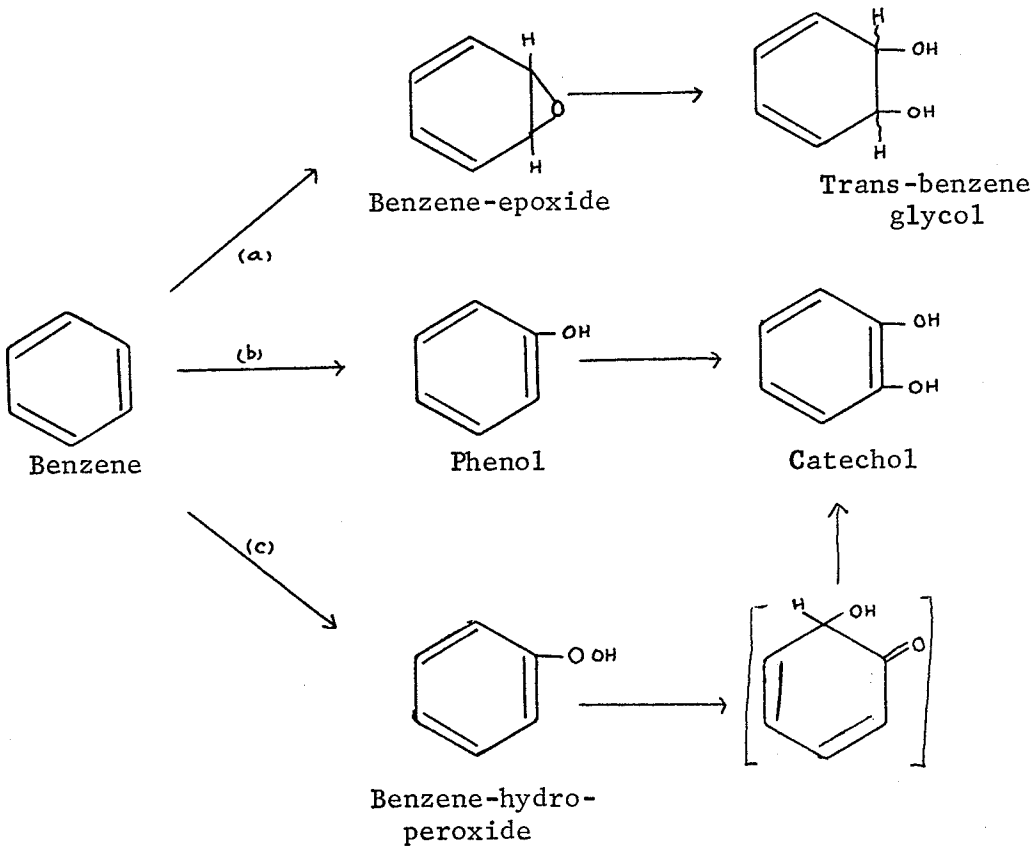


Fig. 1. Hypothetical pathways for the formation of catechol from benzene.

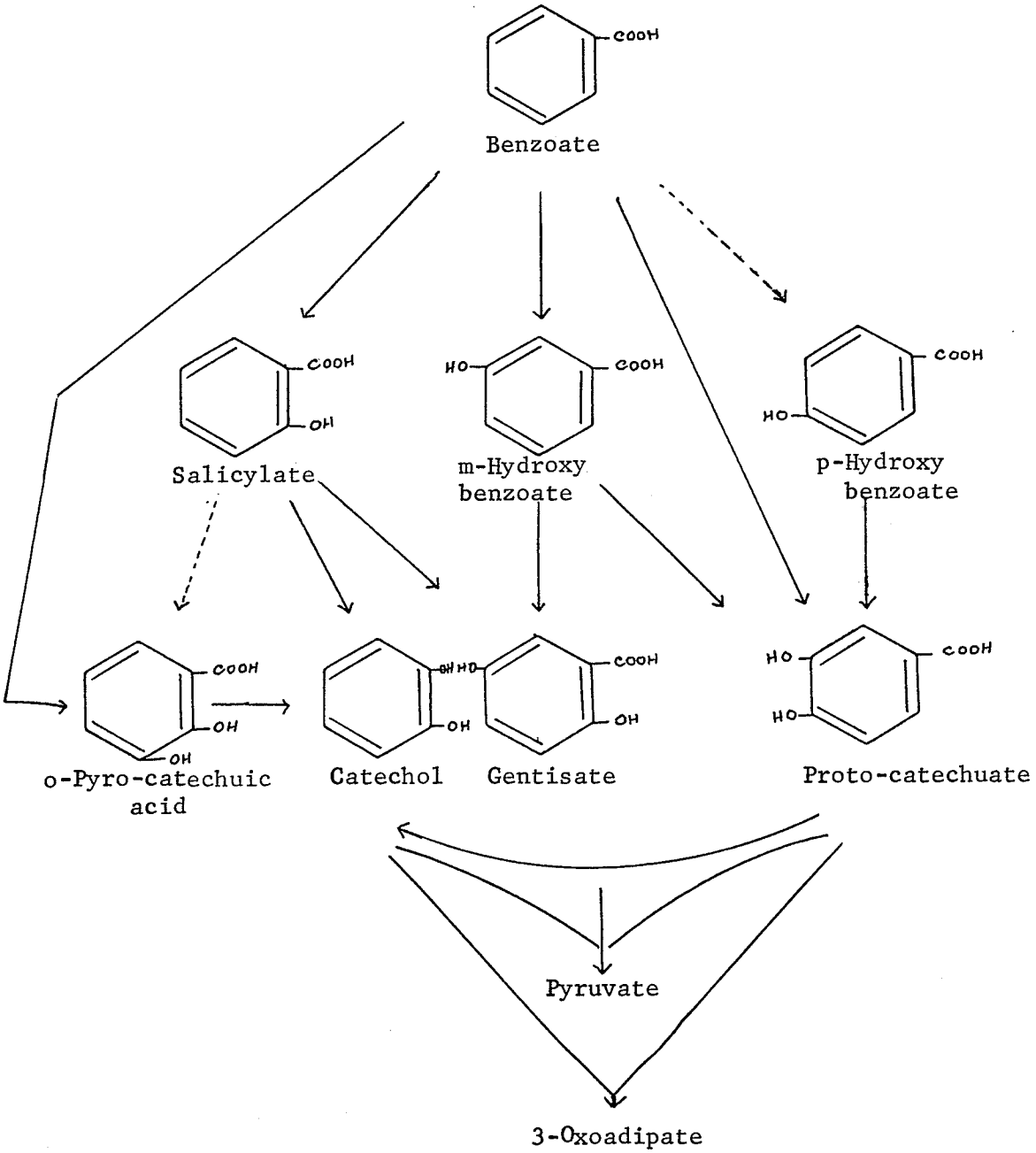
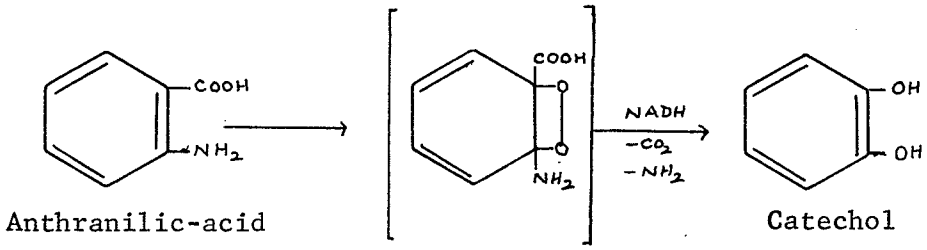


Fig. 2. Diversity in metabolism of benzoate by microorganisms, Dagley, et al. (1960).

mation, Gibson, G. T. (1968).

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



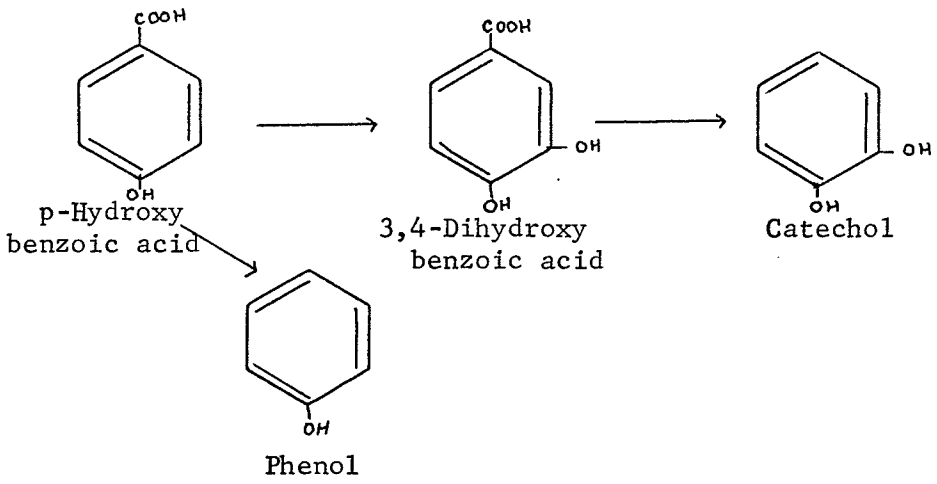
Anthranilic-acid — Hypothetical peroxide — Catechol

Diversity is found commonly in pathways leading to the oxidative metabolism of aromatic compounds prior to ring fission. Hayaishi and Stanier (1951) showed that there were two distinct pathways of tryptophan metabolism among the Pseudomonas. Microbial dissimilation of phenylacetic acid studied by Kunita, N. (1955) revealed that one strain of Pseudomonas fluorescens produced homoprotocatechuic acid from phenylacetate, while another appeared to take the homogentisate route.

The pathways, employed for the utilization of the benzoate among microbial species and strains prior to ring fission, show a remarkable diversity, Fig. 2. Intensive work has been done in microbial metabolism of benzoate. Sloan, N. H. et al. (1951) reported formation of aniline from para-amino-benzoate by Mycobacterium smegmatis, which was further oxidized to para-amino-phenol. Further studies with acid fast bacteria revealed the following sequence.

p-amino-benzoic acid ⟶ p-amino-benzyl alcohol ⟶ p-hydroxy-aniline

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



Phenyl-alanine has been shown by Dagley, S. et al (1953) to be oxidized to homogentisic acid by Vibrio through two different pathways; one via phenyl-pyruvic acid and phenyl-acetic acid and the other through tyrosine and para-hydroxy-phenyl-pyruvic acid. Walker and Wiltshire (1953) demonstrated the presence of salicylic acid and catechol as intermediates during metabolism of naphthalene by a soil bacterium, using simultaneous

adaptation technique.

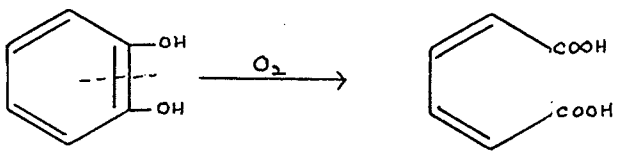
The enzymic transformations involved in these reactions are hydroxylations and decarboxylations. The particular route taken by a microorganism, capable of utilizing an aromatic compound, will presumably depend on its genetic potential coupled with the influence of environment for synthesis of the relevant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

Due to apparent diversity in mechanisms utilized by microorganisms to prepare different aromatic compounds for ring fission, Hayaishi, O. (1964) proposed the term mono-oxygenase for enzymes that incorporate one atom of oxygen into a substrate molecule, and di-oxygenase for enzymes incorporating both atoms of the oxygen molecule. Di-oxygenases function principally in the enzymatic fission of aromatic nucleus.

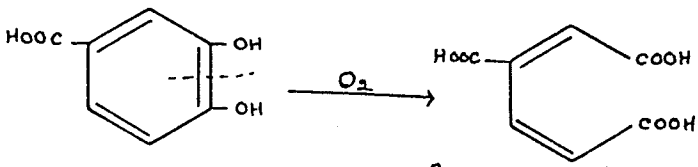
(b) FISSION OF AROMATIC NUCLEUS.

The dihydroxylated benzene derivatives, such as catechol and protocatechuic acid, have been investigate quite intensively, due to the fact that they are metabolic intermediates of many different aromatic compounds.

Hayaishi and Hashimoto (1950) isolated an enzyme which would cleave the dihydroxylated derivatives of benzene between the hydroxyl groups. 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.



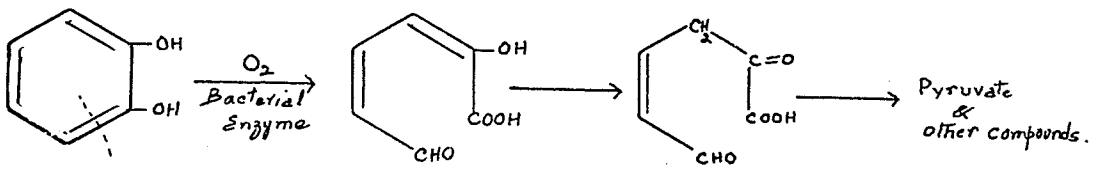
Catechol cis-cis-Muconic acid



Protocatechuic acid β-Carboxy-cis-cis-Muconic acid

An alternate enzymatic cleavage of catechol was reported by Dagley and Stoper (1959) in a Pseudomonas sp., which utilized ortho-cresol as sole carbon source for growth. In this case, catechol was oxidized to ∝-hydroxy-muconic semialdehyde by a reaction catalyzed by catechol-2,3,-dioxygenase; Fig. 3. Dagley, S. et. al (1960) studied the induction of another enzyme, protocatechuic acid-4,5,-dioxygenase, by growing a different Pseudomonas sp., on p-cresol. Protocatechuic acid was oxidized by this enzyme to ∝-hydroxy- γ -carboxy-muconic semialdehyde, Fig. 4.

Ornston and Stanier (1966), reinvestigated the metabolism of these ring fission products. Their studies with Pseudomonas putida showed β -oxoadipic acid enol lactone (γ-carboxy-methyl- Δ^{β} -butenolide) as a common intermediate in the sequence of reactions initiated by catechol-1,2-dioxygenase and protocatechuic acid-3,4-dioxygenase, Fig. 5. Table II shows ring fission substrates corresponding to some aromatic compounds, Gibson, D.T. (1968).



Catechol ∝-Hydroxy muconic-semialdehyde (Keto-form)

Fig. 3. Suggested pathway of catechol oxidation by microorganisms possessing the catechol-2,3-oxygenase system.

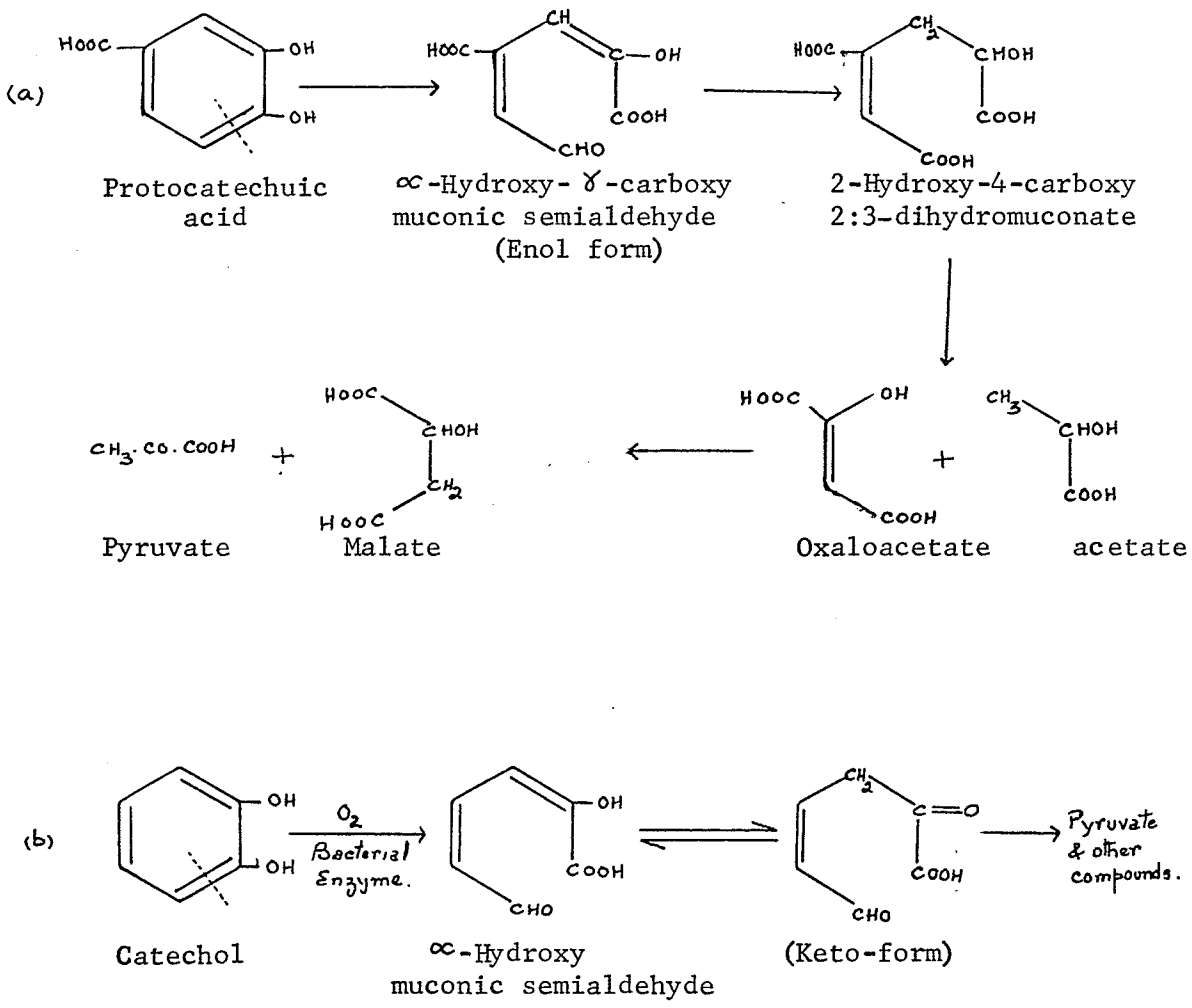


Fig. 4. Proposed pathways for oxidative metabolism of Protocatechuic acid and catechol by Pseudomonas sp., possessing protocatechuic acid-4,5-dioxygenase enzyme system.

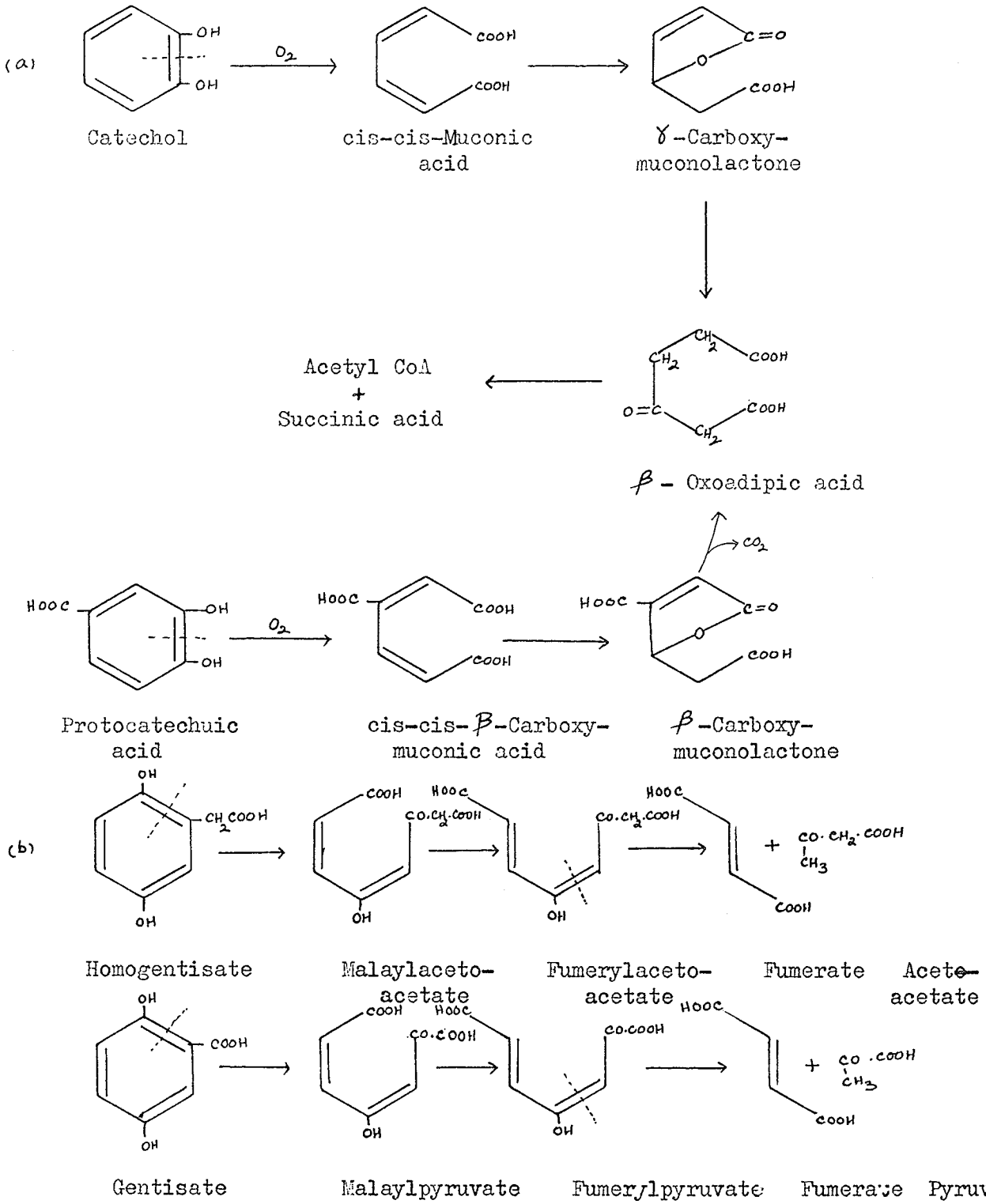


Fig.5. Orson and Staniers suggested oxidative pathways for the fission of the aromatic nucleus by microorganisms:(a) Oxidative fission of the bond between carbon atom bearing the hydroxyl groups of an o-dihydroxyphenol:(b) Rupture of bond betweenthe carbon atom bearing a hydroxyl group and an adjacent carbon atom carrying a carbon side chain or carboxyl group.

TABLE II

Ring fission substrates of some aromatic compounds.

Aromatic compounds	Ring fission substrate
Toluene or ortho-cresol	2,3-Dihydroxy-toluene
β-phenyl-propionic acid	2,3-Dihydroxy-β-Phenyl-propionic acid
Phenyl-acetic acid	3,4- or 2,5-Dihydroxy-phenyl-acetic acid
Naphthalene	1,2-Dihydroxy-naphthalene
Anthracene	1,2-Dihydroxy-anthracene
Phenanthrene	3,4-Dihydroxy-phenanthrene
Estrone	4-Hydroxy-estrone

Diversity in pathways in the enzymatic fission of aromatic nucleus, during oxidative metabolism of aromatic compounds, is found quite commonly in microorganisms utilizing aromatic compounds as sole carbon source for growth. Feist and Hegeman (1969) studied metabolism of both benzoate and phenol by strains of Pseudomonas putida. In the case of growth at the expense of the benzoate, catechol was shown to cleave ortho- and metabolise via the β -keto_adipate pathway, but in the case of cresol or phenol, the catechol or substituted catechol formed were metabolised by a separate pathway, following meta-cleavage of the aromatic ring of catechol.

This diversity in pathways led Dagley, S. et al. (1964) to propose a general scheme for degradation of catechol, Fig. 6. This general scheme has been successfully applied to microbial degradation of several aromatic compounds but microorganisms do not live by a rigid set of rules. It is, therefore, not surprising to find exceptions to the proposed scheme; Table III.

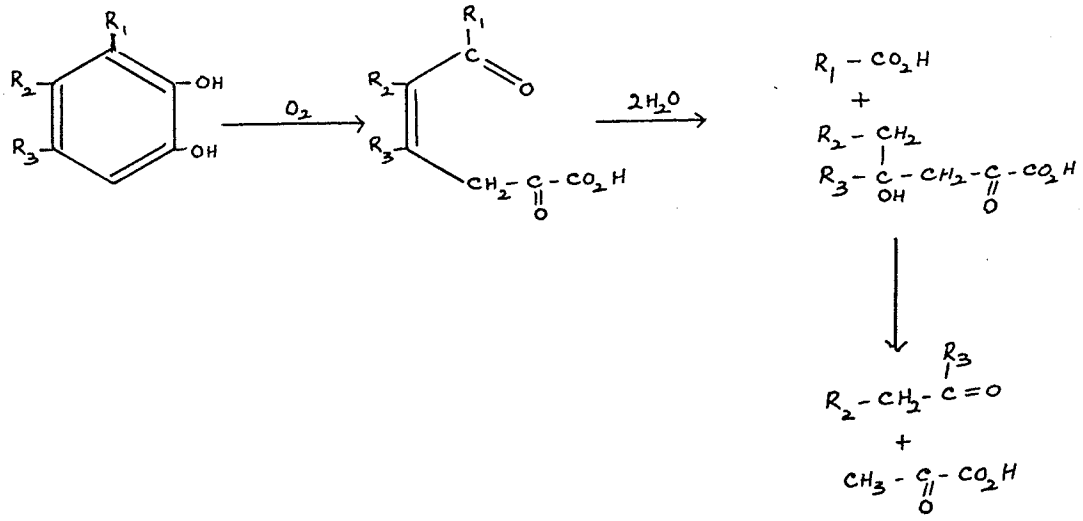


Fig. 6. General scheme for degradation of catechols.

TABLE III

Substrate	Nuclear-substituent	Products
Catechol	$R_1 = R_2 = R_3 = H$	Formate, acetaldehyde, pyruvate
3-Methyl-catechol	$R_1 = CH_3$; $R_2 = R_3 = H$	Acetate, acetaldehyde, pyruvate
4-Methyl-catechol	$R_1 = R_3 = H$; $R_2 = CH_3$	Formate, propionaldehyde, pyruvate
2,3-Dihydroxy-β-phenyl-propionic acid	$R_1 = HO_2C - CH_2 - CH_2$ $R_2 = R_3 = H$	Succinate, acetaldehyde, pyruvate
Protocatechuic acid	$R_1 = R_2 = H$; $R_3 = CO_2H$	Formate, pyruvate (2-moles)

Many alternate routes of metabolism have been discovered. It is, thus clear that catechol, protocatechuate, gentisate and homogentisate are not the only substances susceptible to ring fission. Further, that the oxygenative cleavage of the former two compounds does not always yield a muconic acid.

Although microbial metabolism 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 amines which are essential constituents of several herbicides and pesticides. The elucidation of the metabolic pathways leading to their degradation in soil is important from ecological point of view. Until such studies are carried out, it is impossible to assess meaningfully their toxicity to man.

Bartha, R. et al (1968), studying transformations of aniline and eleven different chloro-anilines in soil, found that no azo compound was formed from aniline, but all monochloro- and some dichloro-anilines were transformed to their corresponding dichloro- and tetrachloro-azobenzene. Sprott and Corke (1971) reported formation of 3,3',4,4'-tetrachloro-azobenzene from 3,4-dichloro-aniline in soil. Acetylation of p-bromoaniline by a soil microorganism as a detoxification mechanism was reported by Tweedy, B. G. et al. (1970). They suggested that acetylation competed with azobenzene formation in utilizing the aniline, formed by metabolism of substituted urea herbicides. Utilization of aniline as sole carbon source for growth has been studied by Walker and Harris (1969). Washed cells oxidized aniline by an induced enzyme system and it was 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 metabolic degradation of acetanilide by microorganisms. The conversion of acetanilide to 4'-hydroxyacetanilide in the mammalian system has been reported by Bernheim and Bernheim (1937). Lester and Greenberg (1947) as well as Brodie and Axelrod (1948) have shown that in man, 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 deacylates to form aniline. Brodie et al. (1953) were successful in hydroxylating acetanilide in the 2'- and 4'-positions with their model system, consisting of ascorbic acid, ferrous iron, ethylenediaminetetra-acetate and oxygen.

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

Theriault and Longfield (1967) reported hydroxylation of acetanilide by several microorganisms. The major conversion product formed was identified as 2'-hydroxyacetanilide; a small amount of 4'-hydroxyacetanilide was also found.

This thesis presents an attempt to elucidate the metabolic pathway for the degradation of acetanilide by a microorganism which utilizes it as sole carbon source for growth.

The study was based on the following objects:

(a) Isolation of an organism which would utilize acetanilide as sole carbon source for growth.

(b) An attempt to elucidate preliminary steps in the metabolism of acetanilide, such as, hydroxylation of aromatic nucleus and preparation for ring fission, assuming that the pathway of ring fission is common to those which have already been reported for other aromatic compounds.

Chapter 2

EXPERIMENTAL PROCEDURES

2.1 MATERIALS.

(a) Reagents.

All inorganic chemicals used were Analar grade. Organic chemicals were redistilled or recrystallized before use, and were shown to be chromatographically pure. Acetanilide, aniline and m-aminophenol were supplied by British Drug House§Ltd., England. Catechol, o-aminophenol and p-aminophenol were obtained from Matheson, Coleman and Bell, Norwood, Ohio; 2'-hydroxyacetanilide and 4'-hydroxyacetanilide were purchased from J. T. Baker Chemical Co., Phillipburg, New Jersey. 3'-Hydroxyacetanilide was supplied by Eastman Organic Chemicals, Rochester 3, New York; and resorcinol by Merck and Co., Inc., Rahway, New Jersey. 2-Amino-resorcinol was purchased from K and K Labs., New York. 4-Amino-catechol was prepared by catalytic hydrogenation of 4'-nitro-catechol obtained from K and K Labs., N. Y., using 5% palladium on charcoal, supplied by Matheson, Coleman and Bell, Norwood, Ohio. Solvents used for extraction or other purposes were generally redistilled.

(b) Organism -

Isolation and cultivation.

An organism, which could utilize acetanilide as sole carbon source for growth, was isolated by screening several aniline utilizing organisms obtained from garden soil. The medium used for isolation and cultivation of the organisms contained: NH_4Cl, 0.3g; K_2HPO_4, 0.1g; $CaCl_2$, 0.2g; $MgSO_4.7H_2O$, 0.2g; $FeSO_4.7H_2O$, 0.005g, and distilled water to one litre. Aniline was added at the final concentration of 0.2 percent.

The medium was autoclaved at 15 lbs pressure for 20 minutes in 200 ml flasks. Each of these flasks, containing 100 ml of medium, was inoculated with 5g of soil sample and incubated at 30° for several weeks. Turbidity was observed in one flask and so a few mls of turbid solution were transferred to fresh medium containing 0.2 percent aniline, and incubated for a week; several transfers were made to obtain pure cultures. Finally, the cultures were inoculated on solid medium (salt medium containing 0.2 percent aniline and 1.5 percent agar). Several different colonies appeared on soil agar medium and thus pure cultures representing each colony were obtained by several transfers on solid medium.

Screening of all these cultures for growth utilizing acetanilide was done by re-inoculating autoclaved medium, containing salt and 0.2 percent recrystallized acetanilide in several 200 ml flasks with several isolated pure cultures grown on aniline. The flasks were incubated for a week at 30°. After the incubation period only one flask showed turbidity, thus a few mls of the turbid solution were transferred to a fresh medium containing acetanilide. Pure single colonies were obtained by further plating on solid medium, containing 0.2 percent acetanilide. The culture was maintained on 0.2 percent acetanilide at 30° in conical flasks filled with liquid medium, and subcultured every week. Stock cultures were preserved on solid medium at 5°, and the inocula grown on acetanilide were used to seed bulk cultures. Cells were grown aerobically at 30° in one litre portions of medium, contained in 3 litre Erlenmeyer flasks.

2.2 EXPERIMENTAL METHODS.

(a) Identification of the organism.

The organism was identified according to the key formulated by Shewan and Hendrie (1966) for the identification of Pseudomonas species using the following methods:

(1) Microscopic examination of cultures grown on liquid and solid media to determine motility, morphology and Gram reaction.

(2) Electron microscopic examination of the arrangement of flagella.

(3) Colonial morphology and pigmentation on nutrient agar.

(4) Examination of nutrient agar and broth cultures by ultra-violet (uv) light for fluorescence.

(5) Oxidase test of Kovacs (1956).

(6) Attack on glucose tested by the method of Hugh and Leifson (1953).

(7) Ability to grow at 42° by the method of Haynes and Rhodes (1962).

(8) Ability to fix atmospheric nitrogen by growth on the nitrogen free medium of Norris (1959).

(9) Ability to break down oxalate by clearing the medium of Bhat and Barker (1948).

Some additional tests such as Indole nitrite, Methyl-Red-Voges-Proskauer, and Koser's citrate tests were done. Growth on urea broth, TSI slants, gelatine agar, starch agar and lactose was also studied to confirm some of the findings.

(b) Growth studies.

The course of growth on acetanilide was followed turbidimetrically in Reflectance spectrophotometer, Unigalvo type 20, Evans electro-

selanium Ltd., Essex; using appropriate reference standards. The cells were grown on one litre liquid medium containing 0.2 percent acetanilide. 5 ml aliquots were taken at regular time intervals, and each aliquot was measured for turbidity in Reflectance spectrophotometer.

(c) Extraction of metabolites.

It was noticed in the earlier experiments that small amounts of metabolites (intermediates) accumulated in the medium during growth, but the amounts were not sufficient for identification and isolation. In order to obtain sufficient amounts of the metabolic intermediates, cells were grown on six litres of liquid medium, containing 0.2 percent acetanilide at 30°, in several Erlenmeyer flasks. Cells were harvested at the end of the logarithmic phase of growth (11 days) by centrifugation at 5000 r.p.m. (G.4080) for 30 minutes, at 5°. Finally resuspended in 200 mls of liquid medium, containing 0.5g of acetanilide. This medium was then incubated at 30° for 10 days. The bacterial cells were harvested at the end of incubation time by centrifugation, and the supernatant was cleared of any soluble proteins by adding few drops of 20 percent trichloroacetic acid and centrifuging to remove any precipitation.

The clear supernatant obtained was then adjusted to pH8 with N NaOH and extracted in a separatory funnel with three portions of 300 mls of ether. The ethereal extract, thus obtained, was dried over sodium sulfate, and evaporated to a small volume with the help of a flash evaporator at 40°. The supernatant, remaining after the first extraction, was then adjusted to pH3 with N HCl and reextracted with three portions of 300 mls of ether in a separatory funnel. Ethereal extract was dried

over anhydrous sodium sulfate and evaporated to a small volume in a similar manner as before. Both ethereal extracts were then subjected to analytical procedures for identification of metabolites.

A control flask, containing 200 mls of liquid medium with 0.5g of acetanilide, was incubated for 10 days, at 30° and extracted with ether by a similar procedure used for extracting the metabolites. Ethereal extracts of control were also analysed along with the metabolite extracts.

(d) Thin layer chromatography.

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

(1) Ethyl-acetate - Benzene (9:11).

(2) Chloroform - Methanol (aqueous 95%) - Amm. hydroxide, (85:15:1); Theriault and Longfield (1967).

(3) Ethyl-acetate - Dibutyl ether - Acetic acid (50:50:1).

The plates were air dried after the chromatograms had developed, and scanned with ultra-violet light. Finally, the plates were sprayed with the following reagents:

(a) Ferric chloride, 1g; Potassium ferricyanide, 0.1g; 10 ml of water. This reagent mixture revealed phenolic products as intense blue spots on a pale yellow background, changing to pale blue with age. The reagent mixture was freshly prepared before use. Although the reagent is not completely specific under the conditions used, it is sensitive and positive for many phenolic compounds. (Hathway, D. E., 1960).

(b) Diazotisation and coupling with N-1-Naphthylethylenediamine - Dihydrochloride. The reagent is specific for compounds having free - NH_2 group; it consists of two reagents which are freshly prepared before use. (Stahl, 1969).

Spray reagent I; 1% sodium nitrite solution in N HCl.

Spray reagent II; 0.2% N-1-Naphthylethylenediamine - Dihydrochloride in methanol.

The chromatographic plate was sprayed with reagent I, left for one minute and sprayed with reagent II. Dried at 60° to intensify the color.

(c) Vanillin sulfuric acid.

This reagent is specific for dihydroxy - compounds. It is prepared by dissolving one gram of vanillin in 100 mls of concentrated sulfuric acid. After spraying with this reagent, the chromatographic plate is heated at 100° until the spots attain maximum color intensity (Kirchner, 1967).

(e) Preparative-plate chromatography.

(Separation of metabolites for gas-chromatographic assays).

The ethereal extract of metabolites obtained at pH8, which showed only one spot on thin layer chromatographic analysis, was subjected to gas-chromatographic assays without any interference due to presence of acetanilide. However, in the case of metabolites extracted at pH3, interference was observed when acetanilide, ortho-aminophenol and 4-aminocatechol showed similar retention times in one of the column systems used. Therefore, to avoid such interference, the ethereal extract obtained at pH3 was subjected to preparative plate chromatography with the object of separating the metabolites.

The ethereal extract was applied on glass plates (20 x 20 cms) coated with 1 mm thickness silica gel layer, through an applicator, and the chromatograms were developed with chloroform-methanol (aqueous 95%)-amm. hydroxide, (85:15:1). The plates were dried in air and examined under UV light, which showed two bands corresponding in the Rf values to ortho-aminophenol and 4-aminocatechol, and were termed band A and band B, respectively. The areas on silica gel where the bands appeared were marked and the silica gel, in areas marking each band, was removed separately. Bands were eluted with absolute ethanol, filtered, and the filtrate was evaporated to dryness. The dry residues thus obtained were then redissolved in ether and used for gas-chromatographic assays.

(f) Gas-chromatographic assays.

Gas-chromatographic analysis of ethereal extracts of metabolites and control was done with Beckman GC-5 gas-chromatograph, using the following column systems; ethereal solutions of pure reference standards were used in this study.

System A.

Column type: Amine 220, 0.5% on 100-120 mesh C' sorb G', HpDmcs, 6' teflon column.

Injection type: Glass-lined.

Detector type: Hydrogen flame.

Flash inlet temperature: 180°

Column temperature: 153°

Detector line temperature: 205°

Detector temperature: 260°

Carrier gas	Flow rate mls/min	Pressure lbs/sq. in
Helium	100	50
Hydrogen	48	50
Air	250	40

System B.

Column type: OV1 3% on 80-100 mesh C' sorb G' Aw/Dmsc, 6' metal column.

Injection type: Metal lines.

Detector type: Hydrogen flame.

Flash inlet temperature: 170°

Column temperature: 120°

Detector line temperature: 220°

Detector temperature: 300°

Carrier gas	Flow rate mls/min	Pressure lbs/sq. in
Helium	30	50
Hydrogen	30	50
Air	300	40

(g) Aniline in bacterial culture.

(i) Analysis.

Aniline production in bacterial culture during growth on acetanilide was followed by analysing aliquots, collected at regular time intervals. For this study, cells were grown on a one litre fluid medium containing 0.2 percent acetanilide, in a three litre Erlenmeyer flask, incubated at 30°. 10 ml aliquots were collected at regular time intervals, and each aliquot was analysed for ani-

line content by Daniel's quantitative method, with reference to a standard calibration curve. Colorimetric measurements for this analysis were done with Spectronic 20 colorimeter, at 545 mμ.

(ii) Isolation.

Aniline was isolated from bacterial culture growing on acetanilide by the following method.

A suspension of bacterial cells (5g/ml), grown on acetanilide, was added to 200 mls of fluid medium containing 0.5g of acetanilide in a 500 ml conical flask, and incubated at 30° for 7 days. The culture was harvested by centrifugation at 5000 r.p.m. for 30 minutes, at 5°; the supernatant was adjusted to pH7 with N NaOH and extracted with three portions of 300 mls of ether. The ethereal extract was dried over anhydrous sodium sulfate and evaporated to a 10 ml volume. To this 10 mls of extract were added 50 mls of petroleum ether (38° - 48°), which precipitated all the acetanilide in the ethereal extract. Acetanilide was removed by filtration and filtrate was evaporated to dryness. Aniline was obtained, which appeared as oily drops; it was dissolved in chloroform and purified by shaking with N HCl. Aniline, thus obtained, was identified by thin layer chromatography, using silica gel plate, developed with chloroform-methanol (aqueous 95%) - amm. hydroxide (85:15:1), and by the preparation of an arylsulfonyl derivative. A control flask contining 200 mls of fluid medium with 0.5g acetanilide, incubated at 30° for 7 days was extracted with ether by a similar procedure.

(iii) Preparation of the derivative.

Aniline isolated from the bacterial culture was used to prepare an arylsulfonyl derivative by Hinsberg's method (Adams and

Johnson, 1960). The procedure used is as follows:

To one ml of isolated aniline in a test tube, were added 10 mls of dilute NaOH (10%). The tube was shaken thoroughly and 2 mls of benzene sulfonyl chloride were added; warming and shaking was continued until the odor of benzene sulfonyl chloride could no longer be detected. The solution was cooled to room temperature, shaken well and acidified with concentrated hydrochloric acid, again cooled and shaken well. A white solid precipitated out, which was filtered by suction and washed with water. The derivative thus obtained was crystallized from 70% ethanol, and the melting point was determined; this was compared with the derivative prepared from pure aniline by a similar procedure, as a reference standard.

(iv) Infra-red Absorption spectra.

Infra-red absorption spectra for arysulfonyl derivatives, prepared from isolated and authentic aniline, were plotted in Beckman IR8, infra-red spectrophotometer, using identical cells of 0.5 mm path-length. 2.5 percent chloroform solutions of derivatives were used and chloroform was also used in the reference cell.

(h) Manometric studies.

(i) Preparation of bacterial cell suspension.

The oxygen uptake experiments were carried out using two types of bacterial cell suspension.

(a) Cells grown on nutrient agar.

The cells were grown on sterilized nutrient agar at 30°, using standard inoculum and harvested after 5 days of growth, by shaking with 0.02M phosphate buffer pH7. The cells were scraped from the agar surface with the help of a glass rod,

and the lumps of nutrient agar were removed by filtering through muslin cloth. Cells were centrifuged at 5000 r.p.m. for 30 minutes, at 5°, resuspended in phosphate buffer and aerated for 12 hours, 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 acetanilide.

The cells were grown on liquid medium containing 0.2 percent acetanilide at 30° in 3 litre Erlenmeyer flask and harvested at the end of the logarithmic phase (12 days), by centrifugation at 5000 r.p.m. for 30 minutes. The cells were then resuspended in phosphate buffer and aerated by passing filtered air for 12 hours. The cells, after aeration, were washed twice with phosphate buffer and adjusted to required dry cell weight.

(ii) Oxygen uptake experiments.

Oxygen uptake by the bacterial cells in presence of various substrates was measured in a Warburg constant volume respirometer, (Umbreit, Burris and Stauffer, 1964), using a 13 ml single side arm flask at 30° $\pm$ 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 is based on the following reaction, which occurs under alkaline conditions:

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

The potassium ferricyanide was used as 0.1M solution, which was stable if kept for several days in the dark. Each ml of 0.1M potassium ferricyanide liberated $\frac{2240}{4}$ = 560 μl. N_2, thus normally 0.4 ml (= 224 μl.) of 0.1M solution was added. The other reagents required were 4N NaOH and hydrazine; the latter was prepared by dissolving 5 gm. hydrazine sulfate ($N_2H_4.H_2SO_4$) 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. 0.1M 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. (The reaction was complete in about 30 min.).

$$K = \frac{x}{h} = \frac{224}{h} \quad (\text{this is } K_{N_2} = K_{O_2})$$

The constant K was calculated for each flask.

For oxygen uptake experiments, the main compartment of each flask contained 0.5 ml of 0.02M phosphate buffer, and 1 ml of bacterial suspension. The side arm contained 0.5 ml of aqueous substrate solution, neutralized to pH7 with N NaOH where necessary. The centre well contained 0.2 ml of 20 percent W/V KOH and filter paper to absorb carbon

dioxide. The flasks were incubated in the water bath at 30° for 15 minutes to equilibrate the temperature of their contents and surroundings. The reaction was started by tipping the contents of the side arm into the main compartment.

Endogenous oxygen uptake was measured in the flasks containing distilled water in place of substrate solution. A flask filled with only distilled water was used as a thermobarometer, measuring any change of temperature or pressure of the room in which the experiment was conducted. Control flasks containing 0.5 ml of aqueous substrate and 1 ml distilled water were used to show any oxygen uptake due to non-enzymatic oxidation (atmospheric oxidation).

Chapter 3

RESULTS

3.1 Identification of the organism.

The organism which utilized acetanilide as sole carbon source was Gram negative, rod-shaped, measuring 3 x 1μ, and usually with flagella, Plate I. Colonies on nutrient agar were smooth and translucent with spreading margins. No pigmentation was observed during growth; fluorescence was not observed under uv 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 did not grow on urea broth; it showed positive Methyl-Red-Voges Proskauer test. The organism also grew on Koser's citrate slant, TSI slant, starch agar and gelatine without liquification; it showed growth on lactose without production of gas. It fixed atmospheric nitrogen and degraded oxalate. The optimum growth temperature ranged between 28 - 32°. No growth at 42° was observed, and pH range of 6.5 - 7.5 was found suitable for optimum growth.

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

3.2 Thin layer chromatography.

The chromatographic analysis of ethereal extracts of metabolites on thin layer silica gel plates revealed two distinct spots with ethereal extract obtained at pH3, and a single spot with ethereal extract prepared at pH8, Fig. 7. All these spots appeared blue when the plates were sprayed with ferric chloride - potassium ferricyanide reagent. Ethereal extracts obtained from control experiments did not show any spot when chromatographed under identical conditions. The Rf values for these spots

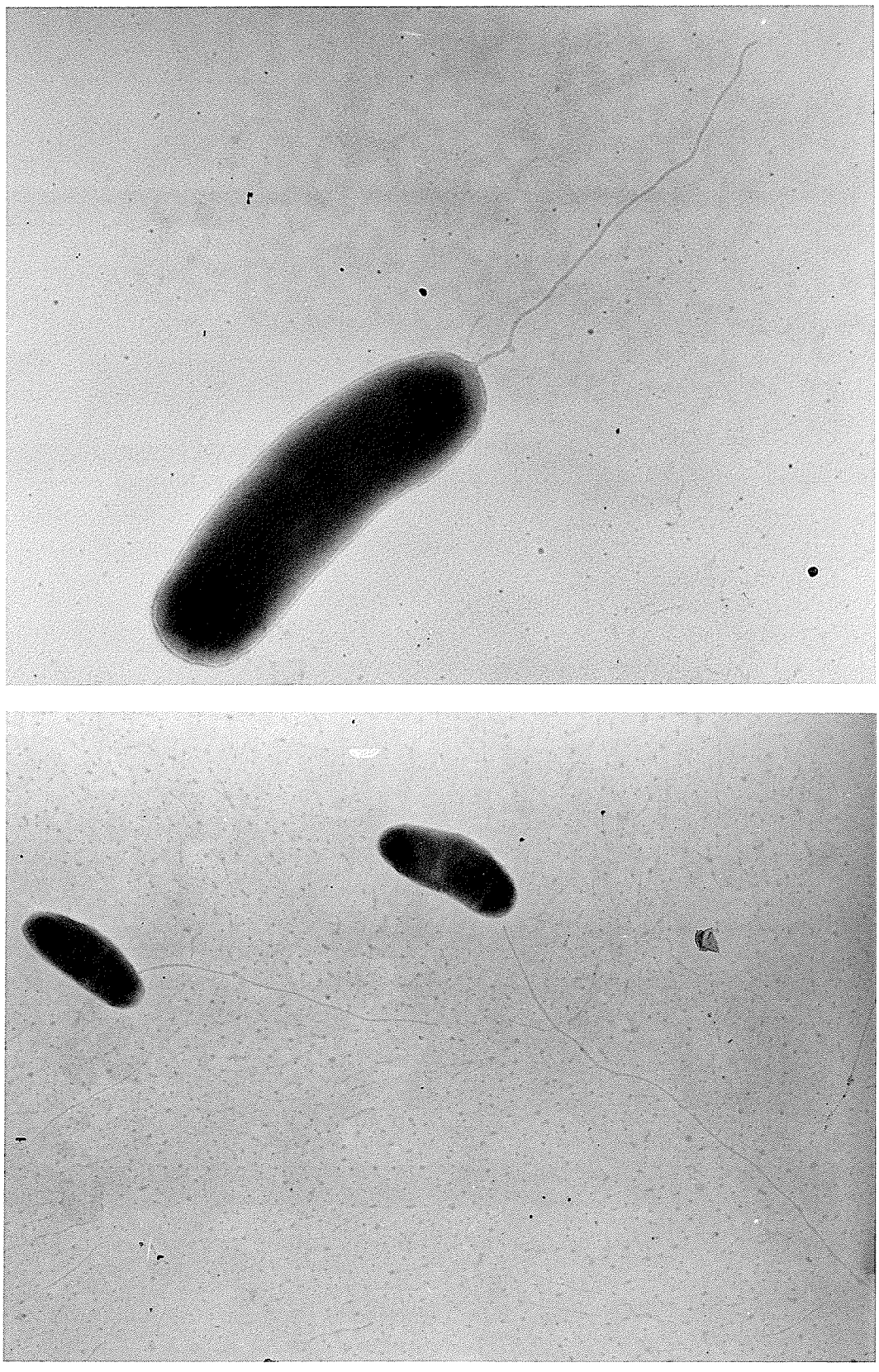


Plate 1. Photomicrograph of acetanilide-decomposing *Pseudomonas sp.*, Top: ×10,000 magnification; Bottom: ×5,000 magnification.

in solvent system (1); benzene - ethylacetate (11:9) are given in Table IV. Spot A had Rf value of 0.56; 0.55 corresponding to standard aniline spot which had Rf value of 0.57; 0.55 under the same conditions, and both appeared pink after the plate was sprayed with the diazotising reagent.

Spot B, which appeared when ethereal extract of metabolites obtained at pH3 was chromatographed, had Rf values of 0.49; 0.50. It was seen as a yellow spot when the diazotising reagent was sprayed and thus resembles ortho-aminophenol in these respects. The second spot C appeared blue when sprayed with ferric chloride reagent and pink with diazotising reagent; it had Rf value of 0.14; 0.13, and corresponded to 4-amino-catechol as far as the Rf value and color reaction were concerned.

Table V shows Rf values for metabolites in solvent system (2); chloroform - methanol - amm. hydroxide, and system (3); ethylacetate - dibutyl ether - acetic acid. The three spots, which appeared on developing chromatograms in these systems corresponded in their color reaction and Rf values to aniline, ortho-aminophenol, 4-aminocatechol, Figs. 8 and 9.

The results of thin layer chromatographic analysis indicated the presence of three metabolic intermediates in bacterial cultures, namely, aniline, ortho-aminophenol and 4-aminocatechol.

3.3 Gas-chromatographic assays.

Gas-chromatographic analysis of ethereal extracts of metabolites and control gave results which are in agreement with those obtained by thin layer chromatographic assays. The results which are given in Table VI, suggest the presence of three major metabolites of acetanilide in bacterial cultures, namely, aniline, ortho-aminophenol and 4-

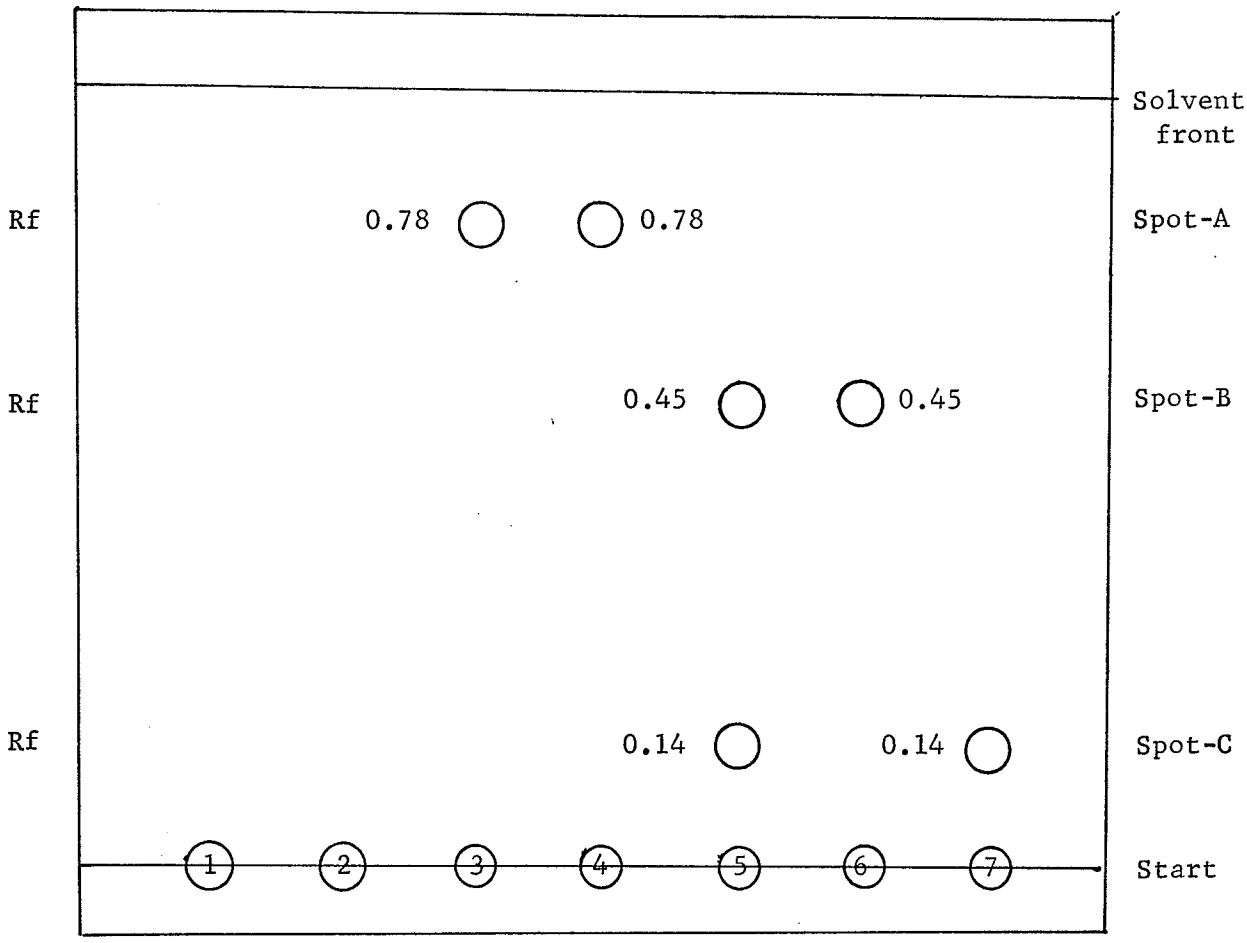


Fig. 7. Thin layer chromatographic analysis of ethereal extracts. (1) Ethereal extract of control at pH8; (2) Ethereal extract of control at pH3; (3) Aniline; (4) Ethereal extract of metabolites at pH8; (5) Ethereal extract of metabolites at pH3; (6) Ortho-aminophenol; (7) 4-amino-catechol. Chromatographed with chloroform-methanol (aqueous 95%)-amm. hydroxide (85:15:1).

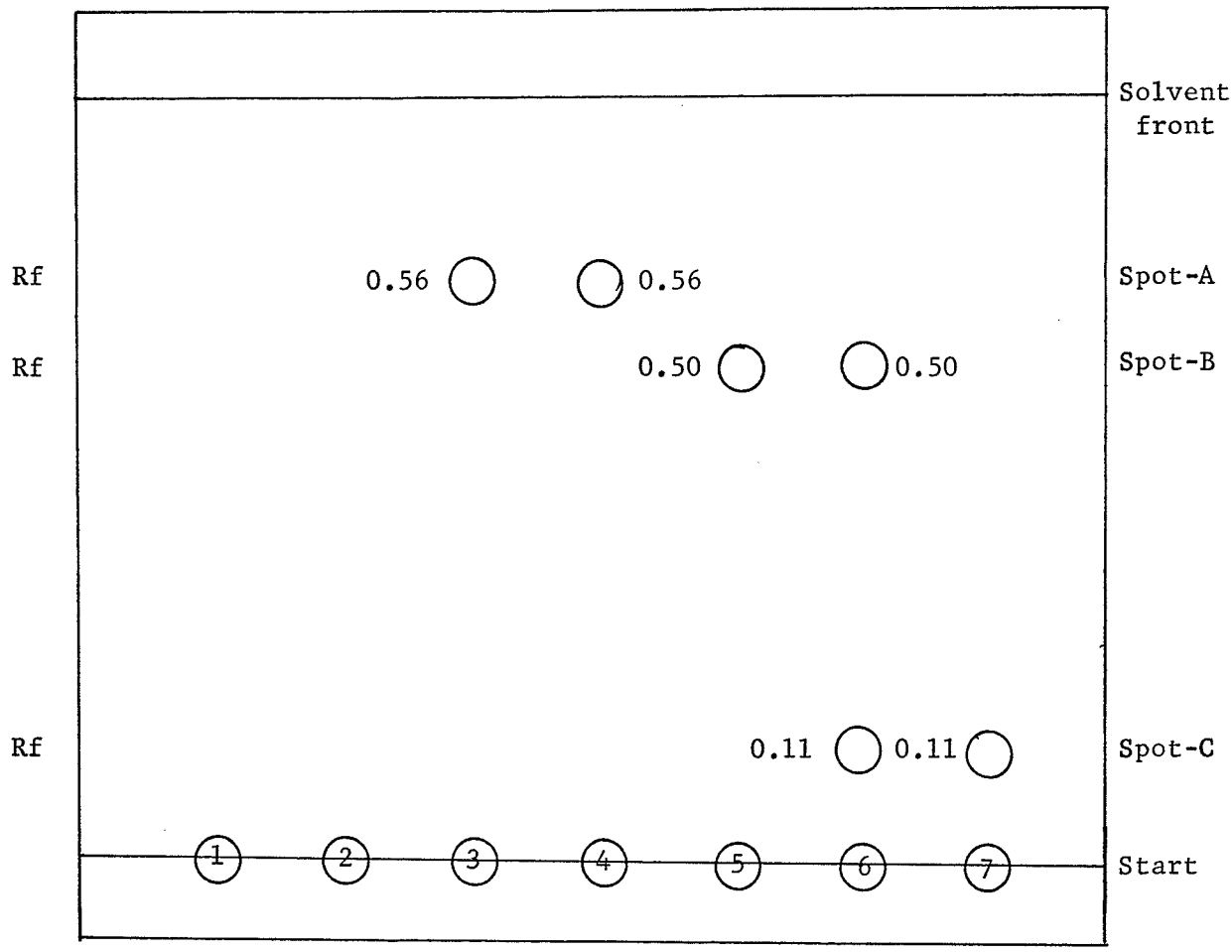


Fig. 8. Thin layer chromatographic analysis of the etheral extracts. (1) Ethereal extract of control at pH8; (2) Ethereal extract of control at pH3; (3) Aniline; (4) Ethereal extract of metabolites at pH8; (5) Ortho-aminophenol; (6) Ethereal extract of metabolites at pH3; (7) 4-amino-catechol. Chromatographed with ethyl acetate-benzene (9:11).

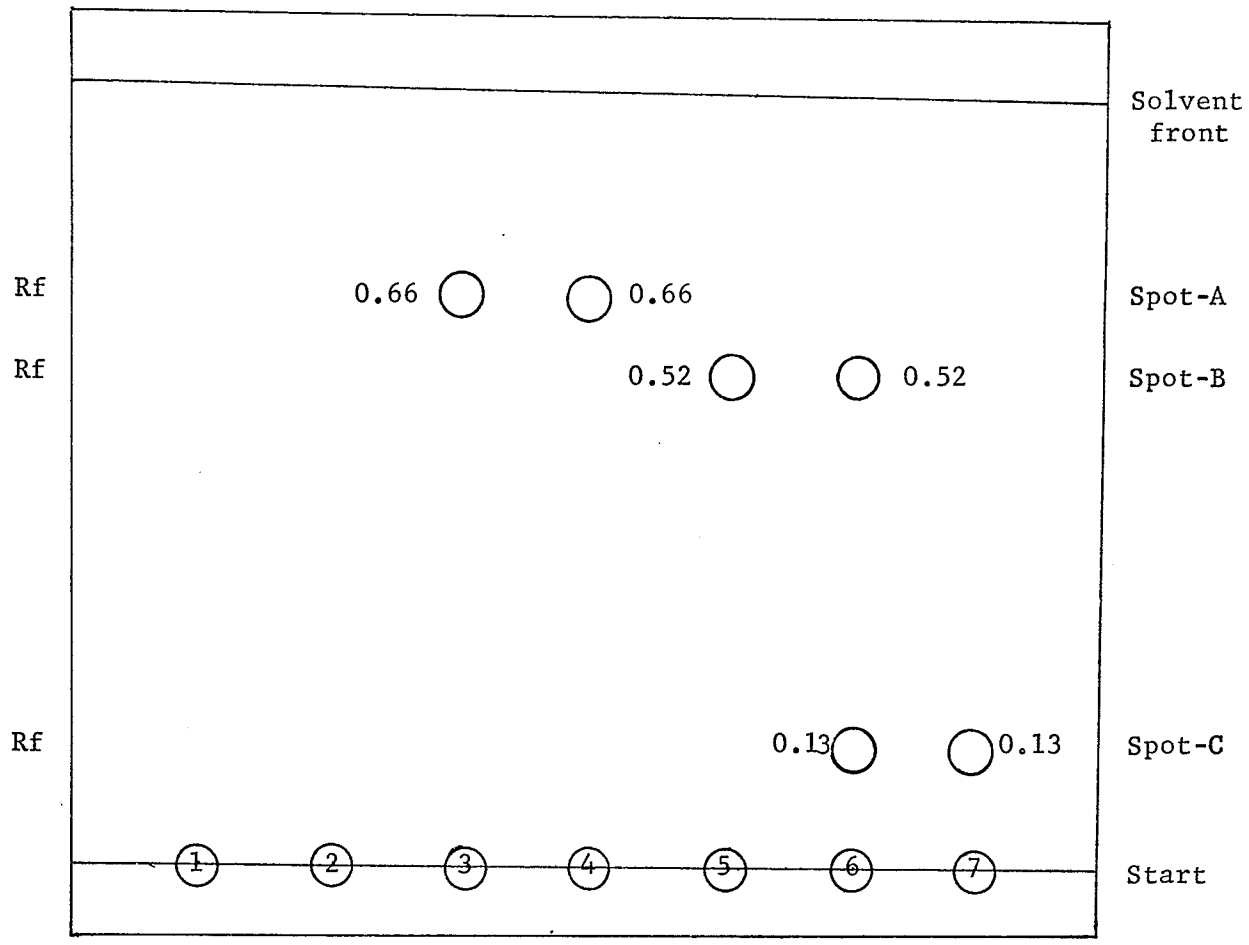


Fig. 9. Thin layer chromatographic analysis of ethereal extracts. (1) Ethereal extract of control at pH8; (2) Ethereal extract of control at pH3; (3) Aniline; (4) Ethereal extract of metabolites at pH8; (5) Ortho-aminophenol; (6) Ethereal extract of metabolites at pH3; (7) 4-amino catechol. Chromatographed with ethyl acetate-dibutyl ether-acetic acid (50:50:1).

aminocatechol.

3.4 Oxygen uptake experiments.

(a) With nutrient-agar grown organisms.

These organisms were used to test whether acetanilide oxidation depended on an induced enzyme system. The results (Figs. 10, 11, and 12) indicate that the organisms failed to show rapid uptake on the substrates tested; however, adaptation, followed by active oxidation, could be observed. The substrates were oxidized after a lag period and the total amount of oxygen consumed was low. Significant oxygen uptake was observed in the case of acetanilide after 15 minutes lag time, whereas aniline and ortho-aminophenol showed a lag period of 10 minutes and 5 minutes, respectively. Catechol consumed very little oxygen after a 10 minute induction period, but 4-aminocatechol and acetate showed a rapid uptake of oxygen, although the total amount consumed was small, Table VII. These results, thus, indicate that the nutrient agar grown organisms do not possess acetanilide oxidizing enzymes.

(b) With acetanilide grown organisms.

The oxygen consumption by Pseudomonas sp., grown aerobically on acetanilide, with various substrates is given in Figs. 13 and 14. Acetanilide, aniline, ortho-aminophenol, 4-aminocatechol, catechol, and acetate were immediately oxidized without any induction period, indicating that the growth on acetanilide induced the formation of an enzyme system specifically capable of oxidizing acetanilide. With m-aminophenol the organism showed a fair amount of oxygen uptake, but with p-aminophenol and resorcinol no oxygen consumption was observed.

TABLE IV

Rf values for aminophenols and metabolites.

Metabolites	Rf		Color reaction	
	A	B	$FeCl_3$-$K_4Fe(CN)_6$	Diazotising reagent
Spot A	0.56	0.55	Blue	Pink
Aniline	o.57	0.55	Blue	Pink
Spot B	0.49	0.50	Blue	Orange
ortho-Aminophenol	0.51	0.49	Blue	Orange
Spot C	0.11	0.12	Blue	Pink
4-Aminocatechol	0.11	0.11	Blue	Pink

TABLE V

Rf values for metabolites and corresponding aminophenols.

Metabolites	Rf			
	Chloroform-methanol-NH_4OH		Ethylacetate-dibutylether acetic acid	
	A	B	A	B
Spot A	0.78	0.79	0.67	0.68
Aniline	0.79	0.77	0.69	0.67
Spot B	0.45	0.46	0.51	0.54
ortho-Aminophenol	0.44	0.46	0.52	0.53
Spot C	0.14	0.14	0.13	0.13
4-Aminocatechol	0.13	0.14	0.12	0.14

TABLE VI

Results of gas-chromatographic analysis of Ethereal extracts of metabolites.

Samples	Retention time	
	Column system A	Column system B
1. (a) Ethereal extract of metabolites at pH8	23", 7'.30"	19", 10'.40"
(b) Ethereal extract of control at pH8	8'.20"	10'.30"
(c) Aniline	23"	19"
(d) Acetanilide	8'.0"	10'.40"
CO-CHROMATOGRAPHY		
(e) Ethereal extract of metabolites at pH 8 and aniline	23" single peak	19" single peak
2. (a) Ethereal extract of control at pH3	7'.30"	10'.40"
(b) Band A	7'.50"	4'.5"
(c) Band B	8'.30"	9'.30"
(d) ortho-Aminophenol	7'.20"	9'.25"
(e) 4-Aminocatechol	7'.55"	4'.0"
CO-CHROMATOGRAPHY		
(f) Band A and ortho-aminophenol	7'.30" single peak	4'.25" single peak
(g) Band B and 4-aminocatechol	7'.50" single peak	9'.55" single peak

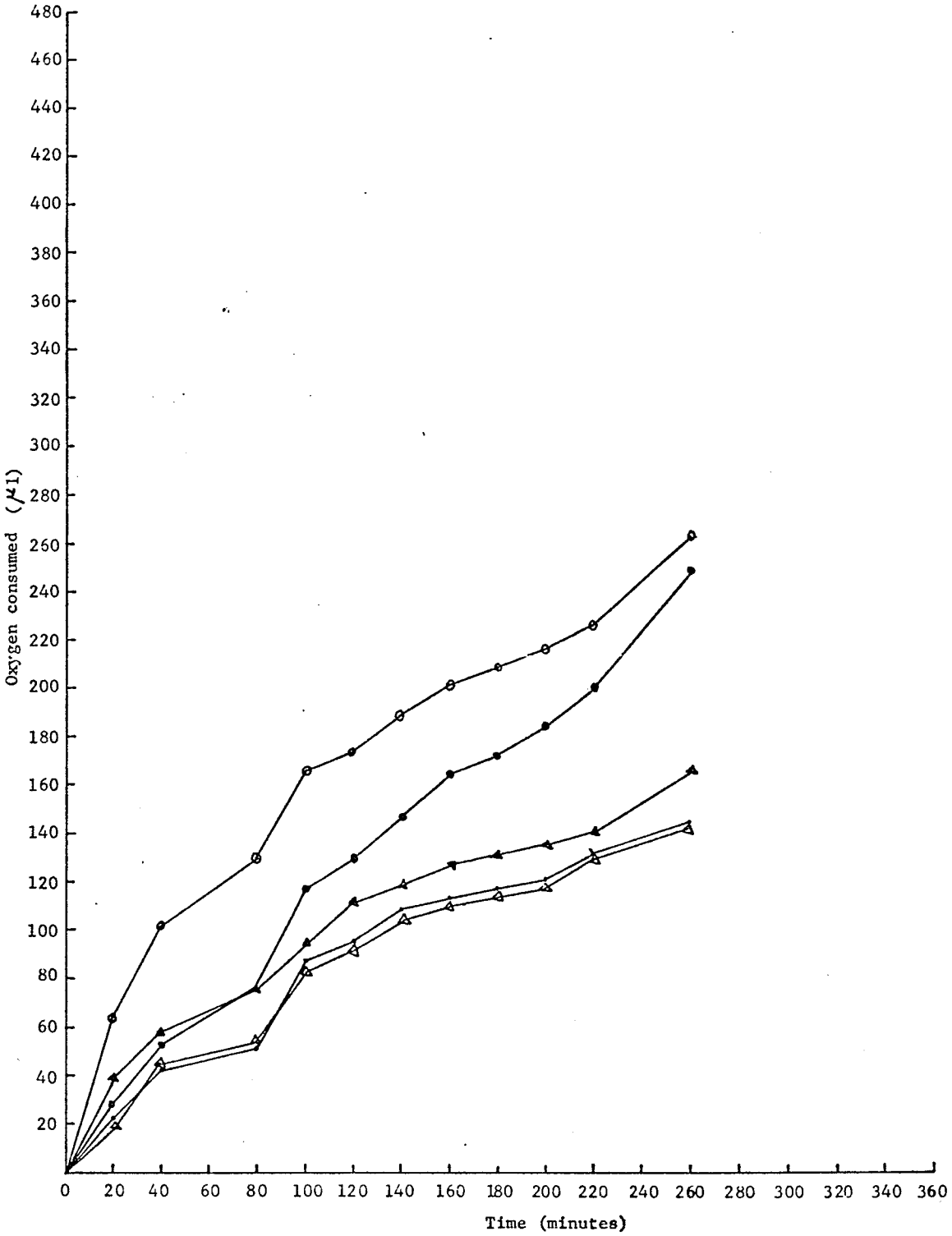


Fig. 10. The rate of oxygen uptake by washed nutrient agar-grown Pseudomonas sp. 16 mg dry weight of cells per ml. • Endogenous: • Acetanilide: ▲ Catechol: o Ortho-aminophenol: △ m-aminophenol.

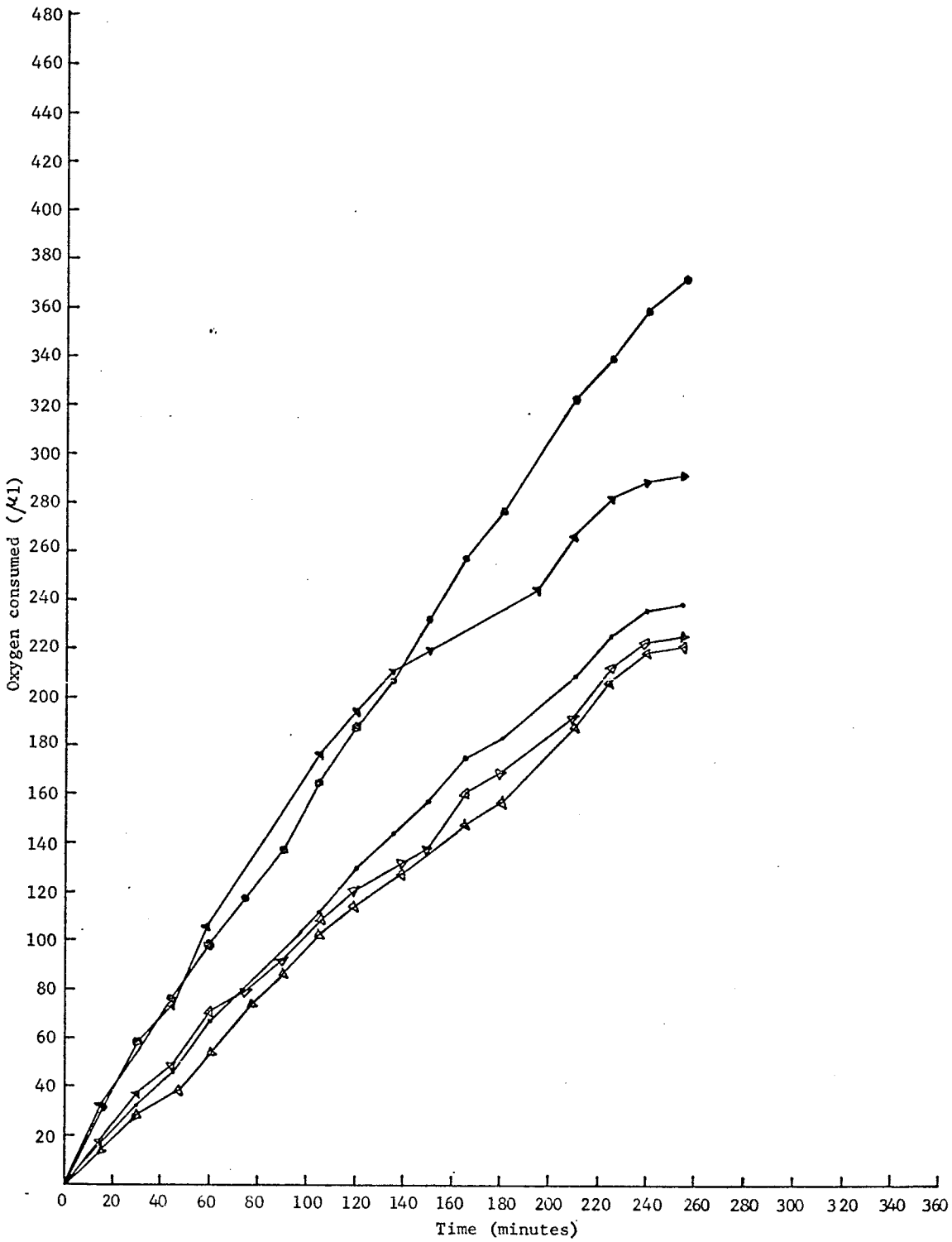


Fig. 11. The rate of oxygen uptake by washed nutrient agar grown Pseudomonas sp. 18 mg dry weight of cells per ml. • Endogenous: ● Aniline: ▲ 2-Amino-resorcinol: ▽ Resorcinol: △ p-aminophenol.

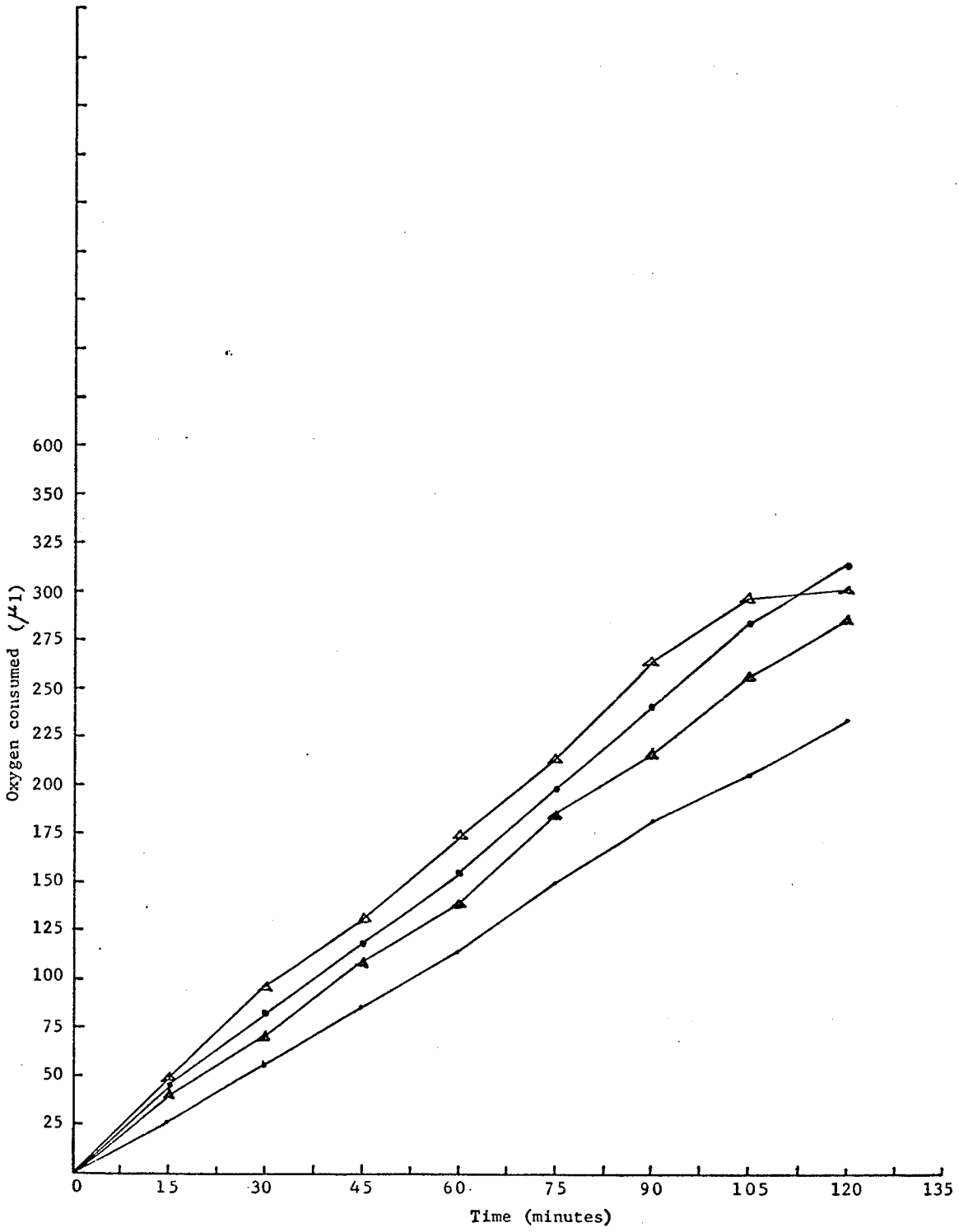


Fig. 12. Oxidation of aromatic compounds by washed cell suspension of Pseudomonas sp. grown on nutrient-agar. 20 mgs dry weight of cells per ml. • Endogenous: • Acetate 2.5 μ mole/ml: ▲ Acetate 5.0 μ mole/ml: △ 4-amino-catechol 5.0 μ mole/ml.

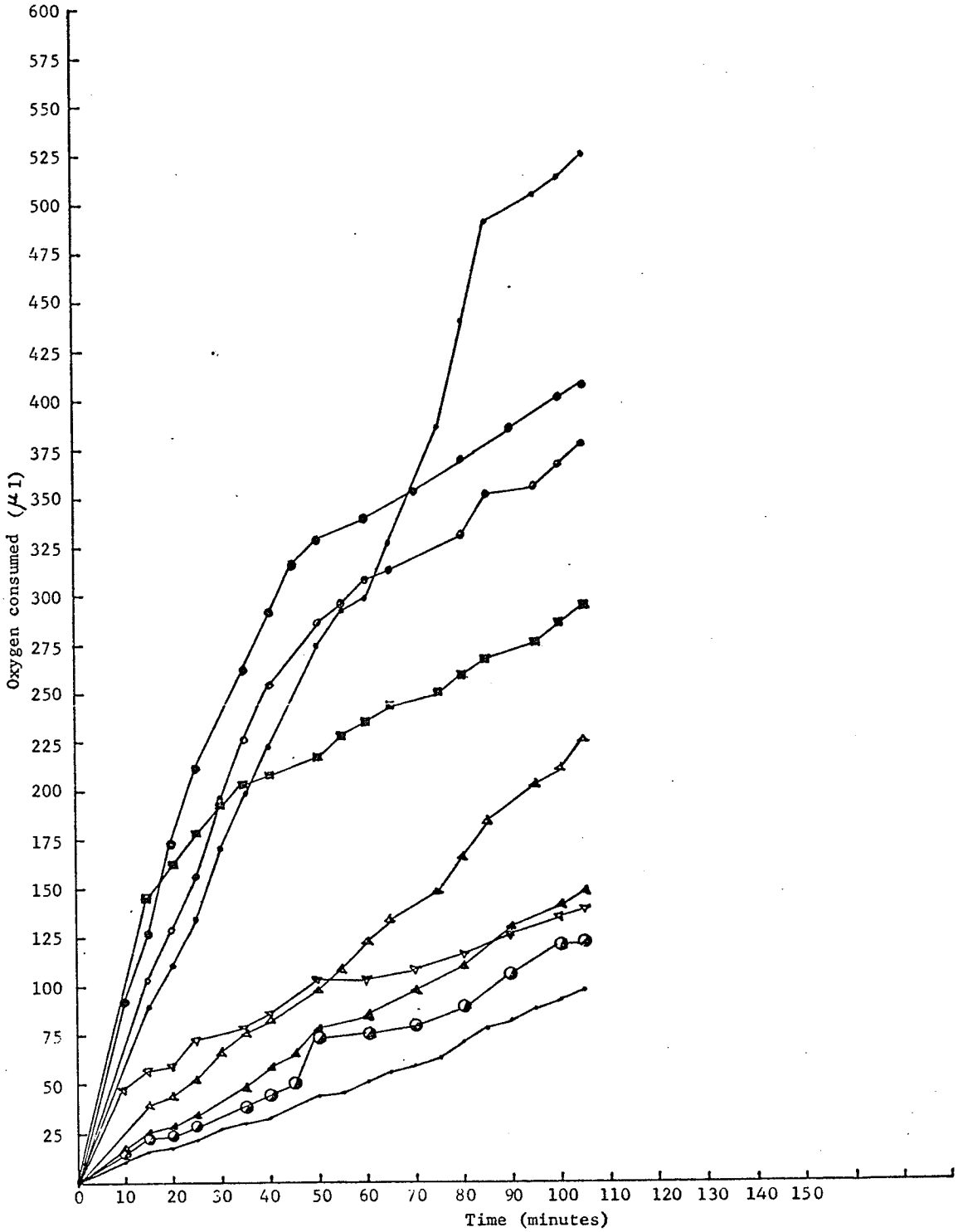


Fig. 13. Oxidation of aromatic compounds by washed acetanilide grown cell suspension of Pseudomonas sp. 18 mgs dry weight of cells per ml. • Endogenous: △ m-aminophenol: • Aniline: o Acetanilide: ■ Catechol: ▲ p-aminophenol: ◐ Resorcinol: ▽ 2-amino-resorcinol: ● Ortho-aminophenol.

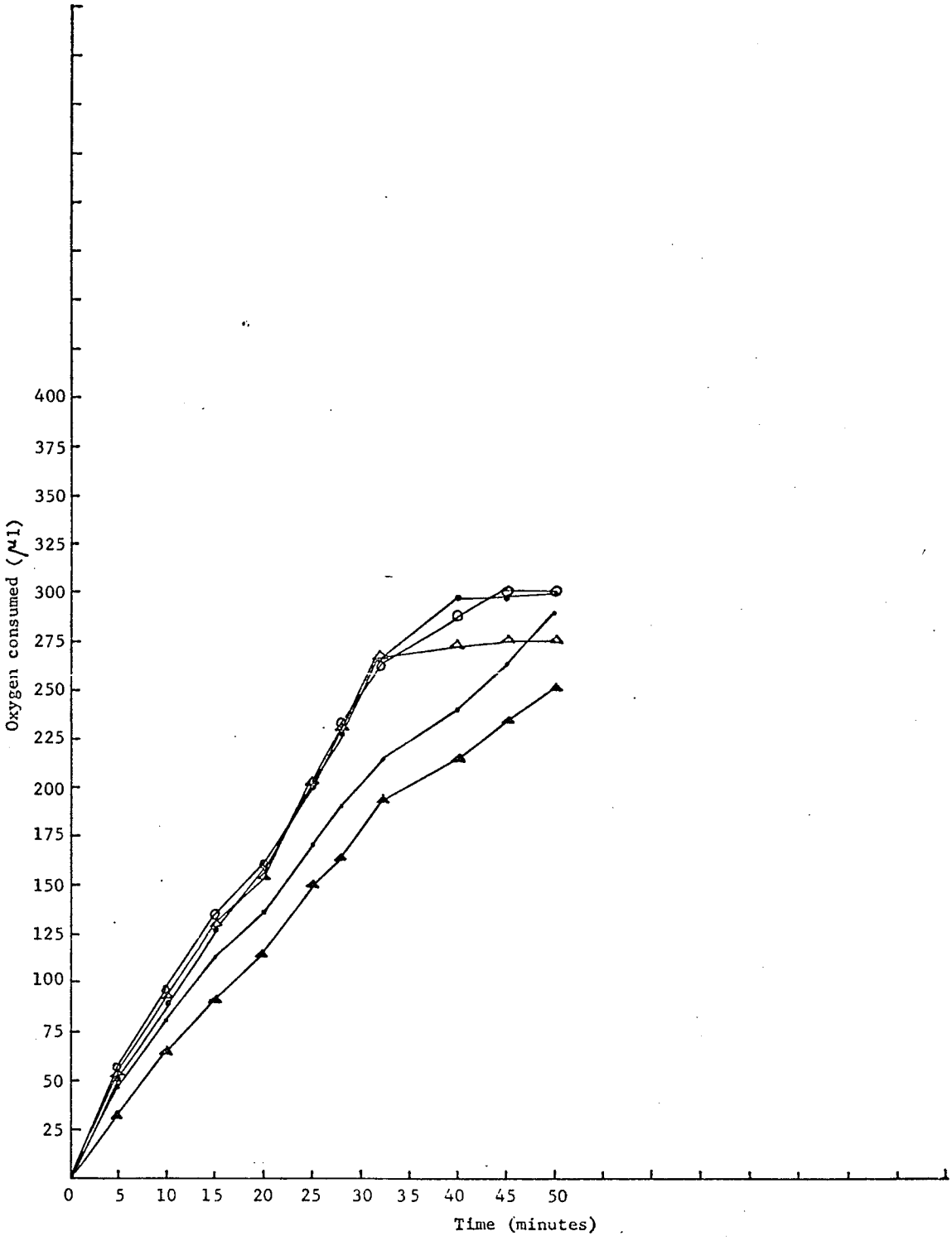


Fig. 14. Oxidation of aromatic compounds by washed acetanilide grown cell suspension of Pseudomonas sp. 26 mgs dry weight of cells per ml. • Endogenous: ○ 4-amino-catechol 5 µmole/ml: △ Acetate 2.5 µmole/ml: • Acetate 5.0 µmole/ml: ▲ Acetate 10 µmole/ml.

These results suggest that the substrates, which show no oxygen uptake, are certainly not intermediates of the acetanilide degradation pathway, whereas the substrates which consume a large amount of oxygen may be the intermediates of acetanilide metabolism. Figures for the oxidation of various substrates are given in Table VII.

The following total quantities of oxygen were consumed by the acetanilide grown organisms (1 mg dry weight) for the oxidation of 1 μmole of the substrates; acetanilide, 3.52, 3.70μl of O_2; aniline, 2.72, 2.80μl of O_2; ortho-aminophenol, 1.98, 2.10μl of O_2; catechol, 2.28, 2.00μl of O_2; 4-aminocatechol, 0.95, 1.00μl of O_2; acetate, 1.5, 1.6μl of O_2. The figures show that aniline consumed less oxygen than acetanilide, which strongly suggests that it is the first metabolic product of the latter. Ortho-aminophenol, 4-aminocatechol and catechol used less oxygen than aniline during oxidation, indicating the possibility of the existance of these compounds as intermediates of acetanilide metabolism.

3.5 Growth studies and aniline production.

Experiments testing the growth of the organisms on different concentrations of acetanilide and aniline in the same basal mineral salt medium, and judging growth turbidimetrically, showed that the organism was capable of growing on both acetanilide and aniline. The organism grew best on fluid medium, containing 0.2 percent acetanilide at 30°. The growth profile showed two distinct logarithmic phases, each followed a lag period during which growth was very slow. The first logarithmic phase of growth started after a 3 day lag period. The organism then grew very well till the 6th day, when growth slowed down, showing a

TABLE VII

The oxidation of phenolic compounds by washed suspensions of Pseudomonas sp.

Substrate	Cells grown in presence of				
	‡ Nutrient agar		* Acetanilide		
	Oxidation of Subs. after lag period (min.)	QO_2E	Oxidation of Subs. after lag period (min.)	QO_2E	O_2 consumed after 15 mins.
1. Acetanilide	15	1.50	0	8.80	89.40
2. Aniline	10	1.90	0	13.60	85.30
3. m-Aminophenol	no uptake	-	0	4.00	25.80
4. o-Aminophenol	5	1.00	0	9.90	110.90
5. Catechol	10	0.10	0	5.70	132.10
6. p-Aminophenol	no uptake	-	15	1.70	10.80
7. Resorcinol	no uptake	-	15	0.88	8.20
8. 2-Amino-resorcinol	10	0.30	0	1.50	43.4
9. 4-Amino-catechol	5	1.70	0	2.60	23.0
10. Acetate	0	1.30	0	3.90	16.0

QO_2E - represents oxygen uptake in the presence of exogenous substrate after substraction of the endogenous uptake. The units being microlitres (μl) of uptake STP/mg dry wt. of cells/hr.

‡ - Substrates used at a concentration of 10 μmoles/ml, except catechol, acetanilide, 4-amino-catechol and acetate which were used at a concentration of 5 μ moles/ml.

* - Substrates used as 10 μmoles/ml, except catechol 1.0 μ mole/ml, ortho-aminophenol, acetanilide, 4-amino-catechol and acetate as 5 μ moles/ml.

second lag period, which lasted for 2 days. After this period, the organism started growing rapidly again and attained maximum growth within 14 days, Fig. 15.

Corresponding aniline production was also logarithmic after a 2 day lag period, and the culture had maximum aniline content after 8 days of growth. Beyond this period, the concentration of aniline in the culture dropped rapidly and within 14 days almost all the aniline disappeared, Table VIII.

3.6 Identification of isolated aniline.

Aniline isolated from culture of Pseudomonas sp., was identified by thin layer chromatographic assay, which showed identical spots with isolated and authentic aniline. These had similar Rf values when chromatographed with chloroform - methanol (95% aqueous) - amm. hydroxide (85:15:1). Both isolated and authentic analine gave similar color reactions with $FeCl_3$-$K_4Fe(CN)_6$ reagent and diazotising reagent. No spot appeared with the ethereal extract of control.

The arylsulfonyl derivative, prepared with isolated and standard aniline, has identical melting points as 105 - 106° and 102 - 104°, respectively. Infra-red spectra of the two derivatives were also identical in all respects, Plate II A & B.

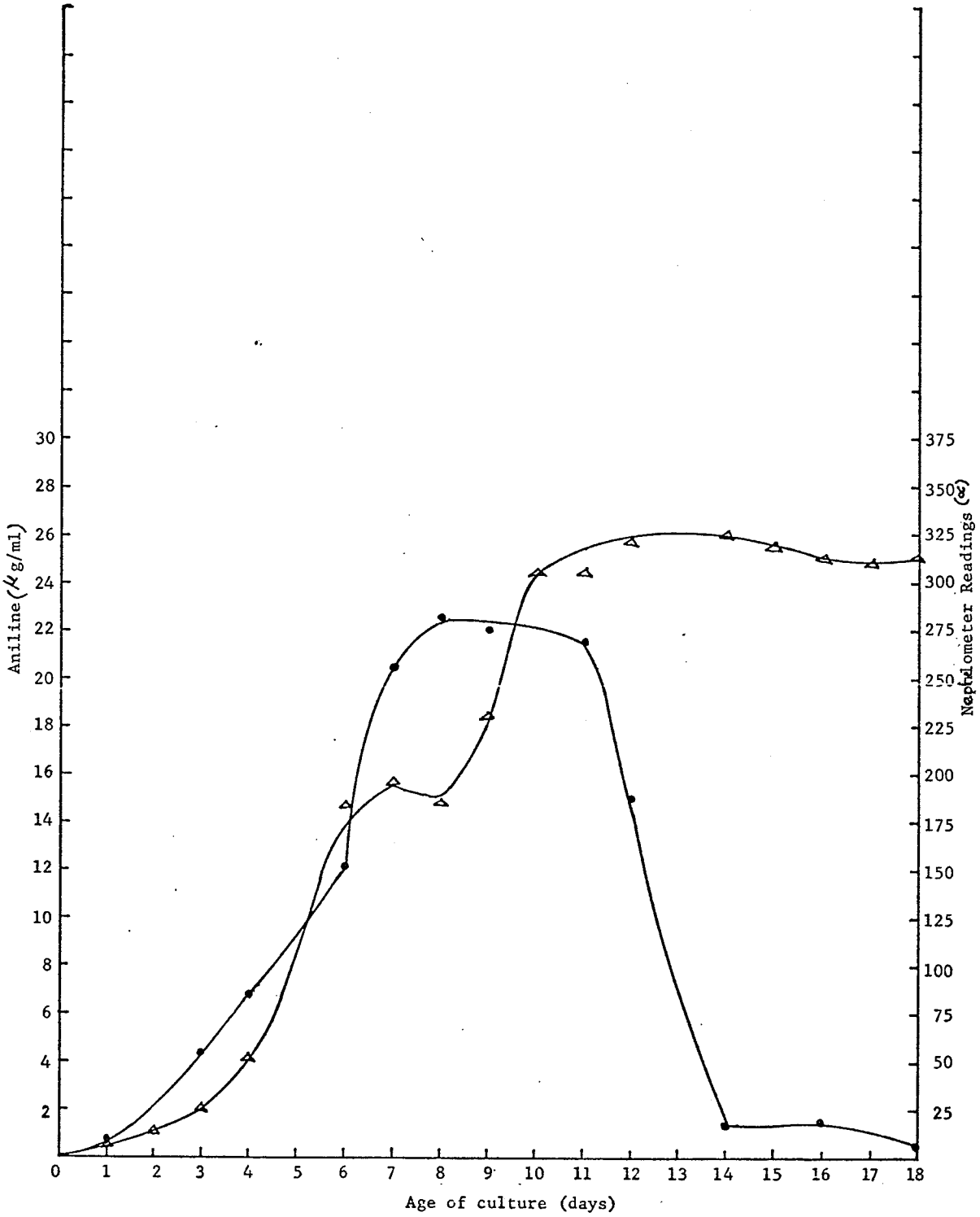


Fig. 15. Growth profile and aniline production during growth of Pseudomonas sp., on acetanilide. △ Growth profile; ● Aniline production.

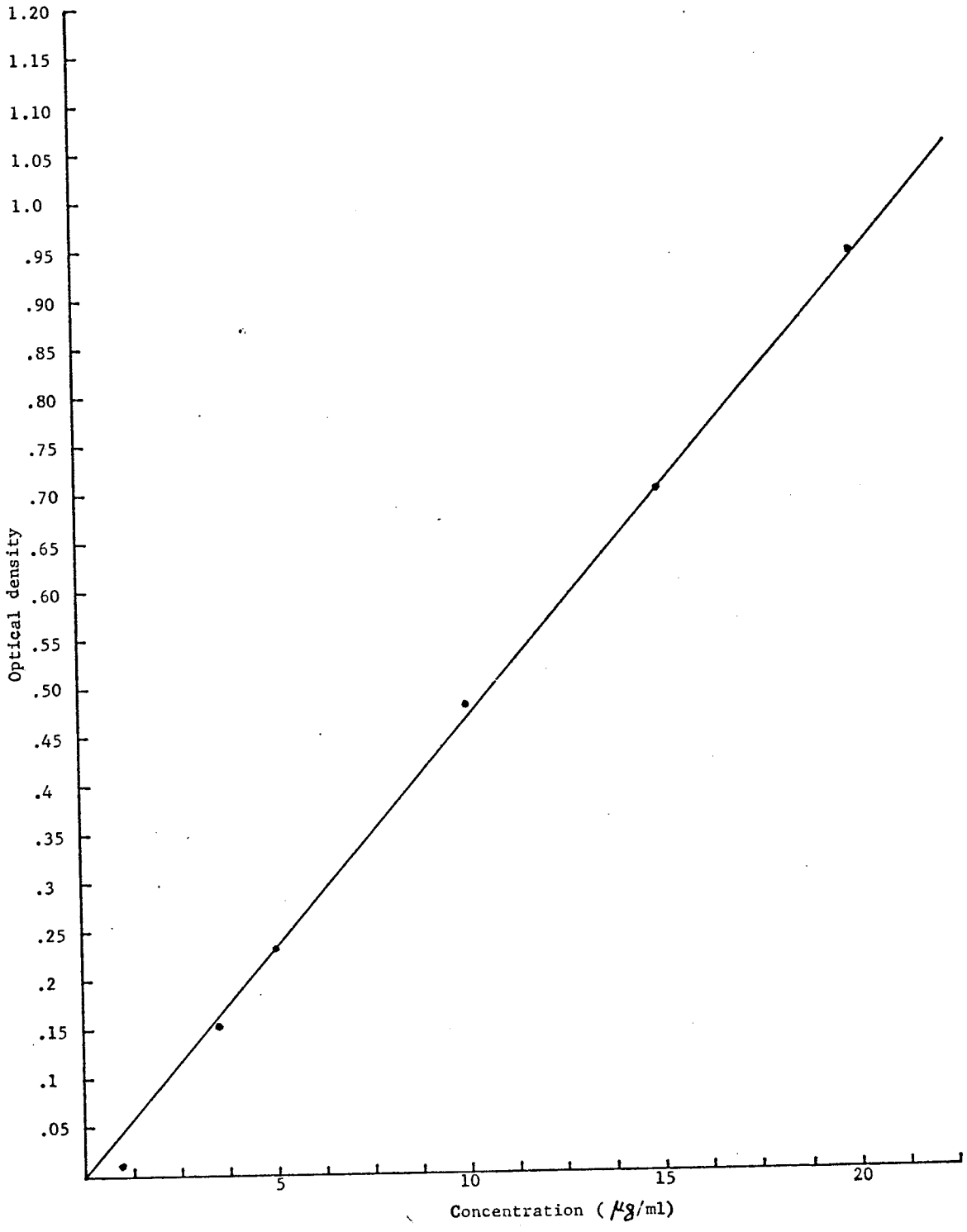


Fig. 16. Standard calibration curve for aniline estimation in bacterial culture by Daniel's method.

TABLE VIII

Aniline production during growth on acetanilide by Pseudomonas sp.

Sample age of culture Days	Turbidity Percentage Reflectance	Optical Density			+Aniline µg/ml.
		A	B	Average	
1	5	0.012	0.014	0.013	0.28
3	26	0.246	0.255	0.251	4.50
4	53	0.320	0.300	0.310	6.80
6	182	0.580	0.560	0.570	12.10
7	195	1.020	0.900	0.960	20.40
8	185	1.040	1.080	1.060	22.50
9	230	1.030	1.040	1.035	22.00
11	292	1.020	0.940	1.030	21.90
12	320	0.710	0.700	0.705	15.00
14	325	0.070	0.070	0.070	1.50
16	320	0.070	0.068	0.069	1.50
18	315	0.020	0.020	0.020	0.42

+ Aniline was assayed quantitatively by Daniel's method with reference to a standard calibration curve, Fig. 16.

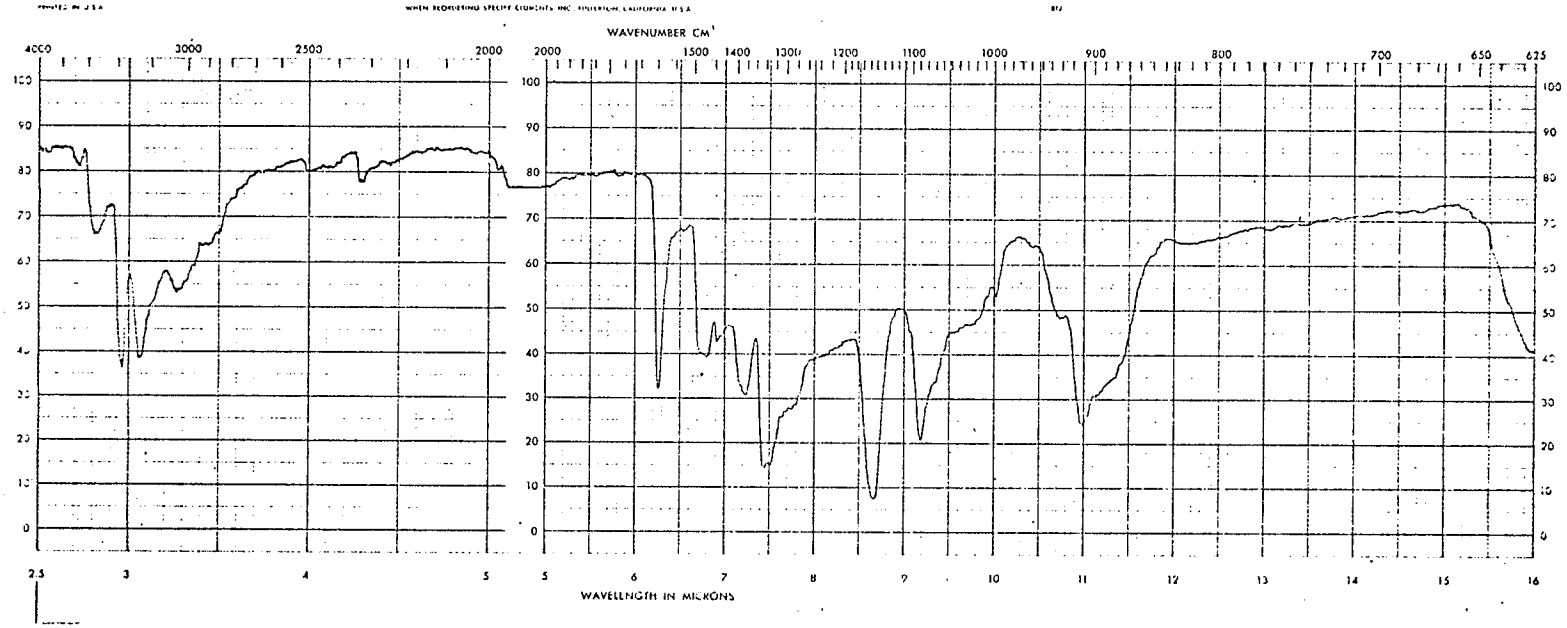
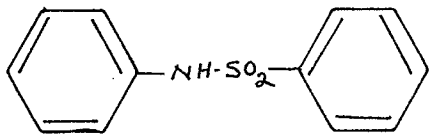


Plate II.A. Infra-red spectrum of aryl-sulfonyl derivative prepared with aniline extracted from bacterial culture growing on acetanilide.



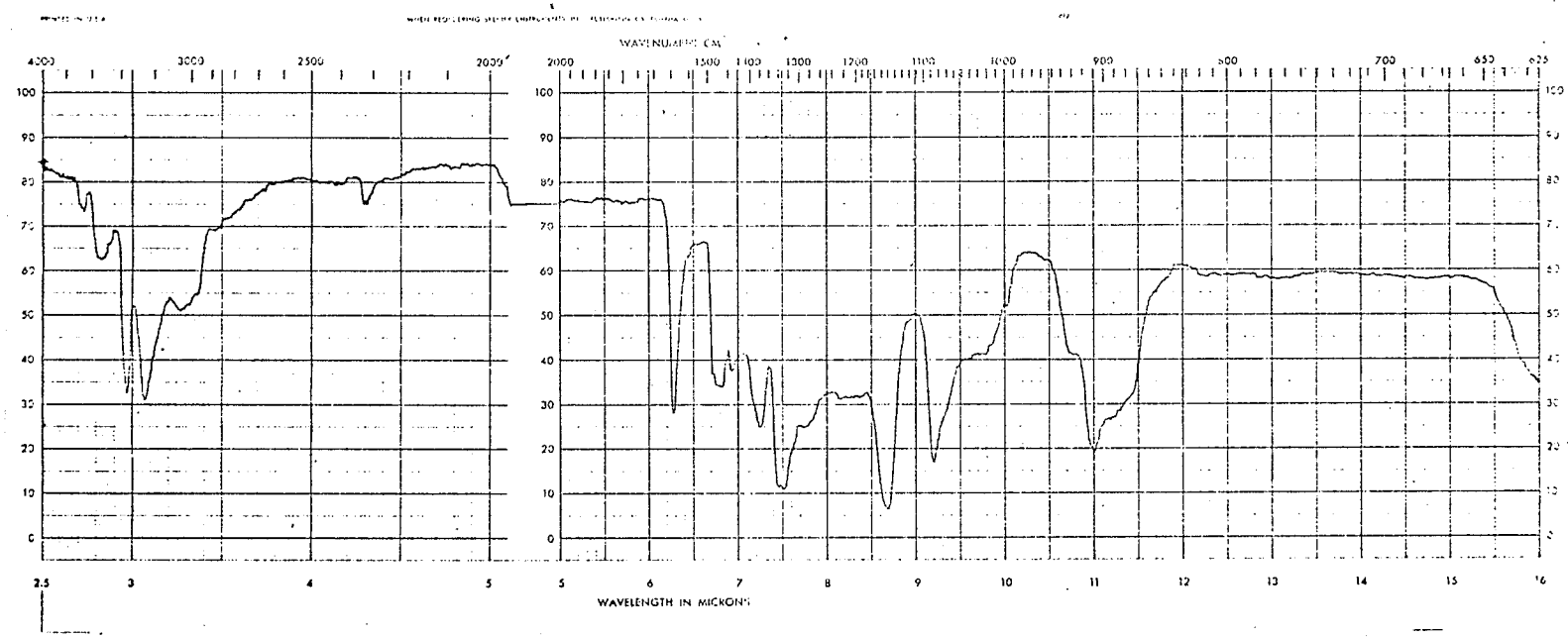
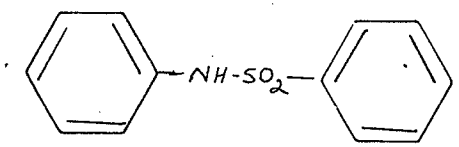


Plate II.B. Infra-red spectrum of aryl-sulfonyl derivative prepared with aniline.



Chapter 4

DISCUSSION

Smith and Williams (1948), working on metabolism of acetanilide in rabbit, reported the detection of aniline, ortho-aminophenol and para-aminophenol. They presented the argument that if an animal can deacylate acetanilide to aniline, then it is to be expected that acetanilide will give rise to ortho-aminophenol and para-aminophenol in such an animal. When acetanilide is fed to a dog both ortho-aminophenol and para-aminophenol can be detected in the urine (Jaffe and Hilbert, 1888). It is, therefore, probable that the dog possesses an active deacylating enzyme for aromatic acetamido compounds. The findings presented in our study of the metabolism of acetanilide by the bacterial species are to some extent in agreement with Smith and Williams' hypothesis, but a difference still exists between the bacterial and mammalian oxidation of acetanilide.

Our results do not seem to agree with those presented by Theriault and Longfield (1967). They reported the conversion of acetanilide to 2'-hydroxyacetanilide and 4'-hydroxyacetanilide, by several bacteria. No indication of the deacylation of acetanilide was given, which seems to be the major metabolic step in our proposed pathway for the degradation. The difference in the results presumably is due to the fact that, in the study by Theriault and Longfield, acetanilide was not used as sole carbon and energy source but on the contrary, it was used as a constituent of the peptone medium, which was the main energy providing source. Under these conditions it may be possible for the organism to convert acetanilide to 2'-hydroxyacetanilide and 4'-hydroxyacetanilide

as a detoxification mechanism, but not to deacylate acetanilide since its growth is not dependent on it.

Thin layer chromatography and gas-chromatographic assays have provided data which indicates the presence of three major metabolites, namely; aniline, ortho-aminophenol and 4-amino-catechol (3,4-dihydroxy aniline) in our Pseudomonas sp., culture grown on acetanilide. Manometric studies have also shown rapid oxygen uptake by the acetanilide grown organisms in the presence of these compounds, which is suggestive of these compounds being intermediates of the acetanilide degradation pathway.

The data presented indicates the deacylation of acetanilide to aniline and the presence of a deacylating enzyme system. This, of course, is inductive rather than constitutive, since the presence of acetanilide in the medium is essential for the maintenance of the deacylase system. Chromatographic and chemical studies, supported by manometric studies, revealed the conversion of acetanilide to aniline, which has been detected in bacterial culture in large amounts. It was isolated and identified by the preparation of arylsulfonyl derivative and by the infra-red spectral analysis.

The further metabolism of the deacylation product has also been pursued in our study, and it has been shown that aniline, which is present in the culture in large amounts during the exponential phase of growth, is metabolised to ortho-aminophenol. Identification of ortho-aminophenol in the culture growing on acetanilide has been done by thin layer- and gas-chromatography. Organisms consume oxygen rapidly in the presence of ortho-aminophenol, which is also suggestive of the possibility of ortho-aminophenol as an intermediate.

Even though ortho-aminophenol could not be isolated in sufficient quantity to carry out infra-red or ultra-violet spectral analysis to confirm its presence, still strong evidence has been presented in our results for the existance of ortho-aminophenol in the culture.

The other metabolite, whose presence was revealed by chromatographic assays, has been identified as 4-amino catechol. The presence of 4-amino catechol along with ortho-aminophenol in culture containing a large amount of aniline provides data, which points to the existance of two major metabolic pathways in the degradation of acetanilide after its conversion to aniline. The overall pathway can be clearly visualized as consisting of two major sets of reactions, Fig. 17.

(a) Deacylation: Conversion of acetanilide to aniline.

(b) Hydroxylation: Hydroxylation of aniline to ortho-aminophenol and 4-amino-catechol.

In Fig. 17, pathway (1) suggests conversion of aniline to ortho-aminophenol, which might be looked upon as a side reaction, but the rate of oxygen uptake by the organisms in the presence of ortho-amino-phenol strongly contradicts this possibility. Hypothetically ortho-aminophenol could convert to 3-aminophenol (2,3-dihydroxyaniline), which acts as a ring fission substrate, and may be quickly broken down to simpler compounds. Therefore, 3-amino catechol could not accumulate in sufficient quantity to be detected by our analytical techniques. No concrete evidence to support this assumption has been gathered during our study.

Reaction sequence in pathway (2) indicates the conversion of aniline to 4-amino-catechol (3,4-dihydroxyaniline), although m-aminophenol or p-aminophenol could not be detected in the culture. Manometric

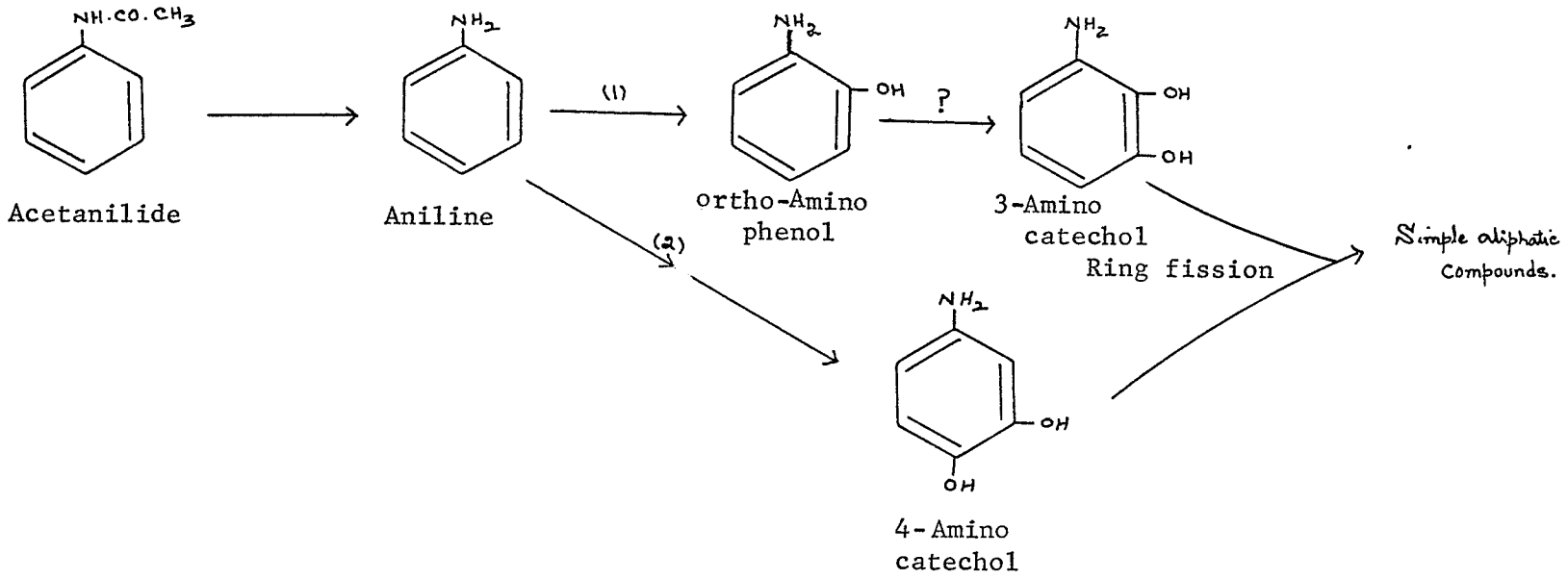


Fig. 17. Proposed pathways for degradation of acetanilide by Pseudomonas sp.

studies reveal moderate oxygen consumption by the organisms in the presence of m-aminophenol and 4-amino-catechol, and points to the possibility of existance of these two compounds as intermediates in an acetanilide degradation pathway, although no direct evidence is available for the presence of m-aminophenol in the culture, at the present time.

Thus, we reach a conclusion that aniline, after its formation from acetanilide by deacylation, undergoes dihydroxylation to 3-amino-catechol and 4-amino-catechol. Both these compounds undergo ring fission to yield simpler compounds for the growth of the organisms. However, as the rate at which 3-amino-catechol is broken down is very high, it does not accumulate in the medium.

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.

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 the existance of catechol. Instead they based their assumption on the results of oxygen uptake studies. We have also been able to demonstrate a large oxygen uptake with our Pseudomonas sp., grown on acetanilide, when catechol was used as a substrate, but we evidently could not show the presence of catechol in our culture. We feel that if the organisms consume oxygen in the presence of catechol, it does not necessarily mean that catechol is an intermediate of the metabolic pathway. This is based on a possibility that the enzymes of the metabolic pathways are non-specific to the substrates and, therefore, they can, in addition to their normal substrate, oxidize catechol.

Cells, grown on nutrient agar, oxidized substrates after a lag period and the total oxygen consumed was very low, whereas the acetanilide grown organisms consumed large amounts of oxygen rapidly, without any lag time. This clearly indicates that the enzyme system, involved in the degradation of acetanilide by Pseudomonas is inductive, and the organisms require an induction before they can start oxidizing acetanilide.

Concentrations of different substrates had to be varied to get an 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 intercellular concentration of substrate would have to be reached before derepression occurs. As a corollary, if a substrate is itself non-inducing but its immediate 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.

The appearance of the second lag period in the growth profile of the Pseudomonas sp., grown on acetanilide can not be satisfactorily explained by the data gathered in our study. However, a possible explanation exists, based on the assumption that during the early stages of the growth, the first step of conversion of acetanilide to aniline is the main carbon and energy providing reaction, which releases a large amount of acetate. This can be readily used as energy and carbon sources by the bacteria for growth. Deacylation, on the other hand, causes accumulation of aniline in the medium, which can not be metabolised

further by the organisms at this phase of growth. The organisms lack enzymes for the degradation of aniline and the amount of aniline is not yet high enough to cause any induction of enzymes; we have already established that a high concentration of aniline is required for growth. As the culture reaches 6 days growth period, the major portion of acetanilide is utilized and the induction of deacylase enzyme cannot be maintained further. Therefore, the growth ceases as the deacylation reaction is the main reaction providing energy in the form of acetate.

At this phase of growth the concentration of aniline is now high enough, and as a result it causes induction of enzymes for its own degradation. This induction is a slow process and so, a second lag period appears in the growth curve. The organisms start growing again rapidly because the energy and carbon for growth is now being provided by aniline, which is broken down to simpler readily utilizable compounds, by ring fission.

This growth study shows that the adaption to utilize acetanilide as sole carbon and energy source is not alone responsible for simultaneous adaptation to aniline. On the contrary, aniline induces enzymes for its own degradation. Thus, we may say that there are two main energy providing steps in the metabolism of acetanilide by Pseudomonas sp.

(a) Deacylation: Which releases acetate, readily used by the organism for the first phase of growth.

(b) Fission of aniline ring after dihydroxylation and formation of simple aliphatic compounds, which provide energy for growth in the second phase.

Our present work does not reveal the mode of cleavage of the aromatic nucleus after dihydroxylation of aniline, by Pseudomonas sp.

Further study of bacterial oxidation of acetanilide is needed to elucidate the mode of ring fission, and some of our results also need to be confirmed by using cell-free extracts and utilizing more sophisticated analytical procedures.

SUMMARY

The bacterial metabolism of acetanilide was studied due to its importance as a constituent of herbicides and pesticides. An organism has been isolated from the garden soil, which is capable of utilizing acetanilide as sole carbon source, and classified as belonging to genus Pseudomonas. The study has also proposed a pathway leading to the deacylation of the acetanilide to aniline which is further hydroxylated, and prepared for the ring fission. Major metabolites of the acetanilide degradation found in the cultures growing on acetanilide were identified as aniline, ortho-aminophenol, and 4-amino-catechol.

The growth of the organisms has been shown to be dependent on two different energy providing steps.

(a) Deacylation of the acetanilide; which provides energy in the form of acetate that can be readily utilized by the bacteria as a carbon source.

(b) Fission of aromatic nucleus; which follows hydroxylation of aniline and produces simple aliphatic compounds that can be quickly used by the organism.

Presence of a deacylating enzyme system is proposed, based on the results obtained. It has also been shown that adaption to utilize acetanilide by the organism is entirely an independent process, and aniline induces enzymes for its own degradation. All the enzymes involved in the acetanilide degradation pathway have been shown to be inductive rather than constitutive.

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