THE BIOCHEMICAL MECHANISM OF THE REDUCTION

OF o-NITROBENZOIC ACID BY

FLAVOBACTERIUM SPECIES

By

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385471

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CHAPTER I

INTRODUCTION

The fact that nitrate nitrogen is assimilated by plants and microorganisms has been known for a long time. The primary step in the utilization of nitrate nitrogen is its reduction. The reduction process and the actual assimilation are so closely associated that the chemical identification of the reduced intermediates is very difficult. It has, however, been possible to note in different organisms the formation of nitrite, a passing occurrence of hydroxylamine or oxime, and ammonia as possible intermediates in nitrate reduction.

The first and most easily recognized reduction product is nitrite. Although present in small amounts, many observers have reported the occurrence of nitrites in higher plants as well as in microorganisms. Steward and Street (50) reported the presence of nitrite in legumes, and Rautenan (38) detected traces of nitrite in the medium when pea plants were growing on nitrate as nitrogen source. Kumada (22) found that nitrate was converted into nitrite in seed embryos of higher plants. The reduction of nitrate to nitrite has also been noted in green algae (17), *Clostridium welchii* (16) and many other anaerobic (63) and aerobic organisms (54). Krasna and Rittenberg (20) have shown that both whole cells and extracts of *Proteus vulgaris* catalyze the reduction of nitrate to nitrite in the presence of molecular hydrogen. Nicholas and Nason (32) were able to prepare a soluble nitrate reductase from *Escherichia coli*

which catalyzed the reduction of nitrate to nitrite by reduced diphosphopyridine nucleotide. A discussion of the properties of the enzyme system involved in the formation of nitrite from nitrate by Bacterium coli was given by Yamagata (66).

Nitrate reductases were also prepared from tomato leaves by Eckerson (9), from soybean leaves by Lemoigne et al. (23) and from soybean nodules (30). These facts confirm the belief that nitrite apparently is the first intermediate in the biochemical reduction of nitrate.

The formation of ammonia nitrogen as a result of nitrate reduction has also been established with different organisms. Bach and Desbordes (3) observed that when Aspergillus was maintained on an acid medium, nitrate disappeared with the formation of ammonia in equimolar amounts. Ammonia production in nitrate media was also noted in Escherichia coli (2, 19), Chlorella and Azotobacter (60) and higher plants (35, 38). Woods (63) reported Clostridium welchii was also capable of reducing nitrate to ammonia with the aid of molecular hydrogen. By using N^{15} isotope, ammonia has been identified as a product of nitrate reduction in Nostoc muscorum (27), Bacillus subtilis (13), and Pseudomonas fluorescens (31). The comparative utilization of ammonia versus nitrate also supports this postulation. Pratt et al. (36) reported that cells of Chlorella vulgaris seem to show preferential absorption of ammonium ions in the presence of nitrate. Lewis and Hinshelwood (24) compared the rate of utilization of ammonia by coliform bacteria with the rate of ammonia formation from nitrate. The results indicated that while ammonia was being utilized by the cells, the reduction of nitrate and nitrite was inhibited. The addition of ammonia to a culture of Bacterium

lactis aerogenes results in an almost complete inhibition of nitrite removal and reduction does not commence until the ammonia concentration becomes negligible. The above studies support the intermediate role of ammonia in nitrate reduction.

Since the reduction of nitrate apparently passes through ammonia, the actual assimilation may take place through the reaction of ammonia with members of the tricarboxylic acid cycle to form amino acids. Kneikova et al. (18) reported the formation of glutamic acid from a-ketoglutaric acid and aspartic acid from malic acid in Escherichia coli. Kritsman et al. (21) showed that enzyme preparations from Bacillus subtilis formed amino acids from ammonia and a-keto acids. Similar reports include those of Nisman et al. (33) and Yakobson et al. (65). A complete review may be found in Virtanen and Rautanen's book (60).

While the formation of ammonium nitrogen during nitrate reduction is evident, there are many findings which reveal that hydroxylamine or oxime nitrogen is also formed in the assimilation of nitrate. As early as 1884 Meyer and Schulze (29) advanced the hypothesis that hydroxylamine is an active intermediate in the assimilation of nitrate and that oximes are primary organic compounds formed in assimilation. Lindsey and Rhines (25) found hydroxylamine in several bacterial solutions containing nitrate as nitrogen source. Wirth and Nord (61, 62) found this compound in Fusarium during nitrate assimilation. Evidence for the occurrence of hydroxylamine as an intermediate in the reduction of nitrate was also found in Clostridium welchii by Woods (63) and in a halo-resistant bacterium by Egami et al. (10). Steward and Street (49) reported both free and bound forms in green plants.

Since hydroxylamine is so highly toxic to the cell, it is, indeed, unlikely that analyzable amounts of free hydroxylamine will accumulate in cells. Lewis and Hinshelwood (24) reported that the addition of hydroxylamine to the medium inhibited growth of coli bacteria completely, and that growth resumed only after hydroxylamine gradually disappeared. Aso et al. (1) demonstrated the formation of hydroxylamine as an intermediate product in the reduction of nitrite in Azotobacter. During enzymatic studies on nitrate and nitrite mutants of Neurospora, Silvers and McElroy (43) 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. Virtanen and Csaky (58) noted the formation of oxime when nitrate was the nitrogen nutrient with Torula, Rhizobium, and Azotobacter. With Torula the formation of oxime was especially rapid. In vigorously aerated yeast cultures the maximum oxime formation was reached within 10 minutes, then a marked decrease took place. The oxime was not detected in short term experiment in ammonium salt solution. In view of these observations it seems that oxime nitrogen is generally formed in the reduction of nitrate. Virtanen and Järvinen (59) have investigated the velocity of oxime formation with suspensions of Azotobacter vinelandii in parallel experiments with molecular nitrogen, nitrate, and ammonium as the N source. The formation was slowest from ammonium. These results support the opinion that oxime nitrogen does not result from oxidation of ammonia in N_2 fixation.

From a study of the comparative effectiveness of various inhibitors on ammonia versus nitrate assimilation, Csaky (7) concluded

that in the case of *Azotobacter*, the assimilation of nitrate does not necessarily proceed through ammonia. However, Novak and Wilson (34) were unable to show that *Azotobacter* could use the oximes of pyruvic, oxaloacetic, and ketoglutaric acids for its nitrogen nutrition. They consider this finding to prove that these oximes as well as hydroxylamine cannot be of significance in N assimilation by *Azotobacter*. Regardless of the above findings in *Azotobacter*, positive assimilation of oxime N has been reported for various organisms. Maurer (28) showed the biochemical reduction of pyruvic oxime to alanine in fermenting yeast culture. Virtanen (56) found that peas assimilate some oximino succinic acid in sterile cultures. Wood *et al*. (64) reported that green plants are able to grow on oximes of pyruvic, ketoglutaric and oxaloacetic acids. Rosenblum and Wilson (39) found that *Clostridium pasteurianum* can utilize oximes. Quastel *et al*. (37) reported that pyruvic oxime was utilized by soil bacteria. These results support the opinion that hydroxylamine is an intermediate in the pathway of nitrate reduction and oximes are primary organic compounds formed in assimilation. However, formation of small amount of oxime N does not prove that hydroxylamine as such is an important factor in the synthesis of amino acids. The realization that hydroxylamine generally reacts with compounds containing the CO group and that a specific enzyme is not known to catalyze the reaction of hydroxylamine with some a-keto acids, e.g. oxaloacetic acid, gives cause to doubt that hydroxylamine plays a significant role in amino acid synthesis (57). It may rather be expected to be reduced to ammonia should it be formed as an intermediate and to combine with the CO group only in case its reduction for some reason is not rapid enough. Oxime, then, may arise as

an insignificant by-product. The existence of a new enzyme "Hydroxylamino Reductase" which catalyzed the reduction of hydroxylamine to ammonia was confirmed by Taniguchi et al. (52, 53) in their studies on cell-free enzyme systems from a halo-tolerant bacterium.

In order to remove some of the controversy that has been raised on hydroxylamine, aromatic nitro analogs have been studied recently. Although the adoption of this model is purposed to eliminate the toxic effect as well as to increase stability of the hydroxylamino group, the over-all objective would be the clarification of the entire nitrate reduction pathway.

The antibiotic Chloramphenicol was found to be reduced to the corresponding amine by bacteria (44). Greenville and Stein (12) showed that various enzymes were capable of reducing dinitrophenol when the reaction was permitted to occur on suitable substrates. Bray et al. (4) found that 2:3:4:5-tetrachloronitrobenzene and p-nitrobenzoic acid were reduced rapidly to their corresponding amines in rabbit intestine. Microbiological studies indicate that the reduction is bacterial in origin. Among other compounds tested, nitrobenzene, m-nitrophenol and o-, m- and p-nitrobenzoic acids and their amides were also reduced at similar rates. The reduction of nitrobenzoic acids and amides by liver, kidneys, heart, muscle, lung, testicles and spleen were found to reduce p-nitrophenol in the above order.

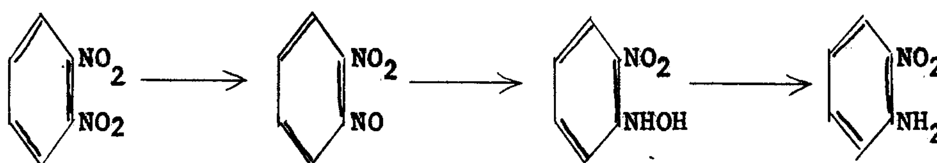
The stepwise reduction of nitro- and nitroso- compounds of chloramphenicol and p-nitrobenzoic acid to their corresponding amino compounds was confirmed by the enzymatic study by Egami (11) with a [illegible] reductase preparation from hemolytic streptocci.

Yamagata *et al.* (68) used cell-free enzyme preparations from a halo-tolerant bacterium which catalyzed the reduction of o-nitroso- and o-hydroxylaminobenzoic acids to anthranilic acid by leucomethylene blue or by a dehydrogenase system in the presence of a H carrier. From studies of reaction velocities and paper chromatography of reaction mixtures, the following scheme of reduction was proposed:

In other experiments the nitro-reductase from *Bacillus pumilus* was shown to catalyze the reduction of nitro- to amino compound similar to the nitrate reduction:

From experiments on competition studies, it was found that nitro-reductase was not identical with nitrite reductase (67).

The cell-free extracts of *Escherichia coli* were found to reduce the nitro groups of chloramphenicol and p-nitrobenzoic acid when L-cysteine (specific) and DPN and L-malate were present, and the enzyme complex reducing organic nitro groups was probably not the same as those reducing inorganic nitrate and nitrite (41). Other experiments showed that DPN and L-malate can be replaced by DPNH (42). In enzymatic studies of a partially purified enzyme preparation from *Neurospora* (70), m-dinitrobenzene was reduced to nitro aniline by this enzyme preparation and reduced nucleotides as exemplified below:



Nitrophenylhydroxylamine has been crystallized and identified as an intermediate in the reduction of dinitrobenzene.

Young (69) studied the biochemical reduction of o-nitrobenzoic acid by a Flavobacterium sp. Quantitative tests revealed the presence of aromatic-hydroxylamine compounds in the media of rapidly growing cultures of this organism utilizing o-nitrobenzoic acid as the carbon and nitrogen source. The fact that the aromatic hydroxylamino concentration rose to a peak and subsequently was depleted, was regarded as indicative of a further breakdown of this compound by the same organism. This assumption may be correct provided there is no chemical or other unexpected influence, however, one must bear in mind that the depletion of aromatic hydroxylamine may also be caused by the active assimilation of the aromatic hydroxylamino group at this stage.

Unfortunately, controversy appeared when attempts were made to clarify the intermediate role of anthranilic acid in the assimilation of o-nitrobenzoic acid. Young demonstrated the presence of very small amounts of aromatic amine in the medium where Flavobacterium sp. was grown on o-nitrobenzoic acid as sole source of nitrogen and carbon. The preferential utilization of anthranilic acid versus o-hydroxylamino benzoic acid led to the suggestion of the possibility of anthranilic acid as the ultimate intermediate in the reduction of o-nitrobenzoic acid. Durham and Gee (8) were unable to implicate anthranilic acid as an intermediate in the biochemical reduction of o-nitrobenzoic acid by the Flavobacterium sp. when employing "simultaneous adaptation". Study was, therefore, initiated to uncover the biochemical mechanism(s) of the reduction and assimilation of o-nitrobenzoic acid by the Flavobacterium sp. with the hope that the knowledge found would contribute

to a clarification of the controversy raised in the previous work and elucidating more completely the inorganic nitrate reductive pathway(s) of bacteria.

CHAPTER II

MATERIALS AND METHODS

Organism: A bacterium capable of rapid growth on o-nitrobenzoic acid as the sole source of organic carbon and nitrogen, was isolated from the soil by enrichment technique. This organism was cultured and tentatively identified as a member of the genus *Flavobacterium* by Durham and Gee (8). Staining and microscopic examinations showed the cells to be Gram negative short rods. An experiment on the assimilation of various nitrogen sources showed that the organism preferred inorganic NH_4^+-N to NO_3^--N or NO_2^--N (see Chapter III). Optimum temperature, biochemical reactions, and other conditions of growth were determined previously (8, 69). Rapid growth has been observed at 37° C on surface of agar medium and in aerated liquid cultures. Stock and subcultures were carried on a synthetic agar medium containing o-nitrobenzoic acid as the sole source of organic carbon and nitrogen.

M/100 phosphate buffer solution: The composition of the M/100 phosphate buffer solution consists of 0.4 ml of 13.6% KH_2PO_4 and 0.6 ml of 17.4% K_2HPO_4 solution in 100 ml of distilled water. The final pH of the buffer reads 7.2.

Mineral salts solution: The mineral salts solution used throughout this study consists of $MgSO_4 \cdot 7H_2O$, 5.0 g.; $MnSO_4 \cdot H_2O$, 0.1 g.; $FeCl_3$, 1.0 g.; and $CaCl_2$, 0.5 g. in 100 ml of distilled water.

Synthetic media: The synthetic medium used to carry stock cultures of Flavobacterium sp. has the following composition: NaCl, 0.2 g.; KH_2PO_4, 0.32 g.; K_2HPO_4, 0.42 g.; and 0.1 ml of the mineral salts solution in 100 ml of distilled water. Ortho-nitrobenzoic acid was incorporated into the medium as a sole source of organic carbon and nitrogen at a final concentration of 0.1%. Agar (2%) was added as the solidifying agent. Sterilization was fulfilled by autoclaving for 15 min. under 15 lb./in.^{2} of pressure (at 240 to 250° F).

The above medium was used as the basic formula to compose media for growing cells enzymatically adapted to various compounds. When cells adapted to a different compound were needed, the o-nitrobenzoic acid was replaced by an equal amount of desired compound.

Cells unadapted to aromatic nitrogen compounds were grown on the synthetic medium with 0.1% asparagine replacing the o-nitrobenzoic acid and 0.1% NH_4Cl to serve as an additional nitrogen supply. NH_4Cl was also used as an inorganic nitrogen source when the compound used as a carbon source lacked an organic nitrogenous group. Salicylic acid and anthranilic acid were used in 0.05% of concn. to avoid the unfavorable depressive effect on the organism.

Growth of cells: Cells were transferred from the stock culture and inoculated by spreading on surface of agar media in Petri dishes, incubated at 37° C and harvested immediately after rapid growth was apparent. The time of harvest varies with substrates upon which the cells are grown.

Preparation of cell suspensions: The cell suspensions used in manometric studies were prepared by harvesting the properly grown cells on

solid agar plates with M/100 phosphate buffer solution, centrifugating to remove supernatant, washing twice and resuspending in the buffer. The turbidity of the suspensions was unadjusted; however, heavy suspensions with % transmittance not higher than 5% at a wave length of 525mu in the B. and L "Spectronic 20" were observed to insure rapid O_2 uptake for successful manometric readings.

Method of study: Simultaneous adaptation (45) was the principal method of investigation.

All respirometric experiments were performed in the Warburg apparatus (55) at a temperature of 30° C with air as the gas phase. Each flask contains 2 ml of the cell suspension in the main chamber, 0.2 ml of 20% KOH in the central well and four micromoles of substrate in the side arm. When o-nitrosobenzoic acid was used as substrate, due to its extreme instability in solution, approximately 0.5 mg of its crystal was placed in the side arm of the flask. o-Hydroxylamine benzoic acid was supplied by the Department of Agricultural Chemistry of this Institute. Other chemicals were obtained commercially.

Tabulation of experimental data: The experimental data have been illustrated graphically in Chapter III. The actual data are included in tabular form in the appendix. All manometric data have been corrected for their endogenous respiration.

CHAPTER III

RESULTS AND DISCUSSIONS

Assimilation of inorganic nitrogen source: A preliminary experiment on the assimilation of different nitrogen sources was conducted by comparing the growth of Flavobacterium sp. in a liquid synthetic o-nitrobenzoic acid medium with that obtained in the same medium with benzoic acid and either NH_4Cl, $NaNO_3$ or $NaNO_2$ replacing the o-nitrobenzoic acid on an equimolar basis. A medium in which glucose and NH_4Cl replaced nitrobenzoic acid as a source of C and N was also included to compare the accessibility of glucose versus o-nitrobenzoic acid and benzoic acid.

Seed cultures were grown on synthetic medium slants and suspensions were prepared aseptically following the same procedure as preparation of enzymatically adapted cell suspensions as described in Chapter II. The final turbidity was adjusted to 50% transmittance using 525mμ wave length on the B and L "Spectronic 20" colorimeter. A standard inoculum consisting of 0.2 ml of this cell suspension was inoculated into flasks containing 100 ml of medium.

The growth studies were conducted at room temperature in duplicate with one set being incubated on the rotary shaker and the other set under static conditions. Plate counts and turbidity readings were made at different time intervals. The results of this study are given in Figures 1, 2, 3, 4, and in Tables I and II of the Appendix.

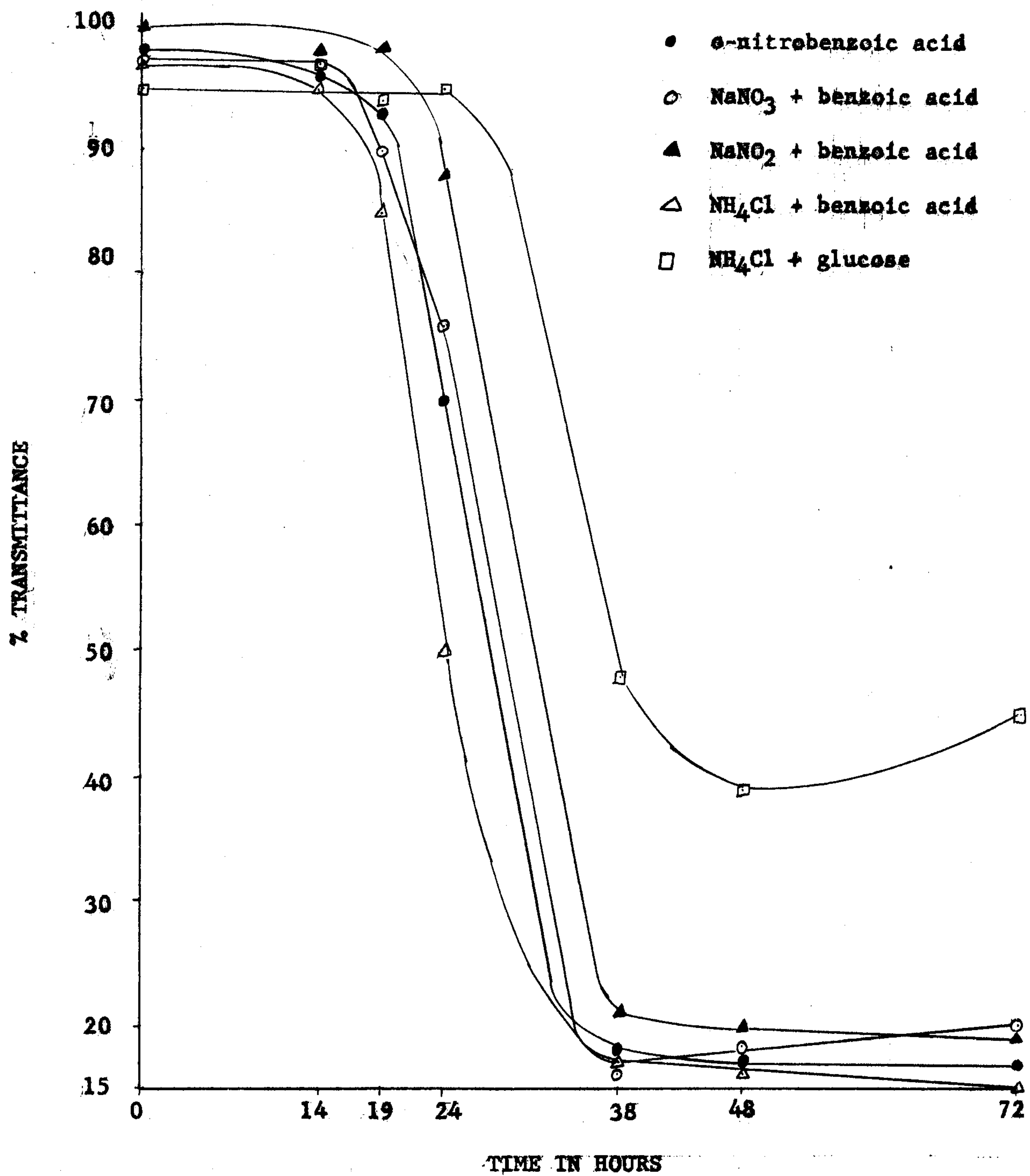


Fig. 1. The Growth of Shaken Cultures of *Flavobacterium sp.* in Media Containing Different Nitrogen and Carbon Sources.

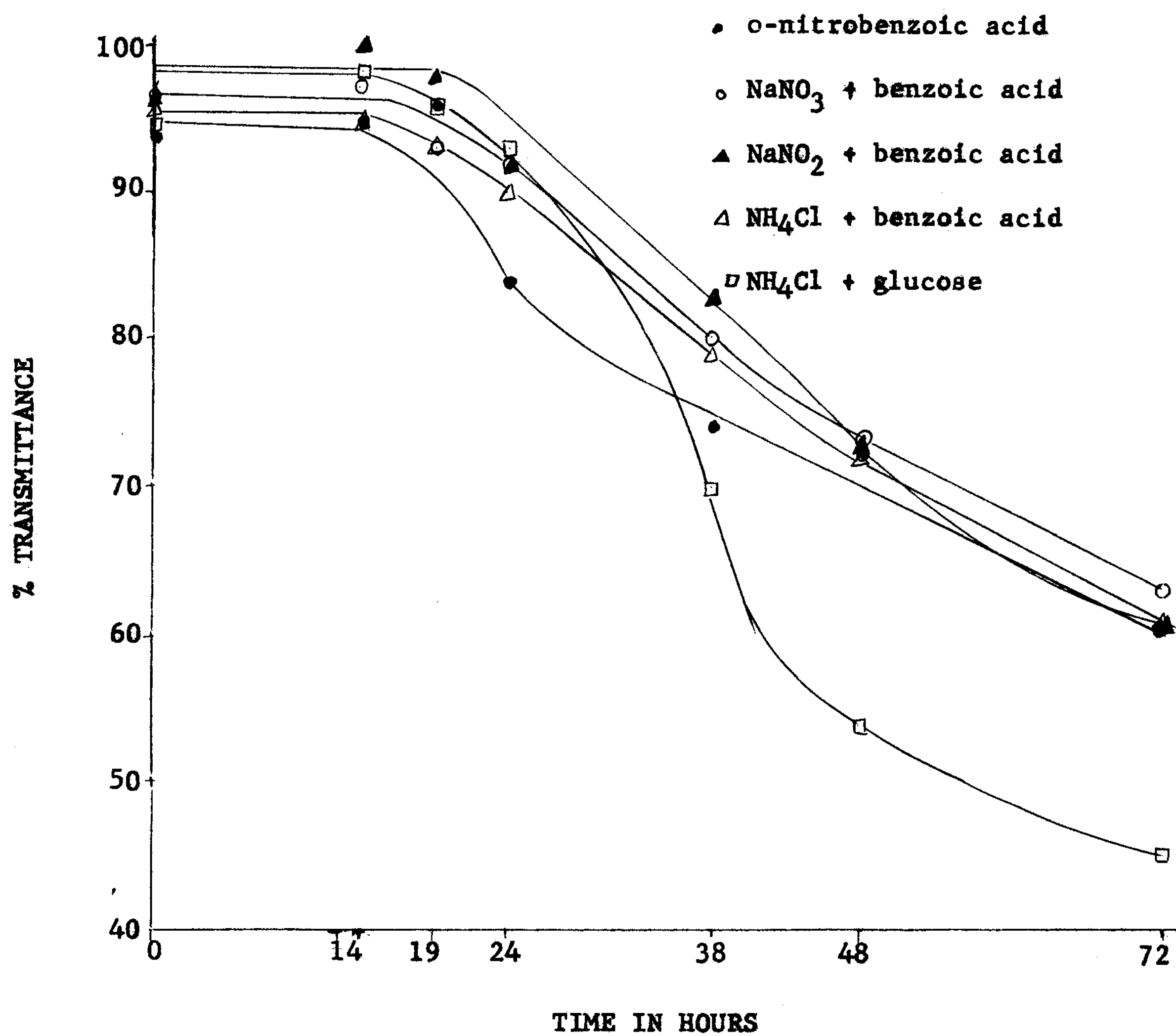


Fig. 2. The Growth of Static Culture of *Flavobacterium sp.* in Media Containing Different Nitrogen and Carbon Sources.

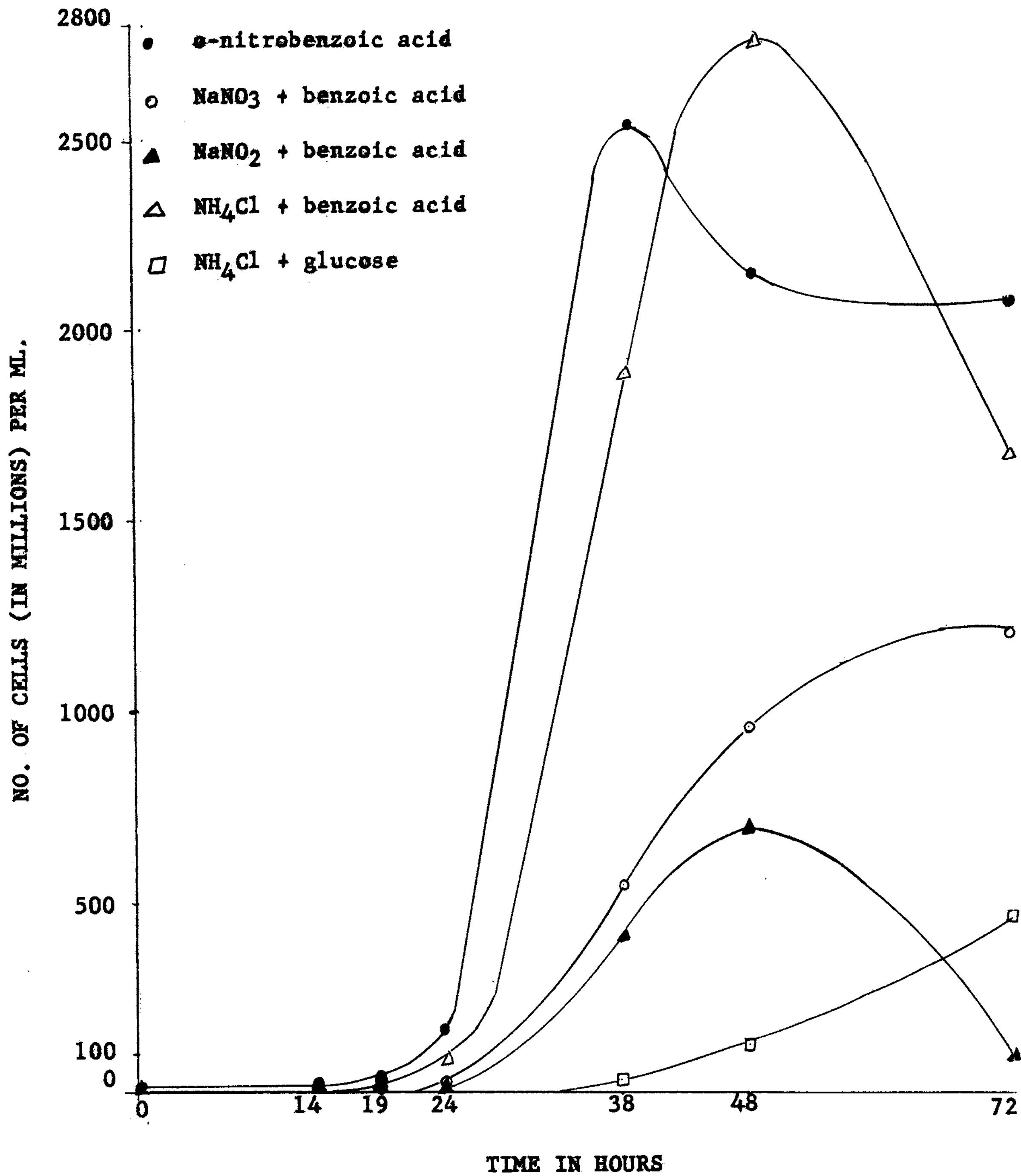


Fig. 3. The Viable Cell Count of Shaken Cultures of Flavobacterium sp. in Media Containing Different Nitrogen and Carbon Sources.

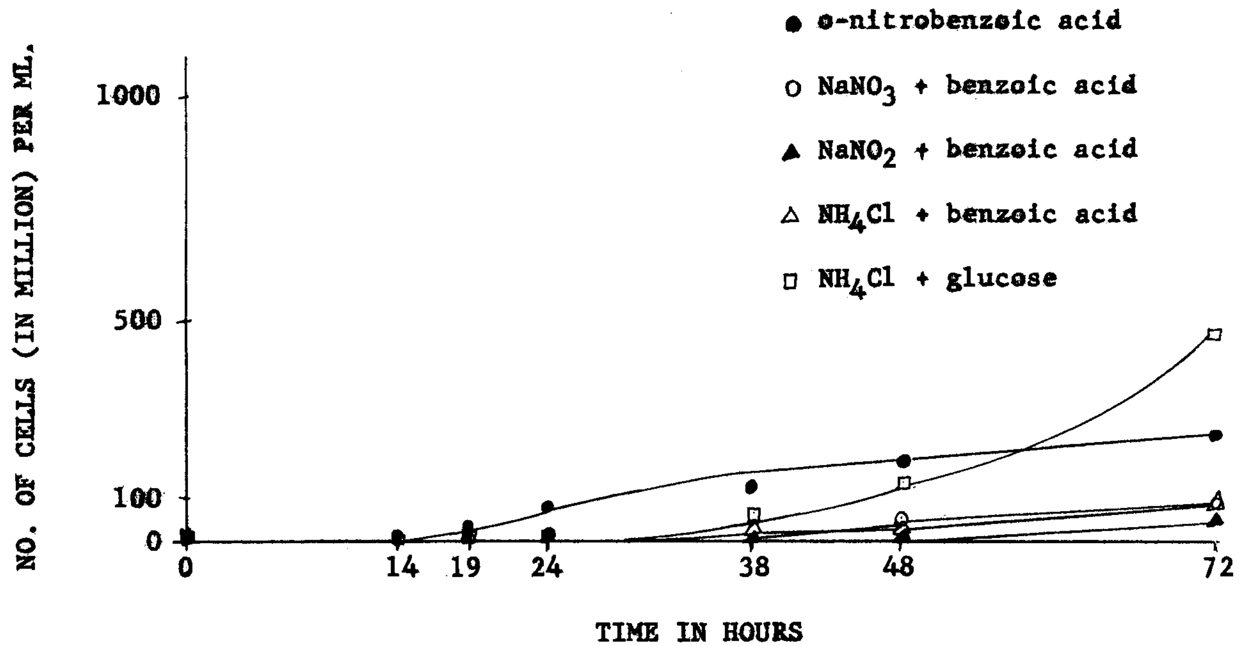


Fig. 4. The Viable Cell Count of Static Culture of *Flavobacterium sp.* in Media Containing Different Nitrogen and Carbon Sources.

These data indicate that the microorganisms grown on o-nitrobenzoic acid utilize this compound and NH_4Cl as a nitrogen source more readily than $NaNO_3$ and $NaNO_2$. Glucose seems to be less favorable than o-nitrobenzoic acid and benzoic acid as a carbon source for this microorganism. Vigorous shaking is necessary for abundant growth in liquid media.

In accordance with the above findings, NH_4Cl was used as the source of nitrogen in the media for growing different enzymatically adapted cells when the compound used as carbon source did not contain a nitrogenous group.

Studies on the adaptive enzymes formation: The experimental method used in this study is based on the simultaneous adaptation technique proposed by Stanier (45). The theory of that technique is as follows: according to the Kluyverian axiom every dissimilation is the result of a series of simple step reactions. It follows that complete reduction of even a small organic molecule will involve the formation of a number of intermediate compounds. In the case of microorganisms the further probability exists that at least some of the intermediate will be attacked by adaptive enzymes. On the general theory of adaptivity, cells adapted to attack the primary substrate should be adapted simultaneously to attack all of the intermediates formed during the reduction of the parent compound, but not to attack other substances which fail to participate in the over-all reduction process in question. Thus by growing cells on the primary compound or on assumed intermediates and then testing for adaptation to related substances, one should be able to obtain convincing evidence of whether or not assumed intermediates do actually occur, together with information about their position in the reaction chain.

Accordingly, suspensions of cells enzymatically adapted to various compounds by growing the cells on the particular compound as a sole source of nitrogen and/or carbon were prepared. Simultaneous adaptation of the cells to various compounds was followed by observing the oxygen uptake when the cells were exposed to different substrates in the Warburg apparatus.

Since a prerequisite for applying Stanier's theory is that the intermediates in doubt should be attacked by adaptive enzymes, an experiment was conducted to study the adaptive enzyme formation of the organism when exposed to different compounds. Results of this experiment were shown in Figures 5, 6, and 7, and in Table III. These data show that the Flavobacterium sp. forms adaptive enzymes to attack o-nitrobenzoic acid, o-hydroxylamine benzoic acid, anthranilic acid, salicylic acid, protocatechuic acid, benzoic acid, catechol, o-nitrobenzalcohol and o-nitrosophenol. Compounds such as o-nitrophenol, o-aminophenol, o-nitrobenzaldehyde, 2,4-dihydroxybenzoic acid, 2,4-dinitrophenol, aniline and nitroso-phenyl-hydroxylamine are not enzymatically attacked by this microorganism. Data on o-nitrosobenzoic acid is lacking in this experiment; however, from the following experiments sufficient evidence is obtained which shows that this compound is also attacked by an adaptive enzyme in this organism.

Adaptation studies for Flavobacterium sp. grown on o-nitrobenzoic acid

The compounds which are attacked by adaptive enzyme systems of Flavobacterium sp. were then tested with the organism grown on o-nitrobenzoic acid as a sole source of nitrogen and carbon to determine their possible intermediate role. Experiments were executed under the

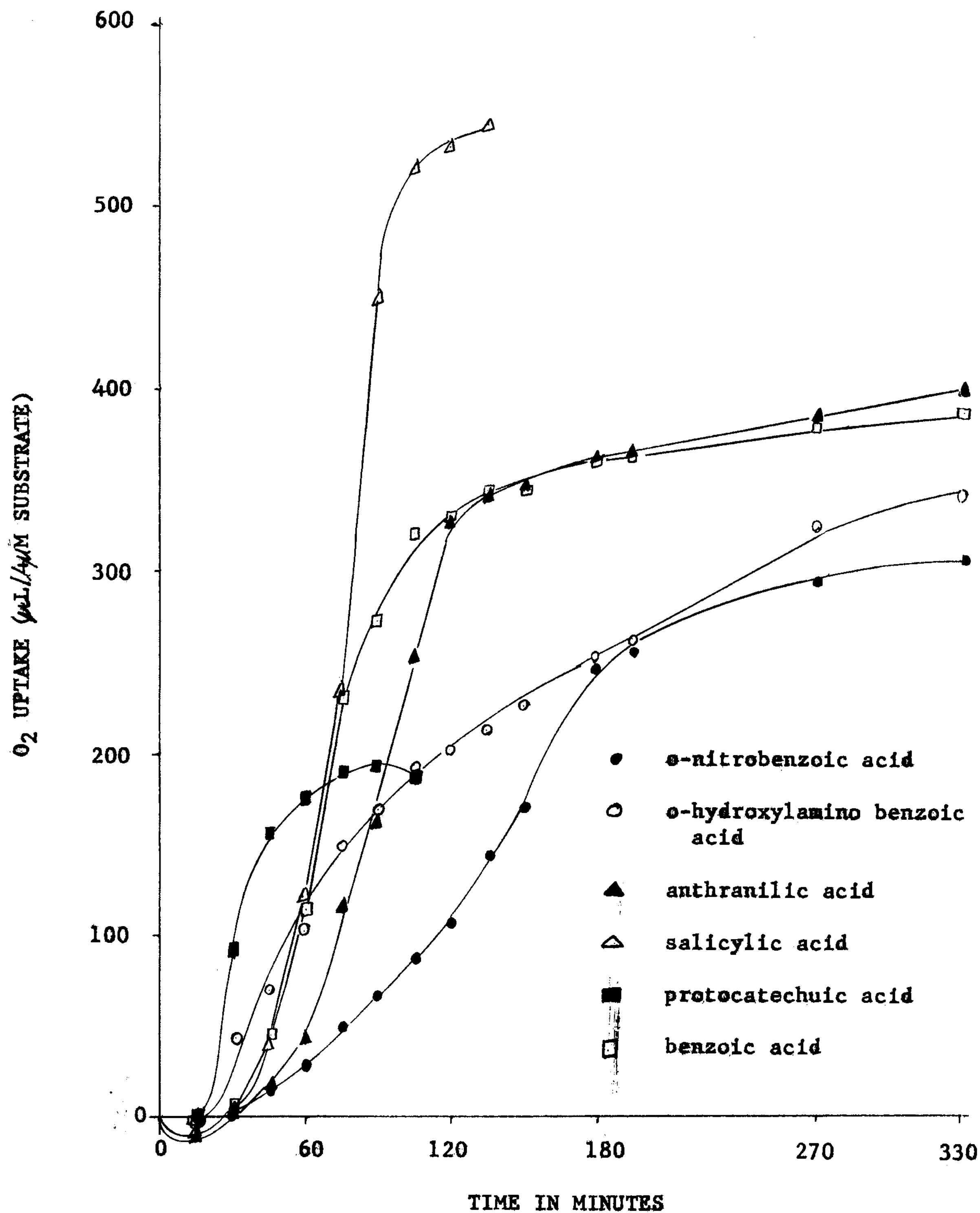


Fig. 5. The Oxidation of Various Compounds by Asparagine-Grown Cells of *Flavobacterium* sp.

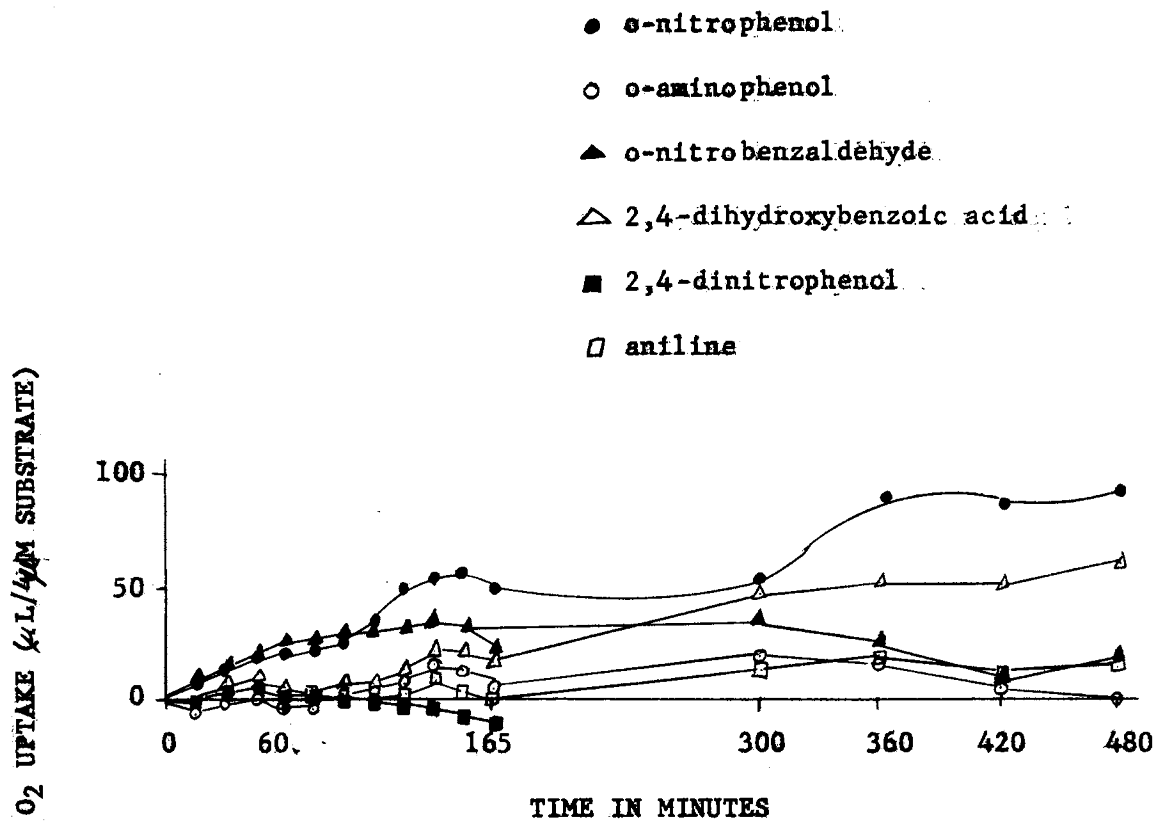


Fig. 6. The Oxidation of Various Compounds by Asparagine-Grown Cells of *Flavobacterium* sp.

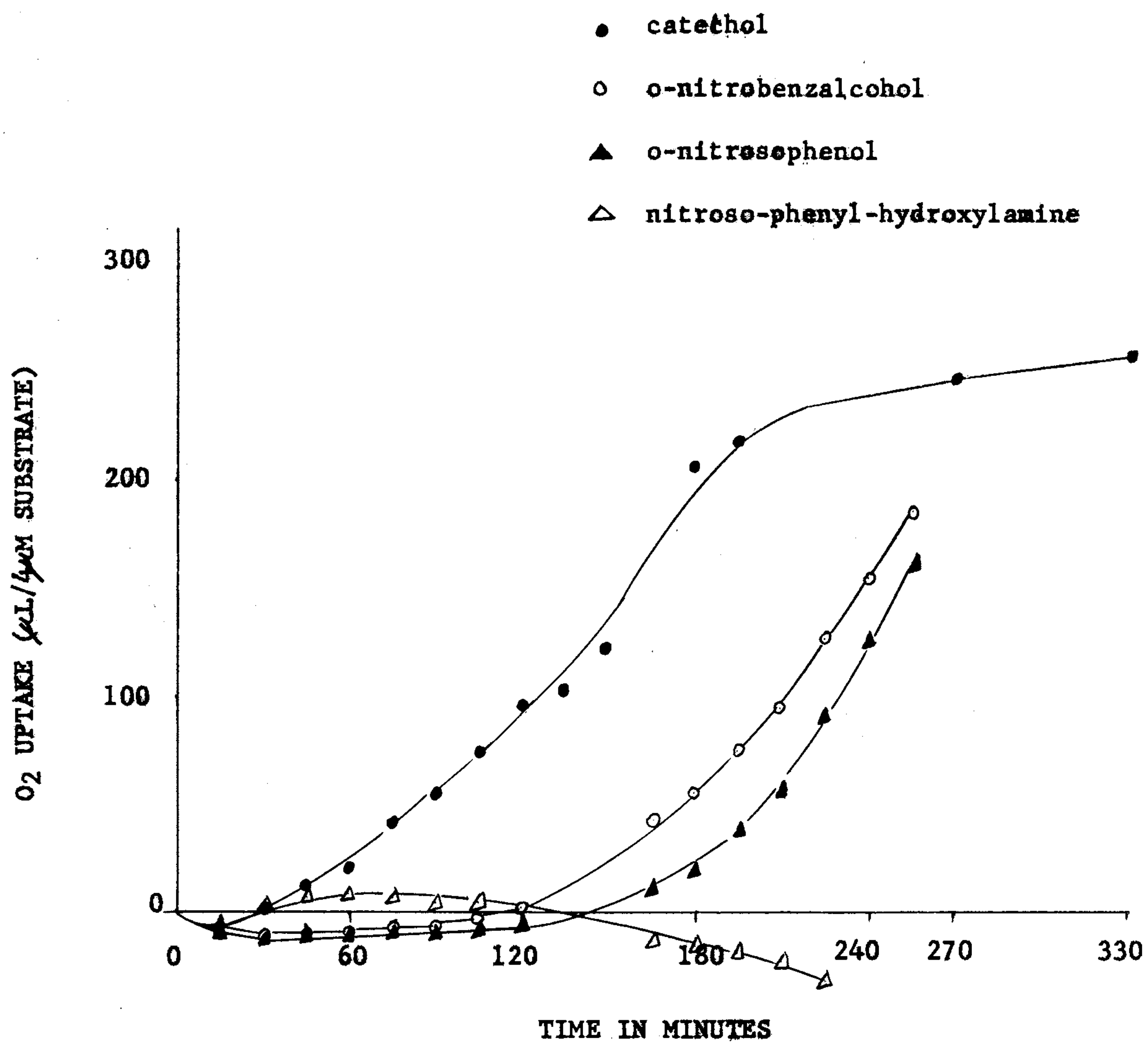


Fig. 7. The Oxidation of Various Compounds by Asparagine-Grown Cells of *Flavobacterium sp*.

same conditions as the initial experiment with cells grown on asparagine. A representative set of results of these experiments are shown in Figures 8 and 9, and tabulated in Table IV. These data show that o-nitrobenzoic acid, o-nitrosobenzoic acid and o-hydroxylamino benzoic acid are attacked simultaneously by o-nitrobenzoic acid grown cells which strongly suggest that these compounds are possible intermediates in the reduction pathway of o-nitrobenzoic acid. Anthranilic acid, salicylic acid, protocatechuic acid, benzoic acid, catechol, o-nitrobenzalcohol and o-nitrosophenol are not attacked simultaneously by the o-nitrobenzoic acid grown cells, thus indicating that these compounds cannot be a principal intermediate in the dissimilation of o-nitrobenzoic acid.

The finding that anthranilic acid does not appear to be intermediate in the dissimilative pathway of o-nitrobenzoic is in opposition to the work of Young (69) and Lively (26). This, together with the rather short lag period required for adaptation to protocatechuic acid by the o-nitrobenzoic acid grown organism, merits further study in this regard.

Studies on the ultraviolet irradiated cells of *Flavobacterium* sp. grown on o-nitrobenzoic acid: Ultraviolet irradiation, at appropriate intensities, has been shown to inhibit the induction of enzymatic biosynthesis without affecting the activity of preexisting enzymes in various microorganisms (6, 15, 51). Experiments were, therefore, conducted to study the adaptive utilization of various compounds by the ultraviolet irradiated o-nitrobenzoic acid grown cells.

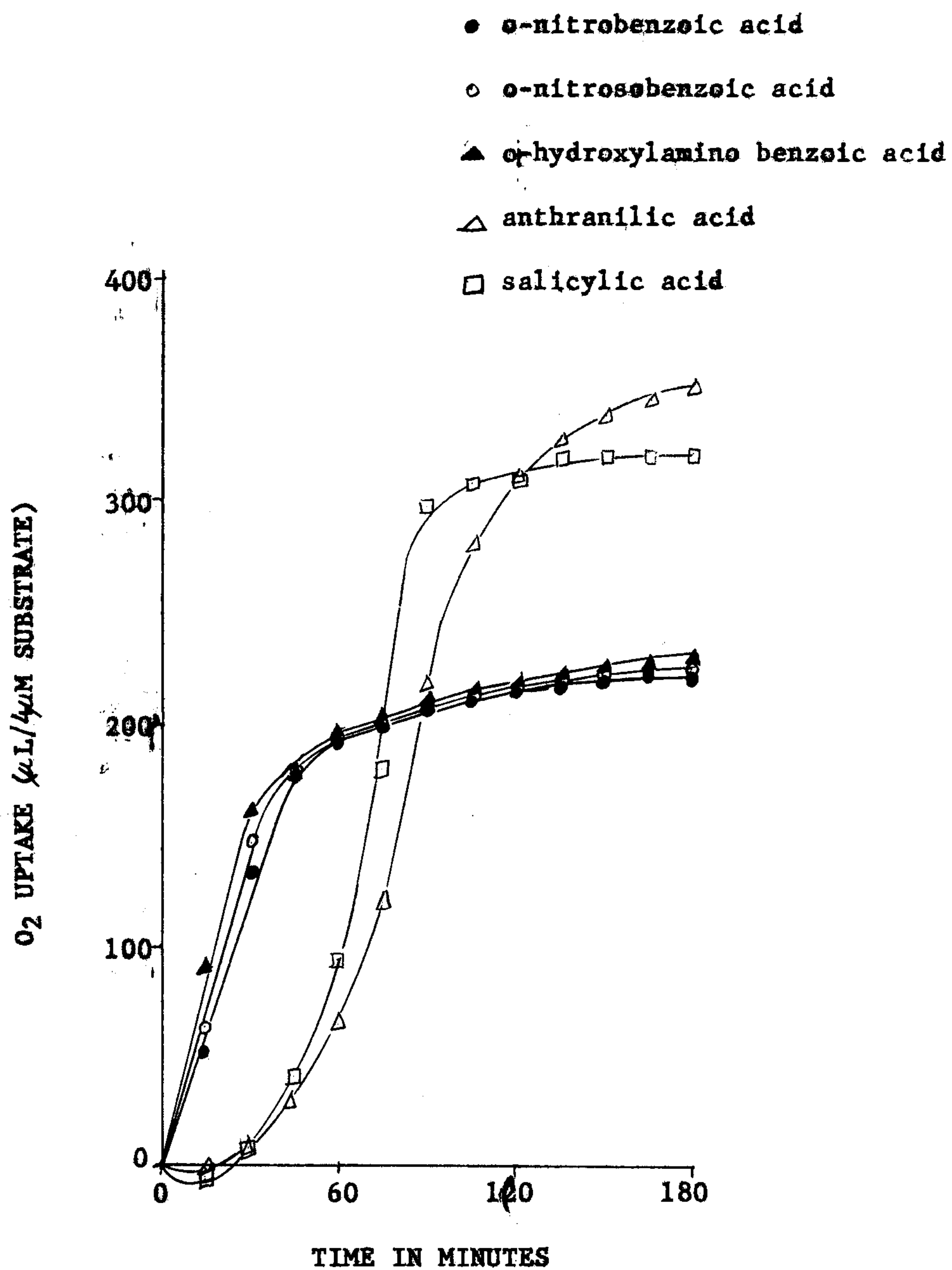


Fig. 8. The Oxidation of Various Compounds by o-Nitrobenzoic Acid-Grown Cells of *Flavobacterium* sp.

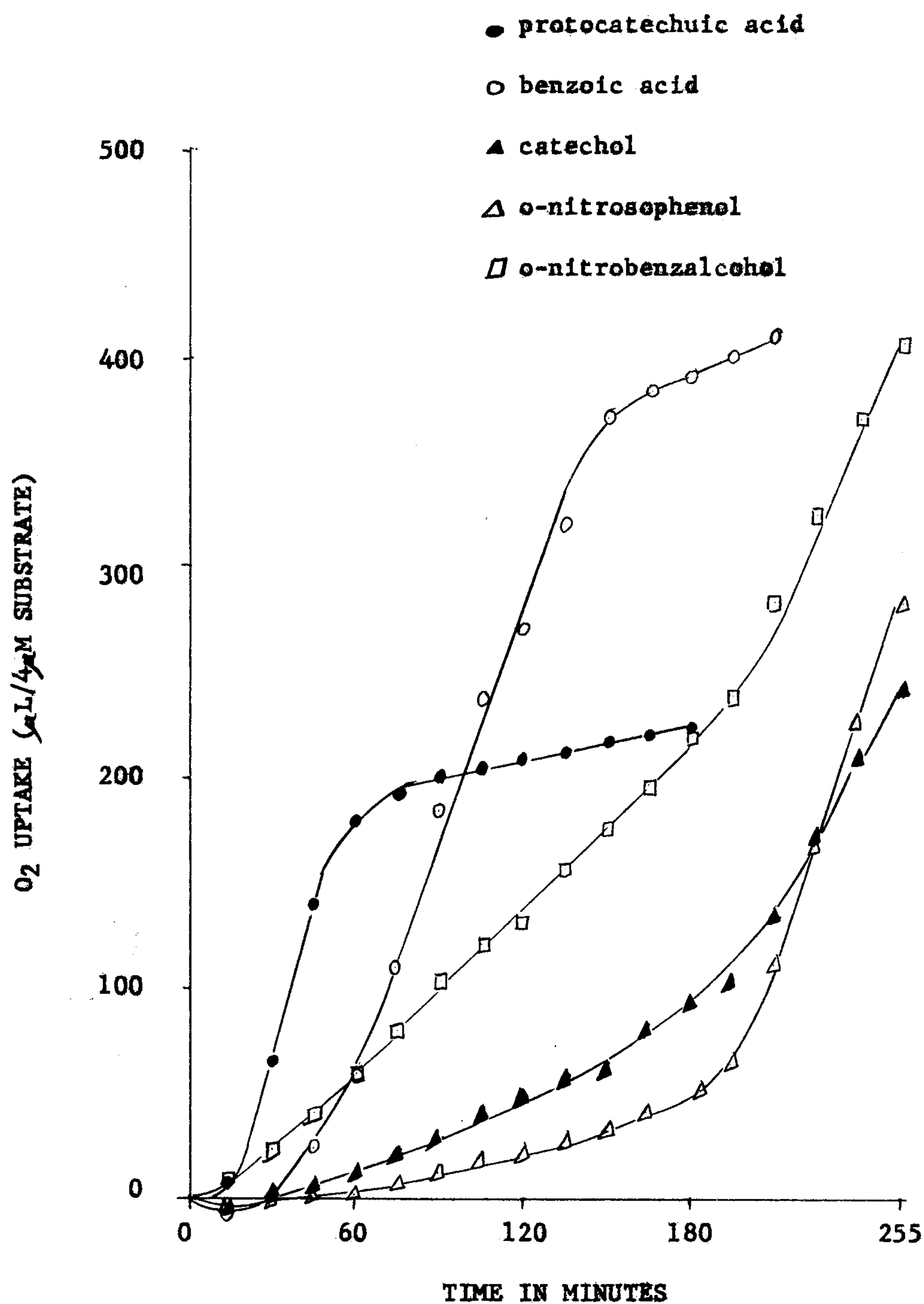


Fig. 9. The Oxidation of Various Compounds by o-Nitrobenzoic Acid-Grown Cells of *Flavobacterium* sp.

Cells grown on o-nitrobenzoic acid were suspended in a phosphate buffer solution, placed in a petri dish, and exposed to a 15 watt General Electric Germicidal Lamp for 10 minutes. The distance from the lamp to the cells was 25 cms. After treatment, the irradiated cells and the non-irradiated controls were added to Warburg flasks, and oxygen consumption was measured as previously described. Results of this experiment are shown in Figures 10 and 11, and in Table V. These data show that the biosynthesis of adaptive enzymes to attack anthranilic acid and protocatechuic acid are inhibited completely by the ultraviolet irradiation at the employed intensity while the preexisting enzymes that attack o-nitrobenzoic acid, o-nitrosobenzoic acid and o-hydroxylamino benzoic acid remain unaffected. The intermediate role of o-nitrosobenzoic acid and o-hydroxylamino benzoic acid, and the non-intermediate role of anthranilic acid and protocatechuic acid in the dissimilation of o-nitrobenzoic acid are further verified by these results.

Studies on cells of Flavobacterium sp. adapted to o-nitrosobenzoic acid and o-hydroxylamino benzoic acid: In order to elucidate the intermediary position of o-nitrosobenzoic acid and o-hydroxylamino benzoic acid in the reaction scheme, cells enzymatically adapted to these compounds were studied under the same conditions as the initial experiment.

Due to the extremely limited supply of these compounds, specific adaptation was achieved by applying Stanier and Tsuchida's method (48) in which the initially "unadapted" (asparagine grown) cell suspensions were exposed to a small amount of the compound in question, rather than growing the cells on the compound. The activation of these resting

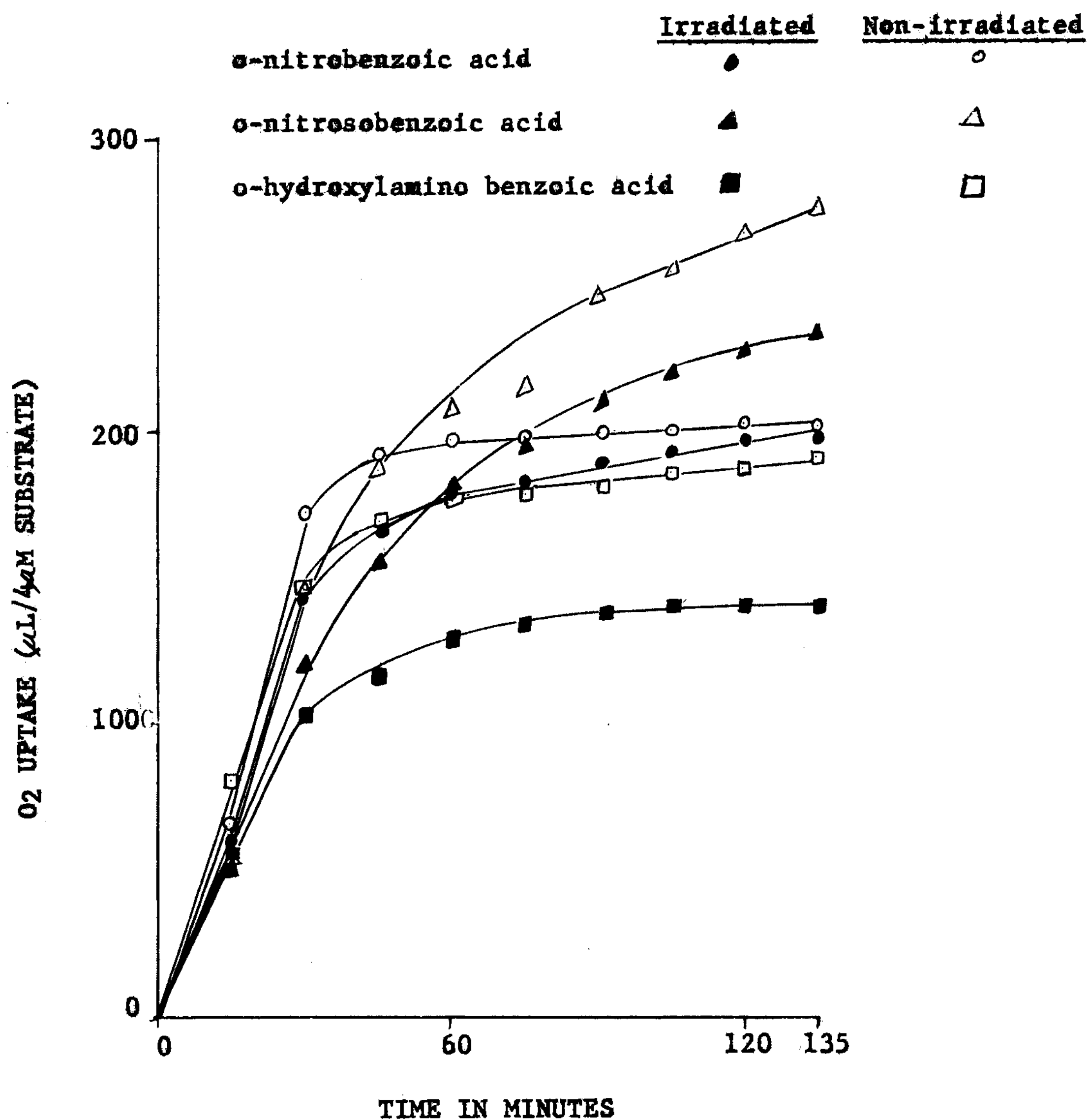


Fig. 10. The Effect of Irradiation on Substrate Oxidation by o-Nitrobenzoic Acid-Grown Cells of *Flavobacterium* sp.

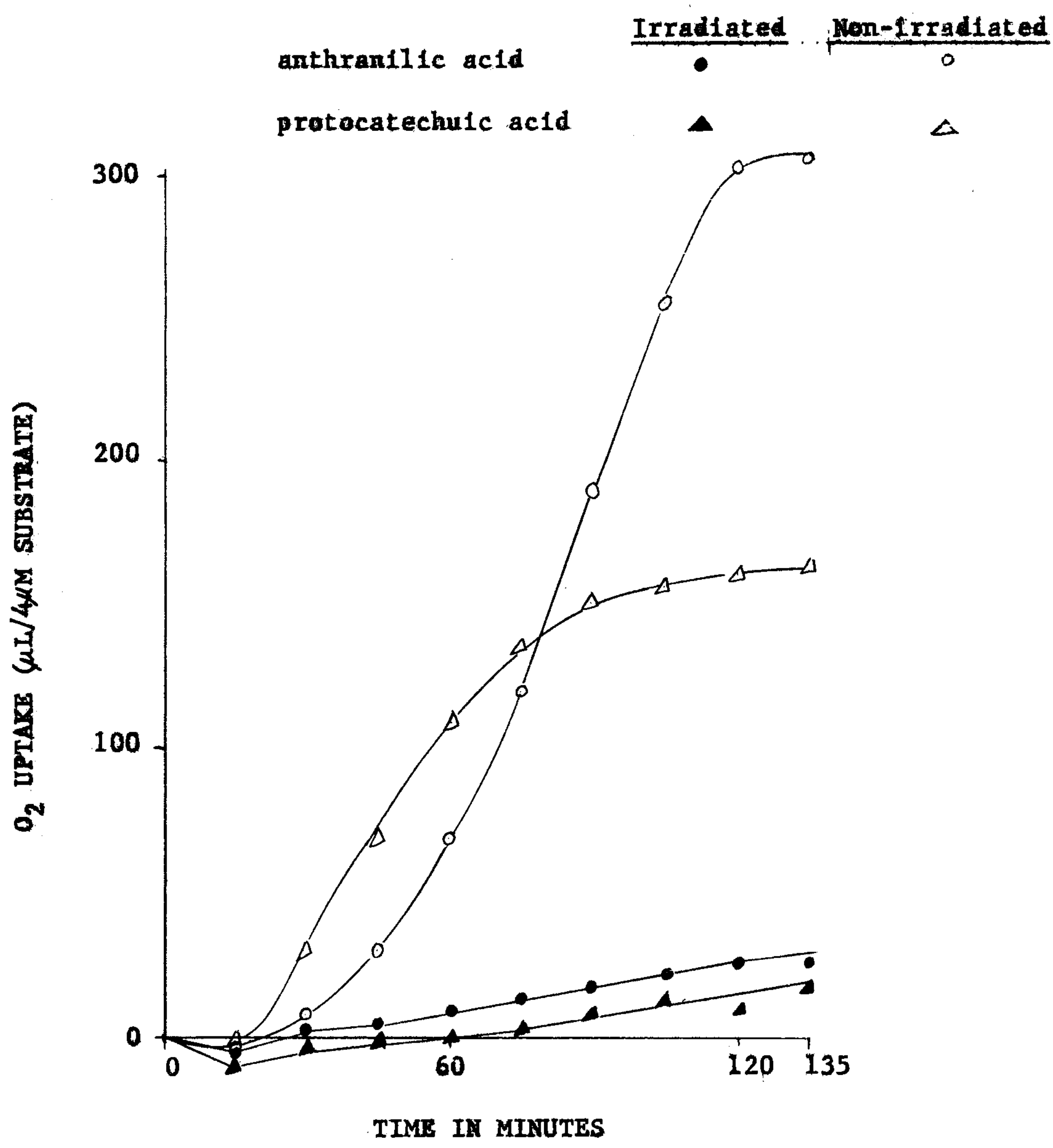
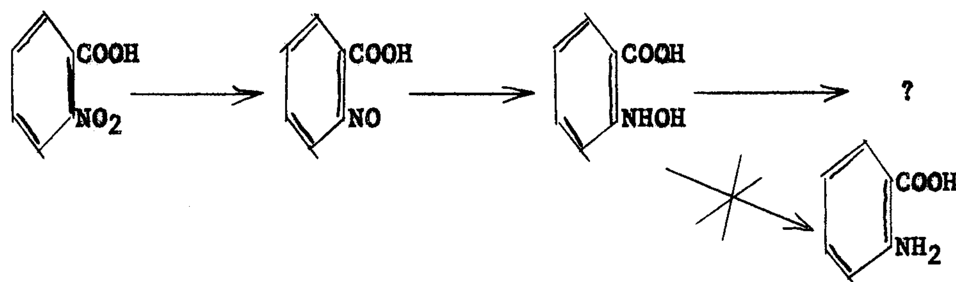


Fig. 11. The Effect of Irradiation on Substrate Oxidation by o-Nitrobenzoic Acid-Grown Cells of *Flavobacterium* sp.

cell suspensions was conducted in Warburg flasks with double side arms. One side arm contained the activating compound and the other, the substrate. After the addition of the activating compound, the course of adaptation was followed by measuring O_2 uptake until the compound was completely metabolized, as judged by a return to the auto respiratory rate of oxygen consumption. The second compound was then added from the other side arm. Representative results of these experiments are shown in Figures 12 and 13, and Table VI. These data indicate that cells previously adapted to o-nitrosobenzoic acid are simultaneously adapted to o-hydroxylamino benzoic acid but not to o-nitrobenzoic acid; while cells previously adapted to o-hydroxylamino benzoic acid rapidly attack o-hydroxylamino benzoic acid and are not simultaneously adapted to o-nitrobenzoic acid. Due to its extreme instability the o-nitrosobenzoic acid compound had probably undergone partial decomposition at the time these experiments were executed, thus accounting for relatively low and somewhat ambiguous manometric readings. However, due to the hardship of securing more of this compound in fresh condition, re-evaluation of these experiments became impracticable.

Although the data so far obtained do not indicate a clear-cut position of o-nitrosobenzoic acid, the intermediary role of this compound is evident as shown in Figures 8 and 10; therefore, a proposed reduction scheme of o-nitrobenzoic acid by the __Flavobacterium sp__. can be drawn as follows:



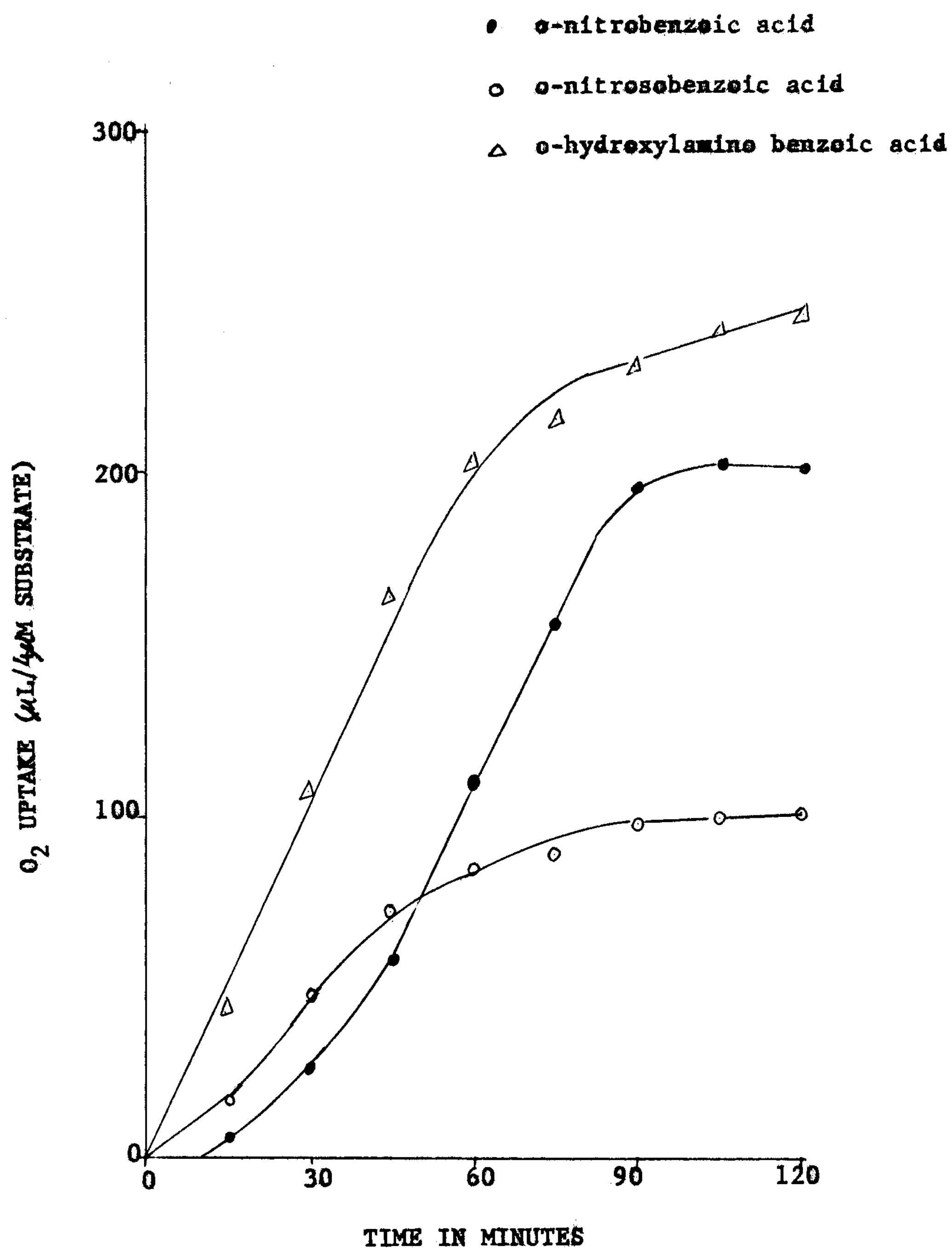


Fig. 12. The Oxidation of Various Compounds by Cells of Flavobacterium sp. Adapted to o-Nitrosobenzoic Acid.

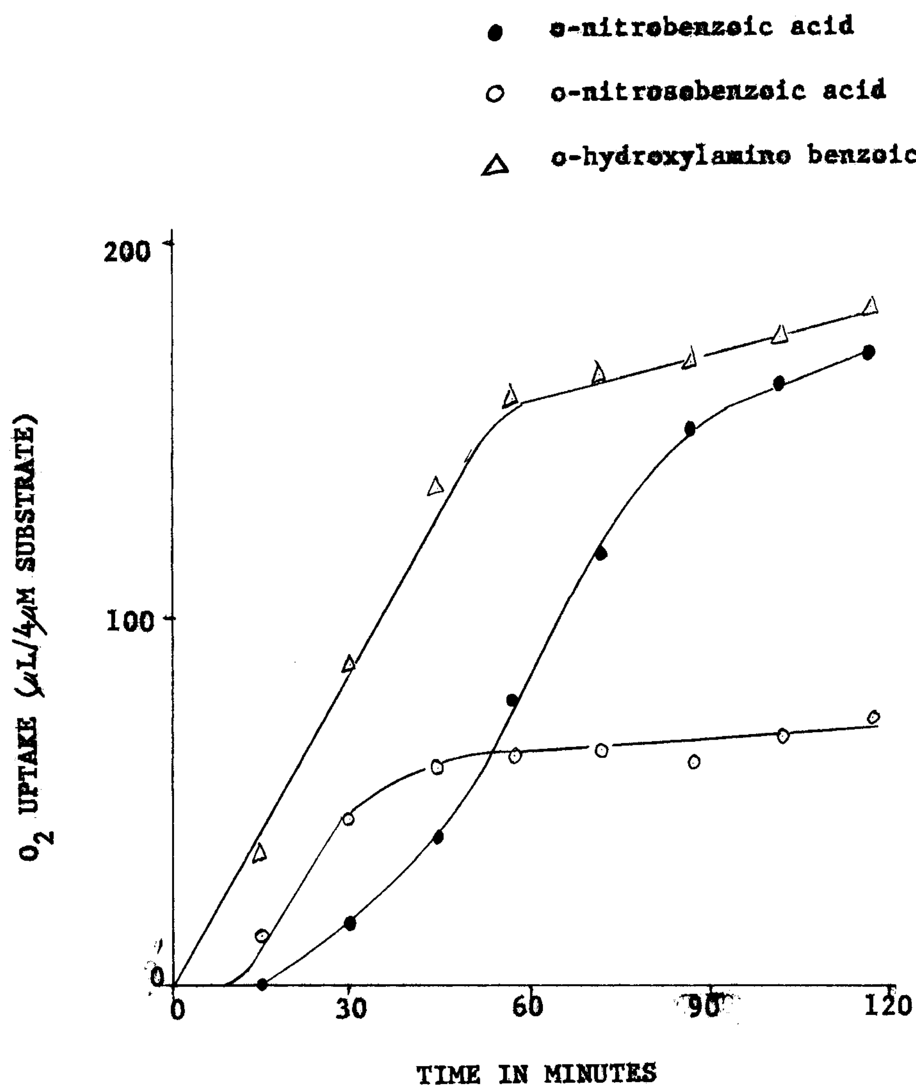


Fig. 13. The Oxidation of Various Compounds by Cells of *Flavobacterium* sp. Adapted to o-Hydroxylamino Benzoic Acid.

Although the possibility of active assimilation of the hydroxylamino compound exists as reviewed in Chapter I, no data toward proving the assimilation of o-hydroxylamino benzoic acid by *Flavobacterium sp.* at this stage is available.

Studies on the CO_2 liberation versus O_2 consumption during the dissimilation of o-nitrobenzoic acid by the Flavobacterium sp. Since o-nitrobenzoic acid is the only source of nitrogen as well as carbon for growth, the dissimilation of the aromatic ring structure is also under speculation. An experiment for the determination of CO_2 liberation versus O_2 consumption during the dissimilation of o-nitrobenzoic acid by this organism was undertaken to study this aspect of the problem. Experiments were conducted following the standard procedure of Umbreit *et al.* (55). Three Warburg flasks containing given amounts of substrate and cell suspensions were run in parallel; the first flask contained KOH to determine O_2 consumption, the second and third flasks contained no KOH but acid was added to liberate the phosphate bound CO_2 at the beginning and end of the reaction respectively. CO_2 liberation may be calculated from the differences in readings from these flasks. Ten replicates of this experiment are tabulated in Table VII.

These data show that when 1 mole of o-nitrobenzoic acid is dissimilated by this organism approximately 4 moles of CO_2 are liberated at the consumption of approximately 2 moles of O_2. This indicates a possible rupture of the ring structure with a three-carbon fragment as a residue. However, without actually detecting the fragments in the reaction mixture and accumulating additional information from enzymatic studies, the above finding is not conclusive.

TABLE VII

THE RESPIRATORY QUOTIENT OF *FLAVOBACTERIUM SP.* DISSIMILATING o-NITROBENZOIC ACID

Replication	MCO_2 / M substrate	MO_2 / M substrate	CO_2 / O_2
1	3.96	2.39	1.66
2	3.70	1.93	1.91
3	4.04	2.14	1.89
4	4.06	2.13	1.91
5	3.86	2.22	1.74
6	3.75	2.11	1.78
7	4.07	2.44	1.67
8	3.71	1.75	2.12
9	3.86	2.26	1.70
10	3.62	2.20	1.64
Average	3.86	2.16	1.80
Standard Deviation	±0.16	±0.17	±0.11

Studies on the *Flavobacterium* sp. cells grown on anthranilic acid and salicylic acid: Although anthranilic acid and salicylic acid do not appear to be intermediates in the reduction pathway of o-nitrobenzoic acid, these compounds are also dissimilated by the *Flavobacterium* sp. as indicated by adaptive enzyme studies. Therefore, the dissimilative pathway of anthranilic acid and salicylic acid by this organism was also studied to a limited extent. The results of manometric studies on the *Flavobacterium* sp. cells grown on anthranilic acid and salicylic acid are shown in Figures 14 and 15 and Table VIII.

Stanier *et al.* (46, 47) in their studies of the bacterial oxidation of tryptophan found that anthranilic acid was oxidized to catechol by a group of Pseudomonads. Sakamoto *et al.* (40) also found the bacterial metabolic pathway of anthranilic acid to be via salicylic acid and catechol. However, the results from this experiment on

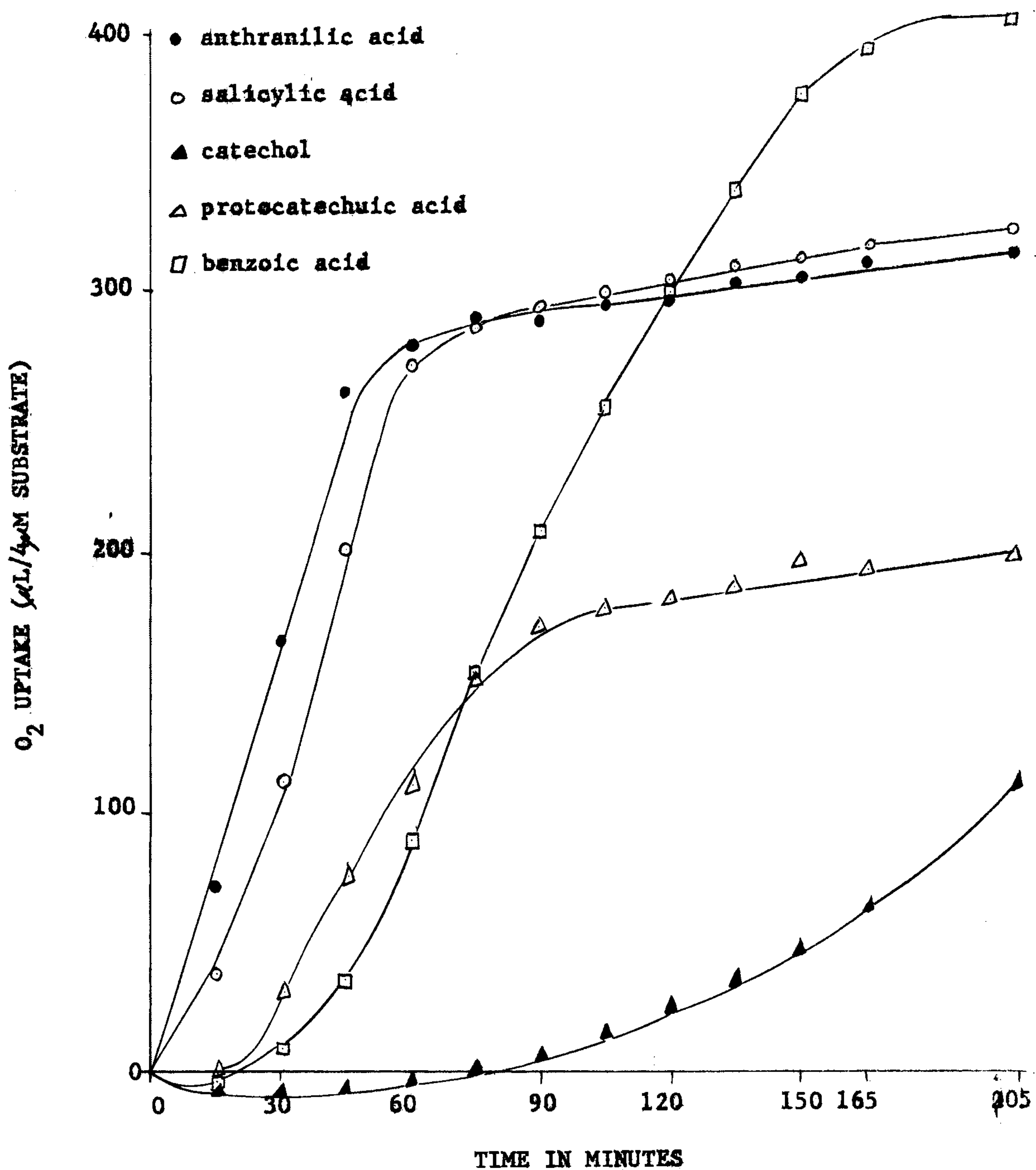


Fig. 14. The Oxidation of Various Compounds by Anthranilic Acid-Grown Cells of Flavobacterium sp.

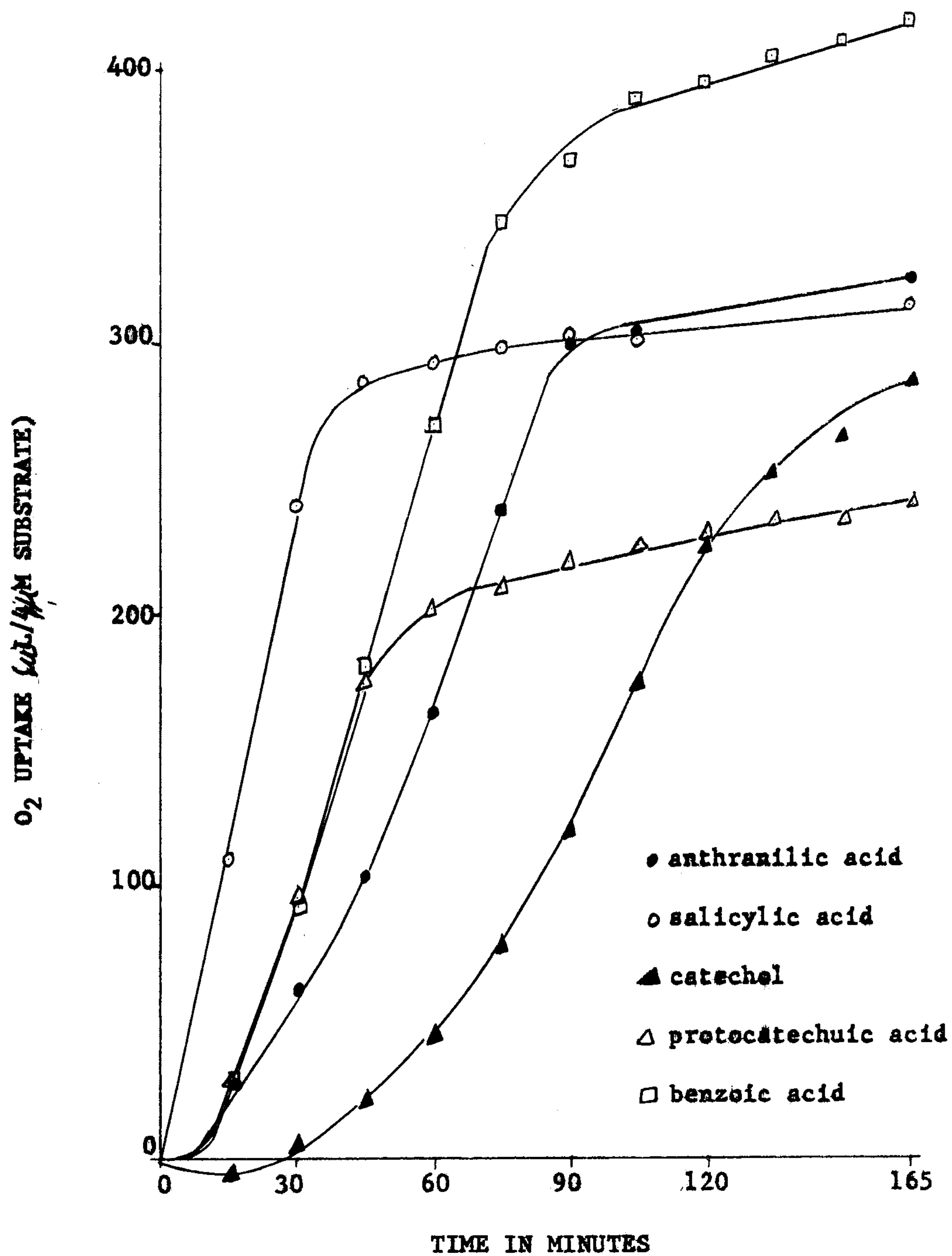


Fig. 15. The Oxidation of Various Compounds by Salicylic Acid-Grown Cells of <u>Flavobacterium sp</u>.

Flavobacterium *sp.* indicate only salicylic acid to be a possible intermediate due to its simultaneously adaptive characteristic. Studies fail to show the intermediary role of catechol, benzoic acid, and protocatechuic acid, in the dissimilation of anthranilic acid by this microorganism.

SUMMARY

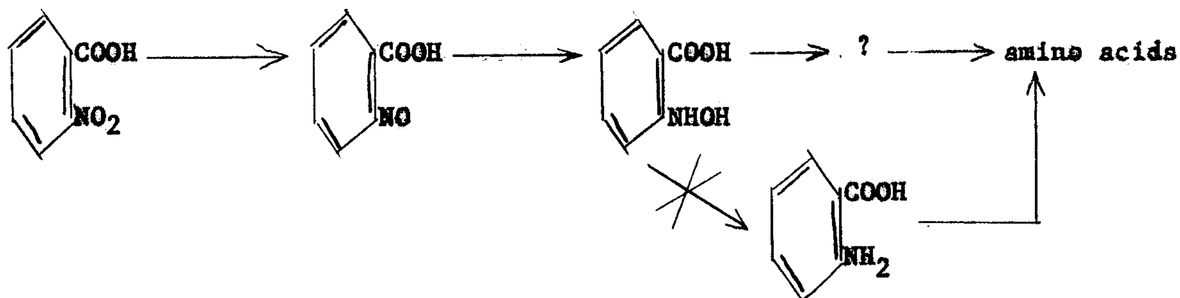
1. This study was designed to help elucidate the biochemical mechanism of nitrate reduction by studying the adaptive utilization of o-nitrobenzoic acid and its reduction products by a Flavobacterium sp. isolated from soil.

2. The microorganism was capable of utilizing o-nitrobenzoic acid as a sole source of organic carbon and nitrogen. Inorganic NH_4-N was found to be assimilated more rapidly by this bacterium than NO_3-N or NO_2-N.

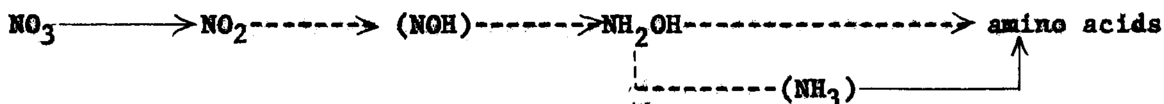
3. Adaptive enzymes were formed to attack o-nitrobenzoic acid, o-nitrosobenzoic acid, o-hydroxylamino benzoic acid, anthranilic acid, salicylic acid, protocatechuic acid, benzoic acid, catechol, o-nitrosophenol and o-nitrobenzalcohol by this bacterium when exposed to these compounds.

4. The simultaneous adaptation to o-nitrosobenzoic acid and o-hydroxylamino benzoic acid by the cells of this microorganism grown on o-nitrobenzoic acid, as indicated by the immediate uptake of O_2 in the Warburg apparatus, suggests the intermediary role of these compounds. Anthranilic acid, as well as salicylic acid, protocatechuic acid, benzoic acid, catechol, o-nitrosophenol and o-nitrobenzalcohol failed to show immediate O_2 uptake by the o-nitrobenzoic acid grown cells indicating that these compounds do not appear to be principal intermediates in the dissimilation of o-nitrobenzoic acid.

5. Cells enzymatically adapted to o-nitrosobenzoic acid showed simultaneous adaptation to o-hydroxylamino benzoic acid but not to o-nitrobenzoic acid. Cells enzymatically adapted to o-hydroxylamino benzoic acid rapidly attacked this compound but did not show simultaneous adaptation to o-nitrobenzoic acid. From these observations, together with other findings, the following scheme of reduction of o-nitrobenzoic acid by the *Flavobacterium sp.* is postulated:



The above scheme supports the view that the pathway of nitrate reduction proceeds via nitrite, hydroxylamine, but not necessarily ammonia:



6. Approximately 4 moles of CO_2 were liberated with the consumption of about 2 moles of O_2 when 1 mole of o-nitrobenzoic acid was dissimilated by this bacterium. These results indicate rupture of the ring.

7. Salicylic acid was found to be an intermediate in the dissimilation of anthranilic acid by the same method of study. Catechol, benzoic acid and protocatechuic acid, were not implicated as intermediates in the dissimilation of anthranilic acid.

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A P P E N D I X

TABULATED DATA FROM WHICH FIGURES ARE PREPARED

TABLE I

THE GROWTH OF *FLAVOBACTERIUM SP.* IN MEDIA CONTAINING DIFFERENT NITROGEN AND CARBON SOURCES

		% Transmittance									
N & C Source		o-nitrobenzoic acid		$NaNO_3$ and benzoic acid		$NaNO_2$ and benzoic acid		NH_4Cl and benzoic acid		NH_4Cl and glucose	
Cultural Conditions		shaken	static	shaken	static	shaken	static	shaken	static	shaken	static
Time (hrs)	0	98	94	97	97	100	97	97	96	95	95
	14	96	95	97	97	98	100	95	95	97	98
	19	93	96	90	93	98	98	85	93	94	96
	24	70	84	76	92	88	92	50	90	95	93
	38	18	74	16	80	21	83	17	79	48	70
	48	17	72	18	73	20	73	16	72	39	54
	72	17	61	20	63	19	61	15	61	45	45

TABLE II

THE VIABLE CELL COUNT OF *FLAVOBACTERIUM SP.* IN MEDIA CONTAINING DIFFERENT NITROGEN AND CARBON SOURCES

		No. of cells (in million) per ml.									
N & C Source		o-nitrobenzoic acid		$NaNO_3$ and benzoic acid		$NaNO_2$ and benzoic acid		NH_4Cl and benzoic acid		NH_4Cl and glucose	
Cultural Conditions		shaken	static	shaken	static	shaken	static	shaken	static	shaken	static
Time (hrs)	0	15	12	9	8	8	8	8	8	7	5
	14	12	7	3	3	3	2	2	3	1	1
	19	31	33	9	5	4	2	18	9	1	1
	24	164	84	23	11	12	8	89	15	4	1
	38	2550	120	557	-	417	9	1890	36	1	30
	48	2150	186	965	48	690	12	2766	30	613	126
	72	2080	237	1213	73	102	57	1670	80	457	487

TABLE III

THE OXIDATION OF VARIOUS COMPOUNDS BY ASPARAGINE-GROWN CELLS OF FLAVOBACTERIUM SP.

Time	O_2 uptake (µl/4µM substrate)					
	Substrates *					
(min.)	o-NO_2 BA	o-NHOH BA	Anth A	Sali A	Prot A	Benz A
15	0	-1	-12	-5	4	-10
30	0	43	3	6	91	6
45	15	70	16	40	158	45
60	27	104	44	123	177	118
75	50	150	118	237	189	235
90	65	170	165	450	192	276
105	85	193	253	523	187	321
120	108	203	327	535	—	331
135	144	216	341	547	—	343
150	170	226	346	536	—	346
180	246	254	364	569	—	365
195	258	263	368	571	—	368
270	295	327	387	589	—	383
330	307	339	397	599	—	389

* o-NO_2 BA   o-nitrobenzoic acid
o-NHOH BA   o-hydroxylamino benzoic acid
Anth A   anthranilic acid
Sali A   salicylic acid
Prot A   protocatechuic acid
Benz A   benzoic acid

TABLE III (Continued)

Time (min.)	O_2 uptake (μl/4μM substrate)					
	Substrates *					
	o-NO_2 Ph	o-NH_2 Ph	o-NO_2 By	24DiOHBA	24DiNO_2 Ph	Aniline
15	11	-9	12	2	2	0
30	15	-4	17	9	0	5
45	20	-1	22	12	4	7
60	19	-4	24	5	1	0
75	21	-3	27	3	3	-2
90	28	2	30	8	3	0
105	35	3	31	7	1	-1
120	50	9	32	15	0	3
135	55	16	36	21	-4	10
150	56	14	32	22	-3	5
165	50	5	25	16	-9	-3
300	53	18	37	49	-17	14
360	89	13	27	55	—	17
420	86	3	18	52	—	9
480	94	-2	16	61	—	14

* o-NO_2 Ph — o-nitro phenol
o-NH_2 Ph — o-amino phenol
o-NO_2 By — o-nitrobenzaldehyde
24DiOHBA — 2,4-di-hydroxyl benzoic acid
24DiNO_2 Ph — 2,4-di-nitro phenol
Aniline — aniline

TABLE III (Continued)

Time (min.)	O_2 uptake ($\mu l/4\mu M$ substrate)			
	Substrates			
	Catechol	o-Nitro-benzalcohol	o-Nitroso-phenol	Nitrosophenyl-hydroxylamine
15	-5	-7	-7	-3
30	1	-14	-15	4
45	12	-12	-11	7
60	20	-12	-13	9
75	41	-11	-12	6
90	54	-10	-12	5
105	75	-5	-11	5
120	99	3	-10	0
135	102	—	—	—
150	123	—	—	—
165	—	43	10	-14
180	207	58	20	-19
195	217	77	38	-20
210	—	97	56	-28
225	—	128	93	-34
240	—	155	126	—
255	—	185	161	—
270	249	—	—	—
330	258	—	—	—

TABLE IV

THE OXIDATION OF VARIOUS COMPOUNDS BY o-NITROBENZOIC ACID-GROWN CELLS OF FLAVOBACTERIUM SP.

Time	O_2 uptake (μl/4μM substrate)									
	Substrates *									
(min.)	OBA	SBA	HBA	ANT	SAL	PRO	BA	CAT	NSP	NBL
15	49	65	90	-2	-5	5	-8	-7	-5	9
30	135	146	161	8	8	66	1	0	-5	23
45	179	178	179	28	39	140	23	4	-1	40
60	195	193	190	64	96	182	59	11	3	60
75	203	201	200	119	183	193	111	19	8	80
90	210	210	209	217	299	203	186	29	12	105
105	212	213	213	282	311	207	239	39	17	123
120	216	216	219	313	314	210	272	46	20	135
135	219	221	225	329	319	215	323	57	27	158
150	221	224	226	340	322	220	372	65	32	178
165	224	226	229	346	325	223	386	78	41	200
180	223	228	231	352	325	227	395	93	51	221
195	—	—	—	—	—	—	404	104	65	241
225	—	—	—	—	—	—	415	137	113	286
255	—	—	—	—	—	—	—	171	170	328
285	—	—	—	—	—	—	—	211	230	372
315	—	—	—	—	—	—	—	244	286	410

* OBA o-nitrobenzoic acid
SBA o-nitrosobenzoic acid
HBA o-hydroxylamino benzoic acid
ANT anthranilic acid
SAL salicylic acid
PRO protocatechuic acid
BA benzoic acid
CAT catechol
NSP o-nitrosophenol
NBL o-nitrobenzalcohol

TABLE V

THE EFFECT OF IRRADIATION ON SUBSTRATE OXIDATION BY o-NITROBENZOIC ACID-GROWN CELLS OF *FLAVOBACTERIUM SP.*

Time	O_2 uptake (μl/4μM substrate)									
	Irradiated					Non-irradiated				
	Substrate *					Substrate *				
(min.)	OBA	SBA	HBA	ANT	PRO	OBA	SBA	HBA	ANT	PRO
15	60	52	58	-7	-11	66	58	81	-5	-1
30	144	122	102	3	-4	172	146	147	9	31
45	166	156	118	4	-2	191	186	169	30	70
60	179	181	129	9	0	197	207	176	70	110
75	184	195	134	13	3	197	215	178	121	136
90	190	209	139	17	8	198	246	182	190	152
105	194	220	141	23	12	200	254	186	255	156
120	197	228	142	26	9	202	268	188	303	161
135	197	234	140	26	19	202	277	191	307	165

* OBA o-nitrobenzoic acid
SBA o-nitrosobenzoic acid
HBA o-hydroxylamino benzoic acid
ANT anthranilic acid
PRO protocatechuic acid

TABLE VI

THE OXIDATION OF VARIOUS COMPOUNDS BY CELLS OF FLAVOBACTERIUM SP. ADAPTED TO o-NITROSOBENZOIC ACID AND o-HYDROXYLAMINO BENZOIC ACID

Time (min.)	O_2 uptake (μl/4μM substrate)					
	Cells adapted to o-nitrosobenzoic acid			Cells adapted to o-hydroxylamino benzoic acid		
	* Substrate			* Substrate		
	OBA	SBA	HBA	OBA	SBA	HBA
15	6	16	43	-1	15	35
30	26	50	109	17	46	89
45	59	73	166	41	60	135
60	110	86	204	78	64	159
75	157	90	218	118	65	165
90	197	99	234	150	61	168
105	202	100	242	162	69	177
120	201	102	247	171	74	185

* OBA o-nitrobenzoic acid
SBA o-nitrosobenzoic acid
HBA o-hydroxylamino benzoic acid

TABLE VIII

THE OXIDATION OF VARIOUS COMPOUNDS BY ANTHRANILIC ACID AND SALICYLIC ACID-GROWN CELLS OF *FLAVOBACTERIUM SP.*

Time	O_2 uptake ($\mu l/4\mu M$ substrate)									
	Anthranilic acid-grown cells					Salicylic acid-grown cells				
	* Substrate					* Substrate				
(min.)	ANT	SAL	CAT	PRO	BA	ANT	SAL	CAT	PRO	BA
15	73	36	-9	1	-2	26	110	-5	27	29
30	167	114	-9	32	8	63	240	4	98	94
45	262	205	-7	77	37	102	285	22	176	180
60	279	273	-4	112	89	164	291	47	203	270
75	289	287	2	153	154	238	298	79	211	346
90	287	295	7	173	209	299	301	121	220	377
105	295	301	15	180	257	303	301	175	226	390
120	297	306	25	184	299	—	—	225	230	396
135	303	311	35	188	340	—	—	261	235	405
150	306	314	47	198	377	—	—	275	235	412
165	312	320	65	195	395	323	314	286	240	418
205	313	325	113	200	408	—	—	—	—	—

* ANT anthranilic acid
SAL salicylic acid
CAT catechol
PRO protocatechuic acid
BA benzoic acid

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