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Title COMPARATIVE STUDY OF AEROBIC AND NITRATE RESPIRATION IN PSEUDOMONAS STUTZERI

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Some forms of bacterial respiration do not involve molecular oxygen but instead utilize other hydrogen acceptors for oxidation of the substrate. Various organic and inorganic acceptors may be used. In the present study, the inorganic radicals, nitrate and nitrite, were investigated. Preliminary evidence indicated that nitrate and oxygen are able to compete effectively as acceptors of hydrogen in respiration. It is the purpose of this dissertation to extend this observation with the hope that the degree of competition may be elucidated and information obtained regarding the conditions under which nitrate and nitrite can most effectively compete with oxygen.

A strain of Pseudomonas stutzeri was used throughout the study. Experiments were conducted with the closed electrolytic respirometer flasks which could be flushed with helium gas for anaerobic studies or with 20% oxygen in helium for aerobic studies. The vessels containing a magnetic bar were set on magnetic stirrers to obtain

maximum aeration of the medium. Samples of the respirometer atmosphere were assayed with a Beckman GC-2 gas chromatograph. Analyses for CO_2, NO_3^-, NO_2^- and fermentation products were performed by conventional procedures. Dissolved oxygen was measured with a Precision Scientific Oxygen Analyzer.

P. stutzeri has a definite requirement for some component supplied by yeast extract. No denitrifying activity is noted in the absence of yeast extract. Neither nitrate nor nitrite can be assimilated by the cell although either can be used as the sole hydrogen acceptor in respiration. Data are presented for nitrite respiration which indicate that this respiratory system may be similar to that of the oxygen system insofar as the atoms of oxygen required for oxidation of the carbon substrate. The nitrate respiring system seems to be less efficient, particularly when using the nitrate to nitrite reduction step. An [O]/C ratio of 2.0 is obtained for nitrite whereas the mean ratio for nitrate is 2.5. Excretion of fermentation products appears to be due to a sluggish acceptor system or to the complete absence of acceptor. Pyruvate, acetate and succinate are metabolized without difficulty.

The cell density greatly influenced the dissolved oxygen content of the agitated medium. This in turn determined the rate at which nitrate and nitrite could be reduced in the aerobic system. Too dense a cell suspension leads to "aerobic denitrification" because the conditions of the medium per se were not aerobic. Attempts to correlate dissolved oxygen with "aerobic denitrification

showed that at a D.O. as low as 0.9 ppm no denitrification occurred. With cell optical densities of 0.5 to 1.0 (0.25 to 0.5 mg dry wt.) very active stirring of the medium was required to maintain the D.O. above 1.0 ppm for a 24 hour period. Although no N_2 gas was produced, nitrate was reduced to nitrite.

The reduction of oxygen uptake by high nitrate concentrations was first noted at 4000 ppm NO_3^--N (0.28 molar) and increased with increase of nitrate. A solution of 8000 ppm (0.57 molar) gave a lag lasting about 3 hours. Solutions of other salts at 0.57 molar (NH_4Cl, KCl, KNO_3, NH_4NO_3 and K_2SO_4) also caused a reduction in oxygen uptake. The effect was greatest with the nitrate salts. A 0.57 M solution of $(NH_4)_2HPO_4$ had no apparent effect on oxygen consumption.

Solutions (0.50 M) of KCl, K_2SO_4 and KNO_3 exerted no apparent influence on the reduction of nitrate to nitrite, but the further reduction of nitrite to N_2 (nitrite respiration) was almost completely inhibited during the 30 hour test period. This was true for both nitrite accumulated from nitrate and for nitrite added in the absence of nitrate.

COMPARATIVE STUDY OF AEROBIC AND NITRATE RESPIRATION IN *PSEUDOMONAS STUTZERI*

by

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COMPARATIVE STUDY OF AEROBIC AND NITRATE RESPIRATION IN PSEUDOMONAS STUTZERI

INTRODUCTION

Bacterial respiration may be defined from both the aerobic and anaerobic viewpoints. Some forms of respiration do not involve molecular oxygen, but utilize instead other hydrogen acceptors for oxidation of the substrate. In certain instances these acceptors are organic compounds whereas others are inorganic. Among the inorganic compounds, nitrate and nitrite are two which are of particular interest in the present study.

There appears to be several ways by which nitrate and nitrite can undergo reduction. One pathway is for assimilatory purposes, i.e., for conversion of nitrate- or nitrite-nitrogen to protein-nitrogen with hydroxylamine as the intermediate. Another route involves the conversion of nitrate to nitrite and then of nitrite to nitrogen gas or oxides of nitrogen. The latter process is referred to as denitrification or more appropriately "nitrate respiration" and "nitrite respiration." In this system, the $[O]$ in the NO_3^- or NO_2^- acts in a similar manner to molecular oxygen for aerobic respiration.

Bhatt (1964) has presented evidence that nitrate and oxygen are able to compete effectively with each other as acceptors of hydrogen in respiration. Additional investigations will be made into this question with hopes that the degree of competition can be

elucidated and information obtained as to the conditions under which nitrate can most effectively compete with oxygen. To be able to equate the individual reduction steps effectively, the stoichiometry of nitrate and nitrite respiration will also be determined.

REVIEW OF LITERATURE

The utilization of nitrate by living cells may be separated into three categories (Taniguchi, 1961), namely nitrate assimilation, nitrate fermentation and nitrate respiration. Nitrate assimilation involves the reduction of nitrate to hydroxylamine or the ammonium ion which is then incorporated into cell protein by transamination and amination reactions. "Nitrate fermentation" on the other hand has been coined for the nitrate reduction process that has been shown to take place in young cotyledons of bean seed embryo which are anaerobic and dissimilative organs (Egami et al., 1957) and accumulate nitrite from nitrate. The enzyme has been shown to be NADH-linked and seems to have its function at the substrate level. The hypothesis presented by Egami et al. (1957) is that the nitrate reductase serves for reoxidation of NADH at substrate level, particularly at triose phosphate. This enables ~P formation at substrate level similar to that in glycolysis with the exception that nitrite is the reduced product instead of lactate or ethanol. A similar phenomenon occurs in the strict anaerobe, Clostridium welchii, which lacks the cytochromes but is an active nitrate reducer. Katsura et al. (1954) and Koyama (1961) have shown that in both whole cells and cell-free experiments an NAD-linked triose phosphate dehydrogenase can serve as electron donor for nitrate reduction. In intact cell experiments, addition of nitrate immediately suppresses the evolution of hydrogen from pyruvate with simultaneous production of nitrite. Koyama has also shown

that C. welchii does carry on an active non-phosphoroclastic decomposition of pyruvate to acetate with evolution of molecular hydrogen which was shown to serve as a physiological donor for reduction of nitrate. As this process of nitrate reduction occurs at substrate level and is similar to that described in 2-day cotyledons it is also referred to as nitrate fermentation.

The third category of nitrate reduction is that described classically as denitrification or nitrate respiration. Nitrate respiration as related to denitrification would also include "nitrite respiration" or the complete dissimilation of NO_3^- to nitrogen gas. This ability is possessed by few microorganisms, some of which are Pseudomonas denitrificans, Pseudomonas stutzeri and Micrococcus denitrificans. On the other hand, Escherichia coli has an enzyme that can activate nitrate and will enable E. coli to grow anaerobically on lactate as the sole carbon source, whereas the organism will not grow if NO_3^- is absent (Quastel, 1925). This was the first demonstration of a type of nitrate respiration in a non-denitrifying organism. According to experiments by Aubel and Egami (1936) aerobes such as Serratia marcescens and Pseudomonas fluorescens also behave in this manner. One of the main differences with this type of nitrate respiration is that it cannot proceed further than the nitrite step with NO_2^- being the final product. This type of nitrate respiration and the enzymic aspects of this respiration are presented in detail in the recent reviews of Taniguchi (1961), Nason (1962) and Nason (1963).

In true denitrifying bacteria, nitrate is reduced to N_2, N_2O or other gaseous oxides of nitrogen while serving as the oxidant of the organic substrate. Some of the organisms possessing the requisite enzymes are Pseudomonas aeruginosa, Pseudomonas stutzeri, Pseudomonas denitrificans, Thiobacillus denitrificans and Thiobacillus thioparus. Other species possessing the denitrifying capacity are found in the genera Micrococcus, Denitrobacillus, Spirillum, Bacillus and and Achromobacter (Delwiche, 1956). Most denitrifiers may be classified as facultative heterotrophs and are able to grow on organic compounds as their energy source with oxygen as the primary hydrogen acceptor under aerobic conditions, and with nitrate or its dissimilatory intermediates serving as the hydrogen acceptor under anaerobic conditions. There are no reports in the literature of denitrifiers growing anaerobically without the nitrate acceptor. Kluyver and Verhoeven (1954) have reported that H_2 can also serve as a hydrogen donor in Micrococcus denitrificans with nitrate being reduced to N_2 or N_2O. Sacks and Barker (1952) and Verhoeven and Goos (1954) have shown that a number of organic substrates are quantitatively oxidized to carbon dioxide. However, no relationship between the nitrate- or nitrite-oxygen required for this oxidation is presented. In this regard Gilmour, Bhatt and Mayeux (1964) recently calculated the contribution of $NO_3^- - [O]$ and molecular oxygen to the oxidation of glucose to CO_2 in an aerobic system.

Oxygen Effect

There have been several conflicting reports as to the effect of oxygen on the denitrification process. The general view is that oxygen is an inhibitor of the denitrification process by virtue of its competition with nitrate as an electron acceptor (Delwiche, 1956). Sacks and Barker (1949), Wijler and Delwiche (1954) and others have shown that oxygen tensions of 1 to 4 percent in the atmosphere suppressed denitrification from 50 to 90 percent. In observations under simulated field conditions, Wijler and Delwiche (1954) found that an oxygen atmosphere of only 5 mm (less than 1%) suppressed denitrification during the early stages of soil incubation to about 12 percent of that obtained under anaerobic conditions. Skerman and McRae (1957) observed that no nitrate reduction by Pseudomonas denitrificans occurred when oxygen was continually present in the culture fluid. They concluded that the competition between oxygen and nitrate for hydrogen electrons so favored oxygen that nitrate reduction occurred only when the supply of oxygen was insufficient to meet the demands of the respiring cells. Nitrate reduction occurred when the oxygen concentration was below the level at which oxygen-utilizing systems were saturated and when it did occur in aerated cell suspensions it was caused by cells deprived of oxygen.

On the other hand, Meiklejohn (1940) studied two organisms of the genus Pseudomonas which she claimed had the ability to denitrify aerobically. A similar observation was made by Korsakova (1941) who made the further claim that the energy yields under aerobic conditions

greatly exceeds that when the organism is subsisting anaerobically with nitrate. More recently, however, Lindeberg, Lode and Sömme (1963) in studies with a halophilic Achromobacter species have obtained results similar to those of Skerman and McRae (1957) and concur in their explanation. It appears, therefore, in spite of the conflicting reports that denitrification takes place only when the oxygen available to the cells is in sufficiently short supply that nitrate can effectively compete as a hydrogen acceptor in a system which is primarily geared to aerobic oxidations.

Characteristics of Nitrate Respiration Enzymes

Because of the physiological and enzymological similarity of nitrate respiration to oxygen respiration, it would be expected that nitrate respiration involves energy-yielding reactions which under given conditions are necessary for the growth of the organism. Recently some evidence has been presented for the coupling of phosphorylation to nitrate respiration (Takahashi, Taniguchi and Egami, 1957; Ohnishi and Mori, 1960; Ota, Yamanaka and Okunuki, 1964). One of the most distinguishing features of the nitrate respiration system is the involvement of one or more cytochromes as electron carriers in the process. In contrast, the assimilatory reduction of nitrate does not include any of the heme proteins as components of the electron transport system.

Fewson and Nicholas (1961) have reported a nitrate reductase system in the denitrifying bacterium Pseudomonas aeruginosa. The purified sulfhydryl flavoenzyme was NADH-specific and contained cytochrome c and apparently molybdenum as a component of the system. The following sequence of electron transport was suggested:

$$\text{NADH} \longrightarrow \text{FAD} \longrightarrow \text{cytochrome c} \longrightarrow \text{Mo} \longrightarrow NO_3^-$$
$$\text{cytochrome c} \searrow \text{cytochrome oxidase} \longrightarrow O_2$$

On the other hand, the nitrate respiring system in E. coli has been reconstructed by Taniguchi et al. (1958) and Iido and Taniguchi (1959), as illustrated below:

$$\text{NADH} \longrightarrow \begin{matrix}\text{FAD} \\ \text{or} \\ \text{FMN}\end{matrix} \rightarrow \begin{matrix}Fe^{2+} \\ \downarrow \\ Fe^{3+}\end{matrix} \longrightarrow \text{cytochrome } b_1 \longrightarrow \text{nitrate reductase} \longrightarrow NO_3^-$$

These workers have shown a b type cytochrome to be active and necessary for the transfer of electrons to nitrate in E. coli nitrate respiration. Yet, the assimilatory nitrate reductase in E. coli is not mediated via cytochrome b_1 or any other cytochrome for that matter. Nicholas and Nason (1955) have presented a scheme for the soluble nitrate reductase in E. coli which is very similar to that found in Neurospora:

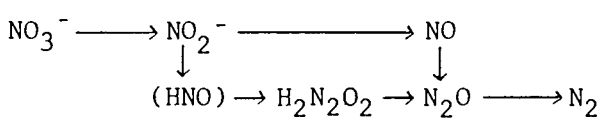
$$\text{NADH} \longrightarrow \text{FAD} \longrightarrow \text{Molybdenum} \longrightarrow NO_3^-$$

Of great interest is the profound effect of oxygen on the E. coli nitrate reductase systems. With aerobically grown cells, lowering

of the oxygen tension decreases nitrate reduction and if the cells are oxygen deficient inhibition of nitrate reduction is complete (Kono and Taniguchi, 1961). Presumably this is the assimilatory nitrate reductase being affected. Complete removal of oxygen causes the onset of anaerobic nitrate reduction and nitrite is scarcely further reduced under these conditions (apparent nitrate respiration). This suggests to the authors that the aerobic reduction requires association with an oxygen-utilizing system and this is essential for further reduction of nitrite rather than nitrate. The enzyme system responsible for aerobic reduction is quite different from the nitrate-respiring system since the latter is markedly inhibited by oxygen in intact cells and supposedly does not function under aeration (Taniguchi, 1961).

Nitrite Respiratory Enzymes

The second step in the reduction of nitrate by denitrifying organisms is the reduction of nitrite. Work on the nitrite reductase has been less extensive than on the nitrate reductase. On the basis of 2 electron steps of reduction the following scheme was proposed:



The reduction of N_2O to molecular nitrogen was first demonstrated *in vitro* by Najjer and Chung (1956) with cell-free preparations

of P. stutzeri. The possibility that enzymatic hydrogenation of hyponitrite (or nitramide) might lead for the formation of N_2 seems to have been eliminated. The original claim by Allen and Van Niel (1952) that P. stutzeri could hydrogenate nitramide to N_2 was ruled out by Kluyver and Verhoeven (1954) who found that nitramide was decomposed too rapidly upon addition to phosphate buffer to be used as an acceptor. The latter workers also showed that neither M. denitrificans or P. stutzeri evolved N_2 from sodium hyponitrite confirming the results of Allen and Van Niel (1952). With regard to the other intermediates of nitrite reduction, Sacks and Barker (1952) concluded that nitrous oxide was not an obligatory intermediate in the formation of N_2.

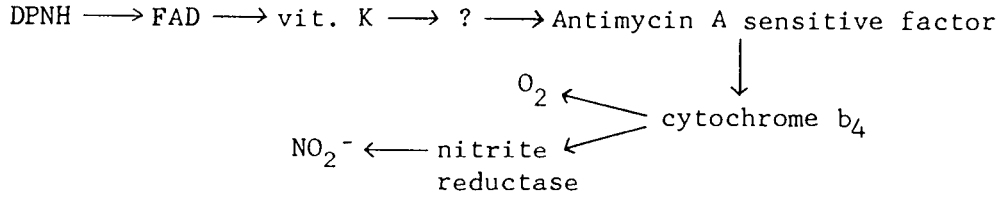
Chung and Najjer (1956a, 1956b) and Najjer and Chung (1956) with crude ammonium sulfate fractions of P. stutzeri extracts showed that TPNH and DPNH served in the reduction of NO_2^- to NO and that FAD or FMN gave a twofold stimulation. Cytochrome c was also shown to be present. They proposed the following scheme of electron transport to yield N_2 and NO in the reduction of NO_2, and N_2 in the reduction of NO:

$$\begin{matrix}\text{NADPH} \\ \text{or} \\ \text{NADH}\end{matrix} \longrightarrow \begin{matrix}\text{FAD} \\ \text{or} \\ \text{FMN}\end{matrix} \longrightarrow \begin{matrix}\text{Cu} \\ \text{or} \\ \text{Fe}\end{matrix} \longrightarrow \text{cytochrome c} \begin{matrix}\nearrow \text{NO}_2 \\ \searrow \text{NO}\end{matrix}$$

In light of the recent report by Wullstein and Gilmour (1964) on non-enzymatic nitrite reduction by transition metals (Mn^{++}, Fe^{++} or Cu^{+}), the data as presented by Najjer and Chung in regard to NO production should be re-examined.

Walker and Nicholas (1961) also reported nitric oxide to be a product of the reaction with P. aeruginosa extracts. One difference was that neither NADH or NADPH could be used as electron donors. In other respects their system was very similar to that presented by Najjer and Chung (1956).

Studies on a denitrifying Micrococcus sp. have shown slight differences in the characteristic of the nitrite reducing system to those studied in Pseudomonas sp. Using a partially purified enzyme from Micrococcus, Asano (1959) observed that almost all of the nitrite reduced appeared as nitrogen gas. The preparation possessed NADH-nitrite reductase activity and was stimulated by FAD and Menadione. The system also included cytochrome b_4 as an electron carrier. Asano suggested the following electron transport sequence for Micrococcus nitrite reductase:



EXPERIMENTAL MATERIALS AND METHODS

Organisms and Growth Conditions

Throughout this study a strain of Pseudomonas stutzeri which was isolated from field soil in this laboratory was used as the experimental organism. The culture was maintained on Najjer's medium (Najjer, 1956) consisting of 1% trypticase (BBL), 0.5% KNO_3, 0.1% yeast extract (Difco) and 1.5% agar in distilled water.

In order to obtain cell masses for respiration studies, several media were used to grow the cells. For aerobic studies in which the amount of nitrate or nitrite reductase in the cell was not critical or wanted to be decreased, the cells were grown on medium 8B which consists of: 4.55g KH_2PO_4 4.72g Na_2HPO_4, 1.5g $(NH_4)_2HPO_4$, 0.001 g each of $FeSO_4$ and MoO_3, 0.1g $MgSO_4$ and 0.5 g yeast extract per liter of distilled water. Glucose was sterilized separately and added asceptically at the time of inoculation at the rate of 5.0g per liter. The cells were grown in 1300 ml of medium in a 2-liter flask which was aerated by pulling filtered air through a glass sparger submerged in the medium.

For anaerobic studies or aerobic studies where a high nitrate or nitrite reductase activity was desired, the cells were grown in medium 8NB which consisted of the following: 4.55g KH_2PO_4, 4.72g Na_2HPO_4, 1.5g $(NH_4)_2HPO_4$, 0.001g each of $FeSO_4$ and MoO_3, 0.1 g $MgSO_4$, 0.5 g yeast extract, and 7.2 g of KNO_3 per liter of distilled water. Glucose was added in the same manner described above.

The medium was dispensed in the amount of 800 ml per liter flask and incubated under stationary conditions. The pH of both 8B and 8NB was buffered at 6.8 and the cells were grown at 28-30°C.

The stationary grown cells were harvested at 30-36 hours while those grown aerobically were harvested after 18-24 hours of incubation. This was accomplished by centrifuging with either the Sharples Super centrifuge or by the use of the 200 ml cups with the Servall SS-2 centrifuge. The cells were washed once in M/15 phosphate buffer at pH 6.8 and resuspended to a concentration to give an $O.D._{500\ m\mu}$ of 1.0 when diluted 1:100. This optical density gave a cell concentration of approximately 0.5 mg/ml on a dry weight basis. The O.D. was measured with a Bausch and Lomb "Spectronic 20" spectrophotometer at 500 mμ.

The respiration studies were carried out in the following medium which is refered to as NFSB (NH_4^+ free salt base): 4.55 g KH_2PO_4, 4.72g Na_2HPO_4, 0.001g $FeSO_4$ and MoO_3, 0.1g $MgSO_4$ and 0.5 g yeast extract in 1 liter of distilled water. The medium was sterilized at 121°C and was dispensed into sterile respirometer flasks as needed. The carbon sources: glucose, pyruvate, acetate or succinate and other additives such as KNO_3, KNO_2 and $(NH_4)_2HPO_4$ were added asceptically from sterile stock solutions to give the desired concentration.

Solutions and Reagents

Sodium hydroxide (1.0 N) was prepared by adding Fisher

Standard sodium hydroxide stock to CO_2-free distilled water and making the volume to 1 liter. Lesser concentrations of sodium hydroxide were prepared by diluting this stock with CO_2-free distilled water.

Potassium nitrate stock was prepared to a concentration of 1 mM/ml by weighing 2.525g into a 25 ml volumetric flask to which 12 ml of distilled water was added. This was then autoclaved at 121°C for 15 minutes and made to final volume with sterile distilled water.

Potassium nitrite was prepared in the same manner by weighing 2.125g KNO_2 into a 25 ml volumetric flask. Water was added and the solution autoclaved before making to final volume. This gave a solution of 1 mM/ml. Addition of 1 ml of either of the latter two solutions to a respirometer flask with a total volume of 100 ml gave a concentration of 140 µg N/ml or a total input of 14 mg N.

Sterile glucose solutions were prepared to a concentration of 1 mM/ml by adding 1.8g of anhydrous glucose per 10 ml of solution. One milliliter of this solution when added to the respirometer flask represented an input of 72 mg of carbon. This input was used unless otherwise indicated.

Solutions of sodium acetate, sodium pyruvate and sodium succinate were prepared to give 3 mM/ml, 1 mM/ml and 0.75 mM/ml, respectively. These were also added to the respirometer flasks to give a final input of 72 mg carbon. Sterilization of these solutions was performed by use of the millipore filter technique with filter pads of 0.5 µ pore size.

Analytical Procedures

Nitrate-N determinations were conducted according to the procedure of West and Lyles (1960) using chromotropic acid (4,5-dihydroxy-2,7-naphthalene disulfonic acid). In some determinations the modified phenoldisulfonic acid method of Eastoe and Pollard (1950) was used. Nitrite-nitrogen was determined by Mayeux's modification (1961) of the N-(1-naphthyl)-ethylene-diamine dihydrochloride procedure of Saltzman (1954).

Glucose was determined according to the method described by Neish (1952). The method had to be altered somewhat as the presence of nitrite interfered with the thiosulfate titration of the iodine. The samples containing nitrite as well as the standards for the curve were treated as follows:

1. Sample contained up to 3 mg of glucose in not more than 4 ml of solution.
2. Add 0.5 ml of 8% sulfamic acid and shake vigorously for 3 minutes. If the pH of the glucose solution is alkaline, one drop of concentrated H_2SO_4 may be added to bring the pH to below 4.0.
3. Add 0.5 ml of 5% NaOH and bring the total sample volume to 5.0 ml.
4. Add 5 ml of $CuSO_4$ reagent and follow the procedure as described by Neish (1952).

Analysis of the atmosphere of the respirometer flasks was performed by the use of a Beckman GC-2 gas chromatograph at 40°C with

a helium carrier gas. Analysis for N_2 and O_2 was performed with the 12 foot molecular seive column and CO_2 and N_2O determinations were made with the 1.5 foot charcoal column.

Carbon dioxide measurements were also made by absorbing the CO_2 in 10 ml of 1 N NaOH and titrating to the HCO_3^- endpoint by use of a Beckman Model K Automatic Titrator.

Determination of ether extractable fermentation acids was performed by the use of the silicic acid partition chromatography as described by Neish (1952).

Apparatus

The electrolytic respirometer described by McGarity et al. (1958) and subsequently modified by Roa (1961) was used for the respiration studies. The respirometer flask was slightly modified by removal of the center well attached to the center of the flask and suspending the CO_2 trap from the center flushing stem of the respirometer top (cover). For aerobic studies, a one inch magnetic stirring bar was added to the 300 ml respirometer flask. The flask with the stirring bar was autoclaved for 30 minutes at 121°C for sterilization. The basal medium (NFSB) was added asceptically along with other additives (glucose, KNO_3, etc.) to give a final volume of 100 ml in the reaction mixture. Ten milliliters of 1 N NaOH were placed in the glass wells for CO_2 adsorption which was then suspended from the central stem of the flask cover. The inoculum was added either at this time (prior to placing cover)

or was added through the serum stopper of the sidearm with a syringe and needle after flushing. The flasks were then seated over a "Magnestir" magnetic stirrer and connected to the electrolysis unit. The flasks were then flushed for 20-25 minutes with a gas mixture of 20% O_2 and 80% He, during which time the reaction medium was actively stirred with the magnetic stirrer. Upon completion of the flushing time the stopcocks were shut in a manner which would prevent entrance of air and the flasks were incubated with active stirring throughout the experiment.

The flasks to be incubated under anaerobic conditions were treated in the same manner with the exception that no magnetic stirring bar was placed in the vessel and the flasks were not actively stirred during the experiment. The flasks were flushed for 20-25 minutes with He gas only and were not connected to the electrolysis unit during flushing or incubation.

Upon completion of the experiment, the flasks were attached to the Beckman GC-2 gas chromatograph and a gas sample was removed from the flask atmosphere into the chromatograph for analysis. The weight of N_2 evolved was determined by comparing the N_2 peak weight recorded with that of a standard N_2 curve.

Dissolved oxygen was measured with a Precision Scientific Oxygen Analyzer. The respiration flask had to be slightly modified by adding a sidearm of sufficient size to insert the D.O. probe into the reaction medium. In this case the volume of reaction medium in the flask had to be increased to 150 ml in order to completely cover the probe contacts.

EXPERIMENTAL RESULTS AND DISCUSSION

Growth Requirements of Pseudomonas stutzeri

In order to study the process of "nitrate respiration" and to be able to compare this phenomenon with conventional aerobic respiration, which shall be considered as normal respiration in this dissertation, it becomes necessary to classify the overall respiratory characteristics of the organism under study. Some of the questions that will have to be answered are: (1) What is the normal respiration picture of this organism? (2) Is it able to grow anaerobically without an inorganic H^+ acceptor? (3) Is the organism capable of assimilating NO_3^--N or NO_2^--N? and (4) Can this organism function in a synthetic medium devoid of yeast extract or a peptone preparation? If the answer to the latter question is yes, other projected studies would be greatly facilitated as they could be performed under defined conditions. Therefore, experiments were devised in order to learn what the minimal conditions were for proper functioning of the test organism and to determine at the same time whether or not the organism could assimilate NO_3^-N for growth.

Yeast Extract Requirement and Nitrate Assimilation

A basal medium consisting of M/15 phosphate buffer (pH 6.8) to which were added 1 mg $FeSO_4$, 1 mg MoO_3, and 100 mg $MgSO_4$ per liter was used for these studies. To this basal medium the following ingredients were added in the combinations indicated below for

each 100 ml of medium:

1. $(NH_4)_2SO_4$ . . . . . 70 mg of N
   glucose . . . . . 500 mg

2. $(NH_4)_2SO_4$ . . . . . 70 mg of N
   glucose . . . . . 500 mg
   yeast extract . . . . . 10 mg

3. KNO_3 . . . . . 70 mg of N
   glucose . . . . . 500 mg
   yeast extract . . . . . 10 mg

4. glucose . . . . . 500 mg
   yeast extract . . . . . 10 mg

5. KNO_3 . . . . . 70 mg of N
   glucose . . . . . 500 mg

6. $(NH_4)_2HPO_4$ . . . . . 70 mg of N
   glucose . . . . . 500 mg
   yeast extract . . . . . 10 mg

Each flask was then inoculated and incubated on a rotary shaker at 28°C and growth of P. stutzeri was measured turbidimetrically at 500 mu with the Spectronic 20.

As can be observed in Figure 1, by contrasting NH_4^+ and glucose curve with the NH_4^+ + glucose + yeast extract curve the organism is unable to grow in a purely synthetic medium. Evidently P. stutzeri requires some factor which is present in yeast extract for growth. Either $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ can serve as an assimilatory N-source for the organism. The remaining curves reveal that the test organism is unable to use NO_3^--N as a nitrogen source neither in a synthetic medium nor in combination with yeast extract. Growth that is obtained in combination with KNO_3 and yeast extract does not exceed the growth that is obtained with yeast extract alone. Therefore one can deduce that all of the nitrogen assimilated in

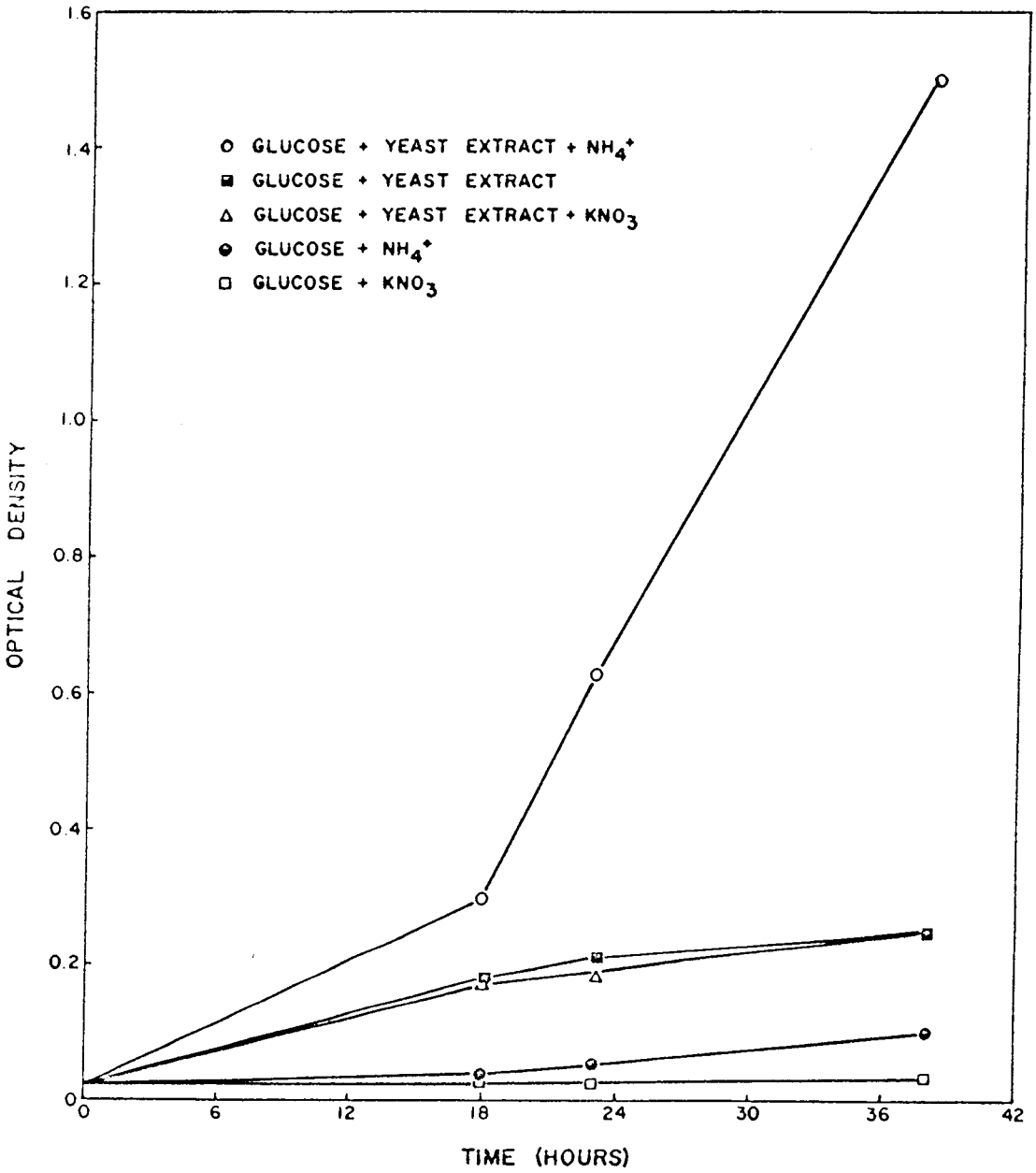


Figure 1. The yeast extract and N-assimilation requirements of P. stutzeri.

either case is obtained from the yeast extract. In the case of the glucose + yeast extract + KNO_3, some of the NO_3^- is reduced to NO_2^- during the growth of the organism but is not further reduced to NH_4^+ or else more growth would have been observed with the NO_3^- than with the yeast extract alone. Thus we may properly conclude that the organism under question cannot assimilate NO_3^--N and NO_2^--N. However, P. stutzeri can use the usual laboratory peptones as a source of nitrogen.

The fact that the above experiment was conducted under what are commonly referred to as aerobic conditions, there is the possibility that NO_2^- reduction is inhibited by molecular oxygen. Therefore, the organism could never have used NO_3^- or NO_2^- for growth under these conditions. Certainly the assimilatory nitrate reductase and the dissimilatory nitrate reductase systems are separate metabolic routes. In order to cover this point identical experiments were performed under anaerobic conditions. The overall conclusions did not change. However, one additional point was brought out, namely that P. stutzeri cannot grow anaerobically without the presence of NO_3^- or NO_2^-- oxygen as the inorganic H^+ acceptor.

The aforementioned data on the presence or absence of a NO_3^- or NO_2^- assimilatory route answers three of the four questions presented in the opening paragraph of this section. Briefly, the answers are: (1) The organism P. stutzeri cannot assimilate either NO_3^--N or NO_2^--N. (2) The organism P. stutzeri cannot grow anaerobically without an inorganic H^+ acceptor. (3) This organism has a definite requirement for some factor which is supplied by yeast extract.

Since either $(NH_4)_2HPO_4$ or $(NH_4)_2SO_4$ can serve equally well as a nitrogen source the ammonium phosphate was subsequently used for growth of the organism. In addition, further experimentation showed that 10 mg of yeast extract per 100 ml was not adequate for optimum nitrate respiration, therefore the concentration was increased to 50 mg per 100 ml. Roa (1961) had shown in earlier studies that there was a direct correlation with the amount of yeast extract added and the subsequent N_2 evolved from NO_3^-.

Utilization of Asparagine

Roa (1961) had also shown earlier that asparagine has an effect on glucose oxidation very similar to that which glucose has on many amino acids, i.e., a sparing effect. This effect proved difficult to explain. An experiment designed to see if asparagine would possibly replace the yeast extract in the medium was conducted. A mineral salt basal medium consisting of the following ingredients was added to 100 ml French square bottles:

KH_2PO_4	4.55 g/l
Na_2HPO_4	4.73 g/l
KNO_3	5.00 g/l
$(NH_4)_2HPO_4$	1.50 g/l
MoO_3 and $FeSO_4$	0.001 g/l
$MgSO4$	0.10 g/l

The bottles containing the medium were sterilized and the following sterile ingredients added:

I	yeast extract	50 mg
II	yeast extract	50 mg
	glucose	.250 mg
III	glucose	.250 mg
	asparagine	.100 mg
IV	glucose	.250 mg
	asparagine	.100 mg
	yeast extract	50 mg

Each bottle was inoculated with P. stutzeri to an O.D. of 0.02 and incubated at 28°C. Growth was followed by measuring the O.D. at selected time intervals.

The results are shown in Figure 2. As can be seen on the graph, the asparagine did not replace the yeast extract. In fact, the only effect that the asparagine seemed to have was to provide an additional carbon source for the organism. Therefore, asparagine was deleted from the medium in subsequent studies.

Effect of NO_3^- and NO_2^- on Aerobic Growth

It has been shown that P. stutzeri cannot assimilate NO_3^--N or NO_2^--N, but that these are required for anaerobic growth of the organism. As this is to be a comparative study of NO_3^- and O_2 respiration and evidence has been presented to the effect that NO_3^- does decrease the rate of oxygen utilization (Bhatt, 1964) it becomes necessary to determine the effect of NO_3^- and its dissimilatory intermediate NO_2^- on aerobic growth. In order to determine the effect, if any, an experiment was designed using the following media:

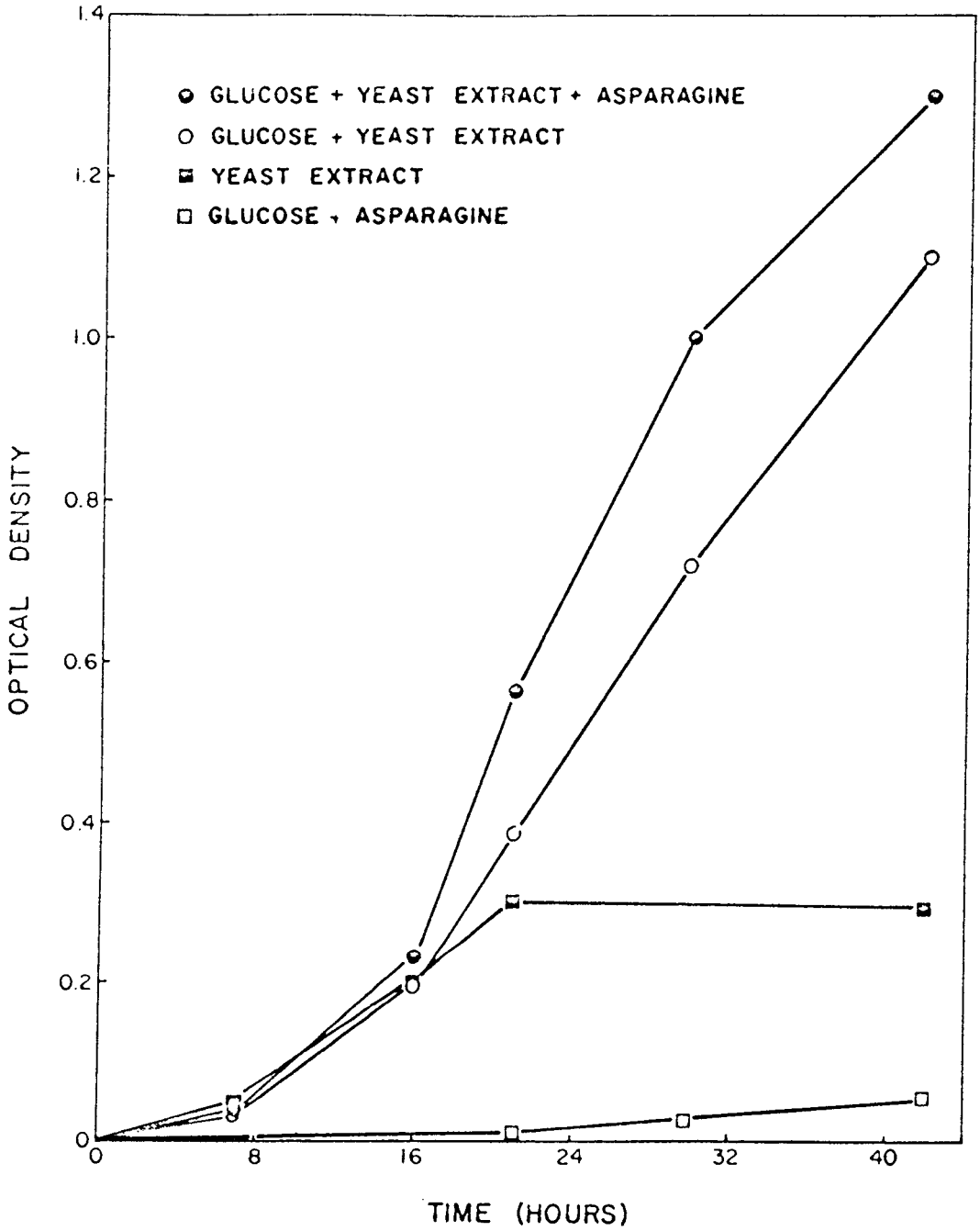


Figure 2. The use of asparagine as a substitute for the yeast extract requirement of P. stutzeri.

I	Medium 8B	100 ml	
	glucose	500 mg	
II	Medium 8B	100 ml	
	glucose	500 mg	
	KNO_3-N	70 mg	(700 µg/ml)
III	Medium 8B	100 ml	
	glucose	500 mg	
	KNO_2-N	5 mg	(50 µg/ml)
IV	Medium 8B	100 ml	
	glucose	500 mg	
	KNO_2-N	15 mg	(150 µg/ml)

The flasks were inoculated to an initial O.D. of 0.02 and incubated at 28°C on a rotary shaker. At intervals samples were removed and the O.D. measured. At the same time that samples were removed for O.D. measurement, NO_2^- and glucose determinations were made.

As can be seen in Figure 3, the presence of 700 µg/ml of NO_3^--N had a slight effect on the growth of the organism. At the same time, however, the presence of NO_2^- adversely affected the growth of the test culture, especially at concentrations approaching 150 µg/ml. It is interesting to note that at no time during the growth of the organism in the presence of NO_3^- did the NO_2^--N concentration exceed 9.5 µg/ml. The NO_2^--N concentrations remained approximately at the initial level throughout the experiment. There was a slight increase most probably due to the evaporation of water from the medium. The glucose disappeared between 33-44 hours in sets I and II; between 44-72 hours in set III; and 360 mg remained in set IV at the end of the experiment.

Accumulation of NO_2^-

It therefore appears that accumulations of NO_2^- may adversely

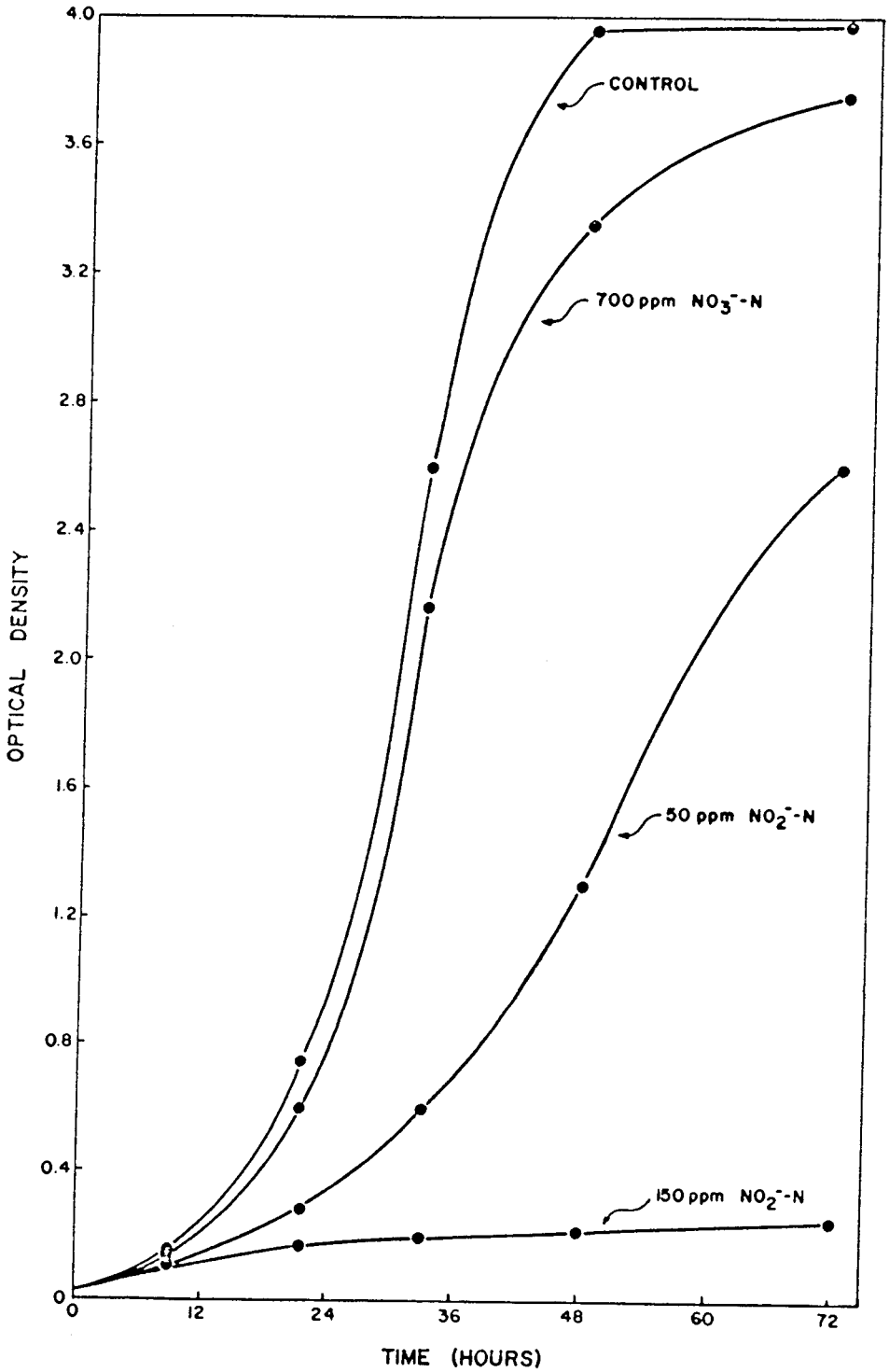


Figure 3. The effect of NO_3^- and NO_2^- on aerobic growth of P. stutzeri.

affect the aerobic growth of P. stutzeri. The question then arises as to how or why does the NO_2^- so act? A second question is at what rate is NO_2^- accumulated by masses of cells in a complete medium. Both Bhatt (1964) and Roa (1961) suspended the cells in the complete medium which was used to inoculate the sand or soil in the flasks. This may have given the cells sufficient time to accumulate NO_2^- thereby inhibiting the respiration of the cells.

An attempt was therefore made to determine the rate at which such a cell suspension would accumulate NO_2^-. Cells were harvested and resuspended in medium 8NB in order to give a final O.D. of 1.0. One sample was placed in an Erlenmeyer flask and placed on the rotary shaker. The other was held under stationary conditions as is usually done prior to inoculation of the flasks. Samples were removed at intervals and the NO_2^- concentration was determined.

As can be observed in Table 1, the cell suspension in the presence of NO_3^- and with an adequate H-donor can cause the reduction of a considerable amount of NO_3^- if allowed to go unaerated. However, under normal conditions a cell suspension so prepared would never be allowed to sit for more than 30 minutes before use as an inoculum. It would be better, however, to add the NO_3^- and the glucose separately and directly into the flask to be inoculated or only add the glucose and NO_3^- to the cell suspension at the time it is being transferred to the respiratory vessel. It would be difficult to obtain good distribution of the glucose and NO_3^- when adding them separately to soil.

Table 1. The rate of accumulation of NO_2^--N from NO_3^- by cell masses

Time in minutes	µg NO_2^--N per ml	
	stationary	shaker
0	0	0
5	8.4	8.4
15	18.2	11.0
30	29.0	12.5
60	46.0	13.0
120	125.0	14.0
180	140.0	14.8
300	178.0	15.0

Molecular Oxygen Versus Nitrate Respiration

Should any differences exist in respect to the fate of the substrate with the NO_3^- and molecular oxygen acceptor systems, it appeared critical to determine the stoichiometry of the substrate oxidation with the different acceptor systems. In addition, by using NO_2^- as well as NO_3^- as independent acceptors it becomes possible to determine the degree of participation of the separate reductive steps of the acceptor. Current evidence points to basic differences between the nitrate and molecular oxygen systems. Bhatt (1964) has shown a reduction in CO_2 evolution with NO_3^- in an aerobic system and has further shown that there is alteration in the $\frac{C\text{-}1}{C\text{-}6}$ decarboxylation ratio of glucose. These data indicate that there may be a concomitant change in the primary pathway of glucose catabolism with different acceptor systems. Further evidence supporting these findings are presented below for the respiration of P. stutzeri.

The respirometer flasks contained in addition to 100 ml NFSB, 500 mg glucose and 1000 µg NO_3^--N per ml, or only 500 mg glucose. The flasks to be incubated aerobically were flushed with $He:O_2$ mixture and anaerobic flasks were flushed with helium only. The flasks were incubated at 28°C and at the termination of the experiment NO_2^-, NO_3^-, CO_2, glucose, and ether extractable acids were determined.

The data in Table 2 provide preliminary information on the participation of the given electron acceptor in the dissimilation of one millimole of glucose. The values indicated for O_2 uptake,

Table 2. Participation of molecular and bound oxygen in the dissimilation of glucose

Hydrogen acceptor	Millimoles of glucose	O_2	NO_3^-	CO_2	meq. acid
O_2	1.0	3.5	-	3.5	0.03
NO_3^-	1.0	-	4.2	3.6	4.56
$O_2+NO_3^-$	1.0	2.1	0.75	3.5	2.02

NO_3^- reduced and CO_2 evolved are calculated on the basis of total glucose utilized which would then include the fraction assimilated by the cells and the portion oxidized to carbon dioxide and water.

However, with the aerobic system, if the calculations are based on non-assimilated glucose, it is possible to quantitate the glucose-C oxidized to CO_2. When the emperical proportions are calculated on this basis, the mM O_2 used and mM CO_2 evolved from six glucose-carbons approaches the theoretical value of 6, (i.e., 5.8), thus indicating complete oxidation of the substrate. From this information we can conclude that complete oxidation of glucose by P. stutzeri can be summerized by the following conventional equation:

$$C_6H_{12}O_6 + 6O_2 \longrightarrow 6CO_2 + 6H_2O$$

The second terminal acceptor system listed in Table 2 is NO_3^- or the anaerobic set. A total equivalent of 4.2 mM of NO_3^--oxygen was utilized for oxidation of 3.6 mM of glucose-C to CO_2. The accumulation of ether extractable acids with this acceptor system indicates that NO_3^- respiration may be of a fermentative type, and the transport system associated with nitrate reduction may be characterized as effecting only a partial combustion of glucose to CO_2 and H_2O. In an attempt to attain a complete fermentation balance, the separation and identification of the component acids produced during respiration was initiated. The results are shown in Table 3. Acetic acid comprises the major portion of the acids identified in either of the acceptor systems. Lactic acid and succinic acids were present in lesser amounts. The absence of lactic acid in the aerobic

Table 3. Observed products of glucose catabolism

Acid	Milliequivalents of Acid/100 ml*		
	Hydrogen Acceptor		
	O_2	NO_3	O_2 + NO_3
Acetic	0.016	0.408	0.323
Succinic	0.008	0.091	0.079
Lactic	0.000	0.133	0.124
Unidentified	0.004	2.480	1.860
Total	0.028	3.112	2.386

*Glucose input = 500 mg/100 ml

system further attests to the absence of a fermentative mechanism. A large portion of the acid fraction from the sets with the NO_3^- acceptor remains unidentified. Further tests on the unidentified fraction as well as on unseparated extract for the following acids proved negative: formic, propionic, butyric, β-OH-propionic, β-OH-butyric, methylmalonic, fumaric, malic, and citric.

It should also be stated that upon the addition of NO_3^- to the O_2 acceptor system there is an obvious decrease in O_2 consumption and a simultaneous reduction of NO_3^- to NO_2^-. Thus both the "oxygen respiratory system" and the "nitrate respiratory system" are operative in the organism used for this investigation. There does not seem to be any marked inhibition in the reduction of NO_3^- to NO_2^-, but no N_2 or N_2O gas was detected in the respirometer atmosphere attesting to the inhibition of NO_2^- reduction in the aerobic atmosphere.

Stoichiometry of NO_3^- and NO_2^- Respiration

Since the NO_3^- value for oxidation of 1 mM of glucose is quite high when compared to the theoretical amount required on the basis of mM [O] per mM CO_2 produced (for oxygen the $\frac{[O]}{C}$ ratio = 2 but for the NO_3^- the [O]/C ratio is 3.5); it seems that the stoichiometry of NO_3^- respiration needs further investigation and clarification. Thus, experiments were designed to allow calculation of the stoichiometric relationship of the nitrate respiratory complex with regard to the substrate oxidized. These experiments were conducted in the respirometer flasks under anaerobic conditions. Each

flask contained 100 ml of NFSB to which was added 180 mg of glucose and the desired amount of hydrogen acceptor, i.e., NO_3^- or NO_2^-. If required, $(NH_4)_2HPO_4$ was also added aseptically.

The first case to be discussed is the NO_3^- acceptor system to which glucose was added with the NH_4^+ ion as the source of nitrogen. The acceptor NO_3^- was added to duplicate flasks in the amount of 1, 3 and 5 millimoles. At intervals a sample was removed by syringe and needle via the stoppered side arm and analyses made for residual NO_3^-, NO_2^-, and glucose. At time intervals based upon the depletion of acceptor in each system the atmosphere of the flask was tested for the presence of N_2 gas. The center-well NaOH was titrated for CO_2 and final analyses were made for residual glucose, NO_3^-, and NO_2^-.

The NO_3^- and NO_2^- analyses are expressed on the basis of N as NO_3^- or N as NO_2^-, i.e., one mM of NO_3^- is equal to 14 mg N, the same as is one mM NO_2^-. Therefore, the following equations stand as a basis for the calculations:

(1) 14 mg NO_3-N $\longrightarrow$ 14 mg NO_2-N + 16 mg [O]

(2) 14 mg NO_2-N $\longrightarrow$ 14 mg N_2 + 32 mg [O]

I now wish to define 1 mM [O] as equal to 16 mg by wt. Therefore:

(3) 1 mM NO_3^- $\longrightarrow$ 1 mM NO_2^- + 1 mM [O]

and: (4) 1 mM NO_2^- $\longrightarrow$ 14 mg N_2 + 2 mM [O]

The following generalized equation may also be used:

(5) 1 mM NO_3^- $\longrightarrow$ 14 mg N_2 + 3 mM [O]

A second point relating to forthcoming data may also be made, namely that 12 mg CO_2-C is equivalent to 1 mM CO_2. Thus for convenience the CO_2 values will be expressed as C since the titration values for CO_2 are expressed as mg CO_2-C.

The results shown in Table 4 indicate that for each NO_3^- concentration used in the experiment an [O]/C ratio of about 2.5 is obtained. This indicates that for some reason NO_3^--oxygen may not be as efficient an acceptor as is molecular oxygen. The data given in Table 5 provide the same type of information obtained without added NH_4^+ to the system. Again an [O]/C ratio of about 2.5 is obtained for nitrate respiration.

Similar experiments as described above for nitrate respiration were conducted for "nitrite respiration", i.e., NO_2^--[O] as the H^+ acceptor. In order to avoid toxicity of the NO_2^- to the bacterial cells the nitrite was added in increments of 1 mM (1 mM NO_2^-/100 ml = 140 µg NO_2^--N/ml. By using a tuberculin syringe and needle, 1 ml of a stock solution containing 1 mM/ml was added through the serum stoppered side arm of the flask. At intervals a sample was removed and assayed for NO_2^-. Upon depletion of the added NO_2^- additional increments were injected over a time period until the total amount of acceptor desired had been added.

Tables 6 and 7 show the results obtained for NO_2^- respiration with and without added NH_4^+-N. The [O]/C ratios for NO_2^- respiration with NH_4^+ vary from a low of 1.67 to a high of 2.42. However, the mean value obtained is 2.12. In the case of the system

Table 4. Nitrate respiration in the presence of NH_4^+-N

	millimoles					
NO_3^- input	1	1	3	3	5	5
NO_3^--[O] used	3	3	9	9	11.7	12
CO_2-C	1.22	1.29	3.37	3.44	4.63	4.85
Ratio $\frac{[O]}{C}$	2.46	2.32	2.67	2.62	2.53	2.48
Average $\frac{[O]}{C}$ Ratio			2.51			

Table 5. Nitrate respiration in the absence of NH_4^+-N

	millimoles					
NO_3^- input	1	1	3	3	5	5
NO_3^--[O] used	3	3	8.78	8.46	9.90	12.2
CO_2-C	1.26	1.22	3.33	3.24	3.84	4.7
Ratio $\frac{[O]}{C}$	2.38	2.46	2.62	2.61	2.58	2.59
Average $\frac{[O]}{C}$ Ratio			2.54			

Table 6. Nitrite respiration in the presence of NH_4^+-N

mM NO_2^- used	1	1	2	2	3	3	4	4
mg NO_2^--[O] used	32	32	64	64	96	96	128	128
mM NO_2^--[O] used	2	2	4	4	6	6	8	8
mg CO_2-C	12.3	14.4	23.9	23.7	29.7	29.8	45.4	47.6
mM CO_2-C	1.02	1.2	1.99	1.97	2.48	2.48	3.69	3.97
Ratio $\frac{\text{mM [O]}}{\text{mM C}}$ (2)/(1)	1.96	1.67	2.01	2.03	2.42	2.42	2.16	2.02
Average [O] ratio			2.12					

Table 7. Nitrite respiration in the absence of NH_4^+-N

mM NO_2 used	1	1	2	2	3	3	4	4
mg NO_2-$[O]$ used	32	32	64	64	96	96	128	128
mM NO_2-$[O]$ used	2	2	4	4	6	6	8	8
mg CO_2-C	11.9	12.6	20.8	22.2	35.6	40.6	49.3	48.4
mM CO_2-C	0.993	1.05	1.74	1.83	2.96	3.38	4.11	4.03
Ratio $\frac{\text{mM } [O] \ (2)}{\text{mM C} \ (1)}$	2.01	1.91	2.3	2.19	2.03	1.78	1.95	1.98
Average $\frac{[O]}{C}$ ratio				2.02				

without NH_4^+-N the values do not vary quite as much, the high and low values being 1.78 and 2.19. The average value is 2.02. The latter data indicate that NO_2^- respiration closely resembles respiration with the oxygen acceptor system, that is, 2 [O] required for each mole of CO_2 evolved. If this is true, then the following equation holds for NO_2^- respiration:

(6) $NO_2^- + 1$ glucose-C $\longrightarrow CO_2 + 1/2\ N_2$

Therefore the portion of nitrate respiration which is not operating efficiently is the step removing the first [O] from NO_3^-. If the above equation holds true for "nitrite respiration", it should also apply to that portion of "nitrate respiration" which involves the reduction of the NO_2^- resulting from NO_3^- reduction. Theoretically the organism cannot differentiate NO_2^- added exogenously from the NO_2^- accumulating as a result of NO_3^- reduction. If this is true, then that portion of NO_3 respiration which follows equation (6) should be able to be subtracted from the total respiration picture to give the true respiration picture of NO_3^- reduction to NO_2^-.

On this basis the following calculations were performed. From equations (3), (4) and (5) we have:

1 mM $NO_3^- \longrightarrow$ 1 mM NO_2^- + 1 mM [O]

1 mM $NO_2^- \longrightarrow$ 14 mg N_2 + 2 mM [O]

Thus: 1 mM $NO_3^- \longrightarrow$ 14 mg N_2 + 3 mM [O]

From previous information we can also write the equation:

(7) 2 [O] + C $\longrightarrow CO_2$

This equation holds for oxygen as well as NO_2^- which was shown

earlier (eq. 6).

Given the following as an actual example:

NO_3^- input : 5 mM (No residual NO_3^-)

NO_2^--N residual : 22.8 mg N

CO_2-C titrated : 4.63 mM

We can calculate the following:

$$5 \text{ mM } NO_3^- \longrightarrow 5 \text{ mM } NO_2^- + 5 \text{ mM } [O]$$

$$5 \text{ mM } NO_2^- - 1.63 \text{ mM } NO_2^- \text{ residual} \longrightarrow 3.37 \text{ mM } NO_2^- \text{ utilized}$$

from eq. (6): $3.37 \text{ mM } NO_2^- + \text{glucose-C} \longrightarrow 3.37 \text{ mM } CO_2 + \frac{3.37}{2} N_2$

as a result of NO_2^- respiration,

then: $4.63 \text{ mM } CO_2 - 3.37 \text{ mM } CO_2 \longrightarrow 1.26 \text{ mM } CO_2$ as a result of NO_3^- reduction to NO_2^-.

From eq. (3): $5 \text{ mM } NO_3^- \longrightarrow 5 \text{ mM } NO_2^- + 5 \text{ mM } [O]$

From eq. (7) theoretically: $5 \text{ mM } [O] + 2.5 \text{ C} \longrightarrow 2.5 \text{ mM } CO_2$

However, actually: $5 \text{ mM } [O] + 1.26 \text{ mM C} \longrightarrow 1.26 \text{ mM } CO_2$

$$\% \text{ efficiency} = \frac{1.26 \text{ mM } CO_2}{2.5 \text{ mM } CO_2} \times 100 = 50.04$$

The same calculations were performed for the data presented in Tables 4 and 5 for nitrate respiration. The results are presented in Table 8. These data show a variation in percent efficiency from 24.6 to 58 for the experiment with ammonia added and a variation from 29.5 to 68 for the set without added ammonia. Interesting is the fact that in either case (with or without ammonia) the percent efficiency values are very close for the same level of NO_3^- input. The extreme levels are between those sets containing

Table 8. Calculation of the percent efficiency of NO_3^- to NO_2^- reduction in respiration

	millimoles											
	with NH_4^+						without NH_4^+					
NO_3^- input	1.0	1.0	3.0	3.0	5.0	5.0	1.0	1.0	3.0	3.0	5.0	5.0
NO_3^- residual	0	0	0	0	0	0	0	0	0	0	0	0
NO_2^- residual	0	0	0	0	1.63	1.50	0	0	0.11	0.27	2.86	1.35
CO_2-C	1.22	1.29	3.37	3.44	4.63	4.85	1.26	1.22	3.33	3.24	3.84	4.7
Calculated CO_2-C from $NO_2^- \rightarrow N_2$ step	1.0	1.0	3.0	3.0	3.37	3.50	1.0	1.0	2.88	2.73	2.14	3.65
% efficiency of $NO_3^- \rightarrow NO_2^- \div [O]$	44.0	58.0	24.6	29.3	50.04	54.0	52.0	44.0	29.5	34.0	68.0	42.0

3 mM KNO_3 and those of the 1 and 5 mM KNO_3.

Production of Fermentation Products

Although considerable information on the stoichiometry of nitrate respiration has been presented, the question of the appearence of organic acids still remains. In order to fully explain their presence, a carefully monitored experiment was performed. To a series of respirometer flasks 1 mM of glucose was added to the basal medium along with 1, 2, 3, and 4 mM of KNO_3. A second series of flasks was prepared and 1 mM of KNO_2 was added to each flask. The flasks were flushed with helium and inoculated through the side arm with the syringe and needle. At intervals the flasks were tested for residual NO_3^- and NO_2^-, whichever was appropriate. In the case of the NO_3^- sets, the duplicate flasks were removed and assayed for extractable acids whenever the acceptor was depleted. When the NO_2^- was depleted, two flasks were removed for acid assay, and an additional mM of NO_2^- added to the remaining flasks. The same procedure was repeated until flasks utilizing 1, 2, 3, 4 and 5 mM NO_2^- had been obtained. In this manner a considerable amount of NO_2^- can be utilized by the organisms without ever reaching toxic proportions in the environment. The control flasks contained 1 mM glucose but no NO_3^- or NO_2^-. The results are presented in Tables 9 and 10.

The results presented earlier in Table 2 had shown considerable accumulation of acid from glucose with NO_3^- as the sole acceptor. The data presented in Tables 9 and 10 indicate no acid production for the NO_3^- or NO_2^- acceptor systems. However, considerable

Table 9. Production of acids from glucose by P. stutzeri with the anaerobic NO_3^- acceptor system

mM KNO_3 input	1.0	2.0	3.0	4.0	0.0
Time of removal (hrs)	6	15	28	65	65
meq of titratable acids	0.018	0.029	0.047	0.034	1.169

glucose input - 1.0 mM

Table 10. Acid production from glucose by P. stutzeri with the anaerobic NO_2^- acceptor system

mM KNO_2 input	1.0	2.0	3.0	4.0	5.0	0
Time of removal (hrs)	6	15	22	28	41	65
meq of titratable acids	0.034	0.025	0.022	0.025	0.037	1.169

glucose input - 1.0 mM

acid is produced by the system with no acceptor. The explanation for this lies in the fact that the acid is produced only in the absence of an adequate inorganic acceptor and no acid is produced during the normal respiratory activities of the organism. This holds true for all 3 acceptor systems, O_2, NO_2^- or NO_3^-.

The above explanation is further substantiated by the fact that a considerable amount of acid was found when this experiment was first attempted. The respiratory activity of the test organism was not adequately noted, and several hours had elapsed after depletion of the acceptor before flasks were removed or more acceptor added. Typical data are presented in Table 11. The cells were allowed 24 hours between additions of mM increments of KNO_2 and were not removed for assay until 24 hours after the last increment had been added. The data in Table 11 show that with time and a limited amount of acceptor P. stutzeri accumulates acid until the substrate is expended and then begins to consume the acids that have accumulated. These data would tend to indicate that the acid produced was dependent on the amount of acceptor present.

Utilization of Fermentation Products

A further experiment was designed to determine if the cells could utilize the acids derived from glucose. The cells were suspended in medium NFSB to which was added 180 mg of glucose. The flasks were flushed with helium and incubated for 72 hours. At that time they were either connected to the electrolysis unit and flushed

Table 11. Observed acid production by P. stutzeri under anaerobic conditions with insufficient acceptor

mM KNO_2 input	1.0	2.0	3.0	4.0	5.0
Time of removal (hrs)	24	48	72	96	120
meq titratable acid	0.472	0.780	1.034	0.570	0.306
mg CO_2-C	13.3	23.7	29.9	46.5	54.0

glucose input - 1.0 mM

with the He/O_2 mixture, or NO_2^- or NO_3^- acceptor was added. At intervals, flasks were checked for residual NO_2^- or NO_3^-. Upon depletion of the NO_3^- or NO_2^-, the flasks were removed and assayed.

Table 12 gives the results for oxidation of the acids with the NO_2^- acceptor system. After 33 hours of incubation with the first added increment of NO_2^-, there was still considerable residual NO_2^- and the reaction seemed to be proceeding slowly, if at all. One possibility was that the organism had a shortage of C_4 fragments for operation of the terminal cycle or that a CO_2 fixation reaction was required for operation of the terminal catabolic pathway. Therefore a small amount of glucose was added to two sets of flasks as indicated in Table 12. Immediately upon addition of the glucose, nitrite reduction resumed and added increments of NO_2^- were readily reduced. Concomitant with the reduction of NO_2^-, CO_2 evolution increased and the amount of ether extractable acids decreased. There was a slight decrease in acid content of the set shown in column 2, but at the termination of the experiment the medium still gave a positive NO_2^- test. The total duration of the experiment was 96 hours.

The situation with the NO_3^- acceptor system was similar to that with NO_2^-. One observed difference was that active respiration started immediately and then ceased after more than one but less than 2 mM of NO_3^- had been used. This occurred during the first 24 hours after adding the acceptor. Addition of 15 mg. of glucose to the flask caused resumption of respiration and a decrease in final

Table 12. Utilization of acids produced from glucose by P. stutzeri

mM KNO_2 added	0.0	1.0	3.0	5.0
mg CO_2-C recovered	0.5	6.8	25.3	48.4
meq of acid	1.55	1.40	1.12*	0.48**

Initial glucose input - 1.0 mM

*Point of additional increment of 15 mg glucose

**Point of additional increment of 30 mg glucose

acid concentration from 1.59 meq to 0.86 meq for those flasks having received 2 mM of KNO_3.

On the other hand, those flasks that were reflushed with He/O_2 and connected to the electrolysis unit commenced utilizing oxygen immediately and did so at the rate of 2 ml O_2/hour for 60 hours. No sparker was necessary to stimulate respiration. The amount of acid in this case decreased from 1.59 meq to 0.18 meq.

Utilization of Key Metabolic Intermediates as Substrates

In order to determine whether or not the accumulation of acid was due to the inability of the TCA cycle to operate with the NO_3^- acceptor system, the ability of the organism to utilize certain key intermediates of the cycle was examined. The data pertaining to the utilization of pyruvate, acetate and succinate by the test organism under anaerobic and aerobic conditions are presented in the next three tables.

Table 13 shows the normal respiration picture of P. stutzeri with the indicated substrates. The theoretical [O]/C ratio is indicated for each substrate and the experimental values are calculated on the basis of the CO_2 recoveries. The values are slightly lower than theoretical in each case. No comparison can be made as to rate of respiration with each substrate as each substrate was tested in a separate experiment.

Table 14 shows the data for anaerobic catabolism of the substrates with the NO_3^- acceptor system. The theoretical [O]/C values remain the same for each substrate. In the case of the nitrate respiratory

Table 13. The aerobic catabolism of key metabolic intermediates by P. stutzeri

millimoles	Substrate		
	Pyruvate	Acetate	Succinate
[O] consumed	4.8	8.6	6.5
CO_2-C evolved	3.7	4.7	3.3
Ratio $\frac{[O]}{C}$	1.3	1.8	1.9
Theoretical $\frac{[O]}{C}$ ratio	1.67	2.0	2.0

Table 14. The anaerobic catabolism of key metabolic intermediates in conjunction with nitrate respiration

	Substrate					
	Pyruvate		Acetate		Succinate	
mM KNO_3 used	2	3	2	3	2	3
mM [O] from $NO_3^- \rightarrow NO_2^-$	2	3	2	3	2	3
mM [O] from $NO_2^- \rightarrow N_2$	4	6	4	6	4	6
Total mM [O] utilized	6	9	6	9	6	9
mM CO_2-C evolved	2.5	3.6	2.4	3.2	2.6	3.2
Ratio $\frac{\text{mM [O]}}{\text{mM } CO_2\text{-C}}$	2.4	2.5	2.5	2.8	2.3	2.8

system, the [O]/C ratios are considerably higher than theoretical whereas the aerobic respiratory system was lower. However, it does seem that the test organism does indeed have a system similar to, if not identical with, an operative TCA cycle during nitrate respiration.

Table 15 shows that in the presence of both NO_3^- and O_2 the intermediates in question can be readily metabolized and that both respiratory systems function simultaneously. One of the surprises of this experiment was the production of N_2 gas in the presence of O_2 by the test organism. However, the N_2 gas evolved was very little when the input of NO_3^--N is considered, i.e., $\frac{3.26}{112} \times 100 = 2.7\%$ of the total N input.

Almost all evidence currently available indicates that the enzyme(s) involved in the reduction of NO_2^- to N_2 is oxygen sensitive. The most likely explanation is that the rate of solubility of the O_2 gas into the liquid phase is insufficient to supply the needs of the bacterial suspension used in this experiment and that the culture would then turn to whatever acceptor is available. Therefore, slight amounts of NO_2^- could be reduced to N_2 under conditions of reduced dissolved oxygen content.

Aerobic Reduction of NO_3^- to N_2 gas

In order to clarify the relationship of cell concentration and nitrogen gas production under aerobic conditions, the following experiment was performed. The respirometer flasks were set up in an identical fashion except that the cell inoculum was varied as

Table 15. The metabolism of key metabolic intermediates in the presence of dual hydrogen acceptor systems (O_2+NO_3^-)

	Substrate		
	Pyruvate	Acetate	Succinate
mM NO_3^- input	8.0	8.0	8.0
mM [O] from $NO_3^- \rightarrow NO_2^-$	0.31	0.18	0.42
mg N_2 evolved	3.26	3.63	3.66
mM [O] from $NO_3^- \rightarrow N_2$	0.70	0.65	0.78
mM [O] from O_2	4.84	5.83	4.25
Total O_2 consumed (mM)	5.85	6.66	5.45
mM CO_2-C evolved	3.17	3.12	2.70
Ratio $\frac{[O]}{C}$	1.84	2.14	2.02

indicated in Table 16. The flasks were flushed for 20 minutes with the He/O_2 gas mixture. The flasks were incubated on a magnetic stirrer and constant stirring of the liquid medium was obtained with the stirring bar. The flasks were incubated 24 hours and then removed for the analysis of nitrogen gas in the flask atmosphere. The results are shown in Table 16.

The set of flasks with the highest optical density were much less active than the other flasks. The only available explanation is that the stirrers must not have operated at the same speed as in the other flasks. Yet the trend is clear, i.e., the increase in cell optical denisity beyond the point where the rate of solubilization of oxygen cannot supply the oxygen demand of the organisms will result in the evolution of nitrogen gas by denitrifying organisms. This does not justify the term "aerobic denitrification", as conditions in the medium are probably not aerobic although there is 20% oxygen in the atmosphere above the liquid medium.

In order to further verify the above observation, the experiment was repeated using more efficient stirring. The magnetic stirring bars were operated at the maximum rate possible in the flasks without losing control of the stirring bars. The results are presented in Table 17. In addition to the nitrogen and oxygen analysis, the amount of nitrate reduced to nitrite and the amount of carbon dioxide recovered are presented. The nitrite value does not include that nitrite

Table 16. The effect of cell density upon N_2 evolution from nitrate under aerobic conditions

Initial cell optical density	0.25	0.50	0.75	1.0	2.0
volume of oxygen uptake (ml)	44.8	45.4	66.6	56.1	37.6
mg of N_2 gas evolved	0	1.90	3.80	6.88	6.09

Succinate-C added = 72 mg and NO_3^--N input = 4 mM

Table 17. The effect of aeration and cell density upon NO_3^- reduction to NO_2^- and N_2

Initial cell optical density	0.25	0.50	1.0	2.0
O_2 uptake (ml)	46.5	49.8	56.2	76.1
N_2 gas evolved (mg)	0	0	2.34	4.91
NO_2^--N produced from NO_3^-(mg)	4.6	3.9	9.1	17.6
CO_2-C evolved (mg)	36.3	34.9	45.8	66.7

Succinate-C added = 72 mg and NO_3-N input = 56 mg.

which was further reduced to nitrogen gas.

It is evident that the increased stirring rate decreased the amount of nitrogen gas produced from nitrite as well as increasing the oxygen uptake of the higher cell concentrations. The nitrogen gas produced by the 0.5 optical density flasks (Table 16) must have been marginal as the increased stirring rate applicable to the data in Table 17 was sufficient to supply the cells with their required dissolved oxygen. In addition to the increase in nitrogen gas production by the higher cell concentrations, considerable nitrite was produced from nitrate; 46 μg/ml in the case of the lowest cell concentration to 176 μg/ml with the highest cell mass.

Dissolved Oxygen and Aerobic N_2 Production

In view of the above information and the use of the term "aerobic denitrification", it became important to determine the exact dissolved oxygen concentration in a medium containing a suspension of respiring bacterial cells. In order to do so the respirometer flask had to be modified in a manner which would enable the flask to take the probe of an oxygen analyzer. This was accomplished by the addition of a "side arm" to the flask through which the probe could be inserted. By connecting the oxygen analyzer to a Bausch and Lomb Lab Recorder the current generated by the dissolved oxygen of the medium could be monitored during the experiment. The level of the medium in the flask had to be increased to 150 ml in order to cover the probe. The medium used in the following

experiments was NFSB with glucose as the substrate.

Line A, Figure 4, shows a typical dissolved oxygen picture of the medium which had been subjected to oxygen aspiration and stirred. The dissolved oxygen of the liquid decreases until an equilibrium point is reached between the oxygen of the flask atmosphere and the oxygen in solution. This value stabilizes at about 8 ppm dissolved oxygen. Line B shows the same "oxygenated" medium which has been inoculated with a suspension of P. stutzeri to give a final optical density of 1.0. The dissolved oxygen drops at a more rapid rate and is only slightly more than half the value of the control after 30 minutes of incubation. When the trend is allowed to continue, the dissolved oxygen value drops to about 2.5 ppm in 4 hours. Line C corresponded with A when the latter was inoculated after 30 minutes of stirring. The dissolved oxygen value drops rapidly to a value of 3.2 ppm. This indicates that the dissolved oxygen level of the medium before inoculation will have considerable influence on the rate at which the dissolved oxygen will be depleted.

Line D shows the dissolved oxygen picture of a culture that was allowed to sit stationary until the dissolved oxygen value approached zero before stirring was started. Upon starting the stirrers, the dissolved oxygen level rapidly increases but levels off at about 3.3 ppm. When the experiment was started with an oxygenated medium (10-15 ppm) plus a cell optical density of 1.0, less than 5 minutes were required for the dissolved oxygen level to drop to zero. The above data show that the dissolved oxygen concentration of a medium

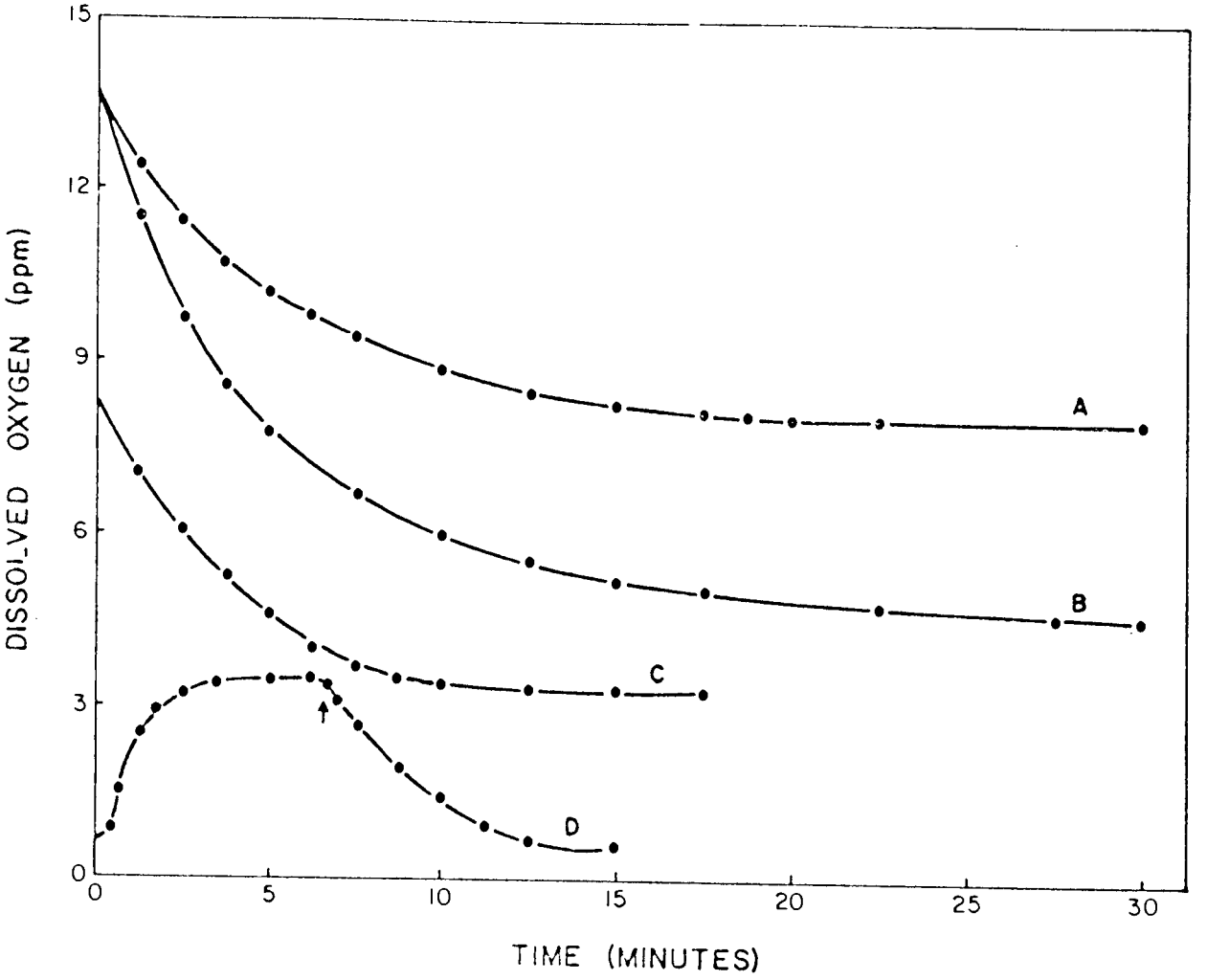


Figure 4. The effect of initial dissolved oxygen content.

before inoculation will have considerable effect on the aerobicity of the medium.

The Effect of Cell Concentration and NO_3^- on Dissolved Oxygen

In view of these observations, an experiment was designed to determine the effect of cell concentrations on the dissolved oxygen of a medium and the effect of the addition of KNO_3 on the dissolved oxygen content of the medium. The results are shown in Table 18 with the dissolved oxygen trends plotted in Figure 5.

Line 1, (Figure 5) attests to the equilibrium obtained between the "oxygenated" medium and the flask atmosphere upon stirring of the medium. The final dissolved oxygen stabilized at 7.35 ppm. Line 2 relates to a similar set of flasks to which had been added a cell suspension to give a final optical density of 0.4. The dissolved oxygen never did stabilize during the monitoring of this set, but continued to drop slowly. With this set as with the following sets to be discussed, the arrows indicate the point at which 12 mM of KNO_3 were added to the system. Curves 3 and 4 show sets with the initial cell optical density of 0.70 and 1.2 respectively. Again the dissolved oxygen drops rapidly to a critical point and then assumes a slower but steady decline. Even the addition of KNO_3 does not cause any increase in the dissolved oxygen concentration although the nitrate is participating as an acceptor as indicated by the accumulation of nitrite (Table 18).

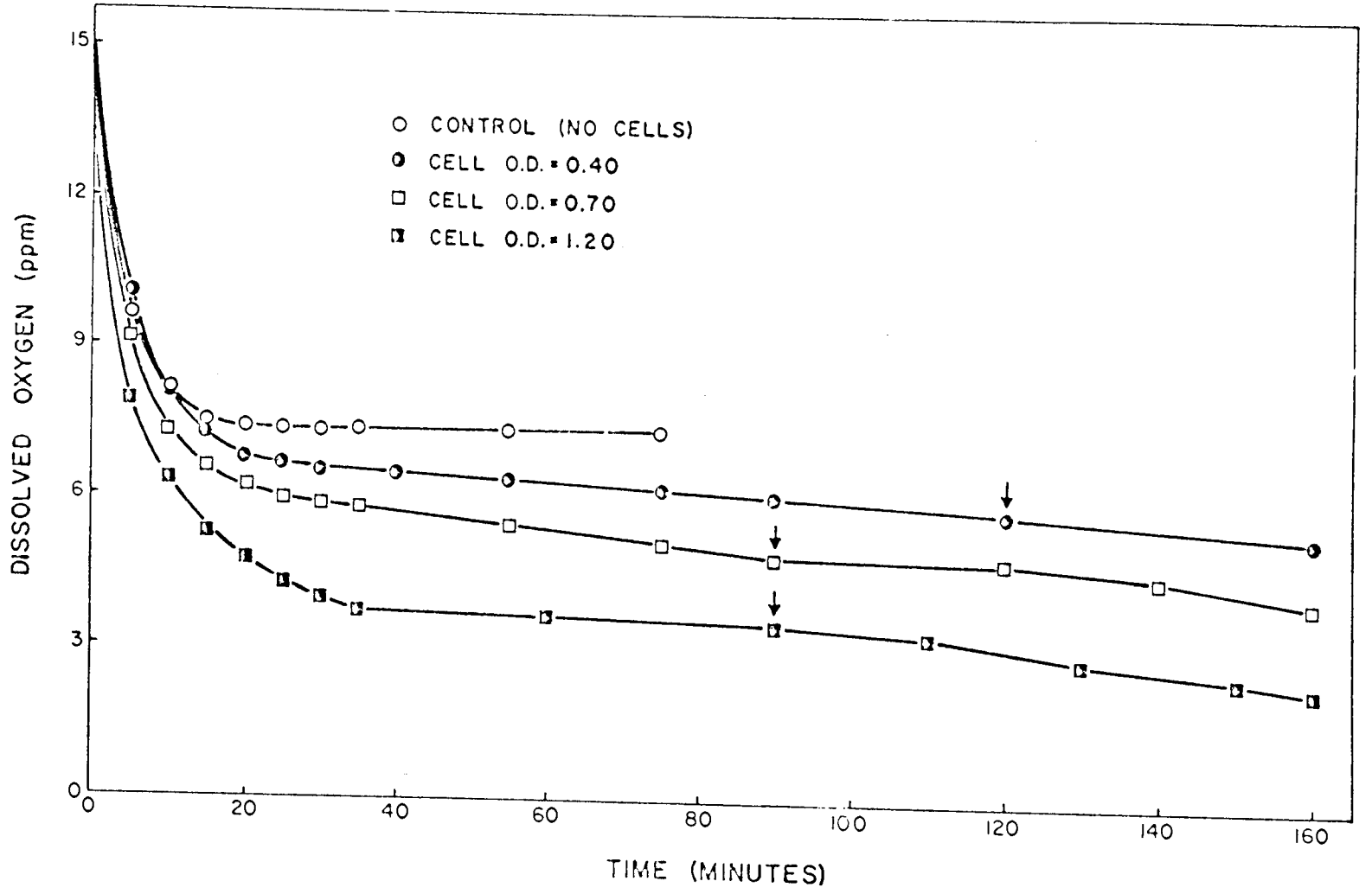


Figure 5. The effect of cell concentration on the dissolved oxygen content of the medium. Arrows indicate addition of 12 mM KNO_3.

Table 18. The effect of cell density on the dissolved oxygen of the medium

	Line 1	Line 2	Line 3	Line 4
Cell optical density	0	0.40	0.70	1.2
Time elapsed (minutes)	75	255	217	269
Final D.O. (ppm)	7.35	4.3	2.9	1.25
O_2 consumed (ml)	-	13.1	15.9	35.3
NO_2^--N from NO_3 (mg)	-	0.316	0.723	1.77

The Effect of Dissolved Oxygen on Denitrification

The trends shown above indicate that under aerobic conditions and a well agitated culture medium, there may be a time when the dissolved oxygen content of the medium and the rate of solubility of gaseous oxygen may not be adequate to supply the needs of the bacterial suspension. In this case the organism may have to change to terminal acceptors other than oxygen. In the case of the denitrifying bacteria, the organisms will turn to nitrate if it is available. If the dissolved oxygen does become sufficiently low, it may be that the nitrate will be reduced thru to nitrogen gas, in which case true denitrification will have taken place. Whether such can be termed "aerobic denitrification" remains open to conjecture.

Thereby, an experiment was designed to determine how low the dissolved oxygen of a medium containing a cell suspension must be in order that nitrogen gas be evolved from nitrate. As the flask containing the probe for measuring the dissolved oxygen would not be feasible for determining nitrogen gas production because of air leaks, an indirect system had to be designed. A series of identically treated flasks were set up with the exception that one flask contained the oxygen probe. Other than that, all flasks contained 150 ml of NFSB with 6 mM KNO_3, 500 mg of glucose, and a cell optical density of 1.0. All flasks were flushed with He/O_2 and incubated with the magnetic stirring bars operating at the same speed. At intervals, flasks were removed and analyzed for nitrogen gas in the atmosphere. The results are shown in Table 19.

Table 19. The effect of dissolved oxygen on N_2 evolution from NO_3^-

Time of incubation (hrs)	22	4	7½	11	22
O_2 consumed (ml)	95.8	10.5	38.0	50.3	92.7
Minimum D.O. at time of removal (ppm)	0.91	3.2	2.7	1.6	0.91
N_2 gas evolved (mg)	-	1.23	1.68	2.16	1.61

The rates of oxygen consumption in all flasks were about the same. In the monitored flask and the last flask removed for analysis, there is only a 3.1 ml difference in oxygen consumption over the 22 hour period. The nitrogen produced in all cases is approximately 2% (±) of the total NO_3^--N added. The important observation here is that with the dissolved oxygen maintaining a range of from 2.1 to 0.91 ppm for 15 hours, little or no nitrogen gas was produced from nitrate.

Effect of NO_3^- on Oxygen Uptake by P. stutzeri

Now that the production of nitrogen gas under aerobic conditions has been investigated it becomes important to look more closely at the effect of nitrate on oxygen uptake. Bhatt (1964) has reported that upon the addition of nitrate at concentrations up to 1000 ppm NO_3^--N there is a proportional decrease in oxygen uptake by P. stutzeri. Concomitant with the decrease in oxygen uptake there is also a decrease in carbon dioxide evolution and an increase in nitrate reduction to nitrite. In order to confirm these observations, the following experiments were conducted in liquid cultures. The experiments were performed under aerobic conditions with 100 ml of the NFSB medium, 1 mM glucose, and NO_3^- at various concentrations. In the first experiment, the NO_3^--N concentrations were 1000, 2000 and 4000 ppm. The results are shown in Table 20. There was little, if any, difference in oxygen consumption between the flasks containing nitrate and the control. There is no lag lasting for several hours as was found in sand by Bhatt (1964). A flask containing sand

Table 20. The effect of NO_3^- on oxygen uptake by *P. stutzeri* during 7 hours incubation

NO_3^--N (µg/ml)	0	1000	2000	4000
O_2 uptake (ml)	48.9	60.5	46.5	37.5
NO_2^--N produced from NO_3^- (µg/ml)	-	26.8	10.2	15.8

and 1000 ppm NO_3^--N which was carried with these for comparison showed no oxygen uptake during the 7 hours of the experiment. The explanation for the difference in observations must lie then in either of the following: (1) The stirring of the medium greatly influences oxygen uptake and offsets the nitrate effect or (2) The nitrate concentration of 1000 ppm NO_3^--N in Bhatt's experiments were based on the sand-water volume which gives a much larger effective NO_3^--N concentration. To obtain a 1000 ppm NO_3^--N concentration 1000 µg of NO_3^--N would have to be added per gram of soil or a total of 100,000 µg. However, this 100,000 µg is dissolved in 15 mls of liquid. Therefore, Bhatt's NO_3^--N concentration in the liquid phase is 100,000 divided by 15 or 6,666 µg/ml NO_3^--N in the liquid phase. This may also be expressed on the basis of molar concentration which is 0.47 molar KNO_3. The 1000, 2000 and 4000 ppm NO_3^--N used in the present liquid culture study could be expressed as 0.075, 0.143, and 0.285 M KNO_3 respectively.

In view of the above information, the experiment was repeated with the following nitrate concentrations: 0.0, 0.285 M (4000 ppm N), 0.428 M (6000 ppm N), and 0.572 M (8000 ppm N). The effect on oxygen uptake is presented in Figure 6. All sets containing nitrate did show a depression of oxygen uptake which increased with the increased concentration of KNO_3. The D.O. in the flask containing 0.57 M KNO_3 was monitored throughout the experiment and is also plotted in Figure 6. The depression of oxygen uptake cannot be said to be due to lack of oxygen in the medium as it

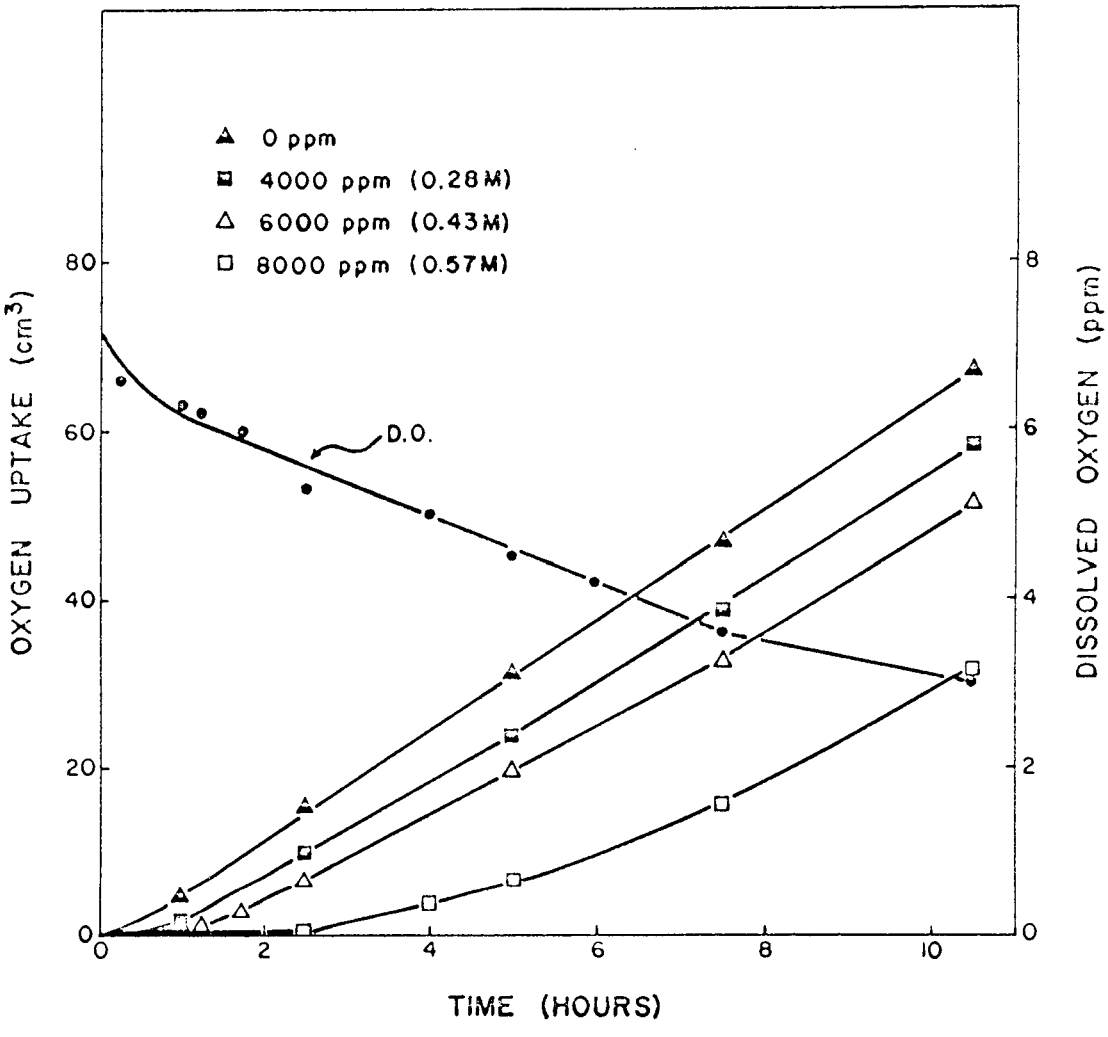


Figure 6. The effect of NO_3^- on oxygen uptake in liquid cultures under vigorous aeration.

remained considerably higher than in previous experiments with a lower nitrate concentration. The lag noticed with the 8000 ppm NO_3^--N set is considerably shorter than was noted in sand by Bhatt (1964). Therefore, it does seem that the stirring does influence the consumption of oxygen, probably by making it readily available and maintaining the concentration in solution at a more adequate level.

As is indicated in Table 21, the nitrite produced from nitrate does not show an increase proportional to the increase of nitrate but rather exhibits a slight decreasing trend. Here with the increase in KNO_3 -N concentration, not only is a decrease in oxygen uptake obtained, but also a decrease in the amount of nitrate reduced. It may be that a competition between oxygen and nitrate occurs but it may also be possible that the effect on the cells is due to the salt concentration.

Upon the addition of NO_3^--N to a suspension of P. stutzeri, there is a linear decrease in oxygen consumed with each increment increase in nitrate added. At the same time, nitrate is reduced to nitrite. Concomitant with the decrease in oxygen consumption, there is an apparent "switch" in the pathway of glucose dissimilation (Bhatt, 1964).

One of the more obvious explanations is that the nitrate in solution is present in greater amounts than is the dissolved oxygen, therefore the chance of the organism using nitrate over oxygen becomes greater because of the great ratio of NO_3^- to O_2 in solution (1000:1 at 4000 ppm NO_3^--N and 4 ppm dissolved oxygen).

Table 21. The effect of nitrate on the respiration of *P. stutzeri*

KNO_3 (moles)	0	0.285	0.428	0.572
O_2 consumed (ml)	68.0	58.8	51.4	31.9
NO_2^--N from NO_3^- (μg/ml)		188	168	130

Supporting this idea is the fact that the amount of depression of oxygen uptake is dependent on the concentration of nitrate. A second possible explanation is that the organism has a constituitive enzyme; those required for the use of oxygen acceptor system and those required for use of the $NO_3^- \longrightarrow NO_2^-$ acceptor system. If this is true, which all evidence of enzyme studies thus far indicates (Taniguchi 1961; Nason 1963), then why should not the organism use both electron acceptor pathways? The nitrate acceptor pathway is probably not poised as high as the oxygen pathway, therefore it would probably be used to a lesser degree. If the organism has a poor control mechanism for shutting off one pathway then both should function simultaneously if both acceptors were present. The organism may never have needed to completely control one pathway over the other as it is unlikely that in the natural evolution of the species, a condition existed where both the nitrate concentration and the dissolved oxygen were high. Thus a species would develop with weak control mechanisms for any one of the terminal respiratory systems.

Next, the possibility that high NO_3^- concentrations force specific alterations upon the metabolism of the cells should be considered. If this is possible, how does it occur? Henneman and Umbreit (1964) have shown that KCl or NaCl at concentrations in the order of 0.2 to 0.5 M inhibit respiration in *E. coli* B cells that have been washed and suspended in distilled water for up to 72 hours. This was determined by the Warburg technique. This treatment with distilled

water seems to be extremely harsh and probably results in osmotic shock to the cells upon the addition of salt in such high concentrations. Concomitant with the observation that respiration is inhibited, these workers observed that the cells became more refractile and seemed to undergo a type of plasmolysis which is, in some cases, reversible with time or by dilution to a lower salt concentration.

At 1000 ppm NO_3^--N based on the sand-water approach the actual NO_3^--N concentration in the liquid phase is equivalent to 0.446 M solution of KNO_3. At 500 ppm, the concentration is equivalent to a 0.223 M solution. With a liquid culture incubated and stirred with a magnetic stirrer, depression of oxygen consumption was easily demonstrable at 4000 ppm (0.285M). Higher concentrations of 6000 ppm (0.428M) or 8000 ppm (0.572M) greatly accentuated the repression of oxygen consumption. How does KNO_3 at these concentrations affect P. stutzeri? Possibly the same way in which KCl and NaCl affected E. coli B.

The Effect of 0.57 Molar Salt Solution on P. stutzeri Respiration

To proceed on this assumption, these salts should first be tested on P. stutzeri in order to determine if they have an effect similar to KNO_3. A similar experiment was designed to test the effect of various salts on oxygen consumption by the test organism. The salts used were NH_4Cl, NH_4NO_3, $(NH_4)_2HPO_4$, KCl, and KNO_3, all at the critical 0.57 molar concentration. Their effect on oxygen uptake is shown in Figure 7. The salts differ in their degree of effectiveness in depressing respiration. Contrary to what Henneman and

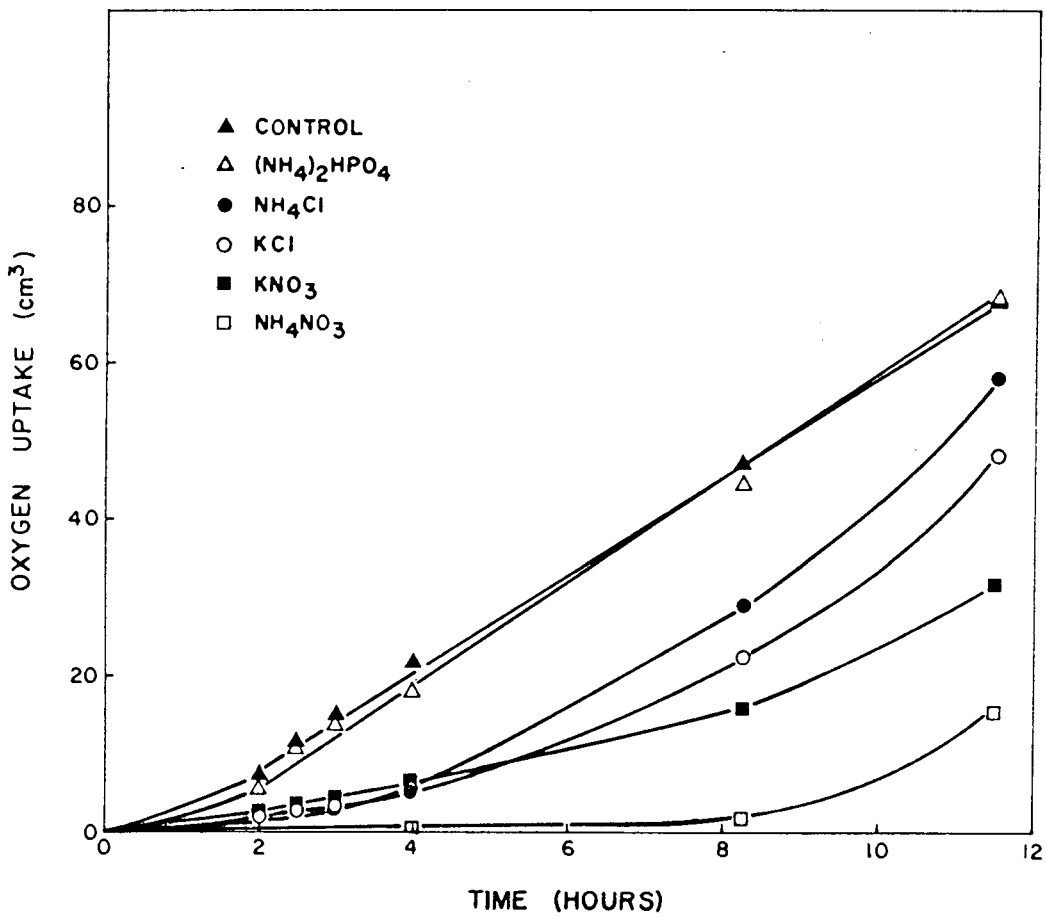


Figure 7. The effect of 0.57 molar salt solution on respiration of P. stutzeri.

Umbreit said, it seems that it may be the anion and not the cation that is effecting the depression of respiration. ...The ammonium ion is twice as abundant in $(NH_4)_2HPO_4$ as it is in NH_4NO_3 or NH_4Cl but yet has no effect on respiration. It would seem then that the Cl^- or NO_3^- ion is the active agent and not the K^+ as Henneman contends. This is beside the point. The point here is that although nitrate salts repressed oxygen consumption more than did Cl^- salts, both were effective. Assuming that part of the effect in the case of nitrate is due to competition with oxygen, what is the cause in the case of Cl^- and could not this same cause be partially responsible in the case of the nitrate salts?

Henneman and Umbreit (1964) mention that the cells become more refractile when exposed to this salt. This is attributed to a type of plasmolysis that takes place upon exposure to the salt. Their explanation for this "salt effect" is that the salt causes expulsion or extraction of substances from the cell. This cannot be doubted when the manner in which the cells are treated before exposure to the salt solution is considered. Henneman and Umbriet (1964) also note that the alteration in the physical state of the cell membrane may have changed the normal spatial arrangement of the various enzymes in the normal respiratory chain. This may be the more realistic explanation.

The high salt concentration probably does cause plasmolysis of the cell and at the same time the shrinking of the cell membrane causes a disorientation of the respiratory enzymes associated with the

the membrane thus temporarily incapacitating the enzymes. The cell is then forced to react in whatever manner possible in order to survive. With its terminal respiratory enzymes out of action, its only alternative is to switch to another means of obtaining energy, namely a more fermentative process. To accomplish this, the glycolytic pathway could be utilized with the result being that pyruvate, lactate, acetate, or some neutral by-product of glucose would result as the "terminal respiratory product". If this is true, then the observations resulting from a Warburg study or other method of studying respiration would be to see (1) a decrease in oxygen uptake, (2) a decrease in carbon dioxide evolved, and (3) an increase in dissimilatory by-products excreted into the medium, that is, assuming glucose can penetrate the cell membrane.

Evidence supporting the above postulates is found in the fact that at 1000 (0.446 M) ppm KNO_3 in soil carbon dioxide evolution and oxygen uptake are reduced considerably. In the meantime, nitrate is being reduced and there is an apparent change in primary pathway of glucose dissimilation (Bhatt, 1964). At the same time ether extractable acids are being secreted into the medium. Worth noting here is the fact that under aerobic conditions with oxygen as the sole acceptor, only traces of acid are found, also when nitrate is the sole acceptor at the rate of 4 mM/mM of glucose. It may be that anaerobically with 0.5 M salt these acids will also be excreted.

Henneman and Umbreit (1964) note that with time or by dilution to 0.2 M the plasmolytic effect on the cell was reversed and that

oxygen consumption resumed to near normal rate. This again attests to the probability that the terminal enzymes are being temporarily disrupted by the plasmolysis of the cell.

In order to obtain further evidence to clarify the above observations another experiment similar to that described (Figure 7) was performed. In this experiment K_2SO_4 was added as a test salt also. All salts were added at a concentration of 0.05 molar and analysis was made for carbon dioxide evolved and the pH of the final medium was tested in addition to measurement of oxygen consumption. The results are presented in Table 22.

Although not evident in the data as presented, the oxygen consumed by the flask containing the NH_4Cl was only about 50-60% of the control during the first 10 hours of the experiment. It then rapidly increased to the point where the oxygen consumed was equal to the control upon termination of the experiment. The pH of each salt solution was not sufficiently low to affect the respiration of the cells. The low carbon dioxide value for $(NH_4)_2HPO_4$ was probably due to high assimilation by the cells.

The Effect of 0.5 M Salt Solutions on NO_3^- Respiration of P. stutzeri

The following work was to study the effect of the salts KCl, K_2SO_4, and KNO_3 at a concentration of 0.50 molar on the rate of nitrite reduction to nitrogen gas. In the case of KCl and K_2SO_4, 4 mM KNO_3 were added as hydrogen acceptor for respiration. Analyses were made at intervals for nitrogen gas evolved, nitrite produced from nitrate, for carbon dioxide evolved and for titratable acids.

Table 22. The effect of 0.5 M salt concentration on respiration of P. stutzeri during 15 hours incubation

Salt	None	$(NH_4)_2HPO_4$	NH_4Cl	NH_4NO_3	KCl	KNO_3	K_2SO_4
pH of solution	6.8	7.6	6.3	6.3	6.5	6.5	6.5
O_2 consumed (ml)	44.6	46.1	47.0	28.4	34.3	32.6	34.5
CO_2-C (mg)	24.1	18.9	26.2	14.2	18.5	17.0	18.3

The nitrogen and carbon dioxide evolution pictures are shown in Figures 8 and 9. The K_2SO_4 study was conducted in a separate experiment but the effect is the same.

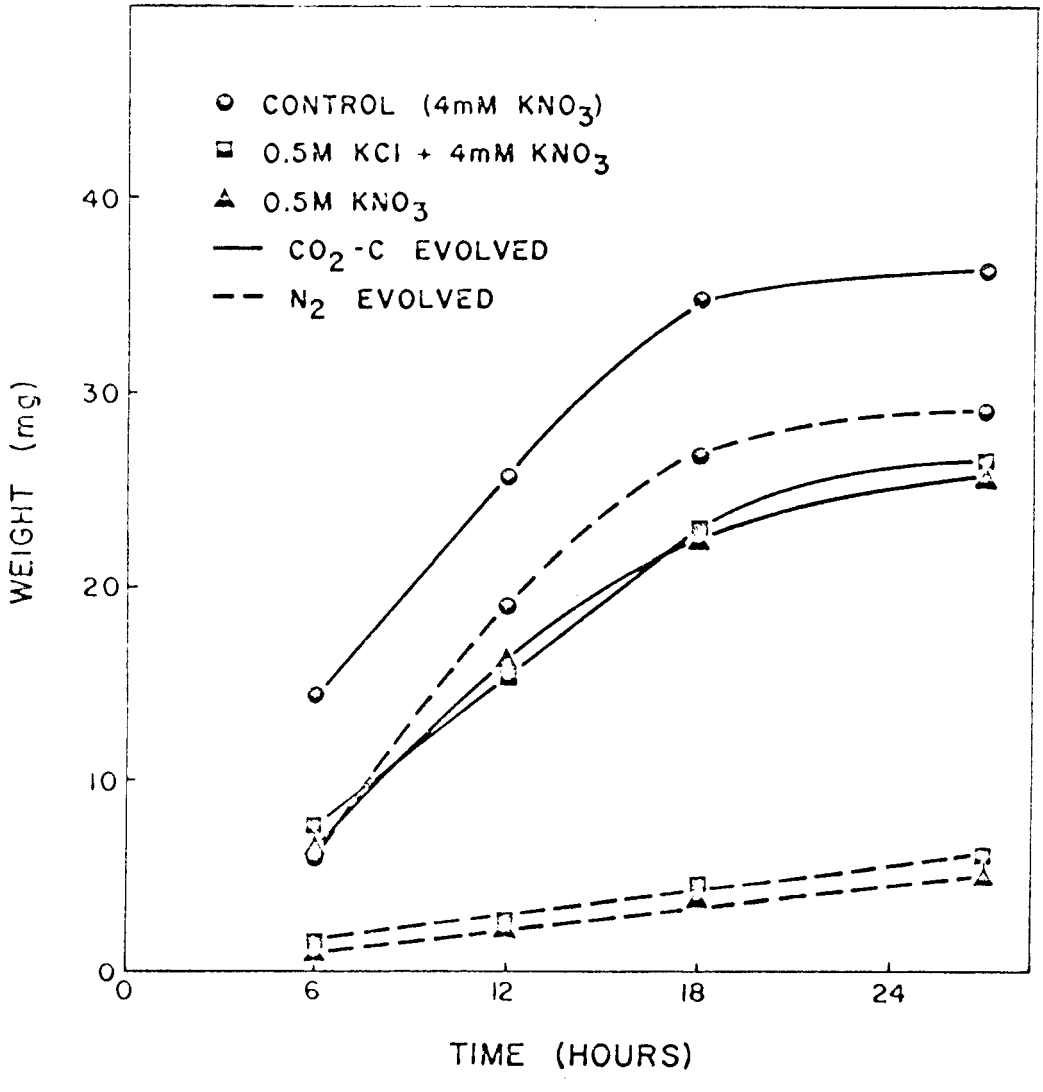
The effects observed here for the 0.5 M salt concentrations on nitrate respirations are similar to those on normal respiration. There is a decrease in the rate of nitrite reduction, the evolution of carbon dioxide is decreased and very little nitrogen gas is evolved. There was no significant difference in the production of nitrate acids. One thing that was noticed was the reduction of nitrate to nitrite which was complete by the termination of the experiment (Table 23). In this case it seems that the inhibition does not affect the nitrate to nitrite reduction step as much as it does the nitrite to nitrogen gas reduction step. This reduction does seem to be directly associated with respiration as calculations of the carbon dioxide values provide a close correlation between the [O] obtained from the nitrate reduced to nitrite and amount of carbon dioxide evolved. An exception to this observation is the set containing the 0.50 M KNO_3. There was much more nitrate reduced than was needed for the carbon dioxide evolved. This again suggests that nitrate may participate at some other point in addition to the terminal hydrogen acceptor pathway. It may participate directly at substrate level being reduced to nitrite by accepting H^+ directly from NADH which may result in no net energy gain for the organism. The reaction may be schematically expressed by the following:

$$2\ NADH + NO_3^- + \text{cytoplasmic nitrate reductase } 2NAD^+ + NO_2^- + H_2O$$

Table 23. The reduction of NO_3^- to NO_2^- by P. stutzeri in the presence of 0.5 M salt solution

Salt	none	KCl	KNO_3	none	K_2SO_4
NO_3^--N added (mg)	56	56	700	56	56
NO_2^--N (mg) produced at 6 hours	3.80	18.7	18.1	0.44*	23.5*
12 hours	0.20	31.5	36.0	-	-
18 hours	0.25	39.0	46.8	3.16*	42.0*
24 hours	6.30	50.0	90.0	12.70*	51.0*

*Samples were taken at 8 hr 15 min, 17 hr 15 min, and 29 hr 45 min, respectively.



The effect of 0.50 M KCl and 0.50 M KNO_3 upon nitrate respiration (CO_2 and N_2 evolution).

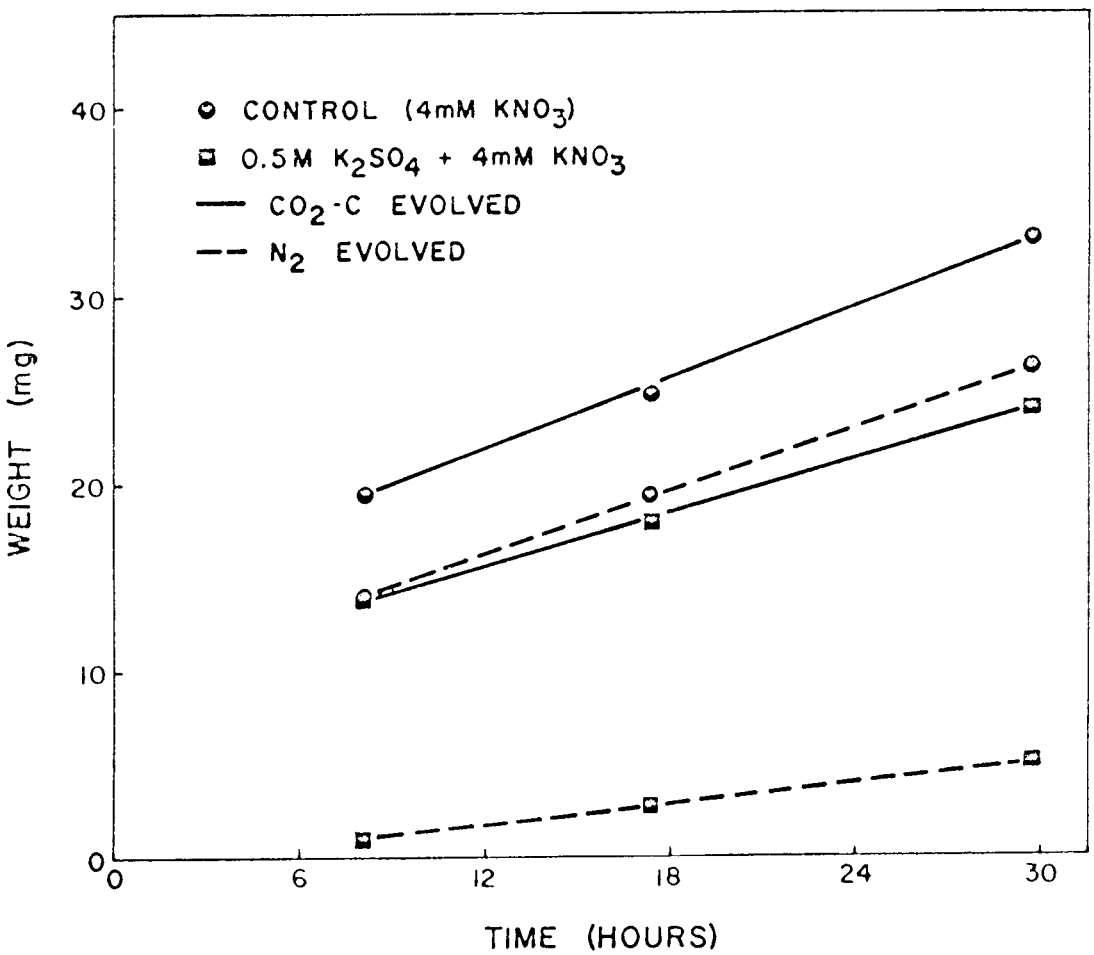


Figure 9. The influence of 0.50 M K_2SO_4 on nitrate respiration (CO_2 and N_2 evolution).

The Effect of 0.5 M Salt Solution on Nitrite Respiration

Before it can be truly said that salts influence nitrate respiration more than the nitrate reducing system, the former will have to be tested in the absence of the latter. An experiment designed similar to the previous one was used for this purpose. The salts KCl and K_2SO_4 each at 0.50 M were added to flasks containing 2 mM KNO_3. The nitrogen and carbon dioxide gases evolved are presented in Figure 10. In 24 hours time most of the nitrogen gas from nitrite was evolved by the control set of flasks. Those flasks containing KCl or K_2SO_4 at 0.5 M exhibited much reduced activity with respect to both nitrogen and carbon dioxide evolution.

Conclusions

The requirement of denitrifying bacteria for yeast extract has been shown again to be one of the factors limiting the growth and rate of denitrifying activity of the organism. The necessary factor which is supplied by yeast extract is still unknown although many workers have noted this requirement (Roa, 1961; Delwiche, 1956; Verhoeven, 1956). The inability of P. stutzeri to assimilate nitrate- or nitrite- nitrogen proved helpful in studying nitrate respiration. As a consequence more accurate nitrogen balances were obtained. Although cultures such as E. coli have both the assimilatory and respiratory nitrate reductases, cell enzyme activity seems to lie at different loci

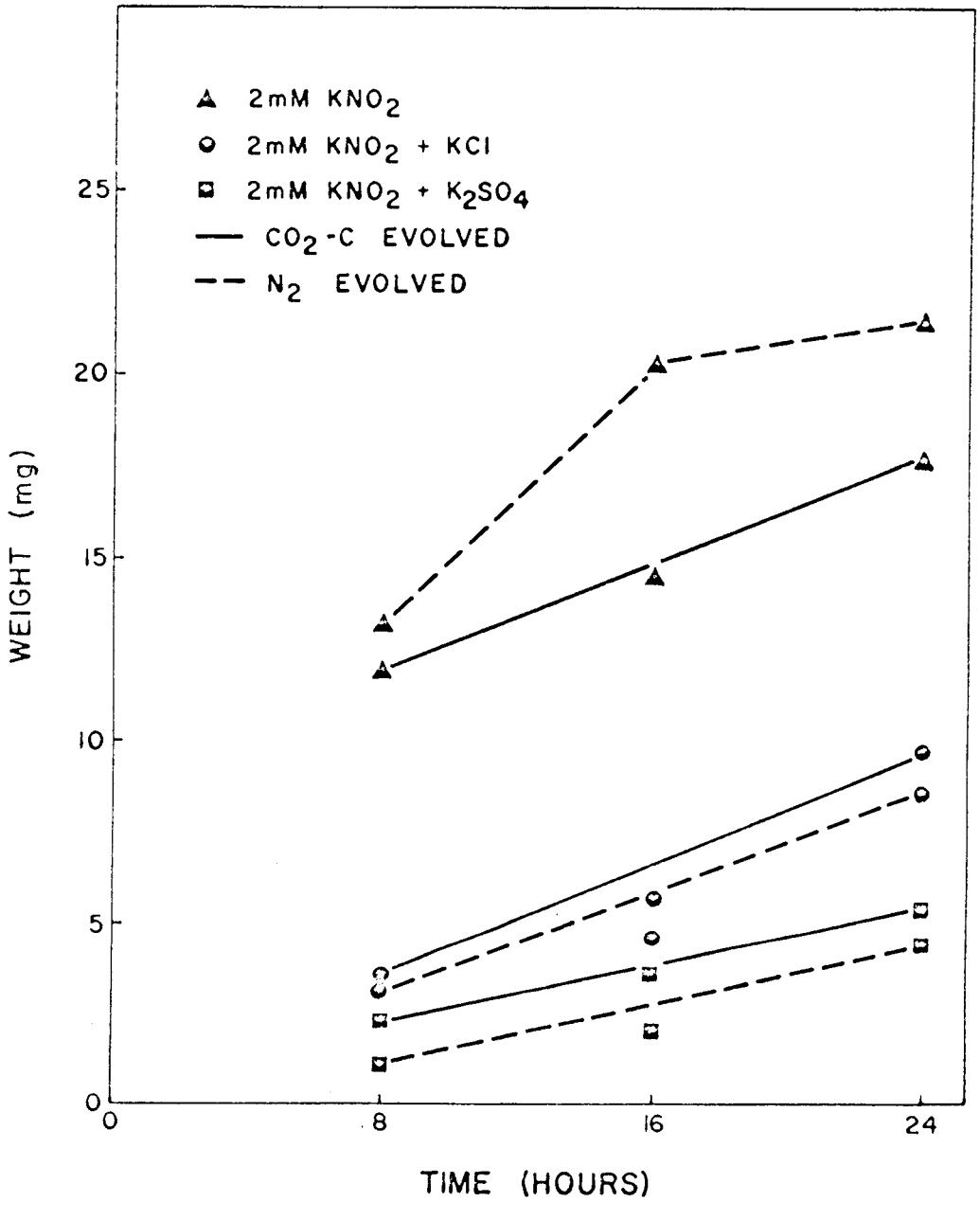


Figure 10. The effect of 0.50 molar KCl and 0.50 molar K_2SO_4 on nitrite respiration.

(Taniguchi, 1961). However, E. coli does not possess the true dissimilatory nitrate reductase system.

Molecular oxygen influences the degree of denitrification by P. stutzeri. Under aerobic conditions, the rate of reduction of nitrate to nitrite and of nitrite to nitrogen gas is determined by the density of the cell suspension. As the cell suspension becomes heavier, the production of nitrogen gas increases. This is undoubtedly due to a deficiency of oxygen in the medium which in turn forces the cells to utilize the nitrate and nitrite thereby causing an increase in nitrogen gas production. The effect of oxygen on nitrite reduction must be considerably more than on nitrate reduction as nitrate seems to be reduced to nitrite with ease in the presence of higher concentrations of dissolved oxygen. The so-called aerobic denitrification reported earlier (Meiklejohn, 1940) probably took place in a culture medium where the rate of solubilization of oxygen was inadequate to meet the demands of the cells. In the studies presented herein no nitrogen gas was evolved if the oxygen content of the medium was adequate for the needs of the cells. Thus, it can be said that under truly aerobic conditions, no denitrification will take place, at least not with Pseudomonas stutzeri.

The question of nitrate competing with oxygen as a terminal acceptor is not yet completely answered. Evidence presented earlier indicated that even at high oxygen concentration (4 ppm) nitrate is still reduced to nitrite. It is probable that at 4 ppm

dissolved oxygen there is sufficient oxygen in the agitated medium to adequately supply the needs of the terminal enzymes requiring oxygen as the final oxidant. However, there is still some reduction of nitrate to nitrite.

Several workers (Koyama, 1961; Katsura et al., 1954) have presented evidence that there is a non-cytochrome dependent, non-assimilatory nitrate reductase present in Clostridium welchii and also in 2 day old bean cotyledons. This nitrate reductase results in the accumulation of nitrite from nitrate. On the other hand, an assimilatory nitrate reductase present in E. coli and P. fluorescens has also shown to be non-cytochrome dependent but was NADH-linked, as was the system in C. welchii. The system in C. welchii has been referred to as nitrate fermenting because it participated at the substrate level.

There are several pieces of evidence presented in this study which indicate that there may also be a fermentative-type nitrate reductase operative in P. stutzeri. This reductase is probably associated with the soluble components of the cell rather than the particulate components. Bhatt (1964) showed that in the presence of oxygen in sand cultures of P. stutzeri nitrate caused a change in the decarboxylation ratio of C-1 and C-6 of glucose. The change in ratio seemed to indicate a switch from almost complete utilization of the Pentose Pathway to partial utilization of a fermentative-type, glycolytic-like pathway in the presence of nitrate.

How could nitrate cause a switch from an oxidative to a fermentative-type pathway? One possibility would be the presence of a soluble NAD-requiring nitrate reductase. The requirement of this enzyme for NAD could cause the organism to alter some of its metabolic activities from a NADPH-yielding Pentose Pathway to a more fermentative pathway yielding NADH.

Evidence was presented earlier that cell suspensions of P. stutzeri could oxidize glucose to acid fermentation products in the absence of an inorganic acceptor system. However, it was shown that this organism could not grow under anaerobic conditions without an adequate acceptor being present. It seems that although the test organism is able to "ferment" glucose it cannot harvest energy for growth from its fermentation products. This would indicate that the organism has the requisite enzymes for dissimilating glucose and that these are probably of the glycolytic-type. If they are glycolytic-like enzymes, then they are most probably associated with NAD for substrate oxidation. If there is present in the cell a soluble NAD-linked nitrate reductase, then addition of nitrate could cause the cells to partially alter their carbohydrate metabolism to a fermentative system in order to supply the NADH demand of the nitrate reductase.

The determination of the stoichiometry of nitrate respiration was not as clear cut as that of nitrite respiration. Calculation of the [O] /C ratio for nitrate to nitrogen gas gave values in the vicinity of 2.5. Calculation of the percent efficiency of the nitrate to nitrite reduction step on the basis of carbon dioxide

production gave values of 24 to 60 percent for different systems. A possible explanation of the high [O]/C ratio and, in most cases, a low percent efficiency may lie in the presence of a non-terminal nitrate reductase in the cell. If the nitrate reductase was participating at substrate level there could be reduction of nitrate to nitrite without the appearance of a proportional amount of carbon dioxide. The reoxidized NAD would continue to accept hydrogen from the substrate, particularly from D-glyceraldehyde-3-phosphate. A substrate level nitrate reductase might result in giving fermentation products such as pyruvate, acetate or acetaldehyde.

Additional and possibly the strongest evidence thus far presented for the participation of nitrate at some point other than in the terminal respiratory system is found in the effect of 0.5 M salt solutions on the respiration of P. stutzeri. Certain salts at the latter concentration inhibit oxygen consumption and nitrite reduction to nitrogen gas. In view of Bhatt's (1964) study with KNO_3 in sand and the present experiments with well aerated liquid cultures, the effect on oxygen consumption seems to be partially dependant on the degree of aeration. With good aeration the effect is partially reversed with time. The effect of the salt on nitrite respiration was similar to that on oxygen respiration. Very little nitrogen gas was evolved, indicating slight respiration. On the other hand, the effect on nitrate reduction to nitrite was not noticeable. All of the nitrate added was

reduced to nitrite during the experiment except in the case where 0.5 M KNO_3 was used. In this case 90 mg of NO_2^--N was produced from the added nitrate. It therefore seems that nitrate reduction is not affected by the 0.5 M salt solutions. Since both nitrite and oxygen respiration are affected and both presumably act as terminal acceptors, it seems possible that nitrate reduction to nitrite may take place at some other loci in the cell. The most logical place would be at substrate level.

The fluctuations of the [O]/C ratios and the percent efficiency of the nitrate to nitrite reduction step leads to the conclusion that in P. stutzeri the reduction of nitrate may be effected by two different enzyme systems, one participating at the substrate level and the second with the terminal cytochrome-associated system. Certainly, the degree the participation of the substrate level system would influence the [O]/C ratios and percent efficiency of the terminal enzyme systems.

SUMMARY

1. The organism Pseudomonas stutzeri has a definite requirement for some component in yeast extract which must be supplied before the organism will grow or respire anaerobically with the nitrate or nitrite acceptor systems. Although the test organism can use nitrate and nitrite as terminal hydrogen acceptors, it cannot reduce these to ammonia or hydroxylamine for assimilation into the cell. Asparagine, which stimulates growth of the organism, cannot replace the requirement for yeast extract.

2. The presence of nitrate and especially nitrite in the medium tends to reduce the ability of P. stutzeri to grow aerobically in an efficient manner.

3. Nitrate and nitrite respiration have been studied with cell suspensions of P. stutzeri. Data are presented which indicate that the nitrite respiratory system may be similar to that of the oxygen system insofar as the atoms of oxygen required for the evolution of carbon dioxide is concerned. On the other hand, the nitrate respiring system seems to be less efficient particularly in the nitrate to nitrite acceptor step. Fermentation products are excreted into the medium but these appear to be due to a sluggish acceptor system or to the complete absence of acceptor for the cell suspension. Pyruvate, acetate and succinate were metabolized without difficulty with either acceptor system.

4. The influence of cell density on the dissolved oxygen content of the medium was studied. As expected, the cell concentration greatly influenced the level of dissolved oxygen which could be maintained in an agitated medium. This also influenced the rate at which nitrate and even nitrite could be reduced in an aerobic system. Too heavy a cell suspension could lead to so-called "aerobic denitrification" although the conditions of the medium, per se, were not aerobic.

5. A study of the effect of the dissolved oxygen content of the medium upon "aerobic denitrification" showed that at a D.O. concentration as low as 0.9 ppm no denitrification occurred. However, with cell concentrations of 0.25 to 0.50 mg dry weight per ml of medium, very active stirring of the medium was required to maintain the D.O. above 1.0 ppm for a 15 to 24 hour period. Although no nitrogen gas was produced nitrate was reduced to nitrite.

6. The reduction of oxygen uptake by high nitrate concentrations was investigated in well aerated liquid cultures. Inhibition was first noted at a concentration of about 0.2 molar. The effect increased with increase in concentration. A solution of 0.57 molar KNO_3 gave a lag in oxygen uptake lasting about 3 hours.

7. Other salts were tested for the ability to reduce oxygen uptake. Solutions (at 0.57 M) of NH_4Cl, KCl, KNO_3, NH_4NO_3 and K_2SO_4 caused a reduction in oxygen uptake. The effect was greatest with the nitrate salts. A solution of $(NH_4)_2HPO_4$ (at 0.5 M) had no

apparent effect on oxygen consumption.

8. Solutions of KCl, K_2SO_4 and KNO_3 (all at 0.5 M) were tested for effects on nitrate respiration. There seemed to be no effect on the reduction of nitrate to nitrite. However, nitrite respiration, that is, the evolution of nitrogen gas from nitrite was almost completely inhibited during the 30 hours of observation. This was true for both the nitrite accumulated from nitrate and for the nitrite added in the absence of nitrate.

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