THE MICROBIAL DEGRADATION

OF

ANILINE

by

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A thesis

submitted to

the Faculty of Graduate Studies and Research

University of Manitoba

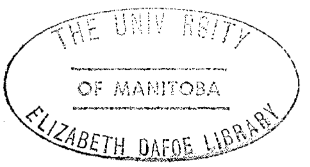
In partial fulfillment

of the requirements for the degree

Master of Science

Faculty of Pharmacy

1972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ACKNOWLEDGEMENTS

The author expresses his sincere gratitude to Dr. J. A. M. Shaw for his guidance and advice throughout the course of this investigation.

Gratitude is also expressed to Dr. L. A. Van Caeseele, Department of Botany, University of Manitoba for the preparation of the electron micrographs presented in this thesis.

The author is indebted to the University of Manitoba for providing the fellowship enabling him to carry out this project.

ABSTRACT

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Two bacterial microorganisms, capable of utilizing the aromatic amine, aniline, as a sole source of organic carbon and energy, when grown in a chemically defined simple mineral salts medium, were isolated from soil samples. One organism was identified as belonging to the family *Pseudomonadaceae*; the other to the family *Thiobacteriaceae*. The utilization of aniline was due to an inducible enzyme system. *o*-Aminophenol was identified by chromatographic techniques as an intermediate in the metabolic pathway of the degradation of aniline by the bacteria isolated. Oxygen uptake experiments indicated the possibility of *o*-aminophenol, *m*-aminophenol, and catechol being possible metabolic intermediates.

INTRODUCTION

INTRODUCTION

The wide use of aromatic compounds and the current concern of ecologists over pollution of the environment by the over-use of such compounds in all fields of human endeavour has stimulated considerable interest in the mechanism of their disappearance and in the mechanism of their biodegradation. Recently, extensive use of aromatics as insecticides has led to investigation of the microbial degradation of such agents as chlorinated hydrocarbons, carbamates, cyclodienes, phenylureas, thiol carbamates, amides, anilides, organophosphates, phenylcarbamates, triazines (Bartha _et al_, 1967) and chloroanilines (Bartha _et al_, 1968).

Tattersfield (1928) reported the isolation of many bacteria capable of destroying aromatic compounds such as phenol, _o_-, _m_-, and _p_-cresol, naphthalene, phloroglucinol, resorcinol and toluene. Pure cultures of these organisms were capable of utilizing the compounds as a sole source of organic carbon when incorporated into a mineral salt medium containing an inorganic nitrogen source. In a study of the distribution of these microorganisms, the above author showed that they were most often found in arable soil, but rarely occured in unmanured or "wild" soil.

It was the purpose of the present project to attempt to follow the microbial decomposition of a simple aromatic amino compound, namely aniline, and to determine to what extent commonly encountered microorganisms might degrade the amine.

Aniline and aminophenol (hydroxyaniline) derivatives have been employed as medicinal agents and are frequently encountered in pharmaceutical

practice. To date, a survey of the literature reveals an abundance of interest in the study of the mammalian metabolic transformations that these agents undergo. Little interest has been shown in the metabolic products formed by microbial activity. These antipyretic, analgesic and sometimes antibacterial compounds are used in ever increasing amounts. As their use escallates, so does their occurence in human waste and social effluent. How are the normal ecological resources able to cope with this onslaught? What role may soil bacteria play in ridding the environment of these potentially harmful agents? Also, to what extent do bacteria, normally encountered in the human intestinal flora, cause the degradation of any aromatic amines present which were introduced for a specific therapeutic effect? How would the bacterial degradation of these aromatics ultimately alter the therapeutic effectiveness or the duration of action expected from the drug? Would metabolic byproducts become involved in any meaningful drug interactions? Table 1 may be referred to for a listing of some commonly encountered medicinally active agents related to aniline and p-aminophenol.

The microbial degradation of aromatic amines could be studied from several different lines of attack. A study could encompass (1) the study of metabolic intermediates, (2) the elucidation of enzyme mechanisms of hydroxylation and ring fission or (3) the investigation of control mechanisms of enzymes involved in the degradation process. Since this project was to form the basis of a major program of research on the microbial degradation of aromatic amines, it was decided to begin with the isolation and identification of bacterial families capable of degrading aniline. Characteristics of the growth of organisms capable of utilizing aniline as a sole source of organic carbon would be determined and the initiation of a study of metabolic intermediates would be undertaken.

TABLE 1

Some medicinally active agents related to aniline

(General structure: R_1–C$_6$H$_4$–N(R_2)(R_3), with R_1 para to N)

Structure			Name
R_1	R_2	R_3	
-H	-H	-H	Aniline
-H	-H	$-CO-CH_3$	Acetanilid
-OH	-H	-H	p-Aminophenol
-H	-H	-CO-H	Formanilid
-H	-H	$-CO-C_6H_5$	Benzanilid
-H	-H	-CO-C$_6$H$_4$(OH) (o-hydroxyphenyl)	Salicylanilide
-OH	-H	$-CO-CH_3$	Acetaminophen
$-OC_2H_5$	-H	$-CO-CH_3$	Phenacetin
-OCO-C$_6$H$_4$(OH) (o-hydroxyphenyl)	-H	$-CO-CH_3$	p-Acetylaminophenol Salicylate
$-SO_2NH_2$	-H	-H	Sulphanilamide
-COOH	-H	-H	p-Aminobenzoic Acid

HISTORICAL

A wide range of aromatic acids, phenols, phenolic acids, heterocyclic compounds and hydrocarbons have been reported to be utilized by nutritionally non-exacting microorganisms as sole organic carbon sources for growth in a simple mineral salts medium. A survey of the available literature resulted in the collection of a vast amount of information relevant to the growth and isolation of soil bacteria capable of such utilization. It was evident that metabolic studies had been undertaken with respect to the microbial degradation of most of the simple aromatic compounds. The notable exception was studies related to the degradation of aromatic amines, specifically aniline and the N-substituted aromatic amines. The only report encountered was that of Walker and Harris (1969), Aniline Utilization by a Soil Pseudomonad. Research into the metabolic degradation of aromatics by microorganisms had been undertaken to an extensive degree with respect to most other simple organic compounds. Several reviews may be consulted, the most recent being that of Gibson (1968).

The disappearance of aromatic compounds as a result of bacterial activity was studied by Sen Gupta (1921). Fowler, Arden and Lockett (1911) suspected the occurrence of phenol utilizing bacteria on sewage filter beds. Tattersfield (1928) successfully isolated and identified several types of soil bacteria capable of destroying phenol, o-, m- and p-cresol, naphthalene, phloroglucinol, resorcinol and toluene. Happold (1930) has studied the capacity of bacterial suspensions to catalyze the oxidation of catechol, guaiacol, resorcinol, phenol, p-cresol and tyrosine. Von Plotho (1948)

isolated two species of Proactinomyces able to decompose aniline. It was stated that both species grew in the presence of phenol; one grew in the presence of naphthalene, and one in the presence of indole. Moore (1949) obtained organisms of the genus Proactinomyces from soil enrichment cultures which were reported able to utilize pyridine, aniline, nicotinic acid, nitobenzene, or phenol + ammonium ion as sole source of carbon, nitrogen and energy. Bacteria of the genera Pseudomonas, Flavobacterium, Achromobacter and Xanthomonas, capable of decomposing various phenolic compounds, were isolated by Tabak, Chambers and Kabler (1964). These investigators reported that a bacterial culture of mixed genera, when adapted to growth on phenol, could be shown to yield measurable rates of oxygen uptake in the presence of aniline. The trials were run with an aniline substrate concentration of 1 μmole/ml. The results were reported to be oxygen utilization of 40 to 50 μl in 180 minutes.

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

McCullough et al (1957), employing cell-free enzyme preparations from E. coli, reported the decarboxylation of p-aminobenzoic acid and anthranilic acid to aniline. The aniline thusly formed was further metabolized through p-aminophenol.

Work has been carried out on the degradation of acetanilid by a soil Pseudomonad (Shaw and Aziz, 1971). It has been shown that deacetyl-

ation of acetanilid results in the formation of aniline. The aniline was then found to be further metabolized through o-aminophenol. Both of the metabolites of acetanilid were isolated and identified by chromatographic techniques.

Walker and Harris (1969) undertook studies on the degradation of aniline by a Pseudomonas sp. isolated from soil. These workers reported the decpmposition and disappearance of aniline with the liberation of ammonia as a result of bacterial activity when the aniline was present as a sole carbon source in a simple salts medium. Aniline grown organisms were reported to yield, without lag, oxygen uptake in the presence of aniline, catechol, p-aminophenol, o-, or m-toluidine. An induced enzyme system was suggested as being involved. The organism isolated was not able to utilize benzene, phenol, monochloroanilines, o-toluidine, or p-aminobenzoic acid as a sole source of carbon.

Anilide herbicides have been shown to be transformed by soil microorganisms (Bartha et al, 1967). Table 2 is presented to illustrate the relationship of the acylanilide herbicides in common use to the aniline moiety of interest. It was demonstrated by respirometry and gas chromatography that microorganisms in soil rapidly degraded the anilide herbicides propanil, dicryl and karsil (Bartha et al, 1968). The aliphatic portion of these molecules was oxidized to CO_2, liberating the 3,4-dichloroaniline moiety, and this was further transformed to 3,3',4,4'-tetrachloroazobenzene. These compounds were isolated and identified by their infrared spectrum and melting point. Since anilines are produced from a variety of herbicides, the relationship of the aniline structure to the formation of azo residues was investigated (Bartha and Pramer, 1967). Soil samples were treated with 10 different anilines and analyzed after 15 days incubation at 28°C. It was

TABLE 2

Acylanilide Herbicides

Chemical Name	Structure	Trade Name
N-(3-Chloro-4-methylphenyl)-2-methylpentanamide	H_3C, Cl-phenyl-NH-C(=O)-CH(CH_3)-C_3H_7	Solan*
N-(3,4-Dichlorophenyl)-cyclopropancarboxamide	Cl, Cl-phenyl-NH-C(=O)-cyclopropyl	Clobber* S-6000
N-(3,4-Dichlorophenyl)-methacrylamide	Cl, Cl-phenyl-NH-C(=O)-C(CH_3)-CH_2	Dicryl*
N-(3,4-Dichlorophenyl)-2-methylpentanamide	Cl, Cl-phenyl-NH-C(=O)-CH(CH_3)-C_3H_7	Karsil*
N-(3,4-Dichlorophenyl)-propionamide	Cl, Cl-phenyl-NH-C(=O)-C_2H_5	Rogue* STAM F-34* Propanil
N-Isopropyl-2-chloroacetanilide	phenyl-N(CH(CH_3)-CH_3)-C(=O)-CH_2Cl	Ramrod*

* Registered trade name

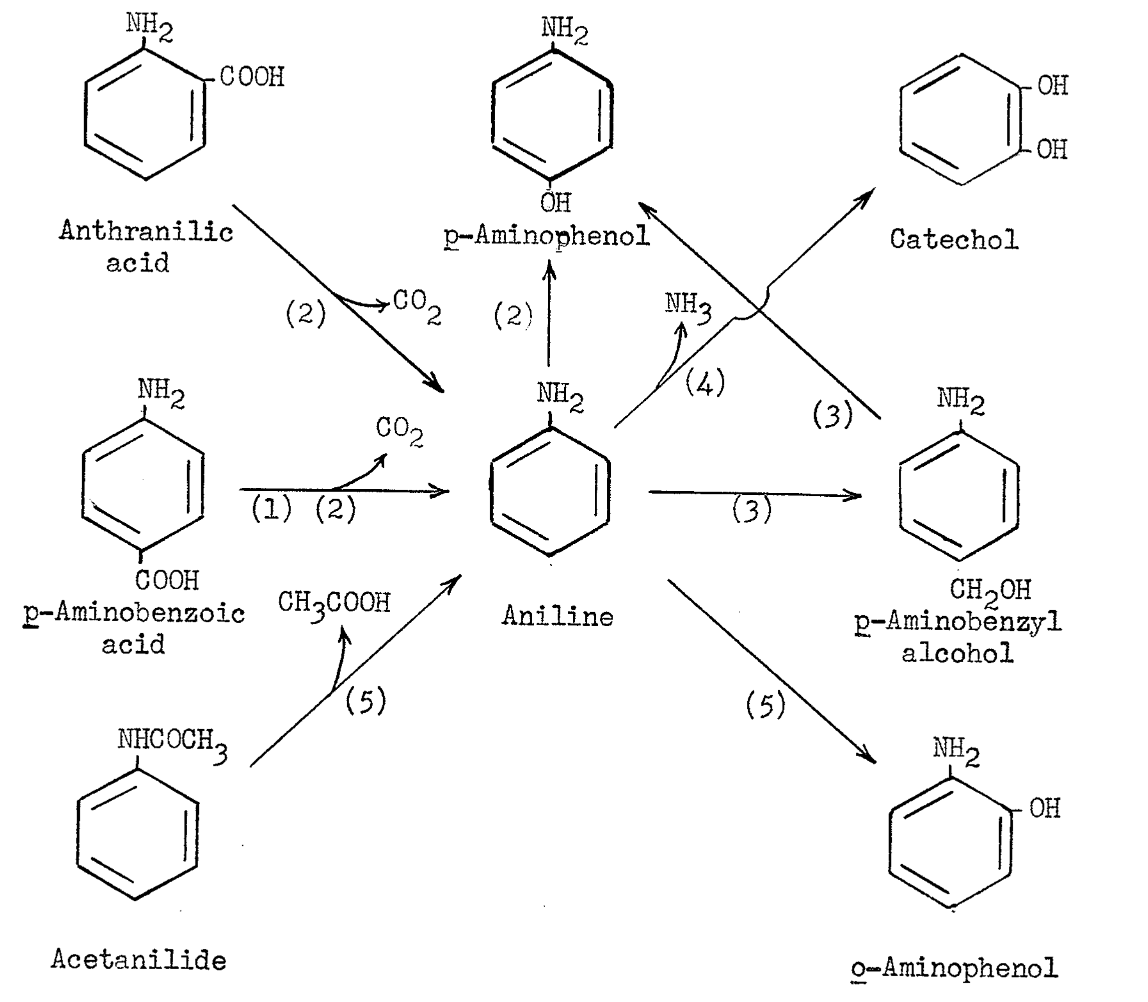
reported that unsubstituted aniline was decomposed without the production of azobenzenes, but o-, m- and p-chloroanilines were transformed to the corresponding dichloroazobenzenes. Further work (Bartha et al, 1968) supported the above findings and confirmed that no azo compound was formed from aniline but all monochloro-, and some dichloroanilines, were transformed to their corresponding dichloro- and tetrachloroazobenzenes. Evidence as to the possible products of the transformation of the acylanilide, N-isopropyl-2-chloroacetanilde, was not presented in a review of the current advances in the study of the metabolism of acylanilide herbicides by soil bacteria (Bartha and Pramer, 1970).

The findings of various workers with respect to the microbial degradation of aromatic amino compounds tends to support the intimate involvement of aniline. Figure 1 is presented to demonstrate the role of aniline in biodegradation and to illustrate the current knowledge, as supported by a survey of the literature, of the possible metabolic pathways for the utilization of simple aromatic amines by microorganisms.

During recent years biochemical investigators of the oxidative degradation of aromatic compounds by several genera of bacteria have revealed that many organisms produce, primarily as a result of induction, a sequence of enzymes which convert aromatic substrates into dihydroxyphenyl derivatives that undergo cleavage to yield aliphatic acids or aliphatic semialdehydes. However, due to the diverse mechanisms by which microbes degrade aromatic compounds one cannot predict the method of attack and mode of fission of any benzenoid compound by any microorganism. A brief review of some of the work done will be presented to illustrate possible biochemical pathways for the entrance of aromatic amines into the tricarboxylic acid cycle, a step necessary if bacteria demonstrate the ability to thrive on

FIGURE 1

Microbial Degradation of Aromatic Amines



1. Sloane et al, 1951
2. McCullough et al, 1957
3. Sloane and Untch, 1964
4. Walker and Harris, 1969
5. Shaw and Aziz, 1971

such compounds when present as sole carbon and energy source.

Benzene, the simplest aromatic compound, is utilized by some bacteria as a sole source of carbon and energy. Marr and Stone (1960) isolated two organisms, a *Pseudomonas sp.* and a *Mycobacterium sp.*, able to degrade benzene. These workers proposed a metabolic pathway for the degradation of the aromatic via 3,5-cyclohexadiene-1,2-diol and catechol (Figure 2). The introduction of two hydroxyl groups ortho to one another to form catechol, rather than the production of a monohydroxylated intermediate prior to ring cleavage, appears to be characteristic of bacterial systems (Gibson, 1968).

Studies on the bacterial metabolism of phenol show that the substitution of a hydroxyl group into the benzene ring still leads to the production of catechol (Evans, 1947).

The effect upon the mechanism of degradation of the introduction of a carboxyl group was found to be dependent upon the relative position of the two substituent groups. *p*-Hydroxybenzoic acid was degraded by *Vibrio* 0/1 via protocatechuic acid (3,4-dihydroxybenzoic acid) (Sleeper and Stanier, 1950). *m*-Hydroxybenzoic acid may be metabolized either through gentisic acid (2,5-dihydroxybenzoic acid) or through protocatechuic acid (Yano and Arima, 1958). Investigations into the degradation of *o*-hydroxybenzoic acid (salicylic acid) revealed the induction of two pathways, the first via protocatechuic acid, and the second via gentisic acid (Yano and Arima, 1958). Earlier, results of investigations by Walker and Evans (1952) had suggested the involvement of catechol in the breakdown of *o*-hydroxybenzoic acid by *Ps. fluorescens*. It is evident that great diversity of pathways in the metabolism of benzoate derivatives by bacteria exsists (Figure 3).

FIGURE 2

Pathway of Benzene Oxidation

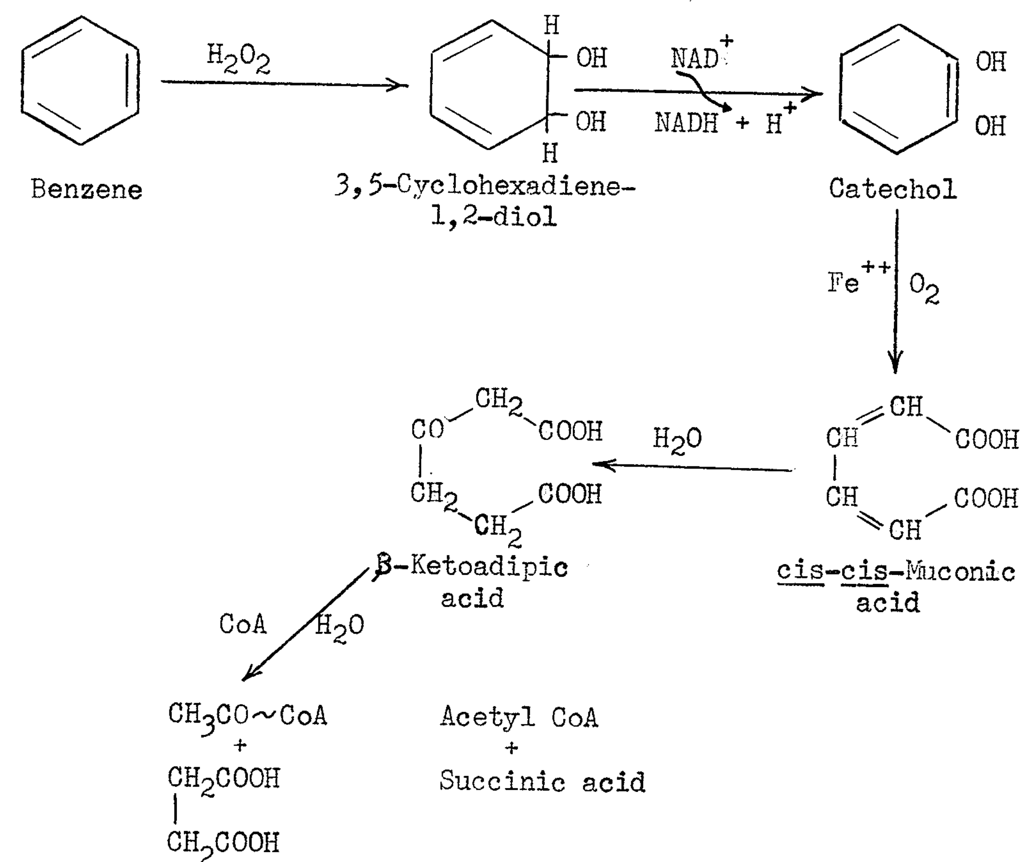
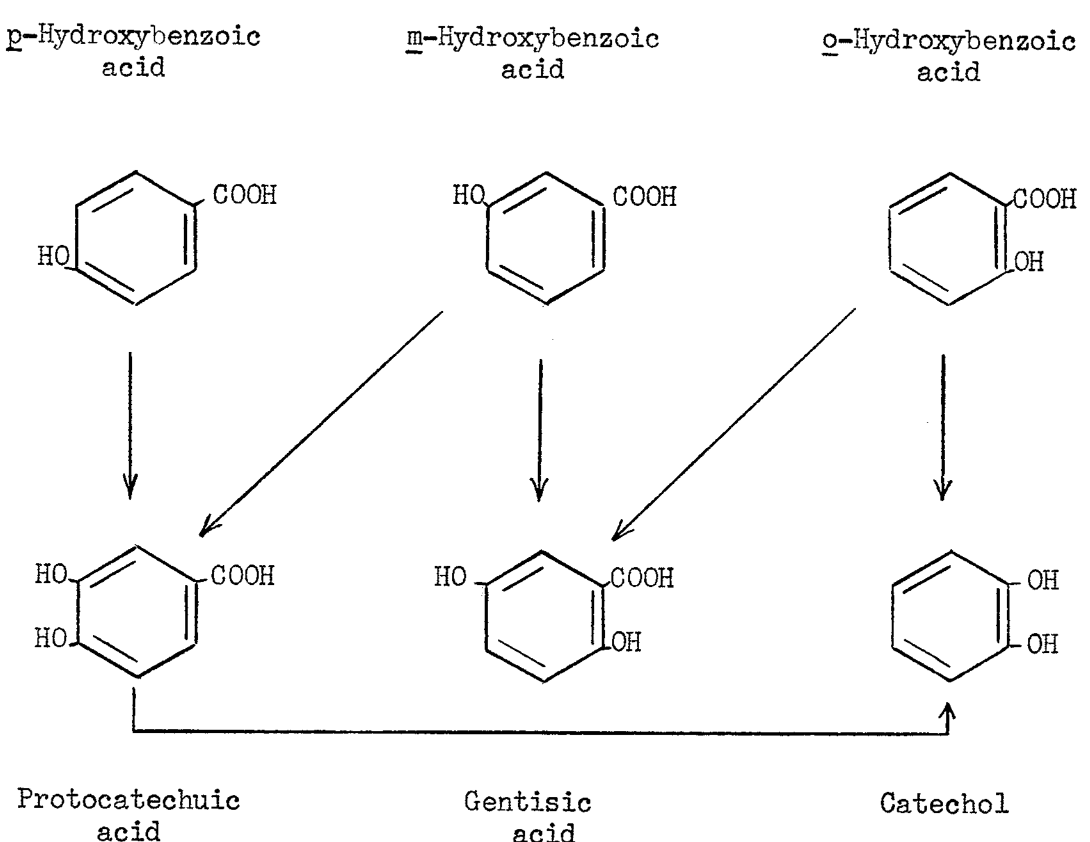


FIGURE 3

Metabolism of Benzoate Derivatives



When an amino group is introduced into benzoic acid ortho to the carboxyl group, the resultant anthranilic acid is metabolized by cell-free enzyme preparations obtained from E. coli to aniline. These same enzyme preparations were also able to decarboxylate p-aminobenzoic acid with the resultant formation of p-aminophenol (McCullough et al, 1957). Anthranilic acid is metabolized by Pseudomonas fluorescens via the intermediate, catechol (Tanuichi et al, 1964). Studies on anthranilate hydroxylase, using O^{18}, revealed that a novel mechanism of double hydroxylation was involved in the conversion to catechol. Kobayashi et al (1964) proposed a scheme for the enzymatic hydroxyation of anthranilic acid (Figure 4). Both atoms of oxygen incorporated into the catechol molecule were shown to have been derived from atmospheric oxygen. Two oxygen atoms, presumably in the same molecule, add to the double bond linking carbons 1 and 2, and the cyclic peroxide intermediate is reductively cleaved with concomitant release of ammonia and CO_2.

When a carboxyl group is the only substituent group in the benzene ring, degradation still proceeds through catechol. Simultaneous oxidative decarboxylation and hydroxylation, resulting in formation of catechol, was observed by Stanier (1947). Proctor and Scher (1960) observed the microbial conversion of benzoic acid to catechol via protocatechuic acid. The degradation of benzoic acid via the formation of salicylic acid has also been proposed (Bhat et al, 1959).

It is apparent that diverse mechanisms are utilized by microorganisms to prepare different aromatic compounds for ring fission. It is generally accepted that dihydroxylation is a prerequisite 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

FIGURE 4

Enzymatic Hydroxylation of Anthranilic Acid

COOH
NH_2
O_2
COOH
O
O
NH_2
NADH
$-CO_2$
$-NH_3$
OH
OH

Anthranilic acid

Catechol

homogentisic acids. Catechol has been shown to be a substrate for ring fission in the microbial degradation of many different aromatic compounds.

Hayaishi and Hashimoto (1950) isolated the enzyme, catechol-1,2-dioxygenase, that catalyzed the incorporation of molecular oxygen into the catechol molecule. The reaction product was identified as cis-cis muconic acid. An alternative enzymatic cleavage of catechol was reported by Dagley and Stopher (1959). A *Pseudomonas* sp. which utilized *o*-cresol as a sole source of carbon for growth, oxidized catechol to α-hydroxymuconic semialdehyde. The enzyme catalyzing this reaction is catechol-2,3-dioxygenase. The above reaction sequences and the subsequent sequences leading to the catabolism and entrance of substrates into the tricarboxylic acid cycle are summarized (Figure 5).

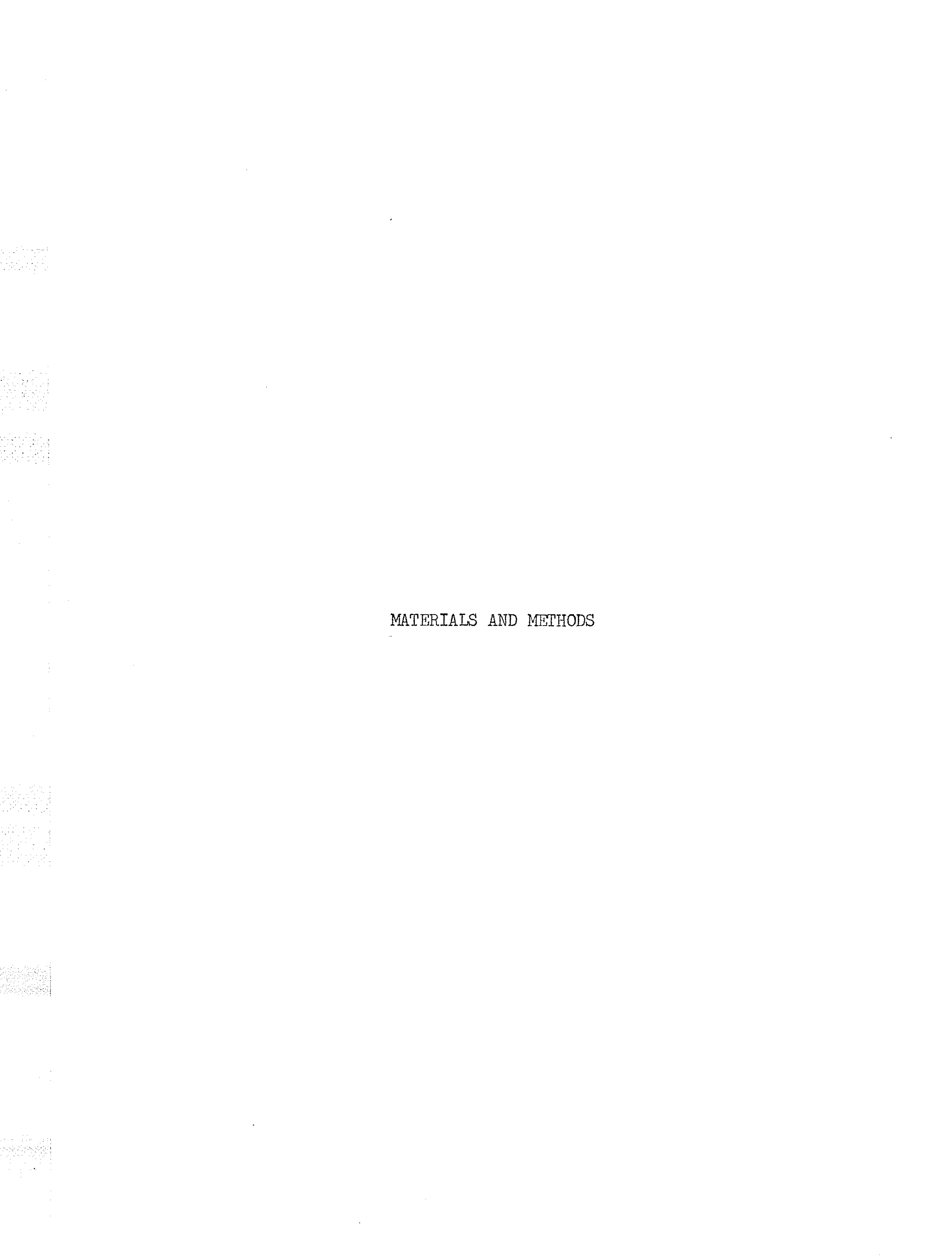
The established mechanisms of disruption of the aromatic ring and subsequent reactions leading from the benzene nucleus to the citric acid cycle are by no means ubiquitous in their occurrence. It must be appreciated that pathways of dissimilation of aromatic compounds exist which may prove to be radically different from those described.

FIGURE 5

Reaction Sequences for the Degradation of Catechol

Sequence (a):

Sequence (b):



MATERIALS AND METHODS

MATERIALS AND METHODS

A. Organisms

Two organisms were isolated by enrichment technique from arable farm soils and pure cultures were obtained. Both bacteria isolated were capable of growth in a mineral salts medium containing aniline as a sole source of carbon and energy.

B. Growth of organisms

Stock cultures and inocula were grown in 250 ml Erlenmeyer flasks containing 100 ml of mineral salts medium (Halvorson, 1970) with 0.2% (w/v) aniline as the sole carbon and energy source. The composition of this medium per litre is as follows:

2.0 g	Organic carbon source (aniline)
0.3 g	NH_4Cl
0.1 g	K_2HPO_4
0.2 g	$CaCl_2$
0.2 g	$MgSO_4.7H_2O$
0.005 g	$FeSO_4.7H_2O$

The pH was adjusted to pH 6.6 to 6.8 with dilute hydrochloric acid to prevent any precipitation of salts on autoclaving. The final volume was adjusted to 1 litre with double distilled water...the second distillation being carried out over glass and the water prepared having been sterilized

immediately after distillation, prior to storage in sealed containers. Any solid growth medium required was prepared by adding to the above liquid medium 1.5% agar (Difco). The culture medium was sterilized by autoclaving at 121°C (15 lbs pressure) for 20 minutes. Growth was carried out by incubation of cultures at 30°C.

Assay of the culture medium according to the method of Daniel (1961), before and after autoclaving, revealed the loss of aniline not to exceed 5% (w/v).

C. Chemicals

Wherever possible, all chemicals used throughout this project were Analar$^{(R)}$, British Drug Houses Limited. The aminophenols, o-, m- and p-, were obtained from the Aldrich Chemical Company and were recrystallized from hot ethanol before use. The aniline used in the studies was Analar$^{(R)}$ which was freshly distilled over zinc and activated charcoal before use. Only that fraction distilling over at 183°C was employed. All reagents and buffers were prepared in glass distilled water. The solvents used were employed as obtained from the manufacturer with the exception of ether. All ether used was redistilled over glass two times before being employed in any extraction procedures.

D. Identification of organisms

The following methods were employed in the identification of the families into which the organisms isolated could be included:

1. Microscopic examination of the cultures grown in liquid and on

solid media to determine motility, morphology, and Gram reaction.

2. Electron-microscopic examination for confirmation of morphology and determination of the presence and arrangement of flagella.
3. Examination of colonial morphology and pigmentation on nutrient agar (Difco).
4. Examination of nutrient agar and nutrient broth (Difco) cultures by ultra-violet illumination for fluorescence.
5. Oxidase test with 0.2% (w/v) aqueous 2,6-dichlorophenol-indophenol, first reduced with dithionite. With this reagent, oxidase positive strains produce a blue colour.
6. Attack on glucose, tested by the method of Hugh and Leifson (1953).
7. Testing for the presence of catalase by determining the organism's ability to destroy hydrogen peroxide.
8. Testing for the ability to use nitrate as a nitrogen source, and the ability to fix atmospheric nitrogen. Such tests were conducted in tubes of basal salt media in which NH_4Cl was either removed or replaced by KNO_3. Lactate (1 g/l) was provided as a source of carbon and energy.
9. Ability to produce indole by way of tryptophan dissimilation.
10. Ability to utilize starch as a growth substrate with resulting hydrolysis and negative reaction to iodine.
11. Ability of the organism to exhibit growth properties of chemo-autotrophic bacteria. The organisms were tested for growth in a neutral environment using reduced sulphur compounds as the source of energy and atmospheric CO_2 as the source of carbon. Starkey's medium No. 2 (Starkey, 1934) was selected. The composi-

tion of this medium per litre is as follows:

1.0 g	$Na_2S_2O_3.5H_2O$
4.0 g	KH_2PO_4
4.0 g	K_2HPO_4
0.05 g	$CaCl_2$
0.30 g	$(NH_4)_2SO_4$
0.02 g	$FeCl_3$
0.02 g	$MnSO_4.2H_2O$
0.30 ml	Phenol red (2%)

The pH was adjusted to pH 7.0 to 7.5 with 10% K_2CO_3 and the volume to 1 litre with distilled water. The medium, minus the thiosulphate, was prepared and adjusted to a volume of 900 ml. The pH was corrected and the solution autoclaved. The thiosulphate was incorporated into the medium as 100 ml of 1% (w/v) $Na_2S_2O_3$. $5H_2O$, pH 7.0 to 7.5, sterilized by bacterial filtration and added to the cooled solution which had been autoclaved. Growth was carried out at 30°C.

The results of the above examinations were interpreted according to Bergey (1957), Hendrie and Shewan (1965) and Stanier et al (1966).

E. Optimum growth temperature

Growth was observed in 20 ml tubes of nutrient broth, on nutrient agar slants and in 20 ml tubes of simple salts medium containing 0.2% (w/v) aniline as sole carbon and energy source. Studies were conducted on the growth of the organisms at room temperature (21°C), at 30°C in a warm air incubator and at 37°C as controlled in a water bath. All inoculations were

made with 2 ml of culture whose turbidity had reached 100, as determined by a nephelometer, in a simple salts medium containing 0.2% (w/v) aniline. Growth was determined visually and was recorded as occurring (+) or as absent (-).

F. Rate of growth

The rate of growth of the organisms in simple salts medium containing aniline 0.2% (w/v) was determined by the measurement of turbidity. Measurements were taken at 24 hr intervals from 20 ml samples of cultures of 1 litre volume which had been inoculated with 100 ml of growth which had attained a turbidity equivalent to 100. The turbidity of the culture was determined using a Unigalvo Type 20 galvanometer fitted with a nephelometer head (Evans Electroselenium Ltd., Halstead Essex, England). An uninoculated control was used to zero the instrument before all turbidity determinations.

G. Utilization of aniline

The disappearance of aniline from the growth medium, as a result of bacterial metabolic degradation, was determined using p-dimethylaminobenzaldehyde. The reaction with aniline produced an intense yellow colour as a result of the following reaction:

$$C_6H_5NH_2 + (CH_3)_2N\text{-}C_6H_4\text{-}CHO \longrightarrow (CH_3)_2N\text{-}C_6H_4\text{-}CH{=}N\text{-}C_6H_5$$

aniline — p-dimethylaminobenzaldehyde — yellow pigment

The procedure adopted for the test was:

1. A drop of culture medium was placed upon a clean filter paper (Whatman NO. 1).
2. A drop of p-dimethylaminobenzaldehyde (1% (w/v) in N/1 hydrochloric acid) was placed on the filter paper such that the spreading margins of the drops just touched one another.
3. A positive indication of the presence of aniline was the appearance of a yellow line at the junction of the margins.

The test may be applied to any compound possessing a free amino group. It was used only to allow determination of the complete disappearance of aniline or any aromatic amino metabolites from the cultures. The ability of an organism to degrade aniline was determined by obtaining a negative reaction for the presence of aromatic amines in the culture fluid.

The rate at which aniline was being utilized as substrate was determined by the measurement of the concentration of aniline in the growth medium at 24 hr intervals. The assay procedure of Daniel (1961) was adopted and all spectrophotometric readings were carried out on a Unicam SP600 spectrophotometer (Unicam Instruments, Cambridge, England) employing 1 cm silica cuvettes. Figure 6 is the standard straight line relationship of a plot of optical density at 545 mμ against the concentration of aniline (mcg/ml) obtained using N-1-naphthylethylenediamine as the coupling reagent in the assay. The slope of the plot was determined and was employed in relating the optical density displayed by a given solution to the concentration of aniline present.

During the assay, the reaction was allowed to proceed for 90 minutes before readings of optical density were taken. The time recommended in the procedure of Daniel (1961) was 15 minutes. The increase in time was found

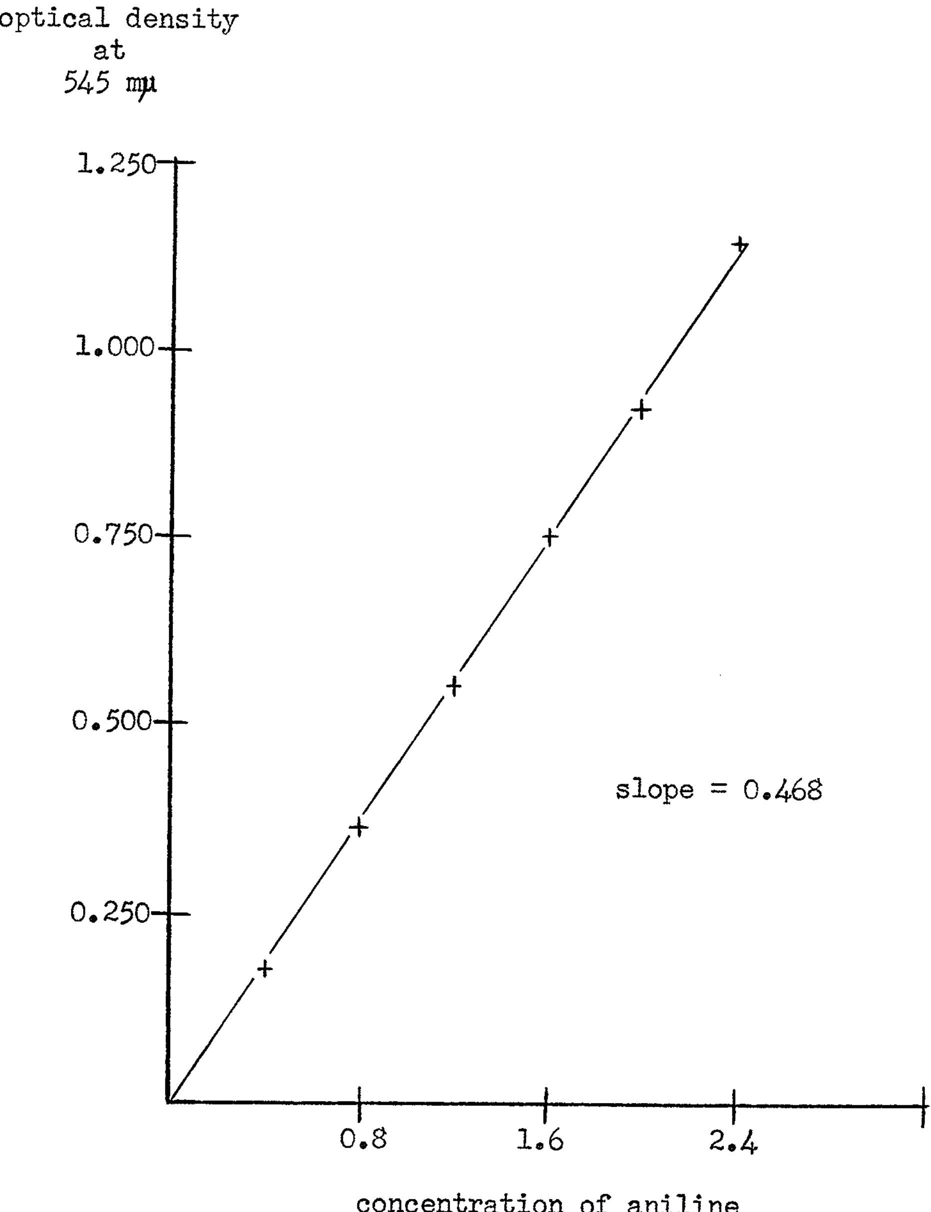


FIGURE 6. Standard curve for the assay of aniline

to be necessary in order to obtain consistent results. All studies were carried out using the standard medium adopted for the project with the concentration of aniline required being adjusted as necessary. The presence of bacterial cells or of the aminophenols suspected as possible metabolites, did not alter the accuracy of the assay procedure with respect to the concentration of aniline present.

H. Effect of aniline concentration on rate of growth

Studies were carried out relating the rate of bacterial growth, as measured by observed changes in turbidity, to initial concentration of aniline in the growth medium. The rate of growth was determined in 20 ml bacterial cultures which had been inoculated with 0.2 ml of a bacterial suspension obtained from a 4 day growth in nutrient broth and in 20 ml cultures inoculated with 0.2 ml of a bacterial suspension obtained from growth in simple salts with 0.1% (w/v) aniline after a turbidity of 100 had been observed on a nephelometer. The initial concentrations of aniline employed ranged from 0.0% (w/v) to 0.4% (w/v), increasing in increments of 0.05% (w/v).

I. Effect of a change of substrate on growth of organisms

Experiments to test for the ability of the organisms to utilize aromatic substrates, other than aniline, as a sole carbon and energy source were conducted employing the same simple salts medium described earlier. Growth was judged turbidimetrically. The organisms were challenged with 0.05% (w/v) and 0.1% (w/v) acetanilide, 0.05% (w/v) and 0.1% (w/v) benzoic

acid, 0.05% (w/v) and 0.1% (w/v) p-aminobenzoic acid, and 0.02% (w/v) and 0.05% (w/v) phenol. Studies were not conducted with catechol, o-, m-, or p-aminophenol because of their inherent rapid rate of oxidation.

J. Extraction of aminophenols

When the culture fluid had attained a turbidity of 100 to 125, the bacterial cells were separated and removed from the liquid medium by centrifugation at 5°C for 15 minutes at a R.C.F. of 12 x 10^3 (RC2-B, Sorvall). The cells collected were retained for subsequent oxygen uptake experiments carried on concurrently with extraction procedures. The cell-free supernatant was adjusted to pH 8.0 with 20% (w/v) $NaHCO_3$ and extracted three times with ether of equal volumes (Brodie and Axelrod, 1948). The ethereal extracts were dried over anhydrous Na_2SO_4, filtered, and evaporated to a volume of 2 ml. The residue was dissolved in 5 ml of absolute ethanol and the solution was applied to silica gel plates for separation by thin layer chromatography. 1 litre volumes of culture fluid were treated.

K. Chromatographic studies

1. Methods

The alcoholic solution of the residue from the ethereal extraction was spotted on thin layer plates of fluorescent silica gel and developed in various chromatographic solvent systems to give a qualitative indication of the aromatic metabolites of aniline that might be present. These metabolites were detected with a potassium ferricyanide/ferric chloride spray reagent. All plates were developed a distance of 15 cm.

Preparative thin layer chromatography was used to remove any aniline present in the alcoholic solution and to allow for the proper separation and collection of any aminophenols present. Once the metabolites had been separated by thin layer chromatography, their presence was confirmed by comparative chromatography in various solvent systems and by gas liquid chromatography.

2. Chromatographic solvents

The following solvent systems were utilized in thin layer chromatographic studies:

- n-butyl ether/ethyl acetate/acetic acid (50/50/5)
- ethyl acetate/benzene (9/11)
- chloroform/methanol 95%/NH_4OH (85/15/1)

3. Detecting reagent

A potassium ferricyanide/ferric chloride solution was employed as the detecting reagent. The solution, used as a spray, consisted of equal parts of an aqueous solution of 1% (w/v) potassium ferricyanide mixed, just prior to use, with a 2% (w/v) aqueous solution of ferric chloride. The resulting blue colour produced by phenols and aromatic amines was intensified by spraying with 2N HCl. Acidification of the silica gel plates tended to bleach the backround, which normally turned blue-green in colour, as well as to intensify the colour of the spots produced.

4. Preparation of chromatographic plates

The thin layer paltes were prepared using fluorescent silica gel (Silica Gel GF_{254} acc. to Stahl-Merck) on a quickfit apparatus, plate thickness being governed and kept constant by a fixed aperture spreader. Qualitative thin layer chromatography was carried out on plates 0.25 mm in thickness. Preparative thin layer chromatography was carried out on plates

1.00 mm in thickness. All plates were dried in a hot air oven at 120°C for 60 minutes prior to use.

5. Gas liquid chromatography

Samples suitable for gas liquid chromatography were obtained by eluting, with ether, any metabolites found to be present in bands appearing on preparative thin layer silica gel plates. Each band of silica gel was scraped from the plate, collected, and the organic components of the band were extracted with three 20 ml volumes of ether. The solution was filtered and the solvent was removed on a Rinco rotary evaporator. The residue remaining was dissolved in 5 ml of ether and was then chromatographed by gas liquid chromatography. For the initial separation by thin layer chromatography, a solvent system of chloroform/methanol 95%/NH_4OH (85/15/1) was employed. The alcoholic solution obtained during the extraction was separated into two bands. The lower band, running between Rf 30 and Rf 45, was removed and its organic constituents were eluted with three 20 ml volumes of ether. After filtration and concentration by the removal of the solvent by evaporation under vacuum, the extract was applied to fresh silica gel plates and was separated in a system of ethyl acetate/benzene (9/11). The band appearing, was removed from the plates and was treated as before with ether. The concentrated extract was then ready to be chromatographed by gas liquid chromatography.

The sole metabolite isolated was detected with a GC-5 gas chromatograph (Beckman) fitted with a flame ionization detector. The column was of teflon and measured 6 ft x $\frac{1}{4}$ in. Separation was carried out with 0.5% Amine 220 (Applied Science Laboratories) on 100 to 120 mesh Chromosorb G Acid DMCS (Johns Manville Ltd.). The carrier gas was helium with a flow rate of 100 ml/minute. The inlet pressure was 50 psi. The temperature of the column

was 153°C; the injection port was 180°C; the detector was 260°C. Recording was done at a chart speed of 0.1 in/minute.

L. Oxygen uptake experiments

Organisms were obtained from liquid culture media which contained sufficient bacterial growth to display a turbidity of at least 100 as determined on a nephelometer. The cells were separated by centrifugation and were collected after the supernatant had been decanted. The bacterial cells were washed once in 0.02M phosphate buffer (pH 7.0) and resuspended in the same buffer solution. The organisms were aerated for 24 hrs. The volume of the cell suspension was adjusted with buffer, using a standardized turbidity *vs* dry weight scale, to a dry weight per ml of 20 mg. The rate of oxygen uptake by 0.5 ml of such a suspension was determined at 30°C in a Warburg constant volume respirometer according to the techniques of manometry of Umbreit *et al* (1957). The main cup of the Warburg flask contained 0.5 ml of cell suspension and 2.0 ml of 0.02M phosphate buffer at pH 7.0. The final volume was 3.0 ml when 0.5 ml of substrate was added from the side bulb of the flask. The centre cup contained 0.2 ml of 20% (w/v) aqueous KOH. Oxygen uptake experiments were conducted with organisms grown in nutrient broth and with those grown in simple salts medium containing 0.2% (w/v) aniline as sole carbon and energy source. The concentrations of each of the substrates, aniline, catechol, *o*-, *m*-, and *p*-aminophenol, used during the trials were adjusted so that each Warburg flask contained 2 µmole of substrate in each 3 ml volume.

RESULTS

RESULTS

A. Bacterial studies

1. Organism designated as AN-3

a. Identification

The organism was aerobic, motile in a hanging drop slide preparation, Gram negative and rod shaped, measuring, 0.75 x 1.25 μ. Individual cells were observed occurring alone or in pairs. The bacteria grew slowly at 30°C on nutrient agar. No growth could be observed at 21°C or at 37°C. Colonies were smooth, circular, raised in a convex fashion and appeared translucent. Margins were regular and spreading with a colony diameter of less than 1 mm after 3 days growth. No fluorescence was observed under either short or long wave ultraviolet radiation. When tested for the presence of oxidase and catalase, positive findings were observed. The attack on glucose was aerobic and oxidative with the formation of alkali (Hugh and Leifson, 1953). The organism was able to reduce nitrate to nitrite; no indole was produced from tryptophan and starch was readily hydrolyzed in an agar medium. Sparse growth was evident in Starkey's Medium NO. 2 (Starkey, 1934) after 3 to 4 weeks incubation. Subcultures produced reasonable turbidity within 2 to 3 weeks. The organism was observed to grow well in a simple salt medium containing 0.2% (w/v) aniline as sole carbon and energy source but was unable to utilize any of the other substrates in the concentrations with which it was challenged.

Initial observation of the bacteria by electronmicroscopy at a mag-

nification of 15 x 10^3 times, revealed a non-capsulated, rod-shaped organism, possessing a single, subpolar flagellum. Attempts to document this finding by subsequent subculturing and re-examining by electronmicrographs failed. The organism was photographed at a magnification of 15 x 10^3 times (Plate 1). The presence of a flagellum could not be documented.

The observations recorded in the foregoing paragraphs reveal the characteristics of the organism, designated as AN-3, to be in keeping with those of an organism belonging to the family Thiobacteriaceae. The displayed ability of the bacteria to derive energy from the oxidation of the inorganic sulphur compound, $Na_2S_2O_3 \cdot 5H_2O$, allows the placement of this organism within the genus Thiobacillus (Bergey, 1957).

b. Growth studies

Growth in nutrient broth was heaviest at the bottom of the tube. The sediment was noted to become slimy after 5 to 6 days of incubation. After 10 days, a slime layer was observed at the surface of liquid cultures. Growth was observed to occur only at 30°C in nutrient broth cultures as well as in cultures grown in simple salts media containing aniline. No growth was evident after 10 days of incubation at either 21°C or 37°C. At 30°C, growth was initiated within 2 or 3 days and appeared to reach a peak on day 5 or 6 of incubation on nutrient media containing no aniline.

When challenged with simple salts media containing concentrations of aniline ranging from 0.0% to 3.0% (w/v), increasing in 0.05% (w/v) increments, the cultures were observed to attain varying degrees of turbidity and the disappearance of aniline from the cultures was noted to occur on successively increasing days in cultures of from 0.05% (w/v) to 0.2% (w/v) aniline. Aniline was detected after 21 days of incubation in cultures con-

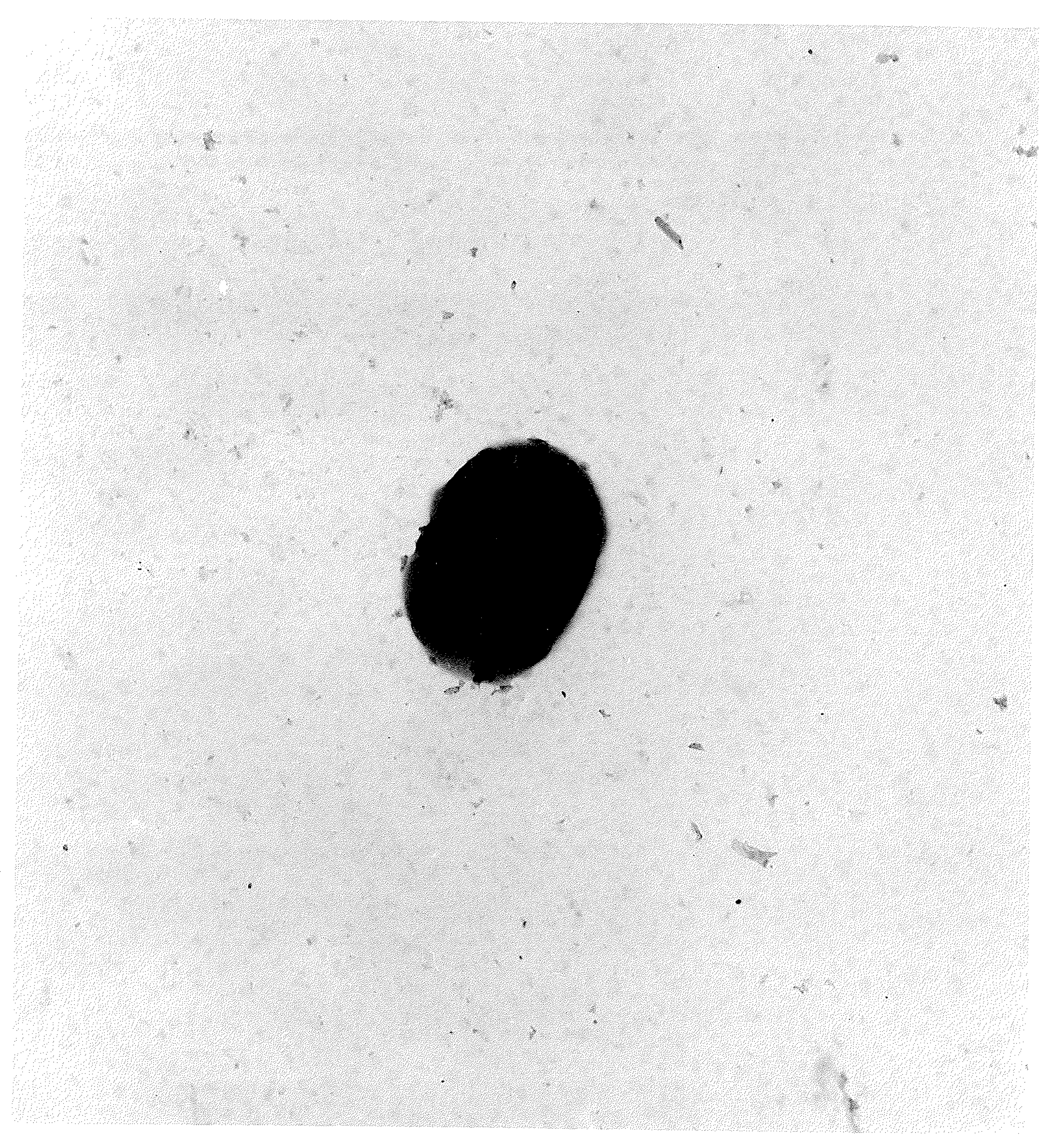


PLATE 1. Electronmicrograph of AN-3
(15×10^3 X)

taining 0.25% (w/v) and 0.30% (w/v) aniline. Figure 7 relates the relative optical opacity (turbidity) attained by the cultures at the time, in days, of the disappearance of aniline from the growth medium. The culture containing 0.25% (w/v) aniline, attained a maximum turbidity of 130, while that of 0.30% (w/v), attained a turbidity of 82 after 21 days of incubation. Figure 8 is a graphic presentation of the maximum turbidity observed in cultures at varying aniline concentrations. It may be seen that, in the growth of AN-3, increasing aniline concentrations, up to 0.20% (w/v), resulted in a corresponding increase in maximum attained turbidity. A sharp decline in maximum turbidity may be observed at concentrations of aniline in excess of 0.20% (w/v).

Increasing turbidity was recorded at 24 hr intervals and, when plotted against increase in time, a reproducable growth curve was observed for AN-3 (Figure 9). At the same time, analysis of the cultures at 24 hr intervals, for aniline content, was carried out. Residual aniline concentrations (mg/ml) were plotted against increase in time. Figure 10 graphically represents the observations respecting the utilization of aniline by a culture of AN-3.

2. Organism designated as AN-4

a. Identification

The second organism isolated, and selected for study, was aerobic, motile in a hanging drop slide preparation, Gram negative, rod shaped and measured 1.25 x 2.50 μ. Individual cells were observed singly or in pairs. The bacteria grew more rapidly than AN-3. It was observed that growth occurred on nutrient agar at 30°C, but could not be demonstrated at 21°C or 37°C. Colonies were smooth, circular, of flat, shiny surface, opaque and white in colour. The margins were regular and spreading, with a colony diameter of

2 to 3 mm after 3 days growth. A slight fluorescence was observed under long-wave ultraviolet radiation. The organism responded positively when tested for catalase and oxidase. The attack on glucose was aerobic and oxidative, with the formation of acid (Hugh and Leifson, 1953). Nitrate was reduced to nitrite. Indole was produced from tryptophan. Starch was not hydrolyzed. No growth could be observed in Starkey's Medium NO. 2 (Starkey, 1934) after 5 weeks of incubation at 30°C. The organism displayed the ability to utilize 0.2% (w/v) aniline as a sole source of energy and organic carbon, but could not be induced to utlize any of the other aromatics with which it was challenged.

Examination of the organism by electronmicroscopy, at a magnification of 15 x 10^3 times, revealed rod-shaped organisms possessing single, subpolar flagellum, occurring at either one, or both ends. An organism, possessing a single, subpolar flagellum, was photographed at 15 x 10^3 magnifications (Plate 2).

The observations recorded in the foregoing paragraphs reveal the characteristics of the organism, designated as AN-4, to be in keeping with those of an organism belonging to the family Pseudomonadaceae, genus Pseudomonas (Bergey, 1957).

b. Growth studies

When grown in nutrient broth, growth was observed to be equally distributed throughout the tube. No odour or slime could be detected. In all media, growth was observed only in cultures incubated at 30°C. Growth was initiated within 24 hours and had attained its peak within 72 hours when grown on a nutrient medium containing no aniline. In media containing aniline, growth was observed to occur at a retarded rate; the initiation occurring within 48 hours and the peak being attained only after 8 days of

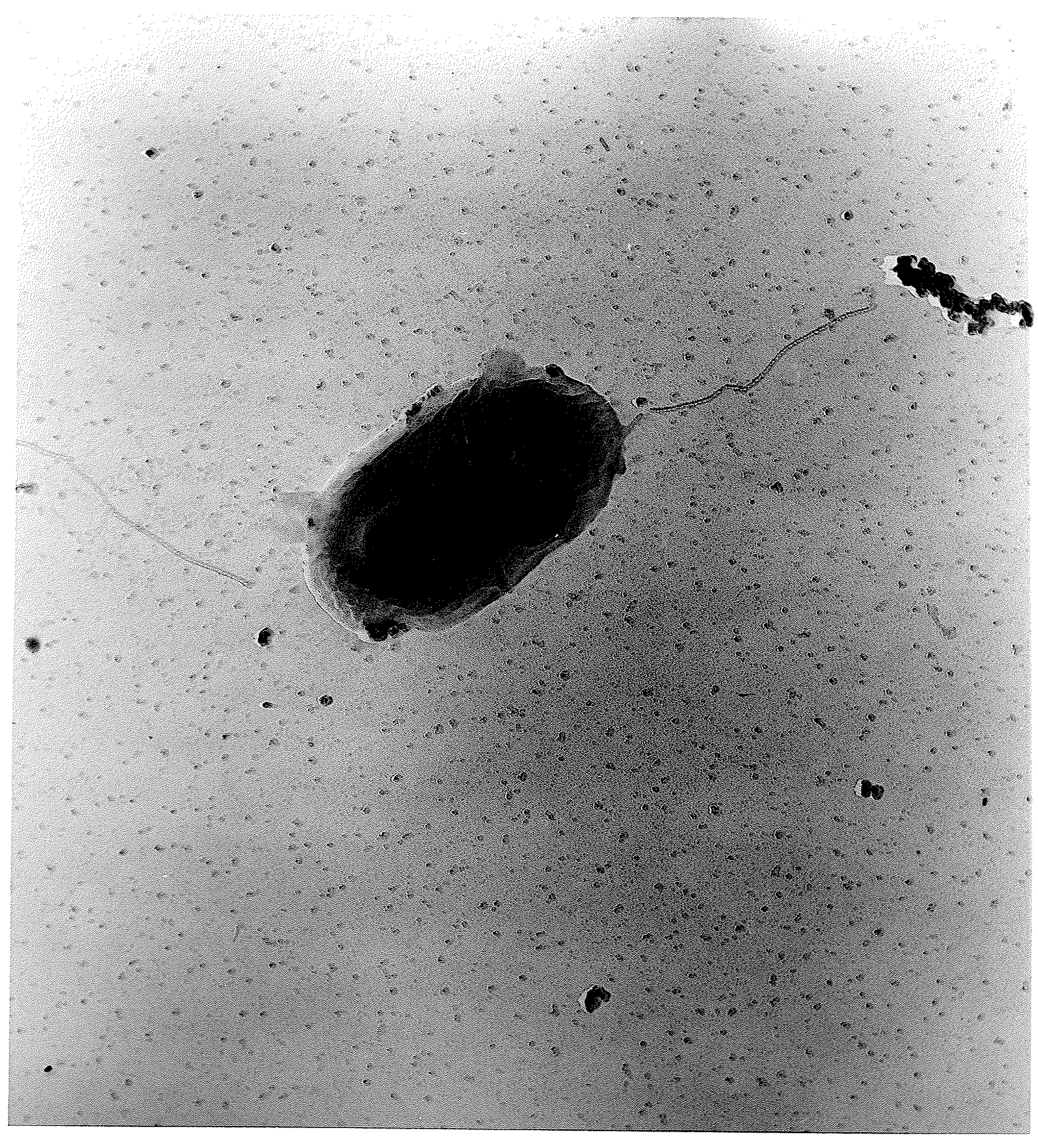


PLATE 2. Electronmicrograph of AN-4
(15×10^3 X)

incubation.

The culture was observed to respond in a fashion similar to that observed in studies of the growth of AN-3 when challenged with media of varying concentrations of aniline (Figure 7). The time taken for the complete utilization of aniline present in the media was proportional to the concentration of aniline initially present in the media, up to a maximum concentration of 0.2% (w/v) aniline. It was found that cultures were unable to utilize completely all aniline present in the media, when aniline concentrations were increased beyond 0.2% (w/v). AN-4 could utilize the available aniline more rapidly than could AN-3. The maximum turbidity attained by the cultures was also found to be dependent upon the initial concentration of aniline employed (Figure 8). A depression of the maximum turbidity attained by a culture was observed in media containing a concentration of aniline greater than 0.2% (w/v). This behaviour was also observed to be characteristic of AN-3. Observations made on the rate of growth of AN-4 and on the rate of utilization of aniline in the standard medium of simple salts and aniline 0.2% (w/v) employed in the project are recorded graphically in Figure 9 and Figure 10. The growth curve (Figure 9) was seen to be composed of an initial lag phase of 4 days followed by a logarithmic phase of 6 days duration. The slowing in growth rate was observed to coincide with the disappearance of aniline from the medium (Figure 10).

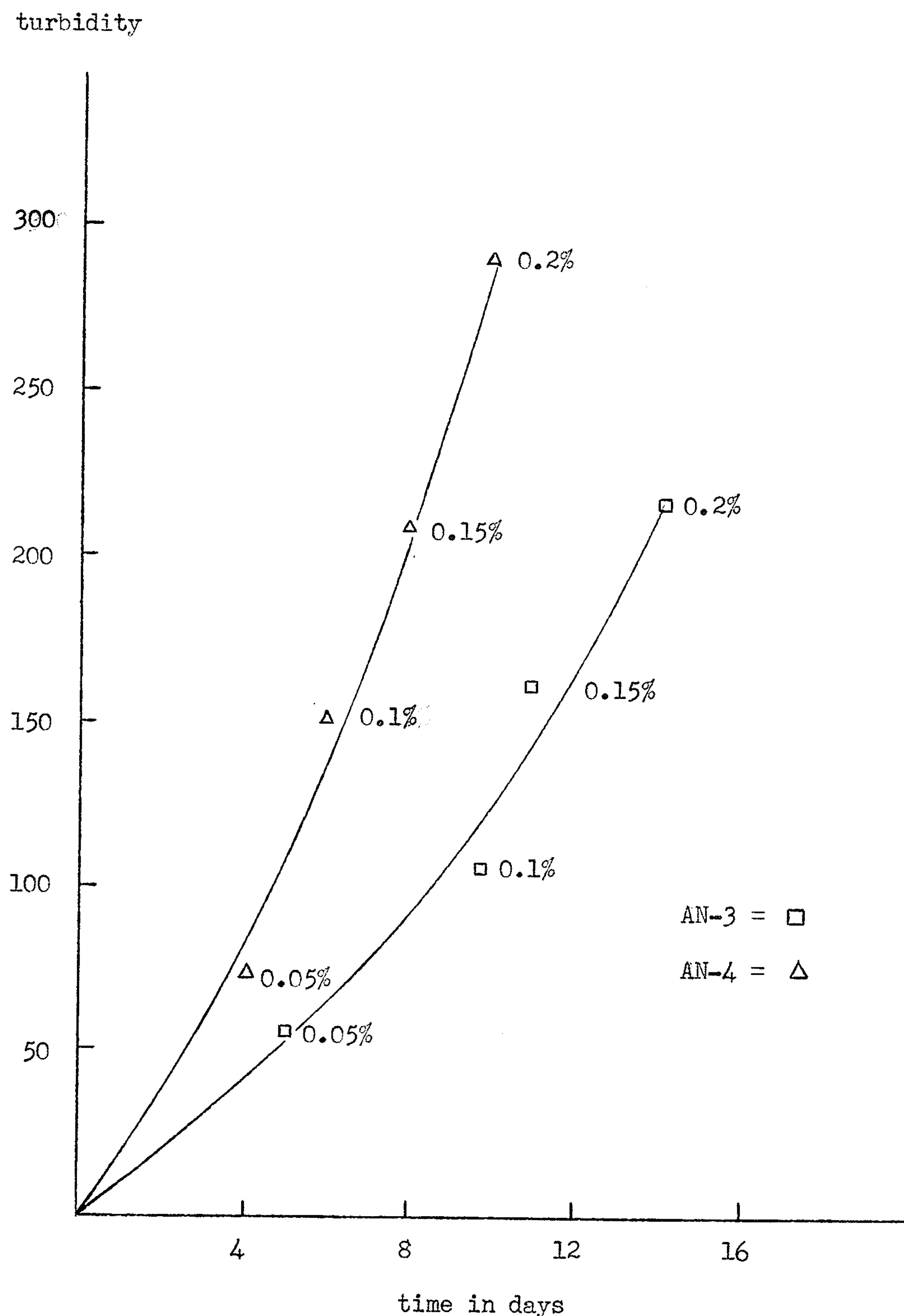


FIGURE 7. Effect of concentration of aniline (% w/v) on time of disappearance of aniline

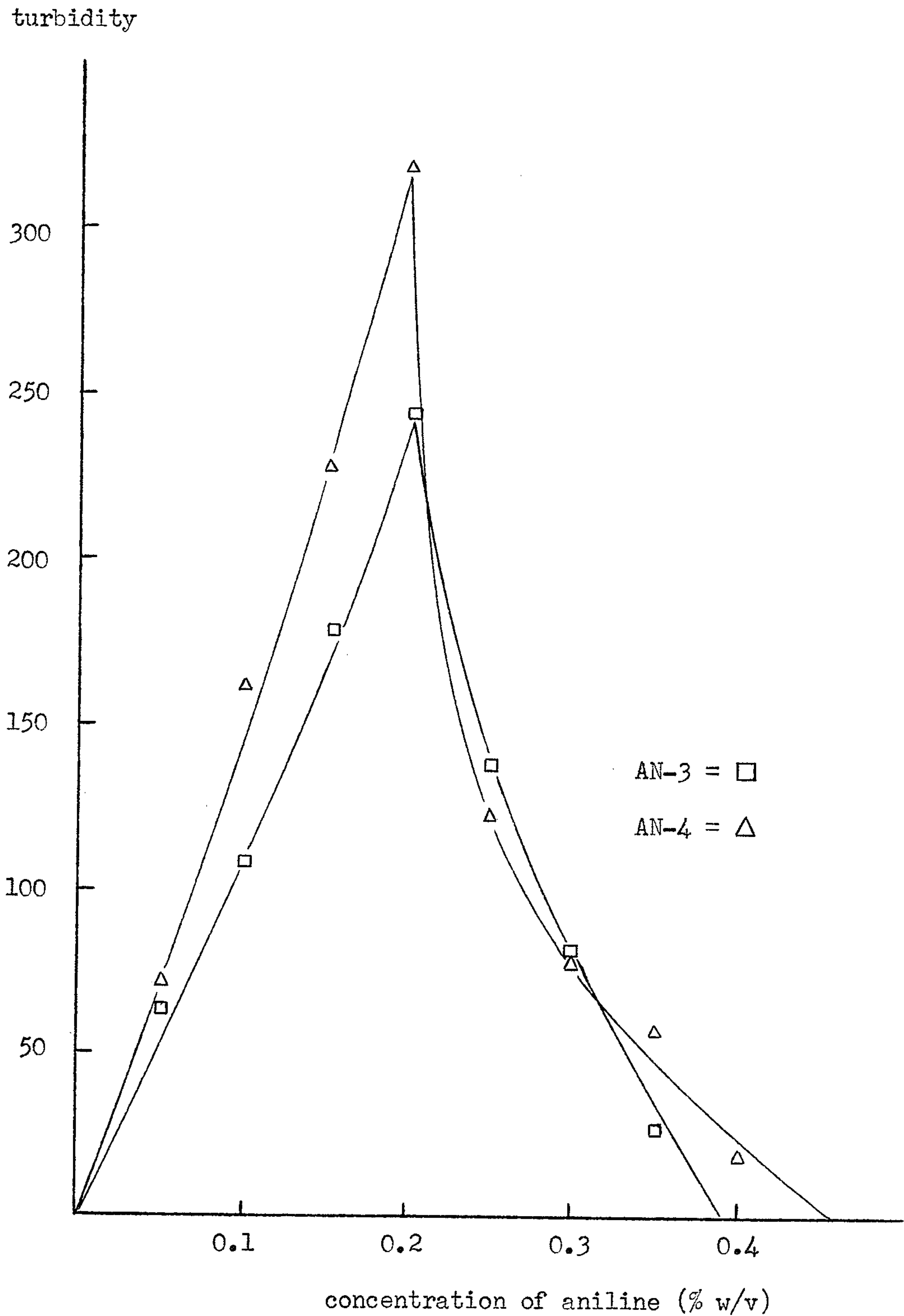


FIGURE 8. Effect of aniline on the maximum turbidity attained

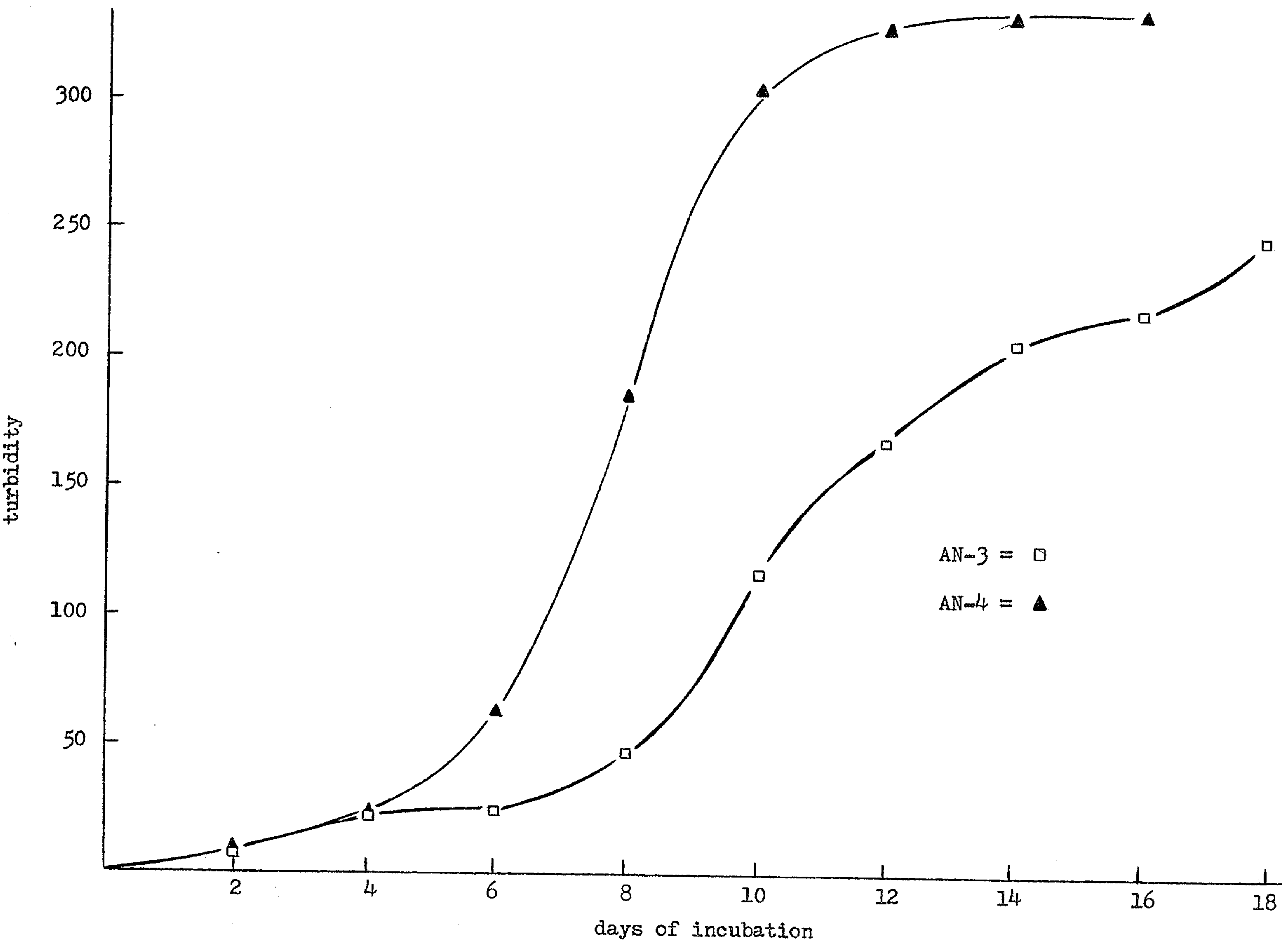


FIGURE 9. Growth in aniline 0.2% (w/v).

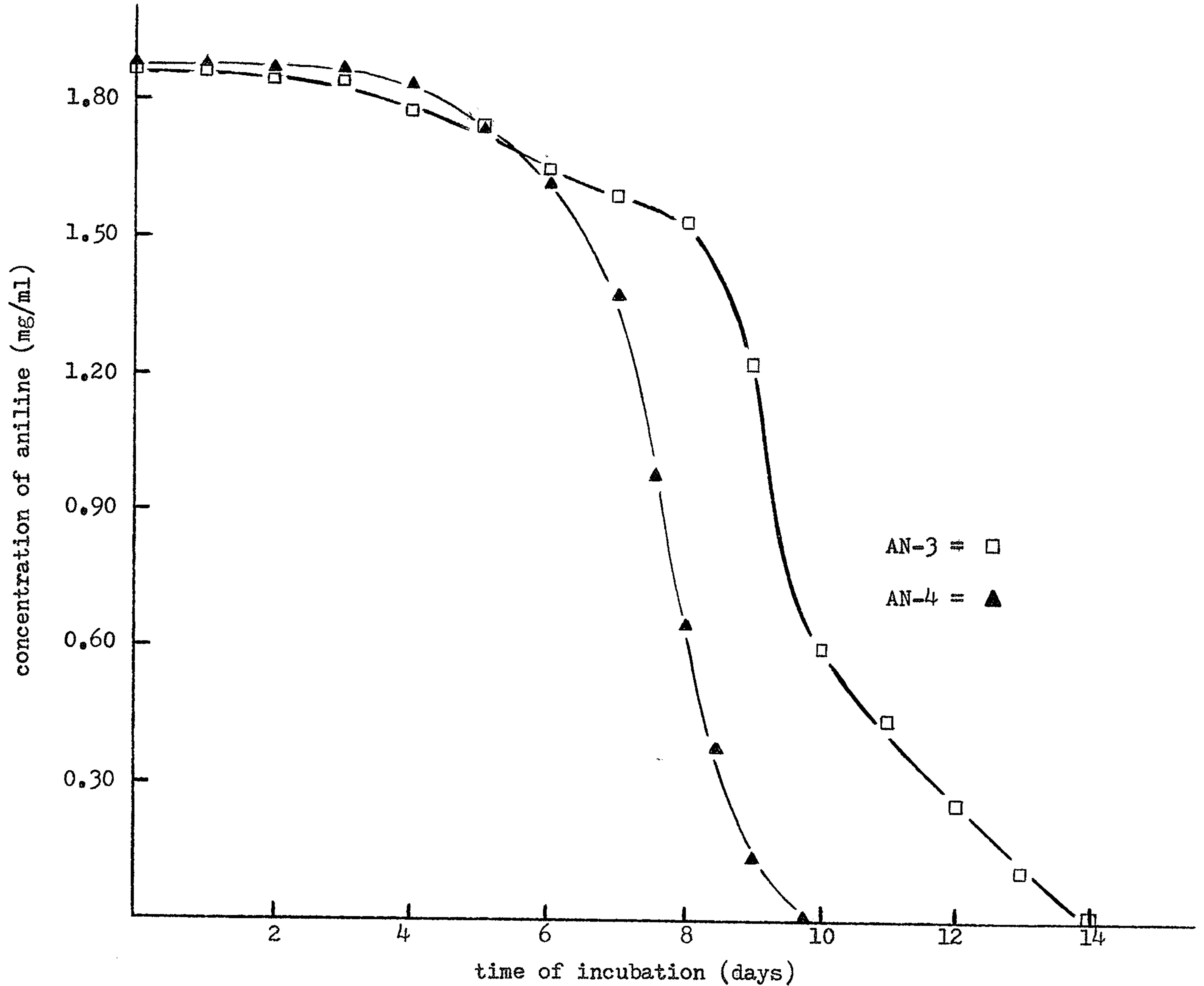


FIGURE 10. Daily utilization of aniline during incubation.

B. Oxygen uptake experiments

A culture of AN-3 was observed to attain a turbidity of 100 on day 9 or day 10 of incubation in media where aniline (0.2% w/v) was the sole source of carbon and energy and on day 2 or day 3 of incubation in nutrient broth. AN-4 grew more rapidly and was observed to attain an opacity equivalent to 100 on the 7th day with aniline and within 48 hours in the presence of nutrient broth. The cells were harvested once this level of turbidity was attained by the cultures. The centrifuged cells were washed once in phosphate buffer, aerated for 24 hours, and standardized to a dry weight of 20 mg/ml.

1. Findings with AN-3

After washing and standardization, the organisms grown on a nutrient broth medium were observed to demonstrate an endogenous oxygen consumption of 30 to 50 µl after 110 minutes at 30°C. Cells grown on aniline were observed to show an endogenous rate of 25 to 35 µl oxygen consumption after 110 minutes. Significantly greater rates of oxygen uptake were observed when the substrate aniline, catechol, or o-aminophenol was introduced from the side arm of the flask. Both m- and p-aminophenol resulted in an oxygen uptake greater than that displayed by the autorespiring organism. When organisms, grown on nutrient medium, in the absence of aniline, were employed in trials, a lag of 100 minutes was observed in the initiation of oxygen uptake in the flasks containing aniline or the aminophenols. A slight lag of 30 minutes was observed when catechol was introduced to nutrient broth grown cells. Figures 11 and 12 are graphical expressions of the observations recorded during oxygen consumption experiments in the presence of various substrates.

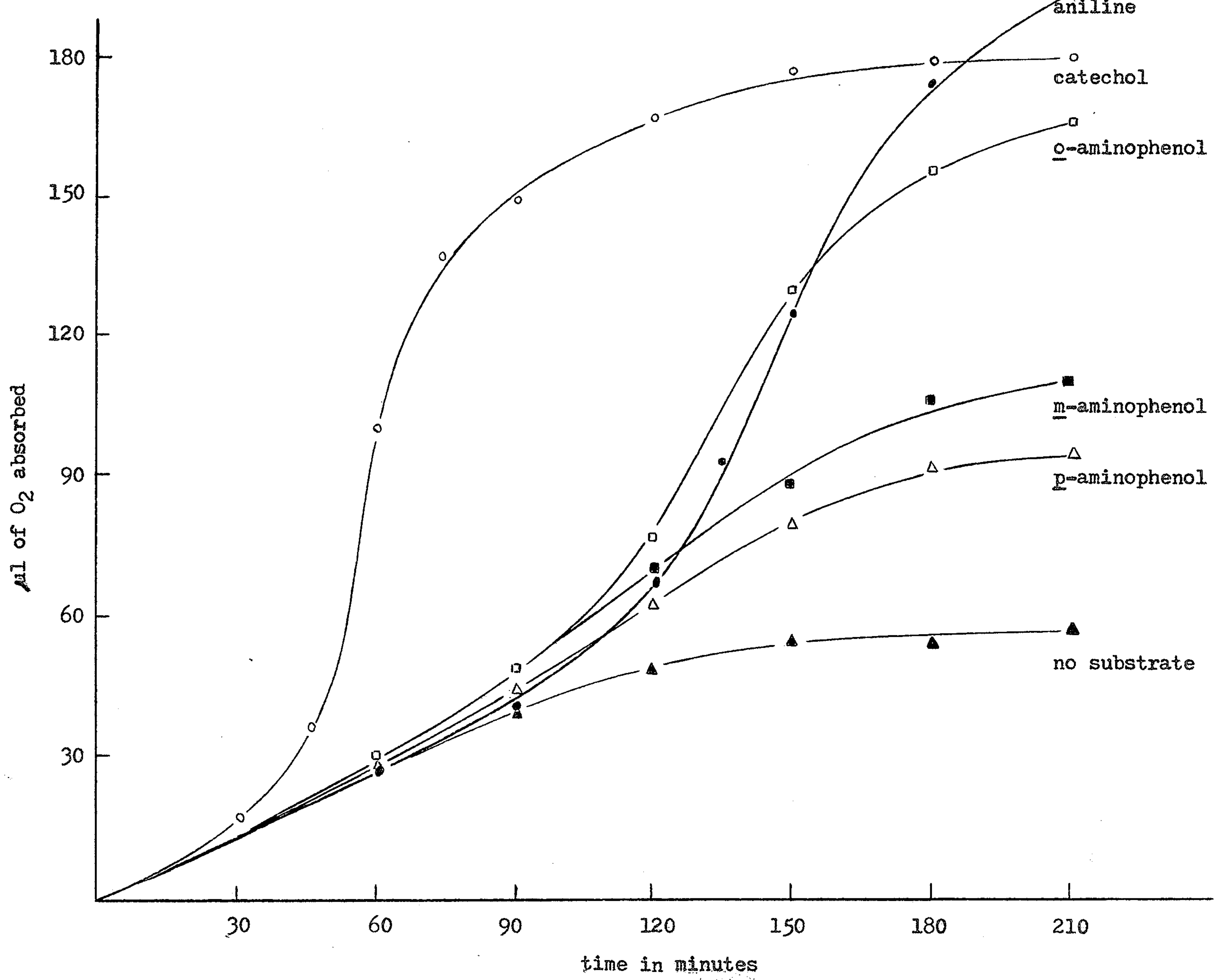


FIGURE 11. Rates of O_2 uptake by washed, nutrient grown cells of AN-3.
(plots are an average of 3 trials)

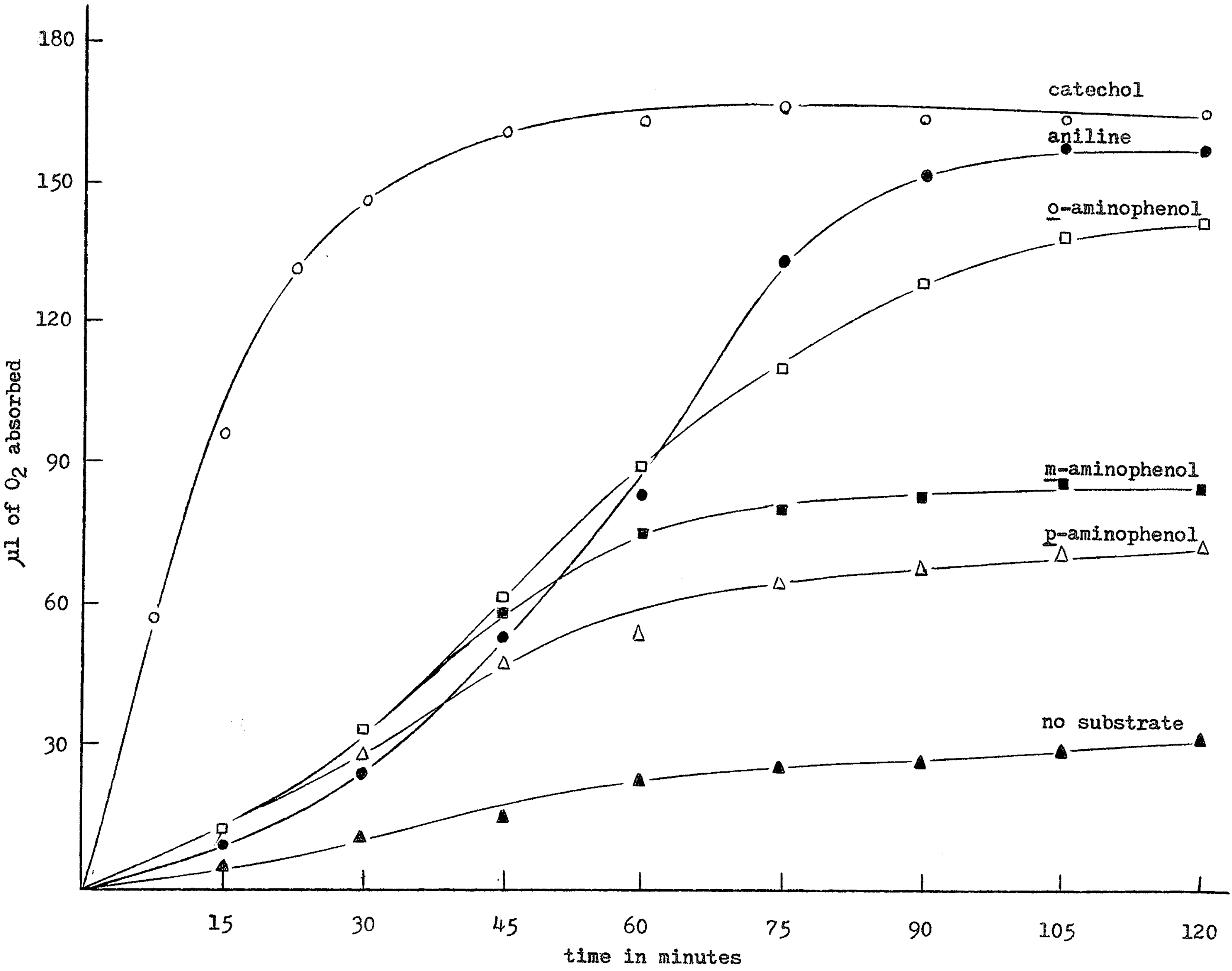


FIGURE 12. Rates of O_2 uptake by washed, aniline-grown cells of AN-3.
(plots are an average of 3 trials)

2. Findings with AN-4

Washed and standardized cells of AN-4 were observed to display an oxygen uptake of 90 to 115 ul due to autorespiration at 30°C for 110 minutes. No difference was observed in cells grown on aniline substrate from those grown on a nutrient medium. Significantly greater rates of oxygen uptake were observed when the substrate aniline, catechol, or o-aminophenol was introduced from the side arm of the Warburg flask. A lag in the utilization of aniline or the aminophenols was observed when cells grown on a nutrient medium, devoid of aniline, were used in the flasks. A short lag was in evidence when catechol was the substrate. Figures 13 and 14 record the observations made during the oxygen uptake experiments conducted with AN-4. Organisms grown on nutrient were seen to be retarded in the initial stages of oxygen uptake by the presence of aniline and to a lesser extent, by the presence of the aminophenols.

C. Chromatographic studies

1. Thin layer chromatography

Three solvent systems were found to be useful in separating aniline and the related compounds suspected as metabolites contained within the culture fluid extracts. The chromatographic behaviour of the various compounds tested are summarized in Table 3. In all cases, the spray reagent resulted in the development of an intense spot, blue in colour, for each of the compounds tested. When phenol was run in the systems, the spot produced, while of intense colour, was inclined to diffuse over a diameter of 10 to 15 mm. Adjustments in the concentrations applied, or in the size of the spots applied failed to alter this situation.

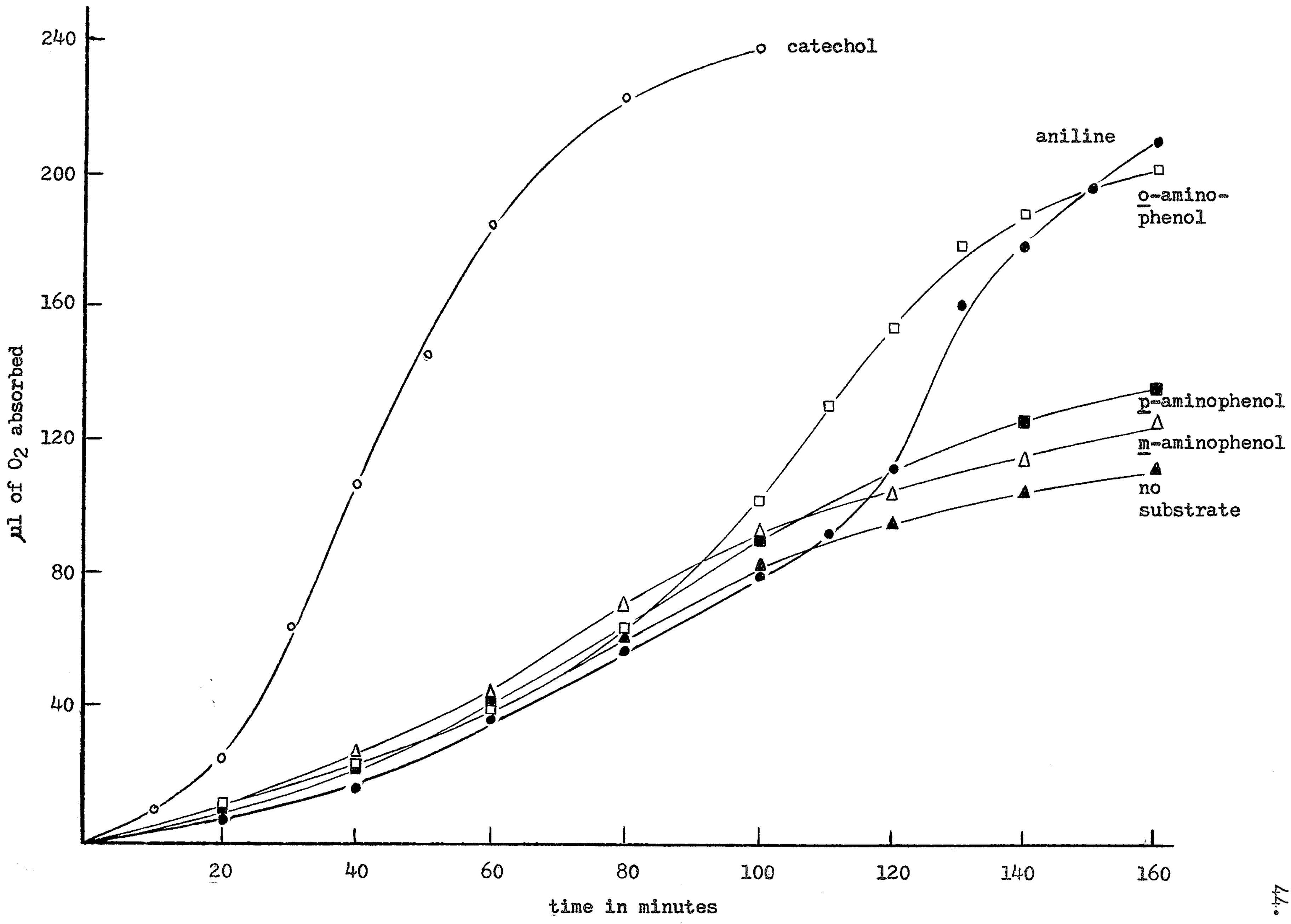


FIGURE 13. Rates of O_2 uptake by washed, nutrient-grown cells of AN-4.
(plots are an average of 3 trials)

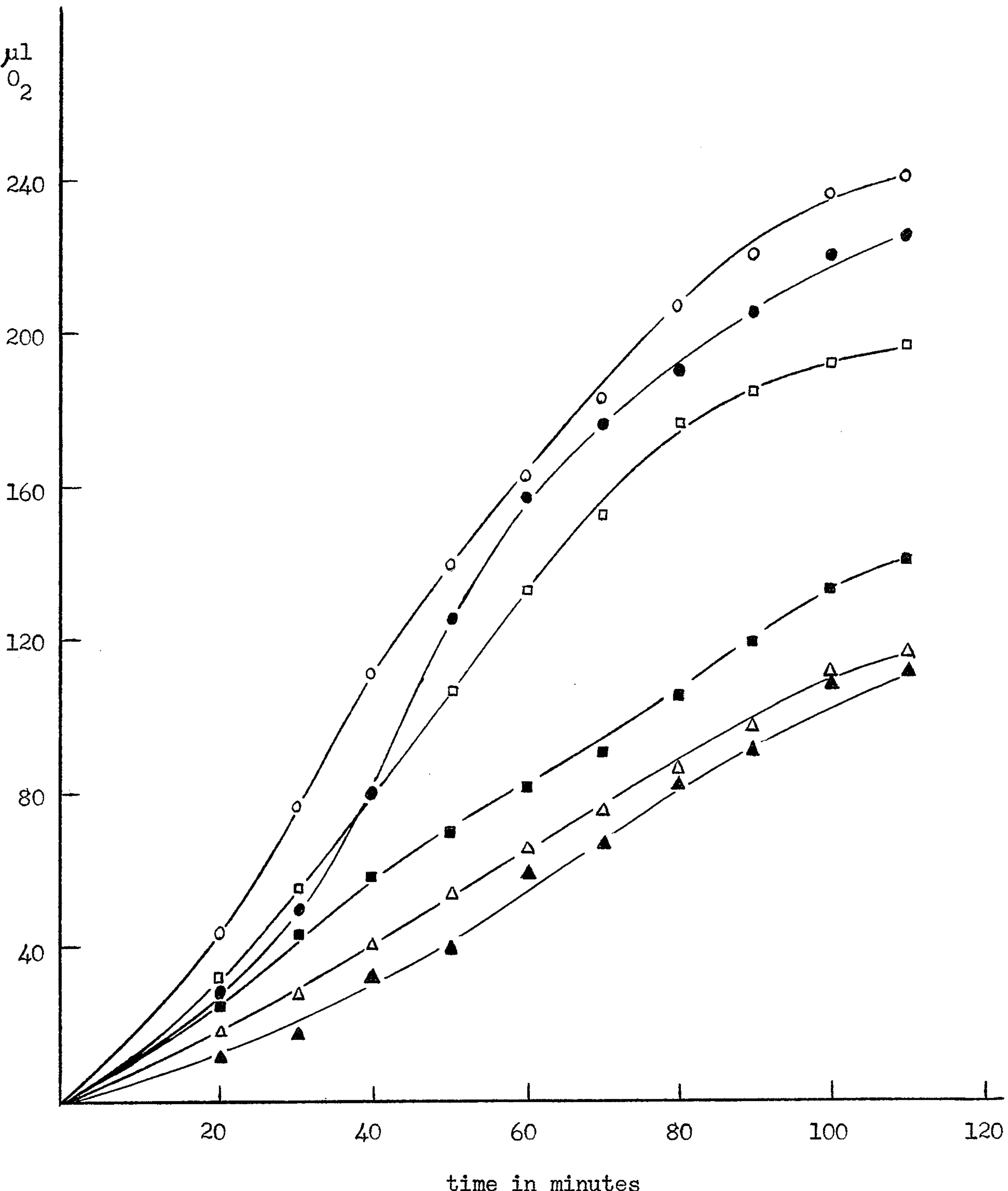


FIGURE 14. Rates of O_2 uptake by washed, aniline-grown cells of AN-4. No added substrate ▲ ; catechol ○ ; aniline ● ; o-aminophenol □ ; p-aminophenol ■ ; m-aminophenol △ .

(plots are an average of 3 trials)

When ethereal extracts of the culture fluid were spotted and run in each of the three systems, the unmetabolized aniline extracted was observed to be present in amounts which caused extreme streaking of spots; a condition which prevented the proper separation of any metabolites in the extract. The aniline, being steam volatile, could be removed from the extract by dissolving the initial residue obtained from the extract of the culture fluid in 200 ml of distilled water, adjusting the pH to 8.0, and, under vacuum, reducing this to dryness. This residue could then be dissolved in 2 ml of ether and spotted on the thin layer plates. It was observed, in prepared test solutions, that the aniline could thusly be removed and a mixture of o-, m-, and p-aminophenols could be separated into their respective characteristics spots. The culture extracts, when treated as above, were found to produce two distinct spots in the case of AN-3 and in the case of AN-4 in each of the three solvent systems used. Good separation of metabolites was also obtained when any aniline remaining, after the initial extraction procedure, was removed by separation and subsequent extraction on preparative thick layer plates. The treated extracts, when spotted and run in the various solvent systems, were observed to be resolved into two spots whose R_f values corresponded with those characteristic of aniline and o-aminophenol. This was true of extracts from cultures of AN-3 as well as extracts from cultures of AN-4. Co-chromatographing of the spots with the suspected metabolites failed to resolve the mixtures into two separate entities. Extracts of AN-3 were observed to consistently produce a spot whose R_f was similar to that of m-aminophenol in each of the three solvents. The spot produced was not well defined and extracts could not be prepared which could yield conclusive confirmation of the presence of m-aminophenol in the extract by gas liquid chromatographic techniques. Table 4 summarizes

TABLE 3

Chromatographic properties of aniline and potential metabolites of aniline

	Solvent System* R_f values x 100 in solvent systems		
Potential Metabolite	1	2	3
Aniline	52	60	78
o-Aminophenol	42	53	45
m-Aminophenol	35	42	34
p-Aminophenol	22	26	39
Phenol	69	73	74
Catechol	64	61	38
Resorcinol	59	63	31

*Solvent Systems:
1. n-butyl ether/ethyl acetate/acetic acid (50/50/5)
2. ethyl acetate/benzene (9/11)
3. chloroform/methanol 95%/NH_4OH (85/15/1)

the characteristic behaviour of the extracts. The control, presented in the table, was incubated, extracted, and purified in exactly the same manner as were the culture fluids. Only aniline was detected in the extracts obtained from the control.

2. Gas liquid chromatography

Attempts were made to separate the crude ethereal extracts from the bacterial cultures into their component metabolites by direct injection onto the gas chromatography column. Consistent peaks could not be obtained using the described column. The gas chromatography system was found to be useful in confirming the presence of a metabolite, after it had been tentatively identified and purified by thin layer chromatographic techniques. The suspected metabolites, as in Table 3, were introduced to the chromatographic column. Table 5 summarizes the retention time found to be characteristic for each compound tested.

The extract prepared from a culture of AN-3, when injected onto the chromatographic column, was observed to appear on the recorder as a tracing of two peaks, a minor peak occurring at 6 minutes, 31 seconds, and a major peak occurring at 7 minutes, 9 seconds. The retention time of o-aminophenol on the column, at this time, was observed to be 7 minutes, 5 seconds. When the sample was co-chromatographed with a standard o-aminophenol, a minor peak was observed after 6 minutes, 21 seconds, and a single, major peak appeared at 7 minutes, 9 seconds. An anthracene standard was observed to display a retention time of 7 minutes, 59 seconds. The above findings are presented in Table 6.

An extract of the culture of AN-4 was treated as above and on injection onto the gas chromatographic column was resolved into a tracing of a single peak appearing at 6 minutes, 53 seconds. o-Aminophenol was seen to

TABLE 4

Thin layer chromatographic results of ether extracts from bacterial cultures

Ether Extract	Solvent System* R_f values x 100			Identification
	1	2	3	
AN-3	50	61	76	Aniline
	42	51	44	o-Aminophenol
	32	42	33	m-Aminophenol (suspected)
AN-4	52	59	76	Aniline
	41	53	46	o-Aminophenol
Control	52	61	77	Aniline

*Solvent System:
1. n-butyl ether/ethyl acetate/acetic acid (50/50/5)
2. ethyl acetate/benzene (9/11)
3. chloroform/methanol 95%/NH_4OH (85/15/1)

TABLE 5

Characteristic behaviour of aniline and suspected metabolites on a gas liquid chromatography column*

Metabolite	Sample (µl in ether)	Sensitivity ($x10^3$)	Retention Time
Aniline	2	1	0'24"
o-Aminophenol	2	1	6'45"
m-Aminophenol	2	1	11'30"
p-Aminophenol	2	1	9'31"
Catechol	3	1	15'59"
Resorcinol	3	1	17'15"
Phenol	1	5	1'01"
Column Standard			
Anthracene	2	5	7'46"

*Column Characteristics:
- teflon column 6 ft x $\frac{1}{4}$ in
- 0.5% Amine 220 on 100-120 mesh Chromosorb G, HpAw DMCS
- column temp. 153°C

display a retention time of 6 minutes, 51 seconds. A co-chromatograph of the AN-4 extract and o-aminophenol was observed to display a retention time of 6 minutes, 57 seconds. A sample of anthracene, used as a column standard, produced a tracing with a peak appearing at 7 minutes, 49 seconds.

Extracts obtained from control cultures and subjected to the above chromatographic technique failed to produce any peaks in the tracing made by the recorder of the gas liquid chromatographic instrument, other than the peak appearing as the solvent front. It was felt that any aniline present in any of the extracts would be masked by the solvent front recorded as the aniline standard was observed to display a retention time of 24 seconds. The characteristic behaviour of the extract prepared from a culture of AN-4 is summarized and presented in Table 6.

TABLE 6

Behaviour of culture extracts on gas liquid chromatography column

Sample	Retention Time			
	Extract	Co-chromatograph	o-Aminophenol	Anthracene
AN-3	6'31" (minor) 7'09" (major)	6'21" (minor) 7'09" (major)	7'05"	7'59"
AN-4	6'53"	6'57"	6'51"	7'49"
	Sample Characteristics			
	2 µl in ether, sensitivity of 5 x 10^3	1 µl o-aminophenol, (ether) 2 µl of extract, sensitivity of 5 x 10^3	2 µl in ether, sensitivity of 1 x 10^3	2 µl in ether, sensitivity of 5 x 10^3

DISCUSSION

DISCUSSION

Both bacteria isolated were observed to be Gram negative, rod-shaped, motile, aerobic and oxidative, and gave positive reactions to tests for catalase and oxidase. The above characteristics are consistent with those of organisms belonging to the family Pseudomonadaceae. On examination, the organism designated as AN-3 was found to possess the ability to utilize $Na_2S_2O_3 \cdot 5H_2O$ as a sole source of energy. Therefore, the organism, AN-3, was classified as belonging to the family Thiobacteriaceae, genus Thiobacillus, in accordance with the accepted classification of Bergey (1957). The organism, AN-4, displayed the biochemical and physical characteristics consistent with those of bacteria of the family Pseudomonadaceae, genus Pseudomonas (Bergey, 1957).

During the growth studies conducted with the bacteria it became evident that the amount of growth obtained, as determined by the maximum turbidity displayed by the cultures, was dependent upon the concentration of aniline present, up to concentrations of 0.2% (w/v). A rapid drop in maximum turbidity was noted in cultures, and an inability to completely metabolize the total carbon source present as aniline when the medium concentrations were increased beyond 0.2% (w/v) was evident. Aniline was found to be increasingly inhibitory, or bacteriostatic, with increasing concentrations of aniline. Growth was both reduced and retarded in rate at a concentration of aniline of 0.25% (w/v) and, was moreso, at a concentration of 0.3% (w/v). The concentrations of aniline employed in the present study were greater than those used in the work of Walker and Harris (1969). Their study was

carried out with a Pseudomonas isolated in a simple inorganic salts medium containing 0.05% (w/v) aniline. The subsequent growth studies were conducted in a medium of 0.1% (w/v) aniline. The present project has shown that bacterial organisms, such as Pseudomonas, can be found which will grow well in culture media containing up to 0.2% (w/v) aniline as the sole source of carbon and energy. In addition, an organism able to utilize an inorganic sulphur compound as a source of energy, a Thiobacillus, has been isolated and been shown to be capable of reasonable growth with 0.2% (w/v) aniline as the sole carbon and energy source.

The growth curves displayed by the bacteria under study were remarkable in that an exceedingly slow rate of growth was observed, with AN-3 showing a slower rate of increase in turbidity than AN-4. The rate of growth was observed to correlate with the rate of aniline utilization. A sharp reduction in growth rate was observed when the carbon source, aniline, could no longer be detected in the medium. At no point did the growth curves displayed allow for the possibility of the presence of a stage at which an accumulation of a metabolic intermediate might occur. The formation of a plateau during the logarithmic phase of growth could have been interpreted as a stage at which such accumulation might occur. AN-3 was observed to produce a growth curve with a minor plateau appearing after 14 days of growth. At this time, no aniline could be detected in the medium. It may be postulated that the slight increase in growth rate observed between the 14th and 18th days of incubation was due to the utilization of metabolic intermediates which had accumulated in the medium. The removal of the aniline from the medium would remove the inhibitory effect on growth characteristic of the amine and would naturally be followed by an increase in growth rate if some energy source were available.

The failure of the bacteria to exhibit a characteristic plateau during growth presented difficulty in determining at which stage a culture should be examined for possible metabolic intermediates. The cultures were examined at several stages of growth but at all times great difficulty was encountered in extracting any aromatic component other than aniline. The most satisfactory results were obtained from extracts of culture medium taken during the early logarithmic phase of growth once a method had been devised for the removal of the aniline which had seriously complicated all thin layer chromatographic separation procedures attempted. The presence of aminophenols, in detectable amounts, during the earlier stages of incubation, was felt due to their having been present in the aqueous growth medium for a relatively short period of time and, therefore, not having undergone a significant degree of chemical oxidation, remaining detectable. No aminophenols could be detected during the latter stages of growth.

Oxygen uptake experiments showed that both the organisms were able to utilize, to a greater or lesser extent, all of the substrates with which they were challenged. Results of the trials implied a preference in the utilization of aniline, o-aminophenol, and catechol. Catechol, a well established intermediate in the breakdown of many aromatic compounds, was immediately oxidized by organisms grown on aniline, as well as by those grown on nutrient. A definate lag was observed in the rate of oxygen consumption when nutrient grown organisms were challenged with aniline and the aminophenols. No such lag was in evidence when aniline grown organisms were used. The utilization of aniline and the aminophenols, by the bacteria under study, would appear to be due to the presence of an inducible enzyme system. The utilization of catechol by these bacteria appears to be independent of this enzyme system. The findings of the present study are in agreement with the

results reported by Walker and Harris (1969). In their study, an inducible enzyme system was implicated in the bacterial utilization of aniline, o-aminophenol, and p-aminophenol.

The present study provides evidence that supports the bacterial utilization of aniline through the formation of aminophenols as metabolic intermediates. A moderate rate of oxygen uptake was observed in all trials of respirometry where aminophenols served as substrate. o-Aminophenol was isolated from the culture medium and was identified. A moderate rate of oxygen uptake by the aminophenols is not convincing evidence that they are intermediates in the breakdown of the aromatic amine, aniline. The isolation and identification of o-aminophenol, however, confirms its presence in the growth medium. The occurence of o-aminophenol may be concluded to result as a product of the bacterial degradation of aniline, since no traces of o-aminophenol could be found when control cultures (growth medium incubated but not inoculated) were subjected to identical extraction procedures as the bacterial cultures.

No precedent could be found in the literature for the occurence of o-aminophenol as an intermediate in the degradation of aromatic substrates by microorganisms. The workers, Walker and Harris (1969), had based their entire presentation upon the findings resulting from trials at respirometry. p-Aminophenol presented a greater total oxygen consumption than had o-aminophenol. Also, the literature provided support for the occurence of the para substituted aminophenol as the intermediate in the degradation of aniline. p-Aminophenol was regarded as a possible intermediate at the conclusion of their report.

Sloane et al (1951), Sloane et al (1954), and Sloane and Untch (1964) working with Mycobacterium smegmatis have demonstrated the formation of p-

aminophenol from aniline during the degradation of p-aminobenzoic acid. McCullough et al (1957) reported the formation of aniline from anthranilic acid by cell-free enzyme preparations of E. coli. The enzyme preparations were reported capable of converting p-aminobenzoic acid to p-aminophenol. The experimental findings of the current investigation are not in agreement with the consensus of a para substitution of the hydroxyl on the aromatic ring.

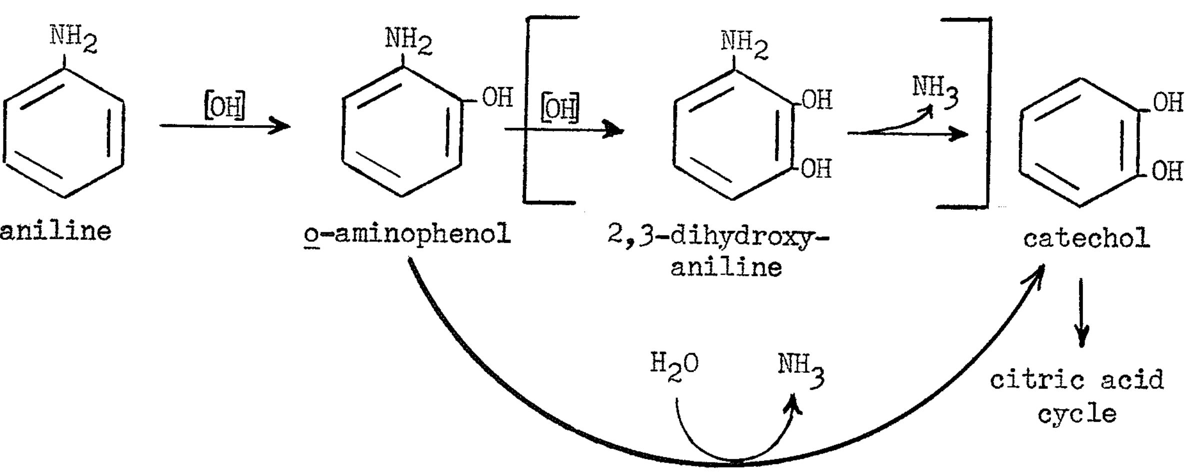
Work of Shaw and Aziz (1971), conducted concurrently with the present project and employing similar experimental techniques, has provided encouraging support for the implication of o-aminophenol as the biochemical intermediate in the degradation of aniline by some bacteria. Attempts to implicate p-aminophenol in the metabolic pathway under study have met with failure in all respects other than the trials at respirometry producing a slight consumption of oxygen by the bacterial cells under test. Attempts during the present project, and the project of Shaw and Aziz (1971), have failed to demonstrate the presence of p-aminophenol in extracts of culture medium. An ortho substitution of the aromatic ring is the suggested means of hydroxylation.

Catechol has been postulated as an intermediate in the catabolism of aniline (Walker and Harris, 1969). Catechol has been identified as an intermediate in the degradation of anthranilic acid (Taniuchi et al, 1964). Both workers reported of a degradation due to the metabolism of an aromatic by bacteria of the genus Pseudomonas. It is generally accepted, and supported in the literature by experimental findings, that dihydroxylation is a prerequisite for enzymatic fission of the aromatic ring. Catechol has been implicated as a definate intermediate.

In light of the support afforded by the literature for the involve-

ment of catechol in the degradation of aromatics by bacteria and, in view of the evidence presented for the ability for the bacterial organisms being studied to utilize catechol as a substrate during oxygen uptake experiments, the findings of this project suggest that catechol may be a metabolic intermediate in the bacterial degradation of aniline by the microorganisms studied.

The presented experimental results may be interpreted as the following scheme for the degradation of aniline by both the bacteria under study. The microbial degradation proceeds via:



The conversion of aniline to o-aminophenol is confirmed by the presence of the aminophenol in the culture fluid extracts. The involvement of catechol in the metabolic pathway is probable in light of the experimental results obtained during the respirometry trials and the support afforded catechol by the literature as an intermediate in aromatic ring fission due to microbial activity.

If deamination were to occur following the monohydroxylation, the resulting metabolite would be phenol. During the study, it was observed

that the organisms were not able to utilize phenol as a sole source of carbon and energy. It would be expected, if phenol were an intermediate, that any accumulation of phenol in the medium would result in a rapid inhibition of growth. The bacteria were observed to completely degrade the available aniline. In fact, the amount of aniline present in the medium was found to be a growth limiting factor, an acceleration of growth occurring in the presence of concentrations of less than 0.2% (w/v) as the concentration of aniline was allowed to approach the optimum. With an increase in the concentration of aniline, an increase in the production of metabolic intermediates would result. An increase in the rate of production of phenol would be expected to produce growth inhibition. Inhibition of growth of the organisms was not observed. Phenol could not be detected in the extracts examined. Phenol was not suspected as a possible metabolic intermediate.

The presence of 2,3-dihydroxyaniline could not be confirmed. Thin layer chromatographic techniques did not provide any evidence to suggest the involvement of this compound as an intermediate. Its formation appears most probable when the status of the monohydroxy compound, o-aminophenol, is considered, and when the formation of phenol is not considered. The chemical supply houses were unable to supply 2,3-dihydroxyaniline. Time did not permit the synthesis of the compound in the laboratory. It was not possible to conduct oxygen uptake experiments utilizing the dihydroxyaniline as a substrate.

Study is currently underway, under the supervision of Dr. J. A. M. Shaw, to determine the extent of involvement of o-aminophenol, 2,3-dihydroxyaniline, and catechol as intermediates in the microbial degradation of aniline and the many compounds available containing the aniline moiety. It is hoped that continuing study will support the experimental findings presented

and will further the understanding of the metabolism of the anilines by microorganisms. Further study of aniline oxidation might render more meaningful results if cell-free preparations are used. A study involving the various enzymes responsible for the degradation of the aromatic amines might better elucidate the mechanism of the oxidation.

CONCLUSION

CONCLUSION

The metabolism of aniline by soil bacteria was studied. Experimental results were presented which suggested the involvement of o-aminophenol and catechol as intermediates in the microbial degradation of aniline. An aminophenol was extracted from culture fluid and was identified as o-aminophenol. A pathway for the degradation of aniline through o-aminophenol, 2,3-dihydroxyaniline, and catechol was suggested.

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