BIOCHEMICAL MECHANISM OF THE REDUCTION OF NITROBENZOIC ACIDS BY BACTERIA

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CHAPTER I

INTRODUCTION

The biochemical mechanism(s) involved in the conversion of nitrate nitrogen to proteins by microorganisms has yet to be fully elucidated. Numerous workers over a period of approximately 75 years have pursued this study from many perspectives. This phenomenon of nitrate reduction, not only by bacteria but by plants, can be considered a basic life process. It is plausible to conclude that this fundamental knowledge might throw light on the related processes of denitrification and nitrogen fixation as well as contribute to a better understanding of the overall metabolic pathways of nitrogen metabolism.

A search of the literature has revealed that inorganic nitrates have been the predominant compounds used in experimental investigations on this problem. A prime objective, in these attempts to uncover this nitrate reduction scheme, has been to elucidate one or more common intermediates which might be considered immediate precursors of amino acids by bacteria. The complexities of such metabolic studies have been innumerable and this major fact could, conceivably, justify the controversy that exists among some workers.

Considerable evidence has been reported in support of the reduction of nitrates to ammonia by the following scheme: nitrates ⟶ nitrites ⟶ hydroxylamine ⟶ ammonia. By relating the above reductive stages to the oxidation states of the nitrogen, a step wise reduction seems apparent (1). There is a net gain of 8 electrons when nitrate is reduced to ammonia, and it is commonly assumed that this should take

place in 2 electron changes as exemptified below:

$$-NO_3^- \longrightarrow -NO_2^- \longrightarrow (X) \longrightarrow NH_2OH \longrightarrow NH_3$$

(+5) (+3) (+1) (-1) (-3)

Speculation as to the identity of (X) has been centered around nitroxyl (HNO), hyponitrous acid ($H_2N_2O_2$), and nitramide (NH_2NO_2); however, no such products have been detected in any system investigated. An enzymatic complexing with these intermediates might occur, rendering analysis for these compounds relatively non-revealing.

Some disagreement has developed concerning the contribution of hydroxylamine to the general pathway of nitrate reduction. One reason for this is that its accumulations in media have been in extremely small concentrations. Efforts by Rosenblum and Wilson (2) and other workers (3) have shown that hydroxylamine is slightly toxic to bacterial growth in concentrations as low as 1-2 micrograms per ml. of medium. In slight contrast to this Lees (4) demonstrated that the utilization of hydroxylamine at 1.5 micrograms per ml. of medium was as rapid as for the same quantity of ammonia. It is a well-established fact that much higher concentrations of ammonia are utilized by microorganisms without any toxicity.

To complicate the situation Suzuki and Suzuki (5) found ammonia in media where bacteria were growing on hydroxylamine whereas Lemoigne and Desveaux (6) and others (7) detected hydroxylamine when ammonia was the source of nitrogen.

Lees and Quastel (8) reported the 0.005 M sodium pyruvate in media completely repressed the toxic effect of 0.005 M hydroxylamine. They arrived at the following explanations:

Toxic Less Toxic

In further support of this Quastel (9) found that many oximes do not exhibit any appreciable toxicity to bacterial growth. Weinrich (10), in contrast to this, was unable to demonstrate a utilization of pyruvic oxime-nitrogen by bacteria.

Silvers and McElroy (11) postulated a pyridoxal phosphate tie up of the free hydroxylamine resulting in oxime production. This could, possibly, account for greater formations of hydroxylamine occurring but not being detected in the media.

Taking in consideration the high toxicity of free hydroxylamine and the lower toxicity of oximes, it was felt that a mechanism study might best be approached through investigating the reduction of aromatic-nitro compounds. In utilizing an aromatic ring it was assumed that increased stability of possible intermediates such as nitroso and hydroxylamino compounds would occur. Nitrobenzoic acids were selected for this study with the prediction that if any bound hydroxylamine was formed by reduction it would be of low toxicity to bacterial growth. Using this line of reasoning it was thought that a greater accumulation of these compounds would appear in the media, permitting more definite identification. The overall objective was to construct a model nitrite reductive scheme which could, conceivably, throw light on the pathway of inorganic nitrate reduction.

Apparently very little work has been done in this direction and because of this, references, other than those concerning bacteria had to be resorted to in order to obtain a basis for initiating the laboratory

investigation.

Bray, Hybs, and Thorpe (12) demonstrated rapid reduction of 2:3:4:5-tetrachloronitrobenzene and p-nitrobenzoic acid to the corresponding amino compounds by bacteria from the intestines of rabbits. In other experiments rabbits were given nitrobenzene, m-nitrophenol, o-, m-, and p-nitrobenzoic acids and their amides. Upon analyzing the intestinal contents of the freshly killed animals the expected amines were identified. Other metabolic studies with the rabbit have produced indications of the formation of hydroxylamine derivatives (13).

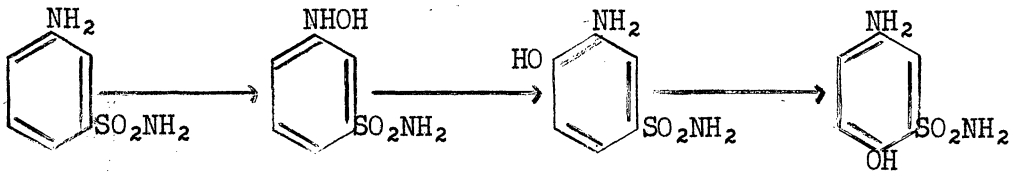
TABLE I

Metabolites of Nitrobenzoic Acids and Amides in the Rabbit

Compound	Percentage of Dose Excreted as		
	Amino Compound, (Free)	Amino Compound, (Acetylated)	Hydroxylamine Derivative
o-Nitrobenzoic Acid	20	0	2.6
m-Nitrobenzoic Acid	4	7	0.9
m-Nitrobenzamide	2	23	1.0
p-Nitrobenzoic Acid	1	10	0.7
p-Nitrobenzamide	2	13	1.0

Bacterial reduction of such aromatic-nitro compounds as Chloramphenicol (D-(-)threo-1-p-nitrophenyl-2-dichloroacetamide-1,3-propanediol) to the corresponding amine was reported by Smith and Worrel (14).

Dobson and Williams (15) studied the metabolism of metanilamide (m-aminobenzenesulphonamide) in the rabbit and found that 4-hydroxy metanilamide was an excreted product. Although no evidence of a hydroxylamine group was found they suggested the following as one possible scheme.



A potential oxidative rearrangement of the hydroxylamino group is not likely; although if it was and actuality it would give some support to aromatic hydroxylamine formations in media from aromatic-nitro compounds.

More work appears to have been done on the reduction of phenyl-nitro compounds by enzymes than by bacteria. A knowledge of the enzymatic action, however, could be very revealing insofar as the actual reductive mechanism(s) is concerned.

Yamashina and others (16) took cell-free enzyme preparations from a halogen-tolerant bacterium. This enzyme system catalyzed the reduction of o-nitroso- and o-hydroxylaminebenzoic acids to anthranilic acid by leucomethylene blue or by a dehydrogenase system in the presence of a hydrogen carrier. From reaction velocity studies and paper chromatographic investigations of the reaction mixtures, the scheme of reduction, $ArNO_2 \longrightarrow ArNO \longrightarrow ArNHOH \longrightarrow ArNH_2$, was purposed.

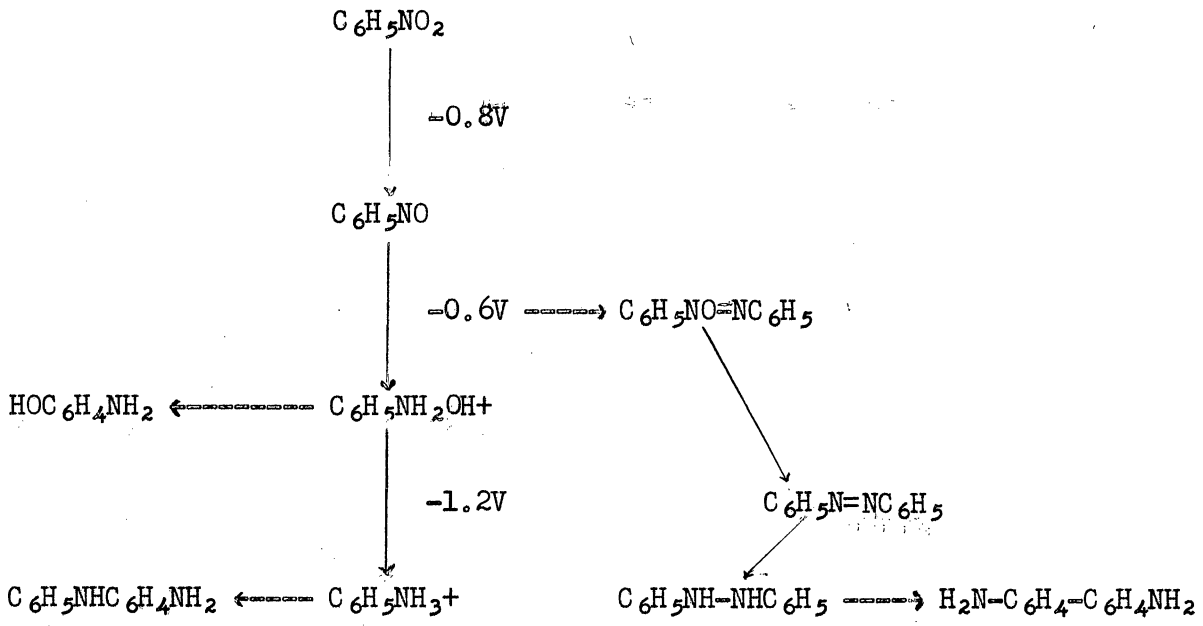
In other experiments the nitrate reductase from Bacillus pumilus was found capable of reduction of aromatic-nitro compounds to the corresponding amines in the presence of a dehydrogenase, a hydrogen donor, and a dye (17). From experiments on competition studies, it was found that the nitro reductase was not identical with the nitrite reductase. Greenville and Stein (18) showed that various enzymes were capable of reduction of dinitrophenols when the reaction was permitted to occur on suitable substrates. Dinitrophenols were reduced by xanthine oxidase, formic dehydrogenase, and lactic dehydrogenase. With xanthine

oxidase no extraneous carrier was necessary.

Saz and Slie (19) found that cell-free extracts of Escherichia reduced the nitro groups of chloramphenicol and p-nitrobenzoic acid when L-cysteine was present. They stated that DPNH could serve as the hydrogen donor. In other enzymatic studies a partially purified enzyme preparation from Neurospora catalyzed the reduction of m-dinitrobenzene by reduced nucleotides to nitro aniline. (20)

The rapid reduction of p-nitrobenzoic acid by ascorbic acid and reduced glutathione was found by Rosenthal and Bauer (21). With ascorbic acid 91% of the aromatic acid was reduced to 4-hydroxylaminebenzoic acid was only a trace to 4-aminobenzoic acid. In comparison, glutathione-SH produced 65% of the corresponding hydroxylamine and 34% of 4-aminobenzoic acid. Such compounds are believed to be contained in microorganisms.

Haber and Schmidt (22), in their studies of the electrolytic reduction of nitrobenzene, found evidence to support the following scheme:

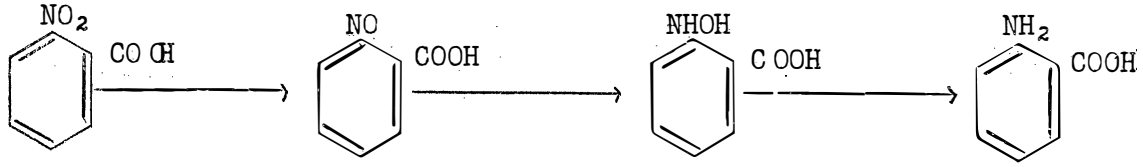


The first step required a relatively high overvoltage. The striking observation was that the phenylhydroxylamine could attain a reversible equilibrium with the nitrosobenzene. Because of the much higher energy barrier, the step from the hydroxylamine to aniline is not reversible. The side products are considered secondary reactions. Conant (23) found that a higher overvoltage favored the complete reduction of nitrobenzene to aniline in acid solutions, and this was accounted for by the fact that if any phenylhydroxylamine was formed it was rapidly reduced, thereby, hindering all side reactions.

Polarographic investigations of the reductions of aromatic-nitro compounds to the respective amines were found to take place in only two steps (24). Basic solution resulted in the reduction stopping at the hydroxylamine stage.

From the above it is plausible to conclude that the effective reduction of similiar compounds by bacteria would depend upon such things as the pH of the media and the reductive capabilities of the participating enzymatic systems. It is conceivable to assume that more energy would have to be utilized in converting possible hydroxylamine intermediates to the corresponding amines than for other compounds of the reductive pathway.

On the basis of the above findings the following scheme for the bacterial reduction of nitro-substituted benzoic acids is postulated:



In summary, bacteria, as well as various enzymatic systems have been found to reduce a limited number of aromatic-nitro compounds to

the corresponding amines with some evidence of aromatic hydroxylamine formation. With this in mind, a study was initiated to uncover the biochemical mechanism(s) of the reduction of nitrobenzoic acids by microorganisms with the hope that the knowledge found would contribute to establishing more completely the inorganic nitrate reductive pathway(s) of bacteria.

CHAPTER II

MATERIAL AND METHODS

Organisms: A bacterium, capable of rapid growth on o-nitrobenzoic acid as the sole carbon and nitrogen source, was isolated from the soil. Durham and Gee, of the Department of Bacteriology at Oklahoma Agriculture and Mechanical College, have tenatively identified the organism as a member of the genus, Flavobacterium. Microscopic examinations showed the cells to be rod shaped and relatively slow moving. Attempts to obtain comparable growth on p-nitrobenzoic acid have been futile. Neumann (25), in his work with the strain, observed fair utilization of inorganic nitrates, and by isotopic means demonstrated that the organism preferentialy used NH_4-N when this form and NO_3-N were available. Optimum temperature and other conditions of growth have not been determined. Rapid growth has, however, been observed at 34-36°C. in still and aerated cultures.

A strain, identified as Pseudomonas fluorescens and also isolated from the soil, was found capable of good growth on p-nitrobenzoic acid as the carbon and nitrogen sources. The motility of the cells was much faster in comparison to the Flavobacterium sp., and appeared as smaller rod-shaped organisms. Another distinguishing characteristic was that the medium became greenish-blue as the cell growth progressed. Some correlation might exist between this greenish-blue pigment and that noted by Cushing and Reid (26), which was produced by Neurospora crassa growing on p-aminobenzoic acid.

Isolation of Organisms: This was accomplished by resorting to enriched-media techniques. In case of the Flavobacterium sp., medium

of the following composition was innoculated with soil: o-nitrobenzoic acid, 0.2%; KH_2PO_4, 0.1%; $MgSO_4$, 0.05%; $CaCl_2$, 1.0 ml. of a 1% solution per liter; and a trace of $FeCl_3$. It was neutralized to a pH of 7-7.2 with dilute NH_4OH. A very thin membrane of cells appeared at the top of the media after 50 hours. Excellent growth was noted at the 72-hour period. Loop transfers of the cells were made to fresh sterile media of essentially the same composition as above with the exception that dilute KOH was the neutralizing agent.

When good growth was apparent, subsequent loop transfers were made to fresh sterile media each succeeding 24-48 hour period. A diminishing intensity of cellular development was noted after the third or fourth transfer. Addition of minute quantities of biotin as a growth factor appeared to rejuvenate the activity of the cells and good growth resulted.

Isolation of the Pseudomonas fluorescens strain was effected using the same techniques as above with the exception that p-nitrobenzoic acid was used instead of the o-nitrobenzoic acid. Biotin, again, appeared to be a growth accelerator.

Some ideas, as to the method used in the above isolations, were obtained from Kameda and Toyoura (27). They isolated an organism from the soil which was capable of using p-nitrobenzoic acid as a carbon source and ammonia as a nitrogen source.

Culture Conditions: Organisms used for assimilation studies had been adapted to growth on liquid media. This adaptation was executed by loop transfers to fresh media every 24-48 hours, and only after rapid growth was apparent was their use as experimental organism considered. Optimum growth appeared to be around 34-36°C in still cultures. Agar slants of pure cultures were maintained. With each transfer to a new slant, incubation at 34-36°C was allowed for 12-24 hours prior to placing

in cold storage. All of the bacteria were trained to grow on simple synthetic media. With the Flavobacterium sp. and the Pseudomonas fluorescens, sterile media of the following compositions were used: nitro-phenyl-carboxylic acids, 0.2%, as the carbon and nitrogen source; $MgSO_4$, 0.1%, $CaCl_2$, 1.0 ml. of a 1.0% solution per liter, and a trace of $FeCl_3$ as the mineral supply; biotin, 0.8 mg per liter, as a growth accelerator; and KH_2PO_4, 0.2% as a buffer. The pH was adjusted to 7-7.2 with dilute KOH. Media was sterilized by autoclaving for 20 minutes at 15 pounds pressure.

Analytical Methods: The procedure for the determination of aromatic-hydroxylamino and aromatic-amino compounds in the presence of each other was a combination and modification of the methods of Rosenthal and Bauer (21), Bratton and Marshall (28) and Marshall and Litchfield. (29) A 10.3-ml. sample, containing both aromatic-amino and aromatic-hydroxylamino compounds, was acidified with 0.4 ml. of 3.65% HCL and treated with 1.0 ml. of a freshly prepared 0.1% $NaNO_2$ solution. After shaking the sample for 3 minutes, 1.0 ml. of a buffer mixture (13.8% NaH_2PO_4 and 0.5% ammonium sulfamate in distilled water) was added bringing the pH to about 2.5. An addition of 4.0 ml. of 0.1% N-1-napthylethylenediamine dihydrochloride permitted the formation of a purple dye. Since the color development appeared to be a function of time, a 30-minute waiting period was allowed for its formation. Then, the optical density of the resulting solution was obtained with an Evelyn colorimeter using a wave length of 540 mu.

The above was a measure of both the aromatic-hydroxylamino and aromatic-amino compounds. To obtain the actual concentration of aromatic hydroxylamines, the above procedure was followed with the exception, that prior to acidification, a 10.0-ml. sample was acylated with 0.3 ml. of acetic anhydride, tying up the aromatic-amino groups, leaving only the

corresponding hydroxylamino groups available for diazo reactions. The acylation step is obviously an important one because any non-acylated amino groups would interfer with hydroxlamino tests. Vigorous shaking, at intervals, over a period of 20 minutes, was found adequate in most instances.

After obtaining the optical density, the magnitude of the aromatic hydroxylamines was determined from a standard calibration plot. By taking the difference in optical densities of the non-acylated and acylated samples, for comparable concentrations, the quantity of aromatic amines was obtained from a standard calibration plot for the same compound.

Much is to be desired over a procedure of this nature but it has served as a practical approach to the analytical problem. The phenyl-hydroxylamino groups did not appear to couple to the same extent as did the corresponding amino groups. This was shown by the occurrance of less intense color development with comparable concentrations. This is as explained by Bauer and Rosenthal.(30) To compensate for this, efforts were made to use larger samples of hyroxylamino compounds to permit better analyses.

The following check was made on the procedure: A calibration plot was obtained with o-hydroxylaminebenzoic acid (1-40 micrograms per ml.). Another was made over the same range using equal quantities of anthranilic acid in combination with the above compound. Results gave comparable hydroxylamine concentrations with satisfactory anthranilic acid recoveries. The magnitudes of both of the above compounds, for individual analyses, were kept under 35-40 micrograms per ml. of sample.

The semimicro nitrogen analyses of nitro-substituted benzoic acids was essentially the same as found in "Methods of Analyses of the A. O. A. C" (31). The quantities were reduced in proportion. Although

this procedure was not specified for aromatic-nitro compounds it has proven satisfactory for those tested. The method consists of treating the sample (1-4.5 mg. of nitrogen in a 100-ml. Kjeldahl flask) with 8.0 ml. of a solution containing 50 grams of salicylic acid in 1 liter of concentrated H_2SO_4. After allowing the sample to stand for several hours, 1 gram of $Na_2S_2O_3$ was added; followed by gentle heating until all danger of frothing was past. Subsequently 4.0 ml. of a mixture (2 grams $CuSO_4$ and 2 grams of Se_2O_3 in 500 ml. water) were added, and then digestion was carried out at higher temperatures for 2 hours after the solution had cleared.

After cooling, 50-60 ml. of distilled water were added. This was followed by the addition of 15 ml of 40% NaOH which was poured down the side of the flask cautiously. The nitrogen was then distilled into 2% boric acid and titrated with N/56 H_2SO_4 using a suitable indicator.

The stepwise mechanism involved by this method in the reduction of inorganic nitrates is as follows: The sulfuric acid converts the nitrates to HNO_3 which in turn nitrates the salicylic acid. $Na_2S_2O_3$ when reacted with H_2SO_4 produces H_2SO_3 which serves a a reducing agent for the nitro-salicylic acid. A possible elimination of the salicylic acid step with nitrobenzoic acids is spectulated; however, if some destruction of the ring should occur with the H_2SO_4, the salicylic would be available for reaction with any resulting free nitro groups. Upon applying this method to o-nitrobenzoic acid good recoveries of nitrogen were obtained as is illustrated in Table II.

The qualitative method used for detection of aromatic-nitroso groups was the procedure described by Fiegl. (32) It involves the heating of a dried sample of the substance with phenol, and then adding concentrated

H_2SO_4; resulting in a reddish-colored compound. Upon treatment with 4 N NaOH a blue color is produced.

TABLE II

Semimicro Determination of o-Nitrobenzoic Acid Nitrogen Using A. O. A. C. Procedure (31).

o-Nitrobenzoic Acid, mg.	Nitrogen Recovered, mg.	Recovery, Percent.
4.190	3.947	94.8
4.190	4.236	101.1
2.095	2.000	99.1
2.095	2.098	100.2

Preparation of o-Hydroxylaminobenzoic Acid: It was synthesized from o-nitrobenzoic acid (Eastman No. 21) using the method of Bamberger and Pyman (33). The reduction was carried out at 10-20°C. with zinc dust and NH_4Cl. The resulting product was recrystallized three times and it gave the characteristic red color with NaOH in the presence of air. Fehling solution test was positive. Melting point was 137-139°C. It was obtained by the oil immersion method using evacuated melting point tubes and by taking the melting point after the bath had been preheated to 130°C. Decomposition occurred before the melting point when this was not done.

CHAPTER III

EXPERIMENTAL AND RESULTS

Reduction Compounds of o-Nitrobenzoic Acid by *Flavobacterium sp.*

Flavobacterium sp. was found satisfactory as an experimental organism as a result of its ability to utilize, rapidly, o-nitrobenzoic acid as the sole source of carbon and nitrogen.

Upon considering the possible step-wise reduction, o-nitrobenzoic acid ⟶ o-nitrosobenzoic acid ⟶ o-hydroxylaminebenzoic acid ⟶ anthranilic acid, it was felt that the mechanism study could be best approached, initially, by analyzing for these reduced nitro intermediates under conditions in which *Flavobacterium sp.* was growing on o-nitrobenzoic acid media. Then, if these postulated intermediates were detected, a utilization study of these compounds by bacteria would be justified. Two experiments were performed using two 2-liter Erlenmeyer flasks with each containing 1 liter of medium. For Experiment 1, the following medium was used: o-nitrobenzoic acid, 0.2%; KH_2PO_4, 0.2%; $MgSO_4$, 0.1%; $CaCl_2$, 1.0 ml. of a 1% solution; trace of $FeCl_3$; and biotin 1.0 mg. The pH of the solution was adjusted to 7-7.2 with dilute KOH. For Experiment 2, the same medium as above was used with the exception that no biotin was added.

After inoculation of the media with comparable liquid-medium volumes of *Flavobacterium sp.*, from separate cultures, growth was allowed to take place under conditions of vigorous aeration at 26° C. At different stages of the growth, samples of the media were collected, filtered through a Seitz filter to remove bacteria, and then were analyzed for

aromatic hydroxylamines.

The results of both experiments are tabulated in Tables III and IV and are graphically illustrated in Figure 1. From these it can be seen that readily detectable quantities of aromatic hydroxylamine, probably o-hydroxylaminebenzoic acid, were found. The magnitudes of

TABLE III

Formation of Aromatic Hydroxylamine in Medium (Containing Biotin) by *Flavobacterium sp.* Growing on 2000 Micrograms Per ml. of o-Nitrobenzoic Acid

Time, Hours	Aromatic Hydroxylamine Micrograms Per ml.
0.0	1.9
0.7	1.9
1.7	4.5
32.5	47.1
49.5	49.5
71.0	15.5
82.0	14.0

these formations indicate much less toxicity to bacteria by aryl hydroxylamines than for free hydroxylamine, of which values of 1-2 micrograms per ml. of media have been reported (2,3). The fact that some hydroxylamino compound showed up in the media at zero time could possibly be attributed to the use of media-cell transfers for inoculation which could have contained some of the compound. Also these values could have resulted from slight reduction of the o-nitrobenzoic acid when it was autoclaved.

Although it is realized that the inoculation quantities of the separate experiments might not have been the same, it does appear by the large difference in growth rates that biotin is serving as a growth

TABLE IV

Formation of Aromatic Hydroxylamine in Medium (Not Containing Biotin) by Flavobacterium sp. Growing on 2000 Micrograms Per ml. of o-Nitrobenzoic Acid

Time, Hours	Aromatic Hydroxylamine, Micrograms Per ml.
0.0	0.5
4.0	0.5
10.3	1.9
13.5	2.0
22.0	3.2
26.0	5.4
33.0	6.0
37.7	7.2
48.0	12.1
54.0	17.8
124.5	32.0
171.5	31.0

accelerator in some way. No speculation will be made as to its absolute necessity.

It was observed that, as the most rapid growth occurred, the pH of the media rose to 7.5-8. The rate of cellular growth appeared to parallel the rise in the aromatic hydroxylamine concentration. Seager and Elving (24) stated that the formation of aryl hydroxylamines by polarographic reduction of nitrobenzoic acids was favored in basic solution. The above might have been in substantiation of that.

The rising of the aromatic hydroxylamine concentration and its subsequent depletion indicates its utilization by Flavobacterium sp. On the basis of these limited experimental findings no conclusion was made as to whether the formed hydroxylamino compound was a major or incidental pathway accumulation in the reduction of o-nitrobenzoic acid.

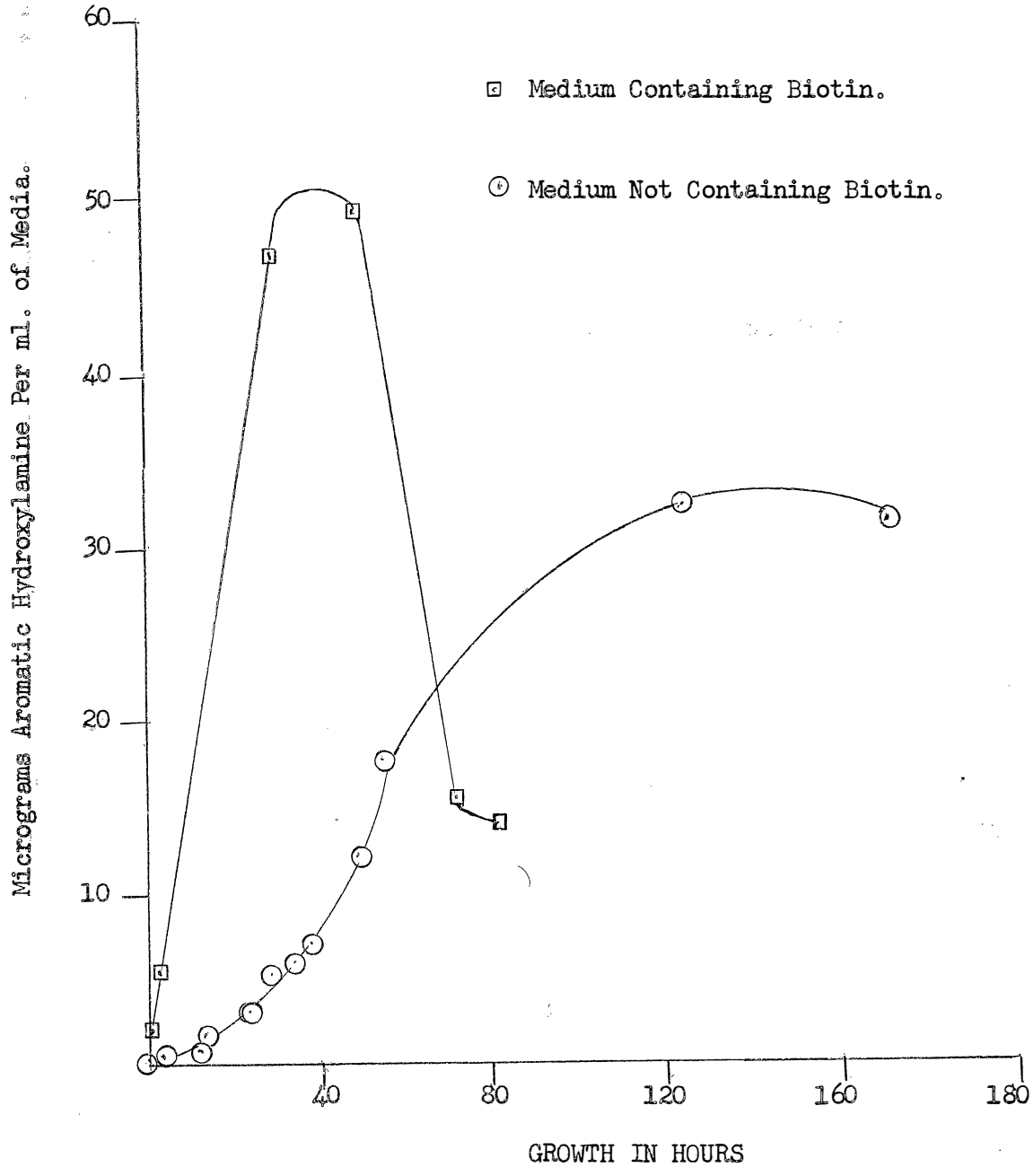


Fig. 1. Cellular Growth Versus Aromatic Hydroxylamine Formation in Aerated Cultures of Flavobacterium sp.. 2.0 mg. Per ml. of o-Nitrobenzoic Acid Used as Source of Carbon and Nitrogen.

After this aromatic hydroxylamine had been detected in bacterial cultures, Silvers and McElroy (11), at John Hopkins University reported that nitrophenylhydroxylamine was found in media where certain mutants of Neurospora were growing on NH_4NO_3 and m-dinitrobenzene.

In an effort to determine the extent of the bacterial reduction of o-nitrobenzoic acid, another experiment was carried out in which analyses for aromatic-amino compounds were made in conjunction with the hydroxylamino derivatives. The same medium, as described on page 15 was inoculated with a media transfer of Flavobacterium sp. Growth conditions and processing of samples were the same as for the above Experiments 1 and 2. The samples, after processing, were analyzed for both aromatic-amino and aromatic-hydroxylamino compounds. The results are tabulated in Table V and are expressed graphically in Figure 2.

As can be seen from Figure 2, the accumulation of the phenyl amine was small in comparison with the phenyl-hydroxylamino compound. This might have indicated that it was more readily available for utilization by Flavobacterium sp. e.g. it was assimilated about as fast as it was produced. However, on the basis of one experiment no conclusion was attempted concerning the role of this compound in the media. The largest magnitude of phenyl hydroxylamine accumulation was comparable to about 3.5% of the original o-nitrobenzoic acid (2000 micrograms per ml. of media). Again, a relatively low toxicity was observed along with an initial rising and final depletion of the phenyl hydroxylamine.

Attempts to detect aromatic-nitroso compound in media where Flavobacterium sp. was growing on o-nitrobenzoic acid failed to indicate their presence.

Fig. 2. Formation of Aromatic Amines and Aromatic Hydroxylamines in Medium with Flavobacterium sp. Growing on o-Nitrobenzoic Acid (2000 micrograms per ml.)

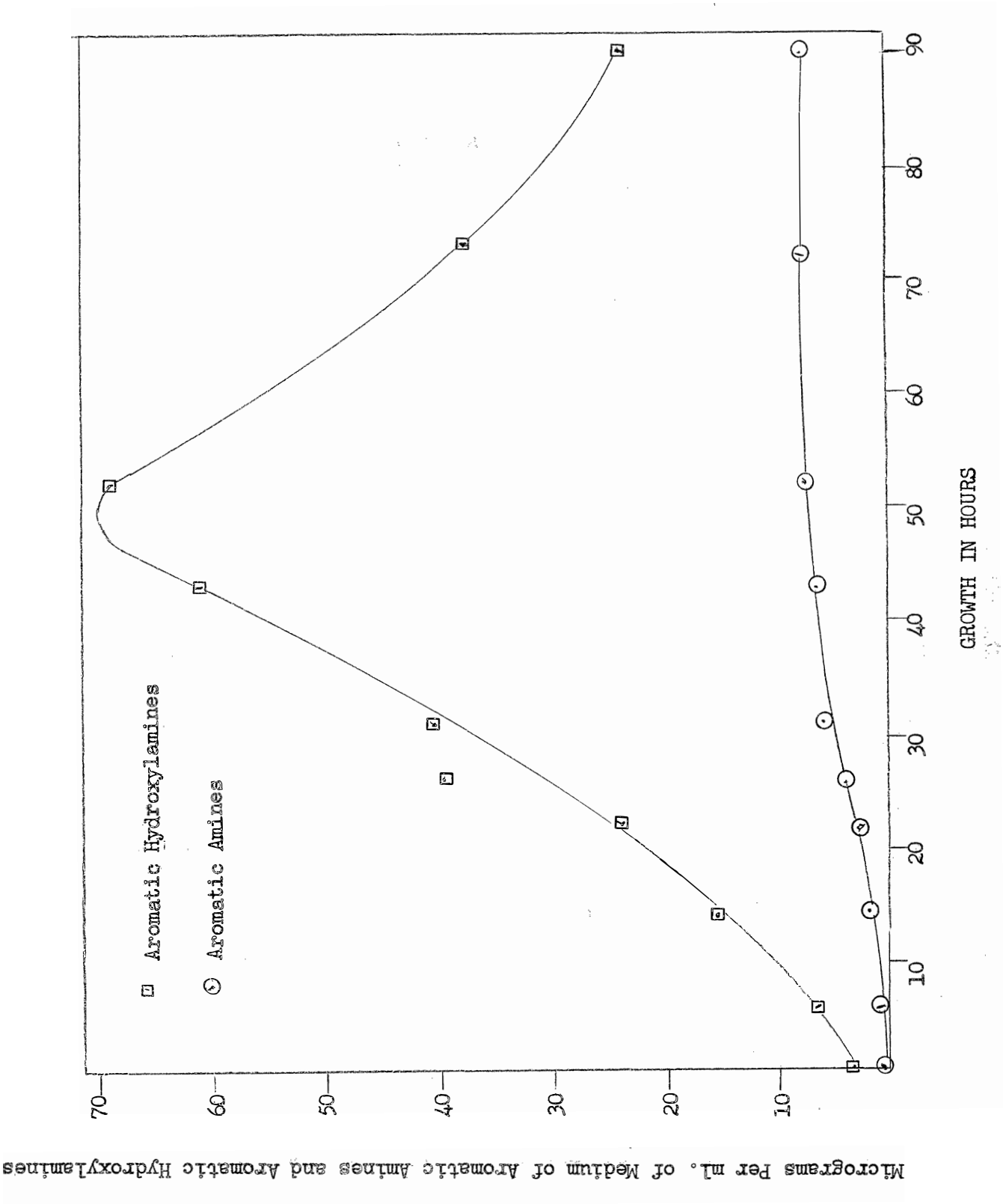


TABLE V

Formation of Reduced Nitro-Phenyl Compounds by *Flavobacterium sp.* Growing on 2000 Micrograms Per ml. o-Nitrobenzoic Acid

Time, Hours	Aromatic Hydroxylamine Micrograms Per ml.	Aromatic Amine Micrograms Per ml.
0.00	1.5	0.3
5.33	6.0	0.4
16.33	15.3	1.1
21.33	23.3	2.4
25.33	39.0	3.7
30.33	40.0	5.8
43.33	60.5	6.0
51.33	68.7	7.0
72.33	37.0	7.0
89.33	23.0	7.6

The Bacterial Reduction Products of P-Nitrobenzoic Acid and Their Utilization by *Pseudomonas fluorescens*

A strain, isolated from the soil and identified as *Pseudomonas fluorescens*, was found capable of rapid growth on both p-nitrobenzoic acid and p-aminobenzoic acids as the sole sources of carbon and nitrogen. In an effort to elucidate possible reduction products of p-nitrobenzoic acid by the same organism, the following experiment was performed. A rapidly growing culture of this strain was transferred to sterile media of the following composition: p-nitrobenzoic acid, 0.1%; KH_2PO_4, 0.2%; $MgSO_4$, 0.1%; $CaCl_2$, 1.0 ml. of a 1% solution per liter; trace of $FeCl_3$; and biotin, 0.8 microgram per ml. The pH of the solution was adjusted to 7-7.2 with dilute KOH.

Growth was carried out at 26° C. with vigorous aeration. Samples were collected via sterilized pipettes at different stages of growth. The bacteria was removed from the media by a Seitz filter and the

analysis for aromatic-hydroxlamino and aromatic-amino compounds were made. Results showed that some aromatic hydroxylamine was formed with little if any aromatic amine accumulations. Approximately 20 micrograms per ml. of medium was the highest phenyl hydroxylamine concentration detected. A reason for this lower value is that only 0.1% p-nitrobenzoic acid was used in the medium as compared to 0.2% for the o-nitrobenzoic acid media used with Flavobacterium sp. A value as high as 68 micrograms phenyl hydroxylamine per ml. of media has been found with this organism. (see page 21 of this thesis). The results of the experiment are as contained in Table VI.

Durham and Gee (34), in Warburg apparatus studies (oxygen uptake) with the same strain of Pseudomonas fluorescens, found that when the cells were adapted to p-aminobenzoic acid they simultaneously adapted to p-hydroxybenzoic acid and protocatechuic acids, with some indications of the ring undergoing oxidative cleavage, forming B-ketoadipic acid. This would, as they stated, indicate that the first two compounds were intermediates in the oxidative scheme. If this were the case the organisms growing on anthranilic acid would first deaminate the ring, then oxidize it to keto acids, and finally by transamination processes amino acids would be produced by the bacterium. The above workers believes that p-aminobenzoic acid can fulfill the role of an oxidizable substrate in addition to a catalytic unit with this strain of Pseudomonas fluorescens.

From the above reasoning one might conclude that p-aminobenzoic acid would be one of the preferred reduced p-nitrobenzoic acid compounds of which Pseudomonas fluorescens could sustain growth. If phenyl amines were formed from p-nitrobenzoic acid this would add weight to the

TABLE VI

Formation in Medium of Reduced Nitro-phenyl Compounds by *Pseudomonas fluorescens* Growing on 1000 Micrograms Per ml. of p-Nitrobenzoic Acid

Time, Hours	Aromatic Hydroxylamine, Micrograms Per ml.	Aromatic Amine Micrograms Per ml.
0	4.0	1.2
22	13.0	1.3
27	13.5	0.8
33	11.6	0.7
45.5	13.0	0.4
57	19.5	0.2
70	15.5	0.9
93	15.5	2.6
121	14.0	0.6

possibility of phenyl hydroxylamines being the precursors. In the above experiment there was not significant accumulation of the amino compound in the media where *Pseudomonas fluorescens* were growing; but, its assimilation by the organism as quickly as it is formed is logical.

The likeliness of phenyl hydroxylamine being an immediate precursor of amino acids by another route(s) is yet to be known.

Utilization of Nitrogen-substituted Benzoic Acids by *Flavobacterium sp.*

This occurrance of small aromatic-amino concentrations in bacterial cultures of *Flavobacterium sp.* prompted the investigation of the availability of the full reduced o-nitrobenzoic acid (anthranilic acid) by the same organism.

It was observed in still cultures that *Flavobacterium sp.*, growing on o-nitrobenzoic acid, readily adapted to growth on anthranilic acid as the sole source of carbon and nitrogen. The difference in the respective media was the change in the nitro-phenyl-carboxylic acid supply.

Simultaneous nitrogen balance studies with Flavobacterium sp., growing on o-aminobenzoic and o-nitrobenzoic acids, were undertaken with the purpose of demonstrating effective assimilation of the nitrophenyl-carboxylic acids and to obtain information concerning the utilization rate of each compound.

Two 1-liter experiments were made with separate media. The sterile medium for Experiment 1 consisted of the following: 167.6 mg. nitrogen as o-nitrobenzoic acid, KH_2PO_4, 0.2% ; $MgSO_4$, 0.1%; $CaCl_2$, 1.0 ml. of a 1% solution; biotin, 0.8 mg. The pH of the solution was adjusted to 7-7.2 with dilute KOH. The medium used in Experiment 2 was of the same composition as above with the exception that 167.6 mg. of nitrogen as anthranilic acid were used in place of the same quantity of o-nitrobenzoic acid. Inoculations were executed by transfers of equal volumes of rapidly growing cultures of Flavobacterium sp., growing on (A) anthranilic acid and (B) o-nitrobenzoic acid, to the above respective media. Growth was carried out at 26°C. under conditions of vigorous aeration. At different stages of growth, media-cell samples were collected. Sterile techniques were at all times employed.

Samples were processed by collecting the media-cell suspensions with sterilized pipettes and then transferring them to Erlenmeyer flasks of appropriate size. Two ml. of 0.8N H_2SO_4 were added for each 20 ml. of sample. The solutions were immediately brought to boil, stoppered, and reserved under refrigeration for further analyses. Separation of cells from media was accomplished by centrifuging the acid solutions in plastic centrifuge tubes. After efficient settling of the cells had occurred, the supernatant was carefully transferred via a pipette to a volumetric flask. The cells were washed three successive times in the same manner, each time adding the supernatant to the original volumetric

flask. The contents of the flask were then diluted to volume and nitrogen analyses were immediately determined on aliquot portions. The procedure used is as described previously in the thesis (see page 12). The entire mass of cells was subsequently subjected to the same analysis. The results of these findings are tabulated in Tables VII and VIII and are graphically illustrated in Figure 3.

TABLE VII

Nitrogen Balance Study With *Flavobacterium* sp. Using Anthranilic Acid as the Carbon and Nitrogen Source

Time, Hours	Cellular Nitrogen Micrograms Per ml.	Medium Nitrogen, Micrograms Per ml.
0.00	No analysis	167.6
4.25	No analysis	78.7
13.25	53.8	----[1]
17.75	95.8	----[1]
20.75	104.9	----[1]
24.75	115.4	61.6
27.75	118.0	50.3
38.75	128.0	31.0

[1] Samples Lost.

TABLE VIII

Nitrogen Balance Study With *Flavobacterium* sp. Using o-Nitrobenzoic Acid as the Carbon and Nitrogen Source

Time, Hours	Cellular Nitrogen Micrograms Per ml.	Medium Nitrogen, Micrograms Per ml.
0.00	No analysis	167.6
4.25	No analysis	162.0
13.25	30.2	-----[1]
20.75	32.8	-----[1]
24.75	65.5	83.9
27.75	107.5	63.0

[1] Samples Lost.

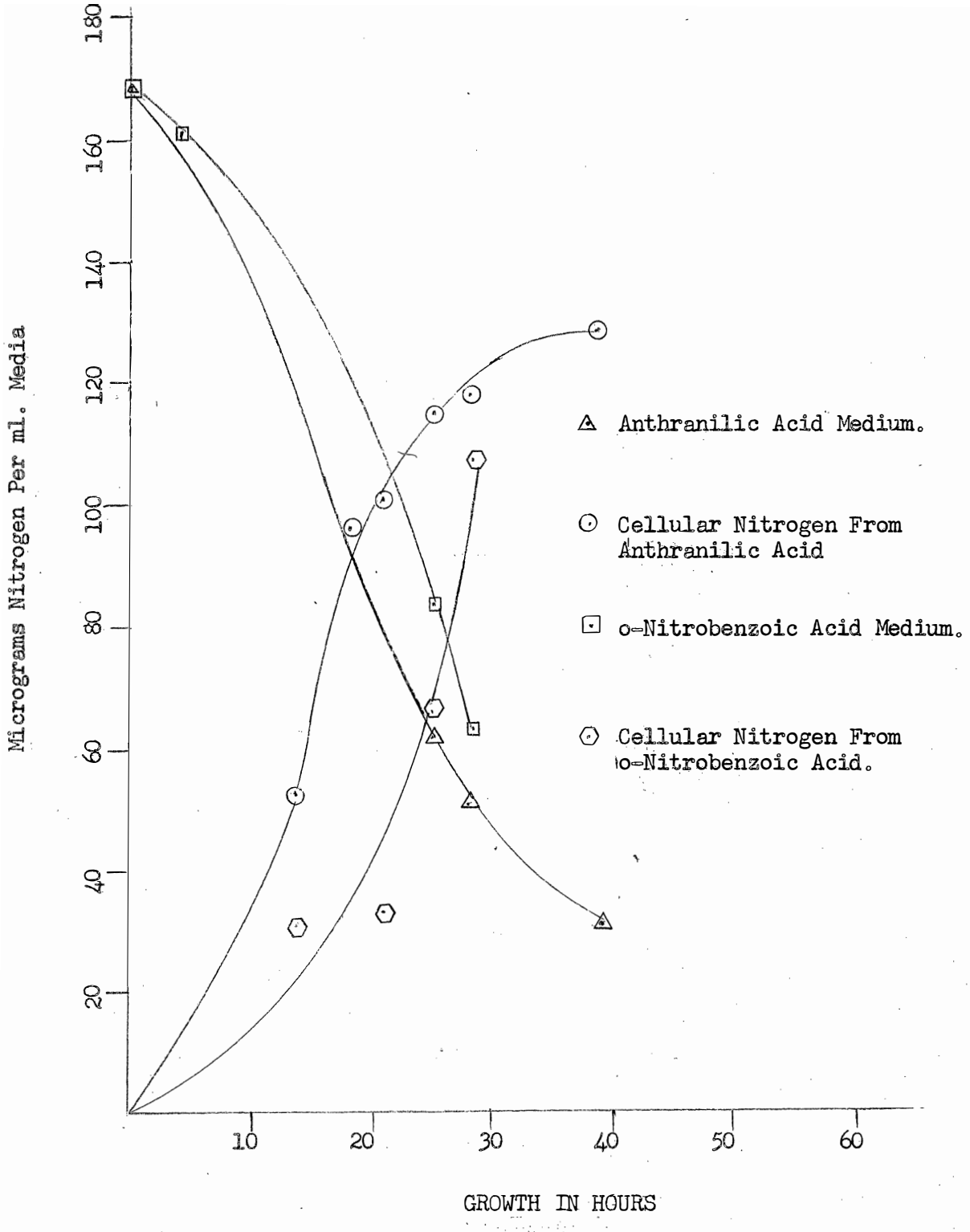


Fig. 3. Simultaneous Nitrogen Balance Studies with Flavobacterium sp. Growing on 167.6 mg. Nitrogen Per Liter of Media as o-Nitrobenzoic and Anthranilic Acids.

Results were gratifying in that over 60% of the nitrogen from each compound was converted to cellular nitrogen. o-Aminobenzoic acid appeared to be utilized faster, but on the basis of only one experiment, no conclusion can be made. Autolysis was thought to have occurred after the major part of the nitrogen was depleted from the media.

Limited investigations, concerning the availability of o-hydroxylaminebenzoic acid by Flavobacterium sp., have shown it is capable of being dissimilated; but, the concentrations in the media appear to be critical. Flavobacterium sp., growing rapidly on o-nitrobenzoic acid medium (2000 micrograms per ml.), was transferred to medium of essentially the same constitutents with the exception that 500 micrograms per ml. of o-hydroxylaminebenzoic acid were used as the carbon and nitrogen source. After 40 hours some noticeable growth was apparent around the surface of the still culture. With subsequent transfers the organism never seemed to take on as rapid growth as with o-nitrobenzoic acid. Simultaneous adaptation at lower concentrations has indicated that the organism is readily adapted to o-hydroxylaminebenzoic acid with little if any lag. This work will be discussed in the next experimental section.

Depletion of Nitrogen-substituted Phenyl-carboxylic Acids From Media by Flavobacterium sp.

It was felt that a study of compound preference of Flavobacterium sp., by simultaneous adaptation techniques, might contribute to elucidating the metabolic scheme e.g. whether the product formations of the previous studied media were major of incidental accumulations. By simultaneous adaptation is meant; for example, organisms growing rapidly on o-nitrobenzoic acid should adapt without lag to growth on o-hydroxylaminebenzoic and anthranilic acids if these compounds are major pathway

productions in the bacterial reduction scheme. In other words the enzymatic systems present should be capable of sustaining rapid growth on these intermediates.

Several efforts were made to determine the compound preference of Flavobacterium sp. which were cultured to growth on o-nitrobenzoic acid at 34°C. The bacterium was transferred to media containing all the usual media constituents (see page 11) with the exception that varying proportions of o-hydroxylaminebenzoic acid to anthranilic acid were used as the nitrogen and carbon supply. In all cases the concentrations of each component were kept under 100 micrograms per ml. of media. The rates of depletion of these compounds were then studied.

Two trial runs were made by inoculating the media with small portions of o-nitrobenzoic acid media containing rapidly growing Flavobacterium sp. organisms. Growth was permitted to take place at 34°C. with vigorous aeration. Samples were collected periodically during the growth period by using sterile pipettes. Subsequently the bacteria were removed from the media by a Seitz filter. The described analytical procedure (see page 11) for these compounds was carried out. Resulting evidence showed a simultaneous depletion of both compounds down to a point and then anthranilic acid appeared to be predominantly the preferred compound. When the anthranilic acid concentration was greatly decreased a step up in the dissimilation of o-hydroxylaminebenzoic acid occurred. The results are tabulated in table IX.

In Experiment 2 of Table IX the depletion study was carried out to a greater extent than in Experiment 1. The results show the o-hydroxylaminebenzoic acid as not being dissimilated initially as quickly as anthranilic acid. For plot of values of Experiment 2 see Figure 4.

TABLE IX

Depletion of o-Hydroxylaminebenzoic and Anthranilic acids from Media by *Flavobacterium sp.*[1] Previously Cultured on o-Nitrobenzoic Acid as the Carbon and Nitrogen Source

Time, Minutes	Phenyl Hydroxylamine Micrograms Per ml.	Phenyl Amine, Micrograms Per ml.
	Experiment 1	
0	79	68
60	66	60
120	59	48
180	57	18
240	52	0
	Experiment 2	
0	90	95
120	78	45
300	65	13
390	41	0
570	9	--[2]

[1] Media inoculation of cells

[2] No analysis.

Two other trials were made by inoculating fresh media containing washed cells and allowing them to continue growth at 34° C. under conditions of vigorous aeration. In Experiment 1 of Table X the anthranilic acid appeared to be more readily available to *Flavobacterium sp.* in comparison to the o-hydroxylaminebenzoic acid. Values contained in Experiment 2 of the same table, in slight contrast to this, show that the o-hydroxylaminebenzoic acid is depleted a little faster than anthranilic acid in the beginning of the growth period.

In summary of this entire depletion study it appears as if *Flavobacterium sp.*, previously cultured on o-nitrobenzoic acid, adapts with little if any lag to both o-hydroxylaminebenzoic and anthranilic

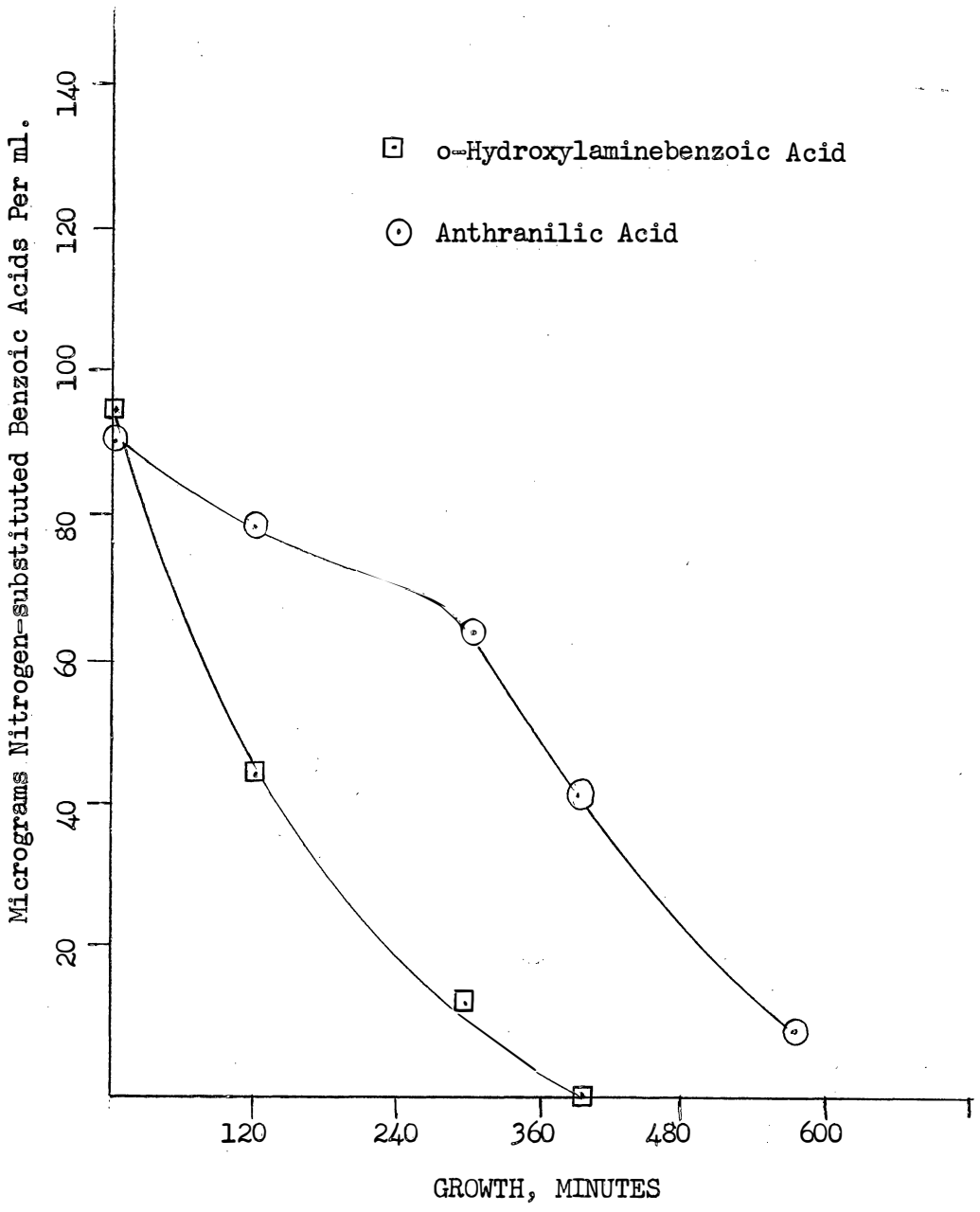


Fig. 4. The Depletion of o-Hydroxylaminebenzoic and Anthranilic Acids from Medium with Flavobacterium sp. Using the Compounds as the Only Sources of Carbon and Nitrogen. The Flavobacterium sp. was Cultured on o-Nitrobenzoic Acid.

TABLE X

Depletion of o-Hydroxylaminebenzoic and Anthranilic Acids From Media by *Flavobacterium* sp.[1] Previously Cultured on o-Nitrobenzoic Acid as the Carbon and Nitrogen Source

Time, Minutes	Phenyl Hydroxylamine, Micrograms Per ml.	Phenyl Amine, Micrograms Per ml.
	Experiment 1	
0	47	30
75	45	19
135	45	12
180	37	0
510	0.3	0
	Experiment 2	
0	92	74
50	78	68
105	75	39
255	35	0

[1] Washed cells for inoculation.

acids with the concentrations used. For the most part, an initial depletion of both compounds was noted down to a point with anthranilic acid then becoming the preferred compound. When the anthranilic acid was almost depleted a step up was noted in the disappearance of the o-hydroxylaminebenzoic acid from the media. On the basis of the limited number of experiments performed no conclusion will be made as to which of the compounds was most readily available to *Flavobacterium* sp. in the initial growth phase. It is, however, realized that a knowledge of the complete picture during this crucial period is of extreme importance to this mechanism study; and because of this it is felt that the situation might best be approached from a statistical manner

Durham and Gee (34) reported that Flavobacterium sp., grown on o-nitrobenzoic acid, showed complete simultaneous adaptation to o-hydroxylaminebenzoic acid by oxygen uptake measurements. In further elaboration of their work, the o-nitrobenzoic acid grown cells exhibited adaptive enzyme formations for benzoic acid, anthranilic acid, salicylic acid, and catechol while oxygen uptake in excess of endogenas was not observed with o-nitrobenzaldehyde, o-aminophenol, p-aminosalicylic acid, and aniline. Cells grown on anthranilic acid showed complete adaptation to catechol and the same cells formed enzymes for o-nitrobenzoic acid, salicylic acid, and benzoic acid while they did not appear capable of dissimilating o-aminophenol, p-aminosalicylic acid, o-nitrobenzaldehyde, and aniline.

From the above observations it appears as if anthranilic acid might serve as an immediate nitrogen source for cellular development by Flavobacterium sp. The lack of adaptation of the same organism, while growing on o-nitrobenzoic acid, to o-aminophenol, indicates that an oxidative rearrangement of possible formations of o-hydroxylaminebenzoic acid to a hydroxyaminobenzoic acid is not likely. This type of rearrangement was hypothetically suggested by Dobson and Williams (15) for the oxidation of metanilamide by rabbits. Further indications, as a result of the above work, suggest that the carboxyl group of the o-nitrobenzoic acid does remain intact up until, possibly, the corresponding aminobenzoic acid is produced as a reduction product.

SUMMARY

This study was designed to elucidate the biochemical mechanism of the bacterial reduction of nitrates by investigating the utilization of nitro-phenyl-carboxylic acids. By using this nitrate analog approach results have been encouraging.

Organisms, isolated from soil, were found capable of utilizing rapidly both o- and p-nitrobenzoic acids as the sole carbon and nitrogen sources. A member of the genus, Flavobacterium, was used extensively in efforts to elucidate the reduction pathway(s) of o-nitrobenzoic acid. A strain of Pseudomonas fluorescens was, also, used to a limited degree in a similar study with p-nitrobenzoic acid.

Aromatic-hydroxylamino compounds were detected by quantitative tests in the media of rapidly growing cultures of Flavobacterium sp., utilizing o-nitrobenzoic acid as the carbon and nitrogen source. The aromatic-hydroxylamino concentration rose to a peak and subsequently was depleted, indicating a possible further breakdown of this compound by the same organism. The accumulation of the hydroxylamino compounds was substantialy greater than amounts of free hydroxylamine which have been found to be toxic to certain other organisms.

Upon analyzing for further reduction products of o-nitrobenzoic acid by Flavobacterium sp., aromatic-amino compounds were also detected in the media. Rapid growth of the same organism has been demonstrated, moreover, when it was supplied with anthranilic acid. The determined rate of growth was as rapid as for the same concentrations of o-nitrobenzoic acid. In contrast to this, growth on o-hydroxylaminebenzoic acid, of the same concentration, was slow in comparison; however, cellular

development appeared improved at concentrations under 200 micrograms per ml. of media. Durham and Gee (34), in their work with the same strain demonstrated, by oxygen uptake measurements, that these cells grown on o-nitrobenzoic acid showed complete simultaneous adaptation to o-hydroxylaminebenzoic acid as well as exhibiting adaptive enzyme formation for anthranilic acid.

Upon transferring Flavobacterium sp. (cultured on o-nitrobenzoic acid) to media containing only o-hydroxylaminebenzoic acid in combination with anthranilic acid, results showed a rapid initial depletion of both compounds down to a point and subsequently anthranilic acid became the preferred compound. A step up in the dissimilation of o-hydroxylamine-benzoic acid was found to occur when the anthranilic acid was almost depleted from the media. Concentrations of both of these compounds for these studies were kept under 100 micrograms per ml. of media.

Limited investigations with the Pseudomonas fluorescens strain, growing on p-nitrobenzoic acid as the carbon and nitrogen source, resulted in the accumulation of aromatic hydroxylamines in the medium. Rapid growth has been observed with the same strain growing on p-amino-benzoic acid.

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