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Title AEROBIC AND NITRATE RESPIRATION ROUTES OF CARBOHYDRATE CATABOLISM IN PSEUDOMONAS STUTZERI

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Pseudomonas stutzeri and other denitrifying bacteria are able to grow under anaerobic conditions, using nitrate-oxygen as the terminal hydrogen acceptor, in a manner analagous to classical aerobic respiration with free-molecular oxygen. This rather unique phenomenon is known as nitrate respiration. Nitrate respiration has been studied with respect to the nitrate reducing enzymes and carrier systems involved in the reduction sequence, but very little emphasis has been placed on the metabolic pathways which are associated with nitrate respiration. This study was carried out in an attempt to establish the metabolic pathways operative, both under aerobic conditions and during nitrate respiration, in order to determine whether there was any shift of pathways under conditions of nitrate respiration.

Primary pathways were determined by the radiorespirometric method using specifically labelled glucose and gluconate. The

results, based primarily on the rate of decarboxylation of the C-1 and C-4 positions of glucose, indicated the operation of the Entner-Doudoroff and pentose phosphate pathways under both aerobic conditions and conditions of nitrate respiration. Evolution of $^{14}CO_2$ from the other labels of glucose, as well as incorporation of these labels into the cell, indicated that terminal pathways such as the tricarboxylic acid cycle or glyoxalate cycle might also be operative under both conditions of oxygen relationship.

The secondary pathways were studied using specifically labelled acetate. These studies indicated the operation of the TCA and glyoxalate cycles. Cell-free enzyme studies also showed involvement of the glyoxalate cycle in the metabolism of acetate.

The TCA cycle was studied further by radiorespirometry, using specifically labelled glutamic acid. The respirometric patterns obtained were typical of those expected for TCA cycle operation. Exclusion of nitrate under anaerobic conditions resulted in cessation of $^{14}CO_2$ evolution from glutamic acid. No label was incorporated into the cells under these conditions and the medium accounted for essentially all label recovered from glutamic acid. This was further supported by the same experiment with glucose and by observation that P. stutzeri will not grow anaerobically in the absence of nitrate.

The cells were shown to fix CO_2 when grown in synthetic medium with acetate, glucose, and pyruvate as the sole carbon

sources. Cell suspensions from these experiments were hydrolyzed. The amino acids were separated by paper chromatography and counted on paper strips by liquid scintillation techniques. The percent recoveries in each amino acid indicated that aspartic acid, glutamic acid, arginine, proline, lysine, threonine, and isoleucine may be synthesized via the TCA cycle. Data for glycine, serine, and cysteic acid showed the implication of the glyoxalate cycle in the biosynthesis of these amino acids.

It was concluded that the Entner-Doudoroff and pentose phosphate pathways constituted the primary routes of glucose dissimilation and that the glyoxalate and TCA cycles served as the terminal pathways in both aerobic and nitrate respiration.

AEROBIC AND NITRATE RESPIRATION ROUTES OF CARBOHYDRATE CATABOLISM IN *PSEUDOMONAS STUTZERI*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

AEROBIC AND NITRATE RESPIRATION ROUTES OF CARBOHYDRATE CATABOLISM IN PSEUDOMONAS STUTZERI

INTRODUCTION

Pseudomonas stutzeri belongs to that class of organisms known as facultative anaerobes. Normally, then, one would visualize an aerobe which might presumably use an EMP or ED pathway as the primary route of glucose dissimilation, as well as the TCA or glyoxalate cycle as terminal pathways to oxygen. Under anaerobic conditions this facultative anaerobe might be expected to metabolize glucose via a primary route alone since the terminal hydrogen acceptor is unavailable. It is here that P. stutzeri differs from those organisms considered to be "normal facultative anaerobes", in that no growth occurs under anaerobic conditions without nitrate. It follows, then, that this organism is a "non-fermenter" i. e. is unable to use a substrate as both the hydrogen donor and hydrogen acceptor. This organism is also a typical denitrifier capable of reducing nitrate to nitrogen gas and nitrogen oxides under anaerobic conditions. Therefore, nitrate must serve as a respiratory-type hydrogen acceptor under anaerobic conditions (nitrate respiration). It would thus seem of interest to determine what metabolic pathways are functioning during this unique form of anaerobic metabolism and to compare these results with those obtained under aerobic conditions.

To this end, the radiorespirometric method of Wang et al. (54, p. 207-216) was used to determine the metabolic pathways operative under aerobic conditions and during nitrate respiration. In addition, growth experiments, CO_2 fixation, and cell-free enzyme studies were used in an attempt to compare the metabolic pathways used under both conditions of oxygen relationship.

Data presented herein should help to clarify the metabolic routes of glucose dissimilation used by P. stutzeri during aerobic growth and under conditions of nitrate respiration.

HISTORICAL REVIEW

The literature to be reviewed will be described in two phases; nitrate respiration and the metabolic pathways, found in other Pseudomonads, which may be associated with nitrate respiration in Pseudomonas stutzeri. Although no work on the reduction sequence of nitrate respiration will be reported in the thesis proper, it is nevertheless an integral--indeed obligatory--part of anaerobic metabolism.

Nitrate Reduction and Nitrate Respiration

The suggestion that the reduction of nitrate to nitrite is an enzymatic process, in living systems, was first proposed by Gayon and Dupetit in 1886 (18, p. 201-307). Since that time this reaction, and the subsequent reactions of nitrate dissimilation, have been the objects of extensive investigation. Quastel et al. (43, p. 304-317) first demonstrated that Escherichia coli could reduce nitrate to nitrite, under anaerobic conditions, if electron donors such as reduced methylene blue were added to the medium. This enzyme activity was studied in cell-free preparations, and designated as "nitratase", as early as 1934 by Green et al. (20, p. 1812-1824). The enzyme (nitrate reductase) was first purified and characterized by Evans and Nason (13, p. 233-254; 37, p. 655-673) using higher plants and

Neurospora. In spite of this early work, there is an obvious lack of literature pertaining to the significance of nitrate reduction linked to established metabolic pathways in bacterial systems. This is especially true of the role of nitrate as a terminal electron acceptor in microorganisms carrying out numerous oxidative reactions under strict anaerobic conditions. It will be the objective of this thesis to report on the biochemical pathways which are linked to such a system in Pseudomonas stutzeri.

Verhoeven (50, p. 61-86) has separated nitrate reduction into three classes; "assimilatory nitrate reduction", "true dissimilatory nitrate reduction", and "incidental dissimilatory nitrate reduction." Assimilatory nitrate reduction involves the reduction of nitrate to hydroxylamine or ammonia which is utilized solely for the synthesis of nitrogenous cell constituents. By contrast, dissimilatory nitrate reduction utilizes nitrate as an obligatory electron acceptor under certain conditions favoring denitrification, i. e. anaerobic conditions. Since the mechanism involves an electron transport system with nitrate playing the role of free-molecular oxygen, Taniguchi (48, p. 341-375) has proposed the term "nitrate respiration" for this process. Incidental nitrate reduction involves the use of nitrate as a non-essential electron acceptor in which nitrate accepts electrons at the substrate level and becomes reduced to nitrite, i. e. "nitrate fermentation." Since this thesis is concerned with the biochemical

pathways associated with nitrate respiration, further discussion of incidental nitrate reduction and assimilatory nitrate reduction would be redundant. Any further discussion of these categories of nitrate reduction will be only for purposes of clarification.

Nitrate respiration is generally considered a property of true denitrifying bacteria which reduce nitrate to nitrogen or nitrogen oxides. The process is the anaerobic equivalent of true aerobic respiration, whereby nitrate-oxygen is used as the terminal electron acceptor, rather than free molecular oxygen, in the stoichiometric conversion of a substrate to CO_2 and H_2O. This type of respiration is carried out by a limited number of microorganisms, examples of which are Pseudomonas aeruginosa, Pseudomonas denitrificans, Pseudomonas stutzeri and Micrococcus denitrificans. In addition, certain marine denitrifiers such as Pseudomonas perfectomarinus, Pseudomonas marinodenitrificans, and Pseudomonas mytili are thought to respire with nitrate (44, p. 799-807), but the enzymes of the reduction sequence for these organisms have not been characterized as thoroughly as those listed above.

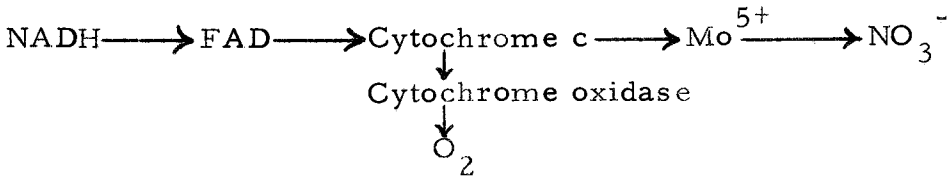
Nitrate respiration has been reported in E. coli by Taniguchi and Itataki (49, p. 263-279) under anaerobic conditions and by Heredia and Medina (24, p. 24-29) under both aerobic and anaerobic conditions. This is an example of nitrate respiration in a non-denitrifying organism whereby nitrate is reduced and phosphorus is

presumably esterified, but the reduction sequence does not proceed beyond nitrite. One would wonder whether this reaction is obligatory for anaerobic growth in E. coli or merely an example of incidental nitrate reduction. Another example of nitrate respiration in a non-denitrifier was recently reported in Aerobacter aerogenes by Hadjipetrou and Stouthamer (22, p. 29-34). In this case ammonia was the end-product and substrate hydrogen was derived via glycolysis rather than by the tricarboxylic acid cycle as shown by Forget and Pichinoty (15, p. 441-444). These reports are cited to indicate that the definition of nitrate respiration is not a standard one and is, indeed, often expanded to include nitrate fermentation under conditions where the TCA cycle is inoperative rather than classical terminal oxidation. There is, therefore, a pronounced need for standardization of the nomenclature applicable to this field of interest.

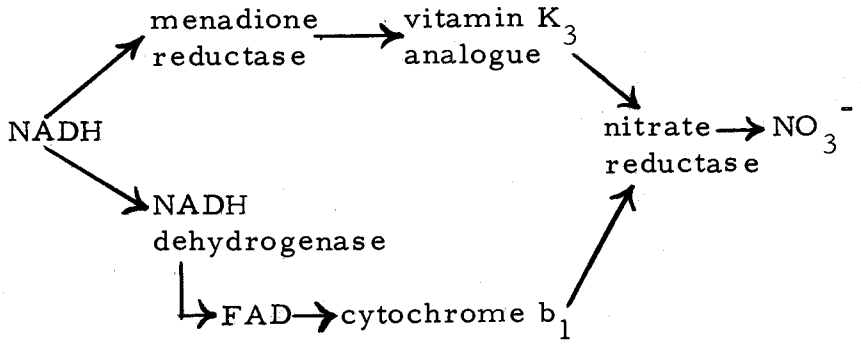
At present, there have been only four bacterial enzymes isolated which are involved in the complete denitrification scheme: nitrate reductase, nitrite reductase, hydroxylamine reductase, and nitric oxide reductase. There is a considerable amount of literature concerning purification and characterization of nitrate reductase, but very little published material concerning the other enzymes of the denitrification sequence.

Since the initial purification of the nitrate reductase from Neurospora by Nason and Evans (37, p. 655-673), numerous workers

have further purified, characterized, and determined the cofactor requirements of this enzyme. Nicholas et al. (38, p. 341-353) first established the requirement for molybdenum by this enzyme and showed a definite correlation between the specific activity of nitrate reductase and its molybdenum content (39, p. 353-360). Experiments with other commonly required micronutrient elements indicated that Mo was specifically required. In a very definitive paper, Fewson and Nicholas (14, p. 335-349) reported a 115-fold purification of nitrate reductase from P. aeruginosa. They found the enzyme to be NADH dependent and that it contains FAD, cytochrome c and molybdenum as functional components. Added mammalian or bacterial cytochrome c was reduced enzymatically by either NADH or reduced FAD and reoxidized by either Mo^{6+} or nitrate in the presence of the pure enzyme. A decrease in cytochrome c content and nitrate reductase activity was noted when the oxygen tension was increased from anaerobic conditions during growth of P. aeruginosa. This was considered indicative of the inducibility of this enzyme by anaerobiosis. The following scheme for electron transport from substrate to NO_3^- was proposed:



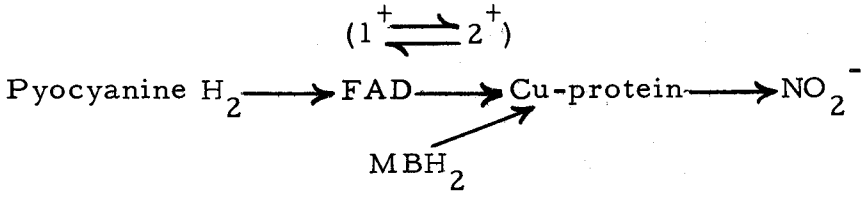
The nitrate reductase system purified from E. coli by Taniguchi and Itataki (49, p. 263-279) is particulate in nature and contains no flavin. Nitrate reductase was solubilized from the system and found to contain one atom of bound molybdenum and 40 atoms of bound iron per molecule. The particulate system seemed to involve transfer of electrons from reduced NAD or formate via cytochrome b_1. A similar nitrate reductase system was reported by Heredia and Medina in a different strain of E. coli (24, p. 24-29). This system reduced nitrates to nitrites under both aerobic and anaerobic conditions. NADH served as the electron donor and vitamin K_3 as the electron carrier under aerobic conditions. No flavin appeared to be involved when the system was functioning aerobically but was detected anaerobically. The dual scheme is depicted as follows:



An assimilatory-type nitrate reductase was recently purified from Aspergillus nidulans by Cove and Coddington (7, p. 312-318). A 300-fold purification was obtained containing cytochrome c

reductase activity associated with the nitrate reductase. The two activities could not be separated during purification after the initial stages and the ratio of the two activities remained constant. This indicated that the cytochrome c reductase is an integral part of the nitrate reductase system in A. nidulans. Evidence was also presented for a cytochrome c reductase, in the crude preparations, which was not associated with nitrate reductase activity.

Nitrite reductase from P. stutzeri was purified by Chung and Najjar (6, p. 617-624). The enzyme appeared to be NAD or NADP dependent and was stimulated by FMN or FAD. Of the metal ions tested, only Cu^{++}, Fe^{++} and Fe^{+++} produced definite stimulation of nitrite reductase activity. More recently, Walker and Nicholas (51, p. 350-360) have achieved a 600-fold purification of a nitrite reductase from extracts of P. aeruginosa. The enzyme reduced NO_2^- to NO when either reduced flavins, reduced, pyocyanine, reduced methylene blue, or reduced napthoquinones served as the electron donor. The enzyme exhibited a cytochrome c spectrum and contained bound iron. Copper was also shown to be present and reduction of activity occurred if Cu or Fe were deficient. It appeared that NADH or NADPH was not involved. The following scheme was depicted for the electron transfer sequence:

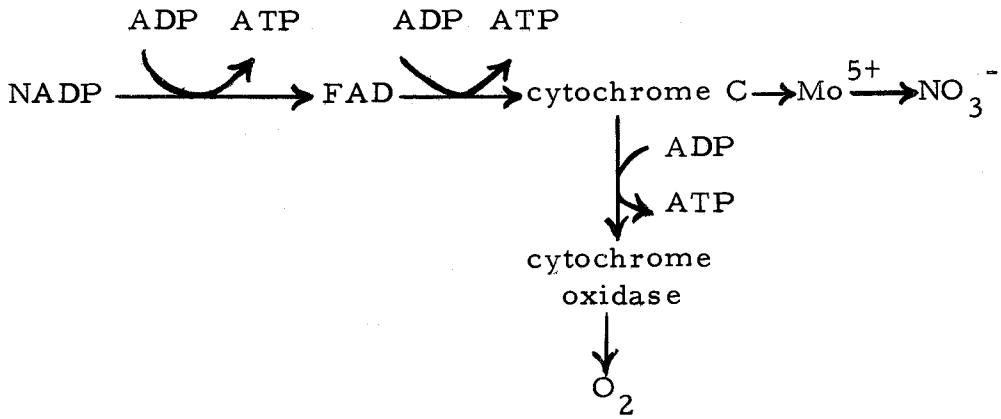


Nitric oxide reductase has been purified from P. stutzeri extracts by Chung and Najjar (6, p. 627-632). The enzyme converts nitric oxide to nitrogen under anaerobic conditions. NADH or NADPH as well as a flavin nucleotide were shown to be necessary for maximal activity. The enzyme appeared to contain cytochrome c as well as copper and iron.

In addition to nitrate reductase, nitrite reductase, and nitric oxide reductase which one would expect to find in typical denitrifiers, P. aeruginosa and P. denitrificans contain hydroxylamine reductase which reduces hydroxylamine to ammonia. Thus, these organisms can produce ammonia for assimilatory purposes with concomitant nitrate respiration for anaerobic metabolism. Hydroxylamine reductase has been demonstrated in a halotolerant Micrococcus (26, p. 419-430). However, it has not been demonstrated in M. denitrificans or P. stutzeri.

Since nitrate respiration involves the transfer of electrons to nitrate resulting in its reduction to nitrite, it is to be expected that oxidative phosphorylation would be coupled with this system and that ATP would be formed in the process. Working with cell-free preparations of nitrate reductase from E. coli, Ota et al. (42, p. 131-135) found a phosphorus to nitrate-oxygen ratio of 1.1:1 when citrate was used as an electron donor and 0.65:1 with glutamate. More recently, Ota (41, p. 137-144) has solubilized three protein fractions causing

coupling of phosphorylation with nitrate reductase. Inorganic phosphate was esterified with the coupling fraction during the oxidative phosphorylation and the incorporated phosphate was transferred to ADP. Since cell-free preparations are known to give characteristically low P/O ratios, Elliot (11, p. 50-58) chose to use growing cell systems to determine the P/O ratios for both aerobic and nitrate respiration in P. stutzeri. By equating oxygen uptake with cell numbers under aerobic conditions and nitrate-oxygen uptake with cell numbers under anaerobic conditions, true P/O ratios of 3:1 aerobically and 2:1 anaerobically were demonstrated. Using the postulated scheme of Fewson and Nicholas (14, p. 335-349), for aerobic and nitrate respiration, Elliot has proposed the theoretical sites of oxidative phosphorylation:



Most recently, Hadjipetrou and Stouthamer (22, p. 29-34) have demonstrated that oxidative phosphorylation in whole-cell preparations of A. aerogenes results in the production of three moles of

ATP/mole of NO_3^- reduced. Under anaerobic conditions with nitrate, this organism converts glucose to acetate via the glycolytic pathway with concomitant reduction of the nitrate to ammonia. The nitrate reductase apparently is involved in the oxidation of formate. These authors equated molar growth yields of A. aerogenes with total ATP produced. Subtracting the amount of ATP known to be produced at the substrate level in glycolysis, a net of 1. 34 moles of ATP were calculated to have been produced. The amount of nitrate used as a hydrogen acceptor was found to be 0. 44 μm/μm glucose, therefore, the reduction of one mole of NO_3^- gave (1/O. 44) 1. 34 = 3 moles of ATP/mole of NO_3^- reduced. It was further indicated that the reduction of NO_3^- to NH_4^+ resulted in no net formation of ATP.

It is not definitely known whether oxidative phosphorylation occurs in the reduction sequence beyond nitrate reduction. There is little doubt that these intermediates are further reduced and electrons transferred. However, the physiological significance of the reduction of nitrate beyond nitrite awaits further clarification.

Metabolic Pathways in Pseudomonas Species

The aerobic metabolism of several Pseudomonas species have been studied and several pathways of carbohydrate dissimilation are known to exist in this genus. With the exception of the work by Forget and Pichinoty (16, p. 364-377), there are no definitive

published data on the pathways which may be functioning during nitrate respiration under anaerobic conditions. All true heterotrophic denitrifiers known, except Micrococcus denitrificans, belong to the genus Pseudomonas; therefore, it was decided to review the known pathways which have been shown to operate under aerobic conditions in various species of this genus. Pathways which will be cited are the Entner-Doudoroff pathway, the pentose phosphate pathway, the tricarboxylic acid cycle and the "glyoxalate bypass".

The Entner-Doudoroff pathway was first detected in Pseudomonas saccharophila (12, p. 853-862). In this study with intact cells and cell-free extracts, these workers showed that glucose and gluconic acid can be dissimilated in such a way that two molecules of pyruvic acid are formed with the carboxyl of one derived from the C-1 position. The other carboxyl is derived from the C-4 position which is in direct contrast to the Embden-Meyerhof-Parnas pathway in which the two pyruvate carboxyls are derived from the C-3 and C-4 positions of glucose. The utilization of glucose by the ED pathway appeared to involve phosphorylation to glucose-6-phosphate with subsequent oxidation to 6-phosphogluconate and cleavage to pyruvate and glyceraldehyde-3-phosphate. The G-3-P could further be converted to pyruvate if the requisite enzymes were present. Data by Wood and Schwerdt (55, p. 501-511) indicated that this pathway was operative in P. fluorescens, which was substantiated by MacGee and

Doudoroff (33, p. 617-626). In this work a new phosphorylated intermediate was found which was produced by the action of phosphogluconic dehydrase. Chemical and enzymatic tests indicated that this intermediate was 2-keto-3-deoxy-6-phosphogluconic acid.

Some definitive radiosotope tracer studies by Lewis et al. (31, p. 273-386), with P. fluorescens, clarified the ED pathway and indicated it could operate concurrently with the pentose phosphate pathway. Using acetate recovered from specifically labelled glucose, these workers found that acetate produced from glucose-1-^{14}C had a low relative specific activity, whereas that obtained from glucose-6-^{14}C had a high relative specific activity. Glucose-2-^{14}C yielded 79 percent of the activity in the carboxyl group of acetate and 21 percent of the methyl group. If the cells were poisoned with arsenite, pyruvate would accumulate with 91 percent of the carboxyl carbon derived from glucose-6-^{14}C. By calculation, the ED pathway accounted for approximately one-third to one-half of the total glucose dissimilated. The remainder was dissimilated via the pentose phosphate pathway.

More recently, using the radiorespirometric technique of Wang et al. (54, p. 207-216), Stern et al. (47, p. 601-611) have studied the metabolism of various species of Pseudomonas. Of the species studied, P. saccharophila was found to metabolize glucose via the ED pathway, whereas P. reptilivora and P. aeruginosa were

found to use the pentose phosphate pathway concurrently with the ED. The primary criterion for the ED pathway was total respiratory $^{14}CO_2$ derived from the C-1 and C-4 positions of glucose being equal. Concurrent operation of the PP pathway is indicated by a C-1 yield from glucose being higher than that from the C-4 position. Data from the other specific labels used further indicated the operation of these pathways as well as the tricarboxylic acid cycle.

Presently, most species of Pseudomonas tested have indicated the operation of the ED and/or PP pathways as the primary routes of glucose catabolism. A notable exception is that found by Eagon and Wang (10, p. 879-886) in Pseudomonas natriegens. In this species the EMP pathway has been shown to be the primary pathway with a minor PP pathway. The ED pathway could only be demonstrated by inductive growth in gluconate medium.

The tricarboxylic acid cycle was studied in resting cells of P. aeruginosa by Campbell and Stokes (5, p. 853-858). Cells grown on acetate as the sole source of carbon, were found to have no ability to oxidize the following TCA cycle intermediates without an adaptation period: citrate, cis-aconitate, isocitrate, alpha-ketoglutarate, succinate, and fumarate. Acetate, pyruvate and malate were oxidized immediately without an adaptation period. When fresh resting cells were dried, it was found that they then had the ability to oxidize all TCA cycle intermediates immediately. The limiting factor appeared

to be cell permeability. The same results were obtained with glucose grown cells indicating that both glucose and acetate were oxidized via the TCA cycle (Note: the enzymes of the glyoxalate bypass had not been reported at this time and this reference is one of the early ones where the glyoxalate bypass was inadvertantly detected).

Meadow and Clarke (34, p. 18P) reported essentially the same results as Campbell and Stokes except cell-free extracts were used to circumvent the permeability problem rather than dried cells.

The TCA cycle was demonstrated by Barrett and Kallio (2, p. 517-525), in P. fluorescens, using cell-free extracts. It was concluded that P. fluorescens could carry out the following TCA cycle reactions: citrate $\longrightarrow$ alpha-ketoglutarate, alpha-ketoglutarate $\longrightarrow$ succinate, citrate $\longrightarrow$ succinate, acetate + oxaloacetate $\longrightarrow$ citrate, cis-aconitate $\longrightarrow$ citrate and D-isocitrate + NADP $\longrightarrow$ alpha-ketoglutarate + CO_2 + NADPH + H^+. The respective enzymes appeared to occur at the same level if either acetate or citrate was the sole carbon source. Simultaneous adaptation studies further indicated that the TCA cycle was functioning in P. fluorescens.

Kogut and Podoski (25, p. 800-811) carried out the same type of study with P. fluorescens KB1. The following enzyme activities were reported: "succinic dehydrogenase, fumarase, malic dehydrogenase, oxalacetic decarboxylase, aconitase, isocitric dehydrogenase, and alpha-ketoglutaric oxidase."

The radiorespirometric studies of Eagon and Wang (10, p. 879-886) were good examples of evidence for the TCA cycle by radiorespirometry. In the case of an ED pathway the following patterns would be expected for the labels converted to $^{14}CO_2$ in the TCA cycle: 2 = 5 > 3 = 6. By contrast, these patterns are 2 = 5 > 1 = 6 for the EMP scheme. Glucose-5-^{14}C is unavailable but the position of the pattern from glucose-2-^{14}C as well as glucose-3-and -6-^{14}C are indicative that TCA cycle is operative in P. natriegens when glucose and gluconate are the carbon sources. In addition, the labels converted to $^{14}CO_2$ via the TCA cycle are indicative that the EMP and ED pathways, respectively, are operative as discussed earlier. In addition, radiorespirometry using specifically labelled pyruvate and acetate has been used by Stern et al. to indicate the presence of an active TCA cycle in whole cells of P. aeruginosa, P. saccharophila and P. reptilivora.

More recently, radiorespirometry using specifically labelled glutamic acid has been used by Wang et al. (53, p. 8) to indicate the presence of the TCA cycle in Saccharomyces cerevisiae. The rationale used was that glutamic would be converted to alpha-ketoglutarate and degraded by successive turns of the TCA cycle to give the following overall patterns for conversion of specific labels of glutamic acid to $^{14}CO_2$ via the TCA cycle: 1 > 2 = 5 > 3 = 4.

Most recently, the operation of the tricarboxylic acid cycle

has been indicated in Micrococcus denitrificans, during nitrate respiration, by Forget and Pichinoty (16, p. 364-377). Using the sequential induction technique, it was shown that cells adapted to one TCA cycle intermediate were then able to oxidize other intermediates. Fluoroacetate inhibited the utilization of acetate and pyruvate and the reduction of NO_3^-, NO_2^-, and N_2O. The inhibition was accompanied by the accumulation of citrate. Finally, the authors were able to demonstrate the presence of all the TCA cycle enzymes in cell-free extracts of M. denitrificans.

In addition to the TCA cycle, most, if not all, species of Pseudomonas have been shown to have the enzymes of the "glyoxalate bypass" or glyoxalate cycle. This cyclic modification of the TCA cycle utilizes, in addition, the enzymes isocitratase (isocitric lyase) and malate synthetase to form C_4-dicarboxylic acids from two molecules of acetate via acetyl coenzyme A. The mechanism was first indicated in the work of Campbell et al. (4, p. 594). In this work, denitrophenylhydrazones of alpha-ketoglutaric acid could not be detected in cell-free preparations of P. aeruginosa using citrate as the substrate. Instead, two unidentifiable spots were obtained which were shown to have the same Rf values as glyoxalate. It was found that cis-aconitate would give succinate and glyoxalate. When the cell-free preparations were incubated with succinate and glyoxalate, citrate was formed. These results were reported as a "deviation

from the conventional TCA cycle." This enzyme was later called isocitratase and was soon detected by Smith and Gunsalas (46, p. 774-775) in P. aeruginosa, P. fluorescens and P. putrifaciens. Kornberg (28, p. 1430-1431) has assayed for isocitratase in P. ovalis and found that of the substrates used in various growth media, only acetate stimulated high levels of isocitratase activity.

Using P. fluorescens KB1, Kornberg and Madsen (29, p. 651-653) showed that washed cell suspensions, grown in acetate as the sole carbon source, readily oxidized acetate and all TCA cycle intermediates. Short-term incubations (3 sec - 15 min) of rapidly growing cultures indicated that ^{14}C-labelled acetate entered the cycle at two points. Later experiments (30, p. 549-557), using E. coli mutants, have shown that acetate enters as acetyl coenzyme A at one site to form citrate from oxalacetate and malate from glyoxalate at another. They found the adol fission of isocitrate, giving glyoxalate and succinate (the isocitratase reaction), to be necessary for malate formation.

Data obtained by Kornberg et al. (27, p. 9-24) indicated that the glyoxalate cycle functions in M. denitrificans when the cells were heterotrophically grown using acetate as the sole carbon source. Autotrophically grown cells used the Calvin cycle for the synthesis of C_4-dicarboxylic acids by the assimilation of CO_2. Later work by French et al. (17, p. 55p) has confirmed this work.

MATERIALS AND METHODS

Culture, Culture Media and Cultural Conditions

The organism used in this study was a strain of Pseudomonas stutzeri originally isolated from soil by Roa (45, p. 58-60). Several cultures of this organism were maintained on a medium consisting of (in grams): KNO_3, 1.0; trypticase, 10.0; yeast extract (Difco) 1.0; agar-agar (Difco), 15.0; and 1000 ml of distilled water. The medium was autoclaved at 121° C for 15 minutes.

Aerobic cells for radiorespirometry were grown in a medium containing (in grams): yeast extract (Difco), 1.0; $(NH_4)_2PO_4$, 0.5; KH_2PO_4, 1.14; K_2HPO_4, 1.45; $MgSO_4 \cdot 7H_2O$, 0.1; MoO_3, 0.001; $FeSO_4$, 0.001; glucose, 5.0; and 1000 ml of distilled water. Cells for nitrate respiration studies were grown in the above medium containing, in addition, 7.3 g of KNO_3 per liter (1000 ppm NO_3^--N). The pH of the media was adjusted to 6.9 and the media were autoclaved at 121° C for 15 minutes. The glucose was sterilized separately by membrane filtration and added aseptically to each medium after autoclaving. The median pore size of the membrane filters was 0.22 mμ.

A synthetic medium was used for CO_2 fixation studies and as a basal medium for testing for the utilization of each tricarboxylic

acid cycle intermediate as the sole carbon source. The basal medium contained (in grams): $(NH_4)_2PO_4$, 0.5; KH_2PO_4, 1.14; K_2HPO_4, 1.45: $MgSO_4 \cdot 7H_2O$, 0.1; $MnSO_4$, 0.01; $CaCl_2$, 0.001; $FeSO_4$, 0.001; MoO_3, 0.001; and 1.0 ml of a vitamin-purine-pyrimidine mixture per 1000 ml distilled water. The vitamin-purine-pyrimidine mixture contained the following (in mg/10.0ml): niacin, 2.0; riboflavin, 1.0; thiamine, 1.0; pyridoxine, 1.0; biotin, 1.0; folic acid, 1.0; adenine, 10.0; guanine, 1.0; thymine, 1.0; and cytosine, 1.0. The vitamin-purine-pyrimidine mixture was sterilized by filtration through a 0.22 μ membrane filter and stored forzen in the dark. Prior to inoculation of the medium, this mixture was added ascepically to the salt medium previously autoclaved at 121°C for 15 minutes. The KNO_3 used as the terminal electron acceptor in nitrate respiration experiments was added, prior to sterilization, at a level of 7.3 g/l.

Aerobic cells were grown in air with agitation at 30°C. Cultures were carried through three successive transfers in liquid medium before inoculation into larger growth flasks to insure an actively growning culture. Cultures to be used for each experiment were harvested in the mid-logarithmic growth phase after 21 hours of incubation.

Cells to be used in nitrate respiration experiments were incubated anaerobically under a 100% helium atmosphere, with agitation

at 30° C. Anaerobic conditions were maintained throughout the growth period by means of a mercury trap attached to the culture flask. This arrangement maintained a slight positive pressure in the flask, allowing for the escape of excess metabolic CO_2 through the mercury trap while simultaneously excluding the entry of atmospheric oxygen. Anaerobic cells were grown for 43 hours before harvesting. In all cases cells were harvested by centrifugation.

Radiorespirometric Methods

The radiorespirometric method of Wang et al. (54, p. 207-216) was used to determine the primary metabolic pathways which may be functioning in P. stutzeri under aerobic conditions and under anaerobic conditions, using NO_3^- -O as the terminal hydrogen acceptor. This method was also used to study the dissimilation of acetate, pyruvate, and glutamate in the study of secondary pathways.

Cells used in these experiments were grown in semisynthetic medium, as previously described, and harvested by centrifugation. The aerobically and/or anaerobically grown cells were washed twice in their respective growth media, minus substrate, and adjusted to a concentration of 20 mg dry weight per 30 ml of medium without substrate.

In general, all radiorespirometric experiments involved the same methodology and varied only in the type and amount of specific

labels used. A series of incubation flasks was placed in a Warburg apparatus at 30° C. Each flask contained 20 mg of cells per 30 ml of medium without substrate. To the sidearm specifically labelled substrate was added (usually 0. 25 μc) followed by carrier substrate. The amount of carrier substrate required was determined by prior experiments using various concentrations of carrier, to determine the level which would be completely exhausted in six to eight hours, and still yield a discrete peak of activity at some period after the first hour.

In the case of the aerobic experiments, the flasks were sparged with air at a flow rate of 50 cc/minute with shaking; for nitrate respiration experiments, the flasks were aparged with 100% helium at a flow rate of 30 cc/minute with shaking. After a 15 minute equilibration period, the substrates were tipped in and metabolic $^{14}CO_2$ from each flask was trapped in 10 ml of absolute ethanol-ethanolamine (2:1). Samples were taken at hourly intervals and diluted to 15 ml with absolute ethanol. A 5 ml portion of each sample was placed in 10 ml of counting solution in a 20 ml glass counting vial. The counting solution consisted of 2, 5-diphenyloxazole (PPO) at a level of 6 g/l and 1, 4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) at a level of 100 mg/l.

At the termination of each experiment the cells and media were separated by centrifugation, and portions of each were counted in

thixotropic gel consisting of: "thixcin," (Thixcin is a product of the Baker Castor Oil Co., Los Angeles, California), 20 g; tween-80-span 80 (1:9), 4 ml; glycerol, 5 ml; and toluene-PPO-POPOP counting solution, 500 ml. Gel samples were counted under the same counting conditions as the liquid $^{14}CO_2$ samples. The efficiency of counting for each sample was determined by use of appropriate internal standards.

The aerobic substrate levels used for the radiorespirometric studies were as follows (in mg/flask): glucose, 10; gluconate, 10; DL-glutamic acid, 5; acetate, 10; and pyruvate, 10. Corresponding anaerobic substrates were (in mg/flask): glucose, 5; gluconate, 5; DL-glutamic acid, 2; acetate, 5; and pyruvate, 5. All specifically labelled compounds were added at a level of 0.25 μc/flask except glucose-3-^{14}C and gluconate-3, 4-^{14}C which were added at levels of 0.01 and 0.007 μc/flask, respectively.

The specifically labelled compounds used in the various radiorespirometric experiments are: glucose-1-, -2-, -3-, -3, 4-, and -6-^{14}C, Gluconate -1-, -2-, -3, 4- and -6-^{14}C; DL-glutamic acid -1-, -2-, -3, 4- and -5-^{14}C; Na-pyruvate -1-, -2-, and -3-^{14}C; and Na-acetate -1- and -2-^{14}C. All labels except the following were purchased from Nuclear Chicago Corp., DesPlaines, Illinois: glucose-3-C^{14}, gluconate -2-, -3, 4- and -6-^{14}C; DL-glutamic acid -1-, -2-, -3, 4- and -5-^{14}C. The latter were obtained through the

generous cooperation of C. H. Wang of this university. Gluconate -2-, -3, 4- and -6-^{14}C had been previously prepared by the method of Moore and Link (36, p. 293-299).

Utilization of Tricarboxylic Acid Cycle Intermediates as Carbon Sources

Utilization of TCA cycle intermediates as sole carbon sources for growth is one of the criteria that may be used to detect the presence of an active TCA cycle in whole cells. However, positive or negative results by no means prove or disprove the presence of a TCA cycle. This type of experiment is, nevertheless, a good probing experiment and can be used as a support for further data.

These experiments were run using the synthetic medium discussed earlier. Each of the following TCA cycle intermediates were added to the synthetic basal medium at a level of 5 g/l (0.05%): Acetate, fumarate, alpha-ketoglutarate, citrate, succinate, pyruvate, and malate. In addition, flasks containing glucose and a control flask containing no substrate were prepared. Nitrate (1000 ppm NO_3^--N) was added to the media to be used for anaerobic experiments. In addition, glucose medium, containing no NO_3^-, was prepared as a check to determine whether or not P. stutzeri could grow anaerobically without NO_3^-.

When visible growth was attained, 0.1 ml was transferred to

100 ml of fresh medium and each flask was reincubated. After three such transfers, each experiment was terminated. Three successive transfers were thought necessary to infinitely dilute other substrates or growth factors which may have been introduced in the initial inoculum.

Carbon Dioxide Fixation

Carbon dioxide fixation experiments were carried out using $^{14}CO_2$ to determine the extent of fixation, if any, when glucose, acetate, and pyruvate were used as the sole carbon sources. Should sufficient fixation occur, the cell proteins could then be hydrolyzed to determine which amino acids had become labelled and to what extent.

The $^{14}CO_2$ fixation reactions were carried out in Erlenmeyer flasks each having a large center well and sidearm adaptable to a rubber serum cap through which the substrate could be added as well as acid for generating $^{14}CO_2$ in the center well. The flasks were fitted with ground glass necks which would accommodate a respirometer head suitable for flushing anaerobic samples, and to safely dispose of residual $^{14}CO_2$ at the termination of the experiment.

For aerobic $^{14}CO_2$ fixation experiments, 20 mg dry wt of cells per 40 ml of medium were added to the flasks. Twenty-five microcuries of $NaH^{14}CO_2$ was added to the center well as a 1 ml solution.

Sufficient $NaHCO_3$ carrier solution (11.9 mg) was added so that a 1% atmosphere of CO_2 could be generated upon the addition of acid. Forty milligrams of substrate were then added to each flask and the heads were immediately placed on the flask and secured. A hypodermic syringe was used to deliver 1 ml of 6N H_2SO_4 through the serum cap, into the center well, for generation of $^{14}CO_2$. The flasks were incubated with constant shaking, in a water bath at 30° C.

Anaerobic flasks were prepared in the same manner except no substrate was added until the flasks had been flushed with 100% helium for 30 minutes. Substrates were then added, $^{14}CO_2$ was generated and the flasks were incubated as discussed above.

After the flasks had incubated for six hours, the reactions were terminated by the addition of sufficient 6N H_2SO_4 (0.5 ml) to lower the pH to 2. Radioactive CO_2 was prevented from reaching the atmosphere by sparging the flasks for 20 minutes. The residual CO_2 was trapped in a 5 N NaOH solution. The medium and cells were separated by centrifugation and washed twice with cold distilled water. After resuspension to a 5 ml total volume, aliquots were counted in thixotrophic gel and the remaining cell suspension was frozen for later hydrolysis and chromatography. The efficiency of scintillation counting was determined for each sample by spiking with an internal standard.

Initial and final cell nitrogen analyses were run, using the

micro-Kjeldahl method (1, p. 987-991) as an index of cell growth during CO_2 fixation.

Chromatography of Amino Acids

The cells from the aerobic and anaerobic CO_2 fixation experiments were sealed under vacuum in pyrex vials and hydrolysed in 6N HCl at 110° C for 20 hours in a toluene bath. After hydrolysis, the vials were opened and the humic acid fractions were removed by vacuum filtration through sintered glass filters. The humic acid-free solutions were then evaporated in vacuo at 35° C. Residual HCl was removed by three successive washings with 10 ml volumes of distilled water followed by vacuum evaporation. Finally, the amino acid hydrochlorides were dissolved in 1 ml of 10% isopropanol in water and chromatographed.

Chromatography was carried out by the two dimensional method of Hausmann (23, p. 3181-3182). Twenty lambda of aerobic hydrolysates and 40 lambda anaerobic hydrolysates were spotted in duplicate on 18 x 22 inch Whatman No. 1 chromatography paper which had been serrated along the bottom edge with pinking shears. The papers were placed in a chromatography cabinet and equilibrated with the first solvent (sec-butanol-3% ammonia 5:2) for three hours. After three hours of equilibration the solvent was added to the troughs and allowed to descend for 40 hours. The papers were removed after

40 hours, dried, and placed in a second cabinet. After three hours of equilibration with sec-butanol-88% formic acid- water (15:3:2), this same solvent was added to the trays and allowed to descend for 12 hours. After drying for 12 hours one of the duplicate sheets, for each hydrolysate, was flooded with 0. 25% ninhydrin in acetone and placed in the dark overnight. Following confirmation of ninhydrin spots, the corresponding duplicate sheets were sprayed with a 0. 1% solution of 1, 2-sodiumnapthoquinone- 4 -sulfonic acid and heated in the oven at 60° C for 20 minutes. The sheets were observed under ultraviolet light immediately following the heating period. The amino acid spots appeared as whitish violet (40, p. 415-424) spots against the dark violet of the paper. The spots were marked and saved for counting of incorporated $^{14}CO_2$.

The spots were cut out as a uniform area (2. 5 x 4 cm), folded down the center of the long axis, and placed in a glass counting vial containing 20 ml of fluor solution. The fluor solution consisted of 4 g PPO and 50 mg POPOP per liter of toluene. The amino acid samples were counted under flat spectrum conditions. Relative counting efficiences were determined by counting 20 lambda samples and 20 lambda spiked samples of the amino acid hydrolysates on filter paper strips.

Determination of Glyoxalate Cycle Enzymes

The assay for malate synthetase was carried out according to the method of Dixon and Kornberg (9, p. 633-637). Cells were grown at 30° C under aerobic and anaerobic conditions using acetate (5 g/l) as the carbon source. The cells from 10 l of medium were harvested by centrifugation after 22 and 43 hours incubation for aerobic and anaerobic cultures, respectively. A slurry was made of the cells, after washing, by resuspension in 0. 02 M phosphate buffer at pH 7. 4, containing 10^{-4} M Na_2EDTA and 0. 01 M mercaptoethanol. The cells were disrupted in a cell press at 10, 000 psi and centrifuged at 35, 000 x g for 20 minutes to separate the cell debris. The extracts were treated with activated charcoal (Darco 6-60, 5 mg/ml extract) to adsorb NAD and/or NADP. The charcoal was removed by centrifugation at 35, 000 x g for ten minutes. One-half of each extract was frozen for use later in the isocitric lyase assay.

The buffer used in the assay of malate synthetase contained: 0. 01 M tris-HCl pH 8. 0, 40 ml; 0. 1 M $MgCl_2$, 1. 5 ml; and 0. 002 M acetyl CoA, 1 ml. The reaction mixture contained 2. 9 ml of this buffer, 0. 02-0. 10 ml of extract, and 0. 07 ml of 0. 02 M Na glyoxalate. The reaction was run against a 30 ml buffer blank. The reactions were carried out in silica curvettes and followed at 232 mμ

by means of a Cary model 12, recording spectrophotometer. The reaction mixture was run against the buffer blank and equilibrated for at least two minutes to determine presence or absence of acetyl CoA deacylase. Since a stable baseline indicated no deacylase activity, the sodium glyoxalate substrate was added. The resulting decrease in optical density was measured indicating that the breakage of the acetyl CoA acyl bond was substrate dependent.

Following the assay of the aerobic and anaerobic cell-free extracts, protein was determined by the method of Lowry et al. (32, p. 265-275).

The assay for isocitric lyase was carried out according to the method of Daron and Gunsalus (8, p. 622-633). The portions of cell-free extracts stored in the freezer during the malate synthetase assay were used in the following assay.

Isocitric lyase activity was measured with isocitrate as the substrate by determining glyoxalate accumulation as the 2, 4-dinitrophenylhydrazine derivitive. The following volumes of reagent were pipetted into a 10 ml conical centrifuge tube containing: 0. 1 ml of 0.1 M Tris buffer, pH 7.9; 1.4 ml, water; 0.3 ml 0.001 M $MgCl_2$; 0.1 ml 0. 2 M cysteine; and 0. 02 or 0. 04 ml of crude enzyme for aerobic and anaerobic preparations, respectively. The amount of enzyme preparation necessary to give 0. 2-2. 0 μM of glyoxalate in ten minutes was determined by prior experimentation. The tubes containing the

reaction mixtures were equilibrated for five minutes in a water bath at 30° C. After equilibration 0. 3 ml of 0. 025 M L(+) - isocitrate was added per tube, the headspace was filled with helium, and covered to exclude the entry of air. After ten minutes the reaction was terminated by the addition of 0. 1 ml of 80% trichloroacetic acid and the precipitate was removed by centrifuging in a clinical centrifuge.

For assay a 0. 5 ml aliquot from each tube was transferred to a second tube, diluted to 1 ml with water, and 1 ml of 0. 1% 2, 4-dinitrophenylhydrazine in 2N HCl was added. After five minutes at room temperature to form the 2, 4-dinitrophenylhydrazone, 2 ml of 95% ethanol, 1 ml water, and 5 ml of 1. 5 N NaOH were added to each tube. Immediately after the addition of the NaOH the optical densities were read in a Bausch and Lombe "spectronic 20" at 540 mμ. The number of micromoles converted to glyoxalate was determined using a standard curve prepared using the same procedure as above, without enzyme, with various known concentrations of glyoxylic acid.

RESULTS AND DISCUSSION

Primary Metabolic Pathways of Glucose Dissimilation in Pseudomonas stutzeri

Nitrate respiration is a rather unique phenomenon in that a hydrogen acceptor other than free molecular oxygen (aerobic respiration) or a substrate more oxidized than the donor substrate (fermentation) is not involved. Because of the unusual nature of nitrate respiration, it was desired to determine what pathways are functioning in the primary breakdown of glucose under aerobic conditions and to compare these pathways with those which function under anaerobic conditions with NO_3^--O as the terminal hydrogen acceptor. Preliminary work by Bhatt (3, p. 77) indicated that there may be a shift in pathways when different hydrogen donors are utilized by P. stutzeri. To determine more accurately what primary metabolic pathways might be operative, with glucose as the carbon source, the radiorespirometric method of Wang et al. (54, p. 207-216) was selected as the method of choice.

Radiorespirometry of Specifically Labelled Glucose and Gluconate

Figures 1 and 2 show the aerobic radiorespirometric patterns when glucose and gluconate are utilized as the respective carbon sources. Total $^{14}CO_2$ recoveries as well as cell incorporation, the

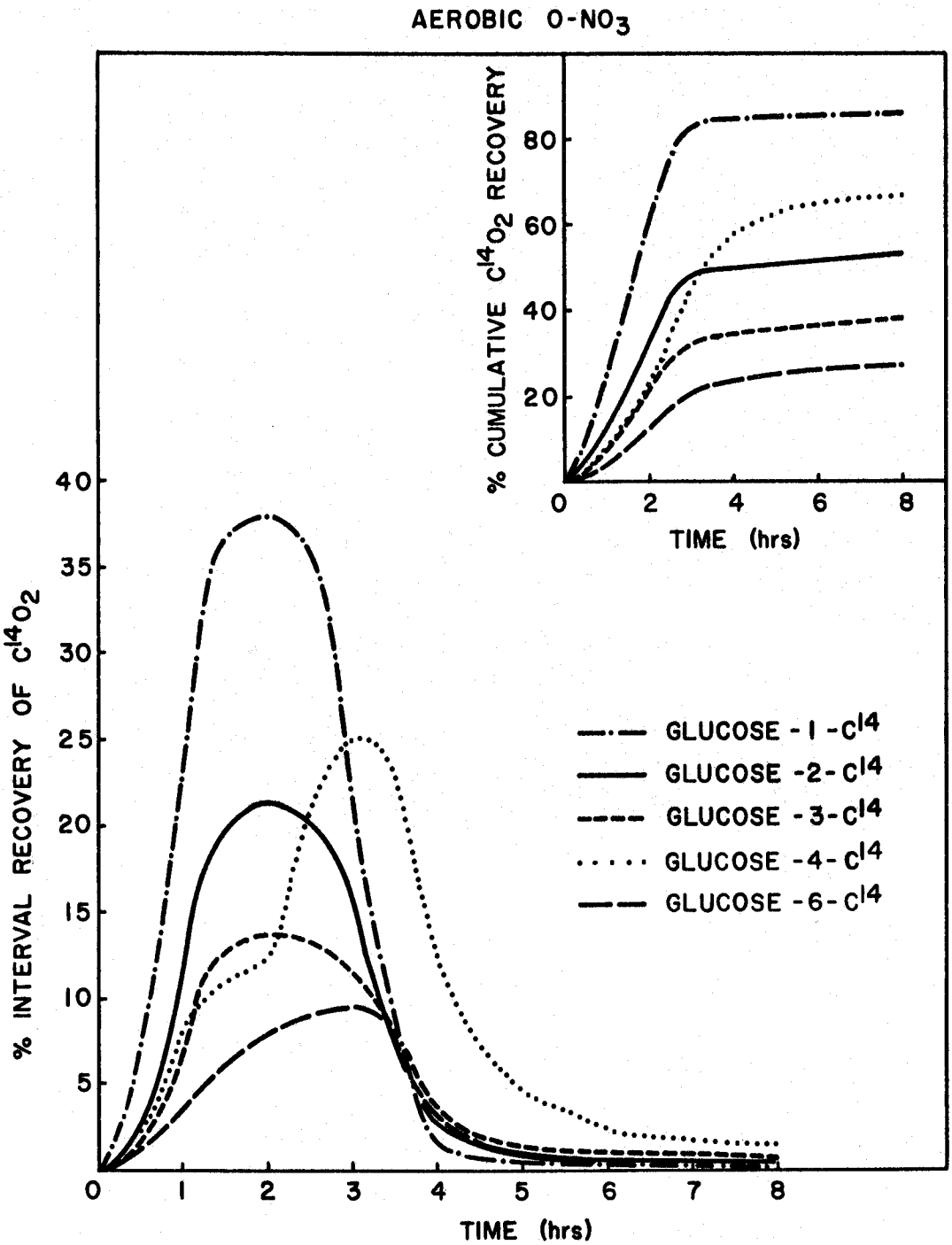


Figure 1. Aerobic radiorespirometric patterns for the utilization of glucose.

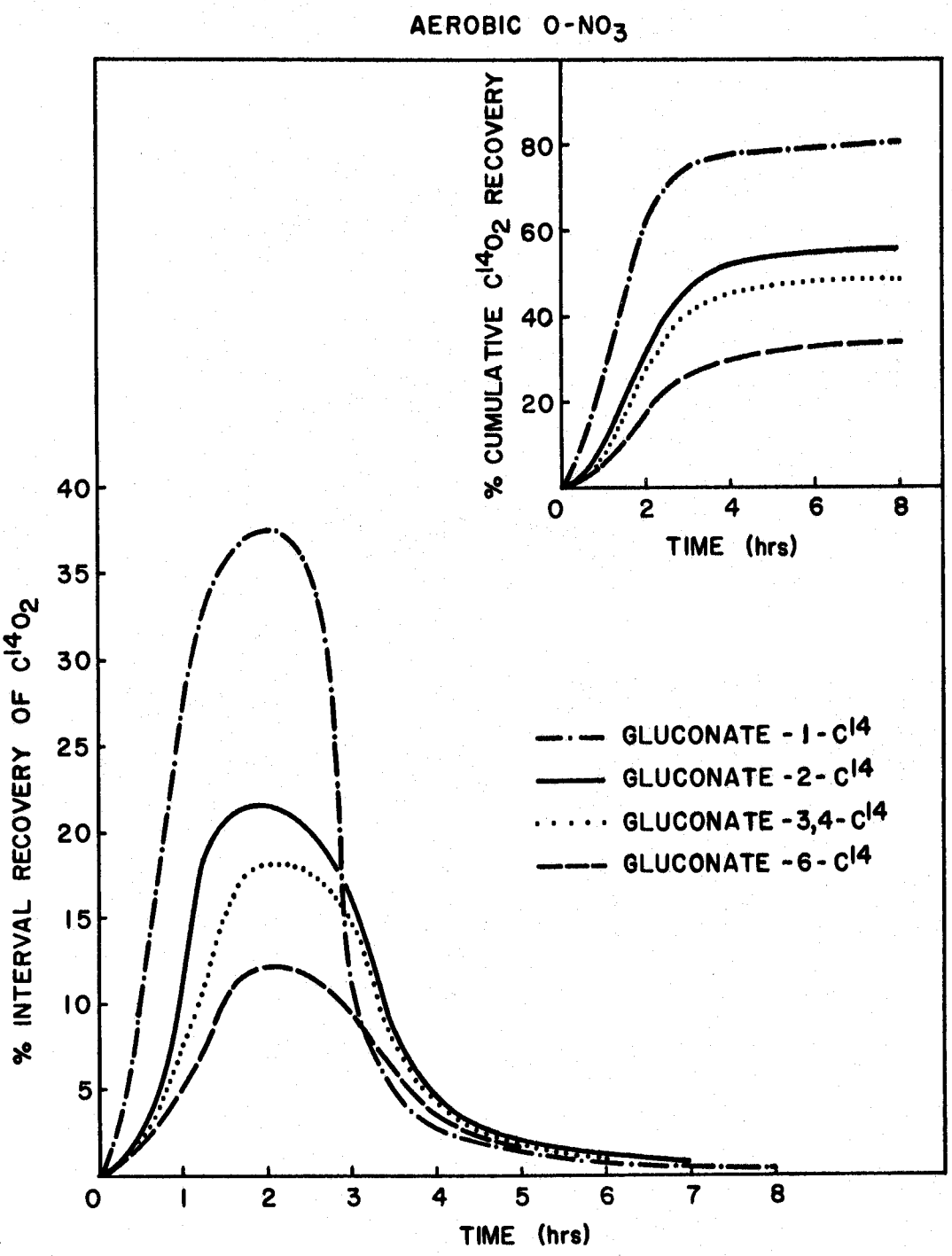


Figure 2. Aerobic radiorespirometric patterns for the utilization of gluconate.

percent activity remaining in the medium, and the percent recovery of total added label are given in Table 1 for glucose and gluconate under aerobic conditions.

Table 1. Aerobic incorporation of ^{14}C label into cells, medium, and CO_2 by P. stutzeri cells

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
Aerobic	%	%	%	%
Glucose-1-^{14}C	86	13	6	105
Glucose-2-^{14}C	53	42	6	101
Glucose-3-^{14}C	38	57	5	100
Glucose-3, 4-^{14}C	53	42	5	100
Glucose-4-^{14}C*	68	27	5	100
Glucose-6-^{14}C	27	59	9	95
Gluconate-1-^{14}C	81	6	9	96
Gluconate-2-^{14}C	56	37	6	99
Gluconate-3, 4-^{14}C	49	43	4	96
Gluconate-6-^{14}C	34	57	5	96

*Calculated from glucose-3-^{14}C and glucose-3, 4-^{14}C recoveries.

The radiorespirometric patterns obtained for glucose and gluconate are typical of cells using a major ED pathway and a minor pentose phosphate pathway (47, p. 601-611). This is shown primarily by the recovery patterns of $^{14}CO_2$ from C-1, C-2, and C-4 of glucose. An organism using an ED pathway in conjunction with the TCA cycle for the catabolism of glucose should give a

radiorespirometric pattern for glucose as follows: 1 = 4 > 2 = 5 > 3 = 6. This is fully in agreement with the ED scheme as it was originally described (12, p. 853-862). When the ED scheme is operative, glucose is converted to 2-keto-3-deoxy-6-phosphogluconate. The keto-deoxy compound is then split into pyruvic acid and glyceraldehyde-3-phosphate which, in turn, can be converted to pyruvate if the requisite enzymes are present. The carboxyl groups of the pyruvate, so derived, originate from the C-1 and C-4 positions of glucose. These positions are then decarboxylated at the highest rate when pyruvate is decarboxylated in the formation of acetyl CoA which is dissimilated via the TCA cycle. This idealized description clearly does not fit the cases depicted in Figures 1 and 2, however, the yields from C-1 and C-4 (86 and 68 %) are clearly higher than those from C-2, C-3, and C-6 (53, 38, and 27%, respectively). The curves for C-3 and C-6 of glucose do fall close enough to indicate an ED pattern. Recoveries from C-1 and C-4 of glucose do not approach coincidence of the two curves. However, the $^{14}CO_2$ yields from C-1 and C-4 of glucose are clearly the highest of all labels used. If a pentose phosphate pathway were also functioning in this organism concurrent with the ED pathway one would expect a pronounced yield from C-1 as compared to C-4 due to decarboxylation of the C-1 position of gluconate to form a C_5 compound. This C_5 compound would be further degraded or rearranged by the pentose

phosphate pathway or incorporated into the cell directly as a pentose. Since part of the gluconate derived from glucose would be utilized via the pentose phosphate pathway with a consequent loss of this fraction of C-1 as $^{14}CO_2$, one would expect the $^{14}CO_2$ yield from the C-1 position to be higher than that of the C-4 position, i. e., 86 and 68%, respectively.

Due to the diverse nature of the pentose phosphate route, it becomes difficult to trace the fate of substrate carbon via the radiorespirometric method, especially if the overall scheme is confused by the concurrent operation of another pathway as is apparent in this case. That both of these pathways are operative, however, may be further clarified by comparing the radiorespirometric patterns for glucose and gluconate. The patterns for each specific label of glucose coincides very well with the corresponding gluconate pattern. Since glucose must be converted to gluconate before either the ED or PP pathways will function and the equilibrium between glucose and gluconate is essentially irreversible, one would expect these patterns rather than patterns typical of the Embden-Meyerhoff pathway. The relatively low yields from the 3, 4 positions of glucose and gluconate (53 and 49%), as well as the apparent ease of conversion of glucose to gluconate and subsequent extensive breakdown of gluconate, rule out any major role for the EMP scheme in this organism.

As a test for fermentation under anaerobic conditions rather

than true nitrate respiration, a series of flasks containing specifically labelled glucose was run under anaerobic conditions without nitrate. $^{14}CO_2$ samples were taken at hourly intervals for 12 hours and the cells and medium were counted at the end of each experiment. The results of these experiments are shown in Table 2. The high yields of $^{14}CO_2$ from glucose -1- ^{14}C (31%) as compared to the very low yields from glucose -2-, -3, 4-, and -6- ^{14}C (4, 2, and 1%, respectively) are quite indicative that glycolysis does not function in P. stutzeri under anaerobic conditions. This is especially evident in the low yield from glucose-3, 4- ^{14}C. If glycolysis were functioning, one would expect a very high $^{14}CO_2$ yield from this label; not 2% as obtained in these experiments. The high yields from C-1 (31%) would indicate that the initial decarboxylation of gluconate, in the PP pathway, is functioning but terminal respiration has ceased as evidenced by the low recoveries from all other labelled positions of glucose.

Table 2. Incorporation of ^{14}C label into cells, medium, and CO^2 by P. stutzeri under anaerobic conditions without nitrate

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
Anaerobic	%	%	%	%
Glucose-1- ^{14}C	31	4	52	87
Glucose-2- ^{14}C	4	3	89	96
Glucose-3, 4- ^{14}C	2	2	76	80
Glucose-6- ^{14}C	1	3	99	103

Enzymatic tests for glucose at the end of each experiment indicated that all activity present in the medium was in the form of glucose. This rules out the possibility of accumulation of fermentation products arising from glycolysis and further indicates that nitrate respiration is necessary for glucose utilization under anaerobic conditions.

The nitrate respiration experiments were carried out using anaerobic conditions and NO_3^- as the terminal hydrogen acceptor rather than free molecular oxygen. The respective patterns for glucose and gluconate are shown in Figures 3 and 4. It is apparent that the radiorespirometric patterns for nitrate respiration are quite similar to those obtained under aerobic conditions for both glucose and gluconate. If glucose and/or gluconate were dissimilated differently under conditions of nitrate respiration, such as a marked change in the ED and pentose phosphate or a shift to the glycolytic pathway (EMP), one would expect a significant change in the overall radiorespirometric patterns. No such major differences are evident which is indicative that the same primary pathways are operative under aerobic conditions and conditions of nitrate respiration.

For the purpose of clarification, the metabolic pathways discussed in this thesis, are described as primary or secondary pathways. The primary pathways functioning in P. stutzeri would be the ED and pentose phosphate pathways and the secondary pathways would

be the TCA and/or glyoxalate cycles. Using this rationale, the C-1 and C-4 positions of glucose would be the principal labels involved in the primary pathways. $^{14}CO_2$ derived from the C-1 and C-4 positions of glucose would result from the decarboxylation of the C-1 position of pyruvate in the ED scheme. Additional $^{14}CO_2$ from C-1 would arise from the initial decarboxylation of gluconate derived from glucose in the pentose phosphate pathway. Comparing the $^{14}CO_2$ yields from the C-1 and C-4 positions of glucose under aerobic conditions (86 and 68%, respectively) and conditions of nitrate respiration (85 and 70%), it would seem that the primary pathways function at the same rate under both conditions of oxygen relationship. It must be clarified that this is a relative rate since only one-half as much substrate was added for nitrate respiration experiments as for aerobic experiments. However, the growth rate of P. stutzeri under anaerobic conditions is one-half that of the aerobic cells, therefore, the rate of the pathways relative to growth is the same.

Secondary pathway activity would then be characterized by $^{14}CO_2$ derived from the C-2, C-3, and C-6 positions of glucose.

Recent work by Elliot (11, p. 54) on oxidative phosphorylation in P. stutzeri, indicates that under aerobic conditions 3 ATP's may be formed for each oxygen atom consumed and 2 ATP's per NO_3^- oxygen consumed under conditions of nitrate respiration. Considering this evidence to be applicable, one would expect that a greater

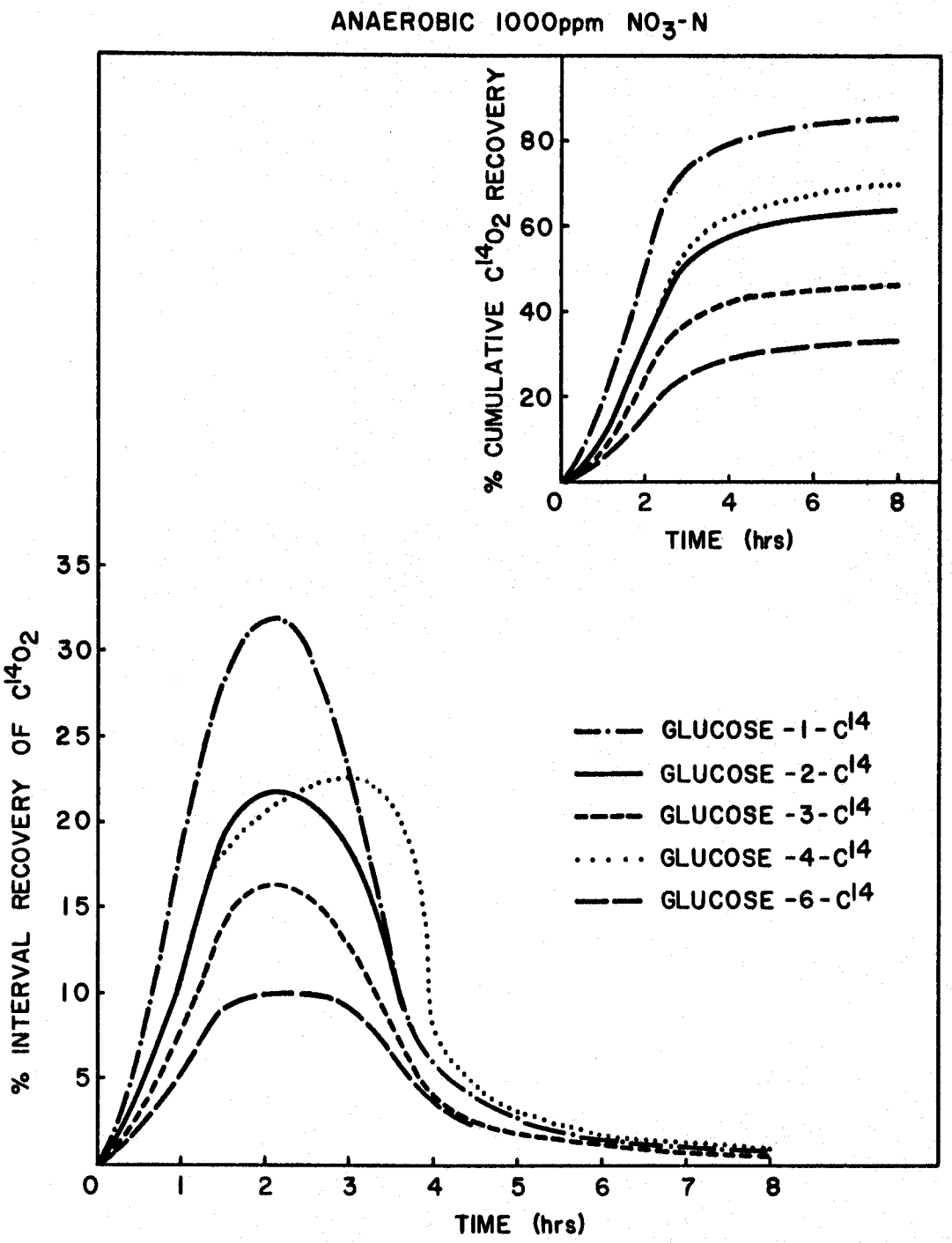


Figure 3. Radiorespirometric patterns for the utilization of glucose under conditions of nitrate respiration.

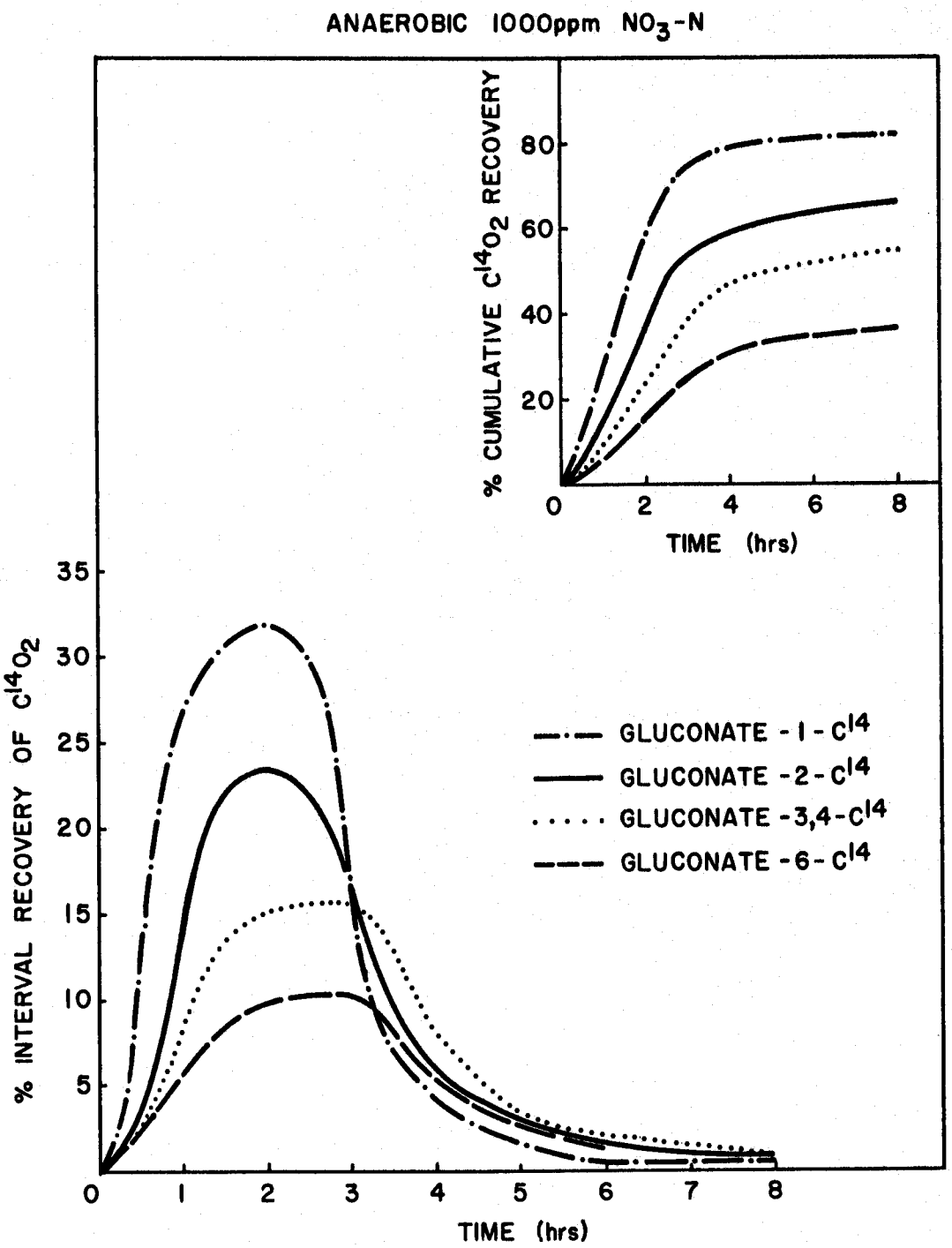


Figure 4. Radiorespirometric patterns for the utilization of gluconate under conditions of nitrate respiration.

Table 3. Incorporation of ^{14}C label into cells, medium,[1] and CO_2 by P. stutzeri cells during nitrate respiration

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
Anaerobic	%	%	%	%
Glucose-1-^{14}C	85	8	6	99
Glucose-2-^{14}C	64	27	8	99
Glucose-3-^{14}C	46	39	13	98
Glucose-3, 4-^{14}C	58	33	9	100
Glucose-4-^{14}C*	70	27	5	102
Glucose-6-^{14}C	34	57	11	102
Gluconate-1-^{14}C	83	1	17	101
Gluconate-2-^{14}C	66	28	9	103
Gluconate-3, 4-^{14}C	55	33	13	101
Gluconate-6-^{14}C	37	57	9	103

[1] Experiments performed in 100% helium atmosphere using NO_3^- as the terminal hydrogen acceptor (1000 ppm NO_3^-N).

* Calculated from glucose-3-^{14}C and glucose-3, 4-^{14}C recoveries.

re-cycling of the TCA cycle would be necessary for an equivalent amount of energy under conditions of nitrate respiration. As a result, more CO_2 should be evolved as a result of secondary pathway activity and less substrate carbon incorporated into cell constituents under conditions of nitrate respiration as compared to aerobic conditions. As previously indicated, the total $^{14}CO_2$ yields and percent cell incorporation (Tables 1 and 3) from the C-1 and C-4 positions of glucose are the same under these conditions.

When comparing total $^{14}CO_2$ evolved and percent cell incorporation derived from the C-2, C-3, and C-6 positions of glucose, it becomes possible to observe a difference in the fate of these carbon atoms. In the TCA cycle the C-2 and C-5 positions would be converted to CO_2 at the greatest rate with less consequent cell incorporation under conditions of nitrate respiration. The C-2 position of glucose does indicate this trend since 64% of the glucose-2-^{14}C was converted to $^{14}CO_2$ with 27% cell incorporation as compared to an aerobic $^{14}CO_2$ yield of 53% with 42% cell incorporation. The C-3 and C-6 positions of glucose would be attacked last and more cell incorporation would be observed under aerobic conditions (57 and 59% aerobically as compared to 39 and 57% under conditions of nitrate respiration). Thus, the general pattern shown by these data indicates that greater secondary or terminal pathway activity might occur under conditions of nitrate respiration than under aerobic conditions.

Secondary Metabolic Pathways in P. stutzeri

Although the respirometric studies on the primary pathways indicated that the TCA or glyoxalate cycles may operate in the terminal respiration of P. stutzeri, it was desired to carry out further experiments more specifically designed to demonstrate the presence of secondary pathways.

Utilization of TCA Cycle Intermediates

As previously discussed, these experiments were carried out using TCA intermediates as the sole carbon sources in synthetic media. The results, shown in Table 4, indicate that all carbon sources used could support the growth of P. stutzeri both under aerobic and nitrate respiration conditions. All substrates, except pyruvate and citrate, gave growth equivalent to that of glucose during the indicated incubation periods. On the first transfer, acetate, pyruvate, and citrate gave visible growth but exhibited a longer lag period than the other intermediates. With successive transfers, acetate and pyruvate yielded growth equivalent to glucose, but the lag with citrate was persistent. It is interesting to note that no growth was obtained when nitrate was excluded from the medium under anaerobic conditions. Even when large inocula were used and the flasks were incubated for as long as 14 days, no visible increase

in turbidity could be detected. This is indicative that nitrate is an obligatory hydrogen acceptor under anaerobic conditions.

Table 4. Growth of P. stutzeri with TCA cycle intermediates

Substrate	Aerobic Growth 24 hrs	Anaerobic Growth 48 hrs
Acetate	+	+
Pyruvate	+	+
Citrate	+	+
∝- ketoglutarate	+	+
Succinate	+	+
Fumarate	+	+
Malate	+	+
Glucose	+	+
Control (no substrate)	-	-
Glucose (no nitrate)	+	-

The results obtained are suggestive, although certainly not conclusive, that the TCA cycle might be functioning under both conditions of oxygen relationship.

Radiorespirometry of Specifically Labelled Acetate

If the glyoxalate cycle is operating in an organism, it can then synthesize C_4-dicarboxylic acids by a cyclic mechanism, the net result of which is essentially the combining of two C_2 units to form a C_4. The organism is thus achieving the same net results as would be attained if pyruvate condensed with CO_2 to form malate. Since

C_4-dicarboxylic acids required for continued TCA cycle operation are normally drained off the cycle for synthesis of amino acids and other cellular constituents, there is always a demand for these C_4-acids. Consequently, either the glyoxalate cycle or CO_2 fixation is a necessity if acetate or pyruvate, respectively, are the sole sources of carbon for cellular growth. Should there be no C_3 available, then the glyoxalate cycle is necessary for the production of C_4-acids from acetate. If the glyoxalate cycle alone were operating no decarboxylation would occur and no CO_2 would be evolved. However, CO_2 is usually evolved when the glyoxalate cycle is operating in intact organisms possessing this pathway. This is because the TCA cycle would also be necessary for terminal oxidation and ATP synthesis. Thus CO_2 is evolved which makes it possible to use acetate as a radiorespirometric substrate for the detection of the glyoxalate cycle operating concurrently with the TCA cycle. When the TCA cycle is operating via pyruvate, CO_2 is first detected from the C-1 position during the formation of acetyl CoA. The next position, decarboxylated in the second turn of the cycle, is the C-2 position followed by the C-3 position, during successive turns of the cycle. The overall $^{14}CO_2$ yield is thus:C - 1>2>3. When specifically labelled acetate is used as the substrate, the carboxyl (Ac-1) position becomes equivalent to the carbonyl group of pyruvate (Py-2) and the methyl group (Ac-2) corresponds to the methyl group C-3 of pyruvate (Py-3). The

overall pattern for the utilization of acetate should then be: C - 1>2.

The results of the radiorespirometric experiments with acetate are shown in Tables 5 and 6 for aerobic conditions and nitrate respiration, respectively. It is quite evident that P. stutzeri degrades acetate under both conditions of oxygen relationship. The radiorespirometric patterns shown in Figures 5 and 6 indicate quite clearly that acetate is being degraded in the manner expected if the glyoxalate and TCA cycles were operating under both aerobic and anaerobic conditions.

Table 5. Aerobic incorporation of ^{14}C label into cells, medium, and CO_2 by P. stutzeri cells

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
Aerobic	%	%	%	%
Glutamic acid-1-^{14}C	67	6	18	91
Glutamic acid-2-^{14}C	64	18	22	104
Glutamic acid-3, 4-^{14}C	28	39	26	93
Glutamic acid-5-^{14}C	62	16	23	101
Acetate-1-^{14}C	78	24	1	103
Acetate-2-^{14}C	47	46	4	97

Table 6. Incorporation of ^{14}C label into cells, medium, and CO_2 by P. stutzeri cells during nitrate respiration[1]

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
Anaerobic	%	%	%	%
Glutamic acid-1-^{14}C	70	3	19	92
Glutamic acid-2-^{14}C	73	10	22	105
Glutamic acid-3, 4-^{14}C	40	26	23	89
Glutamic acid-5-^{14}C	70	10	23	103
Acetate-1-^{14}C	88	12	1	101
Acetate-2-^{14}C	70	25	3	98

[1]Experiments performed in 100% helium atmosphere using NO_3^- as the terminal hydrogen acceptor (1000 ppm NO_3^--N).

Radiorespirometry of Specifically Labelled Pyruvate

These experiments were carried out in an attempt to further substantiate the operation of the TCA cycle using specifically labelled pyruvate. Difficulties were immediately encountered in that very little $^{14}CO_2$ was evolved from any label used. At a carrier level of up to 10 mg/flask under aerobic and anaerobic conditions, a maximum 2-3% of total added label was converted to $^{14}CO_2$ with only trace amounts of cell incorporation. These cells had been grown in glucose, therefore, there was a possibility that adaptation to pyruvate acid would be necessary. Cells were then grown in semi-synthetic medium containing pyruvate (5 g/l). Good growth and cell

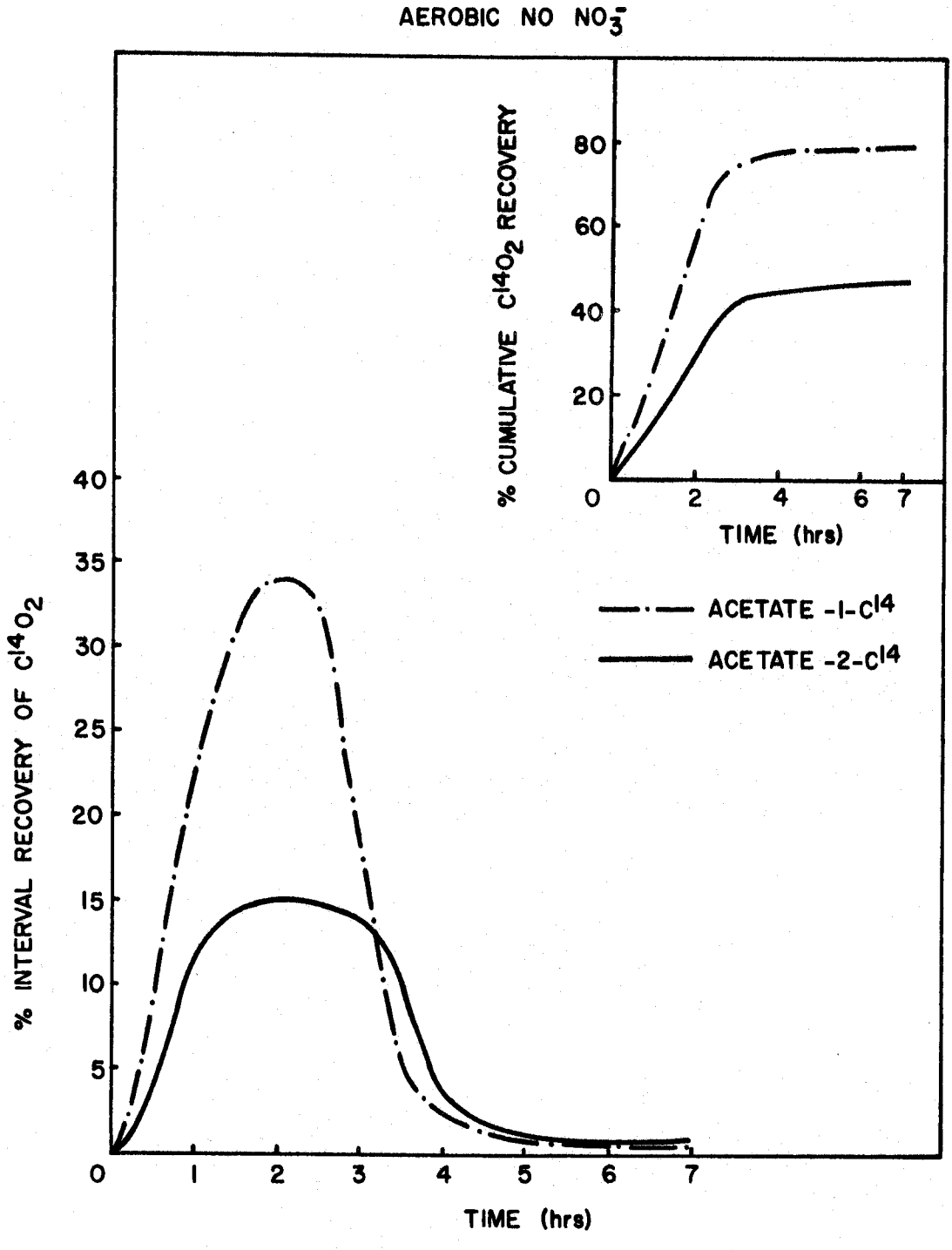


Figure 5. Aerobic radiorespirometric patterns for the utilization of acetate.

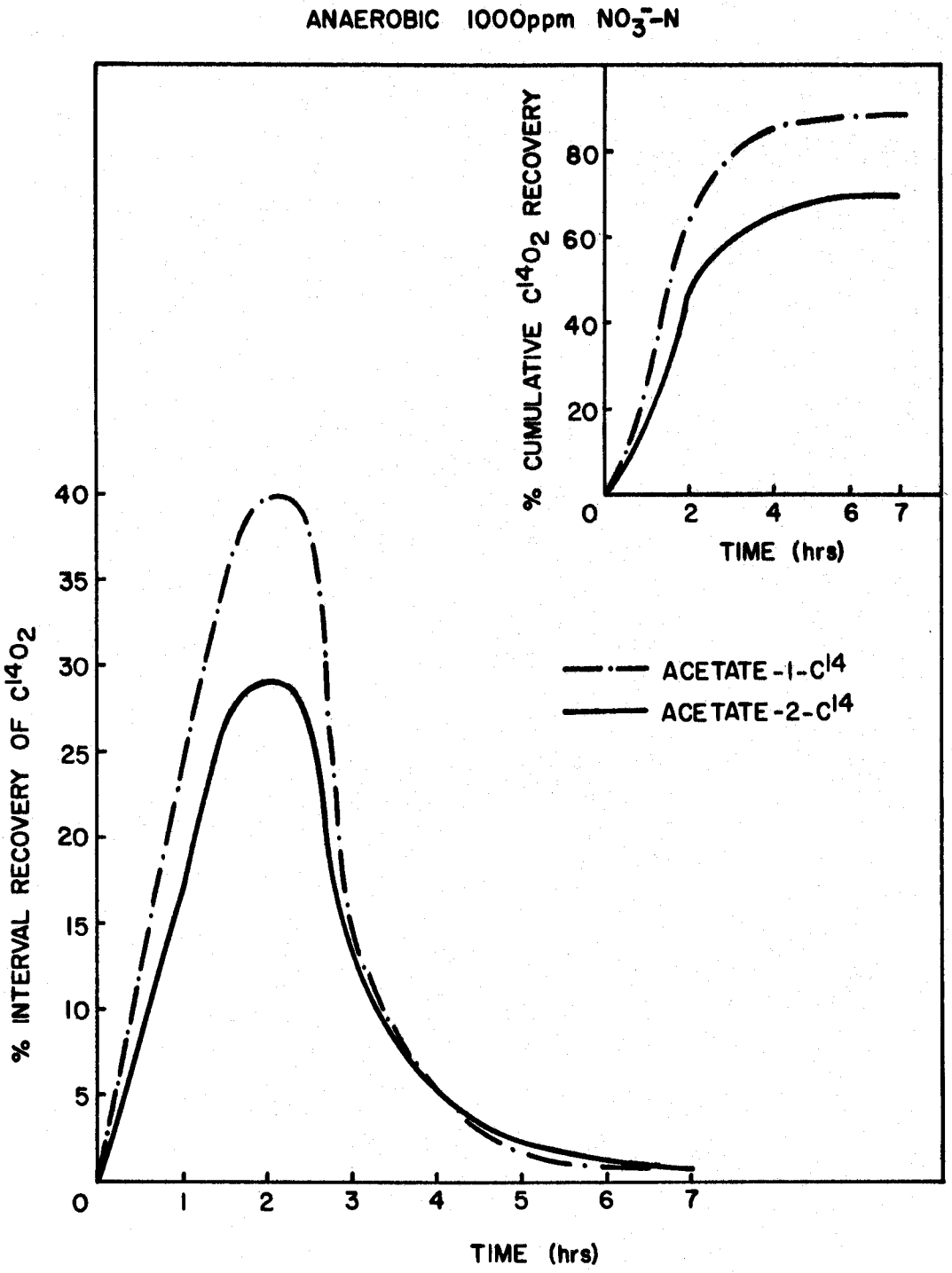


Figure 6. Radiorespirometric patterns for the utilization of acetate under conditions of nitrate respiration.

yields were obtained, but, again the cells failed to metabolize pyruvate during radiorespirometric experiments.

The probable conclusion is that a permeability barrier to pyruvate exists at the low substrate levels required for radiorespirometry. Since cells "adapted" to growth on pyruvate at high levels (5 g/l) would not metabolize pyruvate at low levels (up to 0.4 g/l) this would seem reasonable. However, experiments were not carried out to determine the critical pyruvate concentration necessary for metabolism of pyruvate since sufficient pyruvate concentration to penetrate the cells would be in excess of the amount which could be completely metabolized during a radiorespirometric experiment.

Radiorespirometry of Specifically Labelled Glutamic Acid

A novel approach to the study of the TCA cycle in intact cells has been suggested by Wang (53, p. 14). The approach involves the radiorespirometry of specifically labelled glutamic acid. The rationale is that with glutamic acid as the sole carbon source or, indeed,if glutamic acid is present along with low levels of other carbon sources, such as glucose, alpha-ketoglutaric acid will be formed via a transaminase or the reverse reaction of glutamic dehydrogenase. Since alpha-ketoglutarate is a TCA cycle intermediate,it should be sequentially degraded via the TCA cycle and the rate of oxidation of the carbon skeleton should be C-1>2 = 5> 3 = 4.

P. stutzeri is capable of growth using glutamic acid as the sole source of carbon; therefore, it was of interest to determine whether or not the indicated patterns would be obtained with specifically labelled glutamic acid. The results of these experiments are shown in Figures 7 and 8 and Tables 5 and 6 for aerobic conditions and nitrate respiration conditions, respectively. The respirometric patterns for $^{14}CO_2$ and percent cell incorporation obtained do indicate the TCA cycle is operative. However, the total $^{14}CO_2$ yield from glutamic-1-^{14}C is not as high as would be expected if the C-1 position were decarboxylated first. An indication that glutamic-1-^{14}C is decarboxylated first, however, is evident from the early $^{14}CO_2$ peak shown in the radiorespirometric curves. Another question which arises is; where does the acetyl CoA required for continued operation of the cycle originate? A possible explanation is that part of the glutamic acid is split in some manner to form pyruvate and/or acetate which could then act as the condensing partner for the continued operation of the TCA cycle. Another possibility is that other amino acids, present in the yeast extract, form the condensing partner, i. e., the transaminaton of alanine to form pyruvate. The latter reaction would be more probable from the standpoint of simplicity. Nevertheless, the radiorespirometric patterns and cell incorporation data for glutamic acid do indicate that a TCA cycle is functioning in P. stutzeri under both conditions of oxygen relationship.

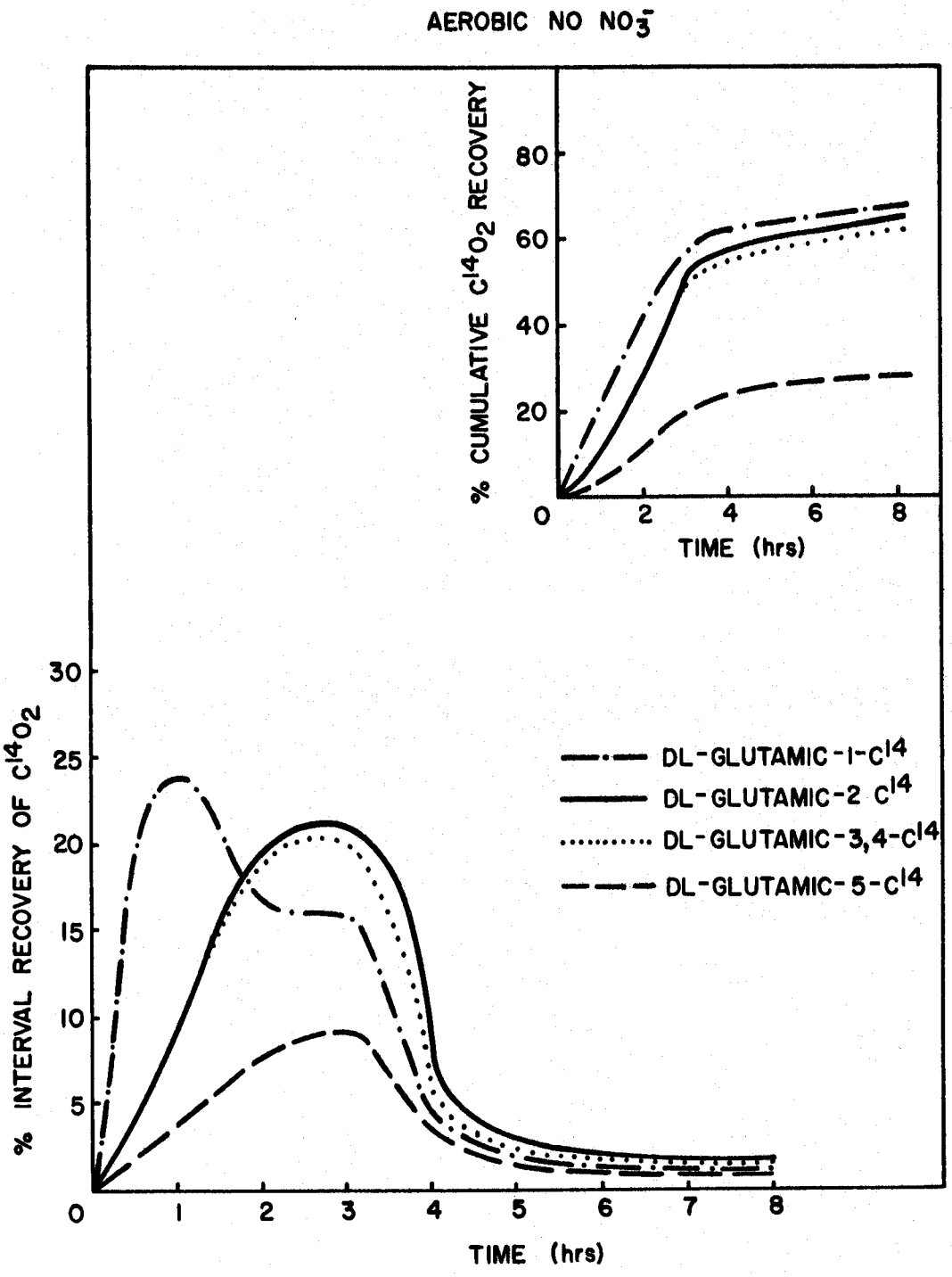


Figure 7. Aerobic radiorespirometric patterns for the utilization of glutamic acid.

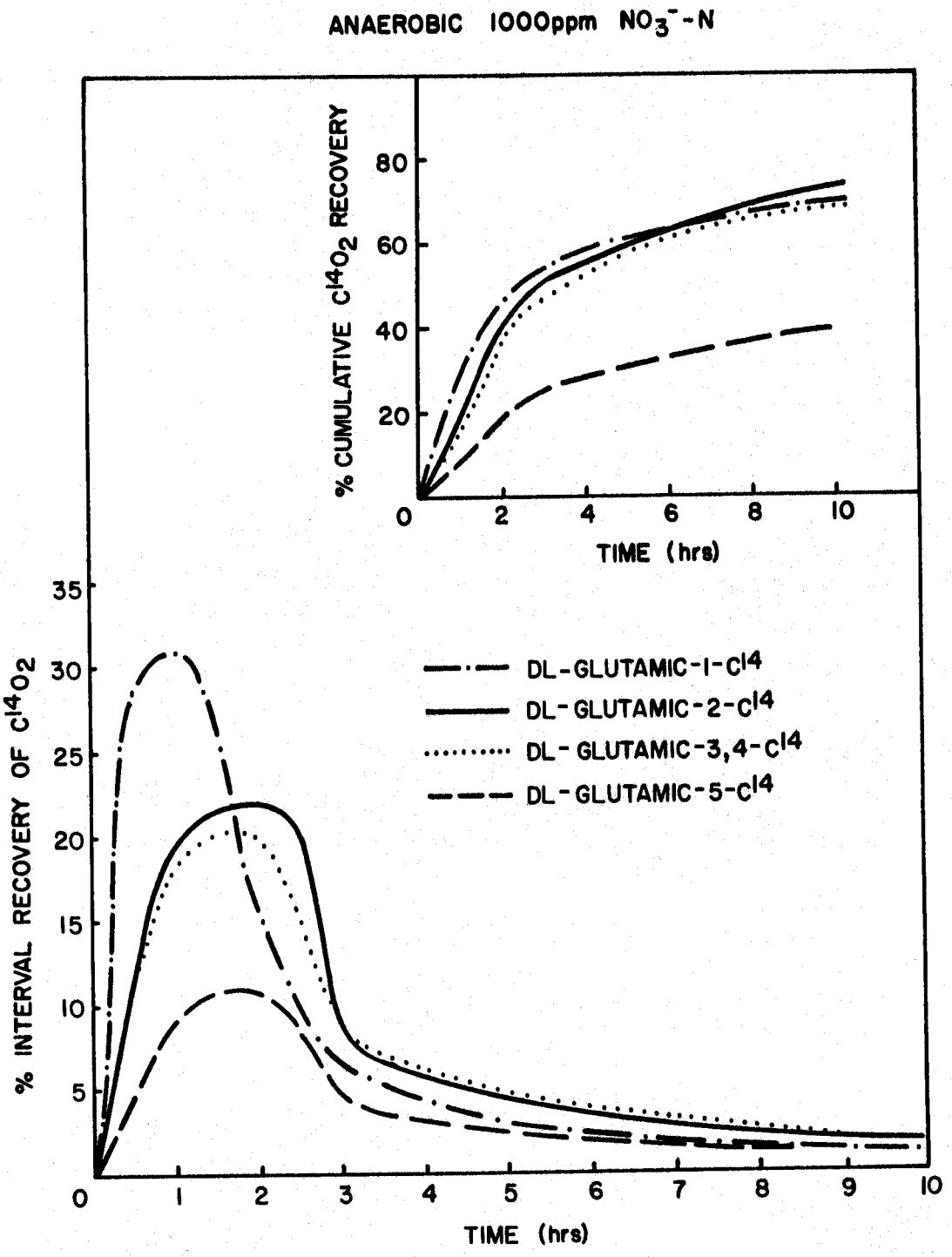


Figure 8. Radiorespirometric patterns for the utilization of glutamic acid under conditions of nitrate respiration.

An additional experiment was performed under anaerobic conditions without nitrate added. Glutamic acid-1-, -2-, 3-, 4- and -5-^{14}C was added at the same level as for the experiments with nitrate. The results shown in Table 7 indicate quite clearly that glutamic acid is not dissimilated in the absence of the terminal hydrogen acceptor (NO_3^-), under anaerobic conditions. Virtually all the added label was accounted for in the medium with only 1% $^{14}CO_2$ evolution in ten hours of incubation with no concomitant cell incorporation. If glutamic acid were dissimilated by some mechanism not involved in terminal respiration, one would still expect some cell incorporation, even if CO_2 was not evolved. Since this is not the case and the dissimilation of glutamic acid does readily proceed under anaerobic conditions with nitrate, there can be little doubt that a terminal oxidative mechanism such as the TCA cycle is functioning under anaerobic conditions with nitrate.

Table 7. Incorporation of ^{14}C label into cells, medium, and CO_2 by P. stutzeri under anaerobic conditions without nitrate

Substrate	Isotope recoveries			
	CO_2	Cells	Medium	Total
Anaerobic	%	%	%	%
Glutamic acid-1-^{14}C	1	-	103	104
Glutamic acid-2-^{14}C	1	-	95	96
Glutamic acid-3, 4-^{14}C	-	-	92	92
Glutamic acid-5-^{14}C	1	-	102	103

$^-$designates only trace amount

Determination of Glyoxalate Cycle Enzymes

The radiorespirometric experiments with acetate, previously discussed, indicate the involvement of the glyoxalate cycle in P. stutzeri under aerobic and anaerobic conditions. Demonstration of the enzymes isocitric lyase, and malate synthetase would further substantiate this assumption.

The protocol used in these assays was previously described in materials and methods. The assay for isocitric lyase was followed by measurement of glyoxalate produced during a ten minute reaction period. The results are shown in Table 8. Units are expressed as micromoles of glyoxalate, measured as the 2, 4-dinitrophenyhydrazone derivitive after ten minutes of reaction time and the final results are expressed as units/mg of protein. The results shown indicate approximately the same specific activity for isocitric lyase under both conditions of oxygen relationship. These results check well with enzyme activities found in other Pseudomonas sp.(46, p. 774-775; 28, p. 1430-1431).

Table 8. Results of isocitric lyase assay for aerobic and anaerobic cell-free extracts

	protein mg/ml	used ml	used mg	(units) μM	units/mg protein
Aerobic	14. 2	. 02	0. 28	0. 395	1. 4
Anaerobic(NO_3^-)	8. 2	. 04	0. 33	0. 390	1. 2

In addition the boiled control and substrate control gave no isocitric lyase activity. When cysteine was excluded the specific activity was reduced by one-third. Exclusion of Mg^{++} likewise reduced the specific activity (one-half).

Malate synthetase activity was measured as the decrease in optical density at 232 mμ when the acyl bound of acetyl CoA is broken in the presence of glyoxalate. The results shown in Table 9 indicate that the activity of malate synthetase is twice as high in aerobic preparations as in anaerobic preparations. Why this might be, when the isocitric lyase activity is the same, is not known.

Table 9. Results of malate synthetase assay for aerobic and anaerobic cell-free extracts

	protein mg/ml	used ml	used mg	Δ O. D. 232/min	Δ O. D./mg protein /min
Aerobic	9. 9	0. 05	0. 455	0. 065	0. 140
Anaerobic(NO_3^-)	5. 9	0. 10	0. 59	0. 04	0. 068

Carbon Dioxide Fixation

Carbon dioxide fixation was studied in an attempt to determine whether this reaction (or reactions) is important in the metabolism of P. stutzeri. The experiments were carried out in synthetic medium using acetate, glucose and pyruvate as the carbon sources under aerobic conditions and conditions of nitrate respiration. The

results, shown in Table 10, indicate that CO_2 fixation does occur to some degree and that almost twice as much CO_2 is fixed under aerobic conditions, as under anaerobic conditions, on the basis of dpm incorporated per milligram net increase in cell nitrogen. It is doubtful whether CO_2 fixation is obligatory, but this would be extremely difficult to prove. Even if all CO_2 could be removed, there would still be CO_2 formed intracellulary, in normal cellular metabolism, which would be readily available for fixation.

Table 10. Fixation of $^{14}CO_2$ by cells metabolizing acetate, glucose, and pyruvate

Aerobic	Total dpm fixed	Fixation %	N net mg	N %/mg	N dpm/mg
Acetate	1230000	2.20	1.10	2.00	615000
Glucose	2450000	4.40	2.60	1.70	1440000
Pyruvate	1180000	2.10	1.60	1.30	908000
Anaerobic					
Acetate	429000	.77	0.80	.96	447000
Glucose	598000	1.07	1.30	.82	729000
Pyruvate	202000	0.36	0.70	.50	404000

More pronounced CO_2 fixation under aerobic conditions is difficult to explain. One possible explanation would be that if more ATP is formed under aerobic conditions per substrate hydrogen pair

donated in terminal respiration, then more cyclic activity would occur in the TCA cycle under anaerobic conditions per ATP produced. Since a $C_3 + C_1$ condensation would yield a TCA intermediate, then this fixed CO_2 would be turned over at a greater rate to satisfy the cell's energy requirements. By contrast aerobic cells would require less cyclic activity per ATP molecule produced and thus more fixed CO_2 might find its way into cellular material. If this were true, just as much CO_2 could be "fixed" under both conditions. However, there would be more turnover under anaerobic conditions, the end result being more retention of the fixed CO_2 under aerobic conditions.

Quantitation of Radioactive Amino Acids Containing Fixed Carbon

Since CO_2 is definitely fixed by P. stutzeri it was considered of interest to attempt to separate the cellular amino acids and study the relative incorporation of label as an indication of their biosynthetic origin. It is well-known that alanine, glutamate, and aspartate can originate from pyruvate, oxalacetate and alpha-ketoglutarate of the TCA cycle by transamination. In addition, the oxalacetate (21, p. 173-235) is now recognized as the precursor of lysine, threonine, and isoleucine as well as aspartic acid. Furthermore, glutamic acid can give rise to proline and arginine. Serine, glycine, and cysteine, are interconvertable to glyoxalate and can conceivably arise via the glyoxalate cycle (35, p. 636-640). Histidine, phenylalanine and

tyrosine are generally considered to originate via the pentose phosphate pathway. The most common pathway of non-photosynthetic CO_2 fixation is via the malic enzyme reaction or phosphenol pyruvic carboxykinase. In either case, a C_4 dicarboxylic acid (malate and oxalacetate, respectively) is formed via a $C_3 + C_1$ condensation. Since these acids are TCA intermediates, one would expect alpha-ketoglutaric acid to become labelled as the TCA cycle "turns". This being the case, the following amino acids would be labelled to a significant degree: aspartic acid, glutamic acid, arginine, proline, lysine, threonine and isoleucine. If the glyoxalate cycle were active, then glycine, serine and cysteine might also be labelled significantly. Amino acids having pyruvate as the precursor would not be expected to be labelled to any degree, i. e. alanine and possibly valine and leucine. Likewise, amino acids such as histidine, phenylalanine and tyrosine, which are probably derived through the pentose phosphate pathway, would be expected to contain little or no label from $^{14}CO_2$.

This trend is indeed indicated in the results depicted in Tables 11 and 12 for aerobic and anaerobic conditions, respectively. Due to the extent of labelling in most of the amino acids detected, as well as striking but consistent differences in the various amino acids, this could hardly be considered an artifact or a result of isotope exchange reactions. The high degree of incorporation into those amino acids arising from the TCA cycle and the low degree of incorporation

Table 11. Percent recovery of labelled amino acids synthesized via CO_2 fixation under aerobic conditions

Amino acid*	Acetate %	Glucose %	Pryuvate %
Cysteic acid	2	3	3
Lysine	7	14	10
Arginine	36	30	27
Histidine	-	-	-
Aspartic acid	10	13	16
Glutamic acid	7	9	12
Serine	7	3	4
Glycine	10	3	6
Threonine	4	13	8
Alanine	4	-	-
Proline	2	5	5
Tyrosine	3	-	1
Valine	2	-	-
Phenylalanine	3	-	2
Leucine	-	-	-
Isoleucine	-	4	4
Methionine	-	-	-
Total	97	97	97

*Recoveries are expressed as percent recovery of labelled amino acids relative to total labelled amino acids recovered.

Table 12. Percent recovery of labelled amino acids synthesized via CO_2 fixation during nitrate respiration

Amino acid*	Acetate %	Glucose %	Pyruvate %
Cysteic acid	3	3	4
Lysine	10	9	11
Arginine	29	35	26
Histidine	-	-	-
Aspartic acid	11	14	17
Glutamic acid	8	10	14
Serine	9	7	6
Glycine	13	8	11
Threonine	3	6	3
Alanine	6	1	1
Proline	3	4	4
Tyrosine	3	-	-
Valine	-	-	-
Phenylalanine	-	-	-
Leucine	-	-	-
Isoleucine	-	-	-
Methionine	-	-	-
Total	98	97	97

*Recoveries are expressed as percent recovery of labelled amino acids relative to total labelled amino acids recovered.

into those amino acids, not considered to result from TCA cycle activity, is highly indicative of operation of this cycle in P. stutzeri. Indeed, the mere fact that the amino acids are synthesized in a synthetic medium without any added amino acids is reminiscent of TCA cycle activity.

It is interesting to note that CO_2 fixation occurred with acetate as the sole carbon source. This is difficult to explain on the basis of known CO_2 fixation reactions. One possibility would be the carboxydismutase reaction whereby ribulose-1, 5-diphosphate reacts with CO_2 to form two molecules of 3-phosphoglyceric acid. This is unlikely since the carboxydismutase reaction is characteristic of autotrophic metabolism. Moreover, 3-PGA would enter the PP pathway and amino acids such as phenylalanine, tyrosine and histidine would presumably be heavily labelled. That this is not the case is shown by the amino acid data in Tables 11 and 12. Phenylalanine and tyrosine show a slight increase with acetate as substrate, but not a significant increase, while the other amino show the same general pattern as with glucose and pyruvate. There is a more significant increase in alanine activity with acetate as compared to glucose and pyruvate. Although not highly significant, this would warrant consideration of a possible $C_2 + C_1$ condensation. A $C_2 + C_1$ condensation however, has never received formal acceptance although it has been reported by Wood (56, p. 41-117) and others. Further study would be of value in clarifying this point.

CONCLUSIONS

The metabolic pathways operative in P. stutzeri which may be associated with aerobic and nitrate respiration, have been studied in some detail. When grown under anaerobic conditions nitrate-oxygen is used as the terminal hydrogen acceptor in a manner apparently analogous to classical aerobic respiration. Therefore, pathways were determined for both conditions of oxygen relationship in order to determine if there is any apparent change in metabolic pathways under conditions of nitrate respiration.

The initial experiments using the radiorespirometric method indicated that the same primary metabolic pathways were operative under aerobic and nitrate respiration conditions, i. e. the Entner-Doudoroff and pentose phosphate pathways. Furthermore, the ratio of decarboxylation of the C-1 and C-4 positions of glucose and gluconate, indicated that the rate of operation of these pathways, relative to growth may be the same. The same studies indicated a possible TCA cycle and glyoxalate cycle which were studied further.

Initial experiments using TCA cycle intermediates as the sole source of carbon indicated that all intermediates used could serve as the sole carbon source for growth of P. stutzeri under aerobic conditions and during nitrate respiration.

Further studies on the secondary pathways were carried out

by the radiorespirometric method. Radiorespirometric studies with acetate-1- and -2-^{14}C indicated that the glyoxalate and TCA cycles were functioning concurrently as indicated by the evolution of $^{14}CO_2$ from positions 1 and 2 of acetate as well as cell incorporation of the respective labels. Cell-free preparations of aerobic and anaerobic cells grown on acetate showed activity for isocitric lyase and malate synthetase.

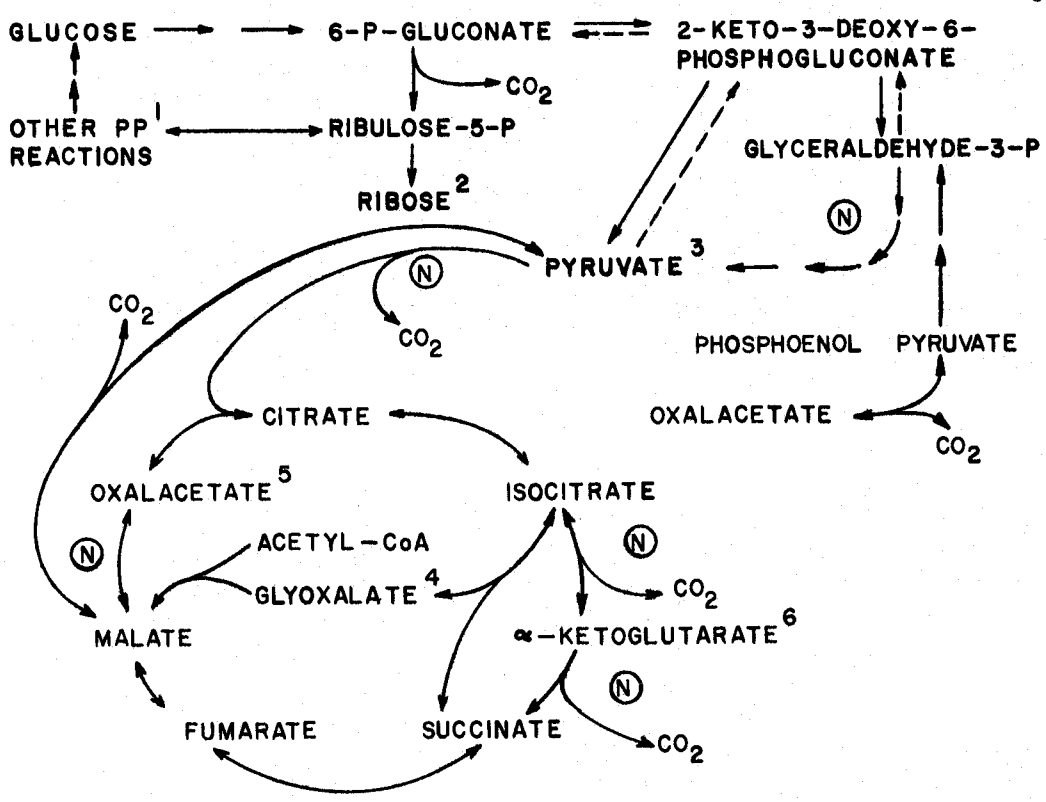
An as additional indication of TCA cycle activity, specifically labelled glutamic acid was used. Cell incorporation yields and $^{14}CO_2$ evolution patterns from glutamic acid -1-, -2-, 3, 4- and -5-^{14}C further substantiated the operation of the TCA cycle during aerobic and anaerobic metabolism. The exclusion of nitrate from flasks used in nitrate respiration experiments resulted in the complete cessation of TCA cycle activity under anaerobic conditions, as indicated by no evolved $^{14}CO_2$ or cellular incorporation of label. This is further supported by the same type of experiment with glucose and by the fact that P. stutzeri will not grow under anaerobic conditions without nitrate.

Carbon dioxide fixation was found to be a capability of this organism but an obligatory role, for this reaction,was not established. More CO_2 was fixed under aerobic conditions which is discussed in relation to the possibility that there is more turnover of fixed CO_2 during nitrate respiration due to differences in efficiency of energy

production. Amino acids were separated from cell hydrolysates and studied in respect to the extent of incorporation of $^{14}CO_2$ into the individual acids. The percent recoveries of label in each amino acid indicated that aspartic acid, glutamic acid, arginine, proline, lysine, threonine and isoleucine are synthesized from TCA cycle intermediates and consequently reflect TCA cycle activity. Data for labelled glycine, serine and cysteic acid indicate implication of the glyoxalate cycle in the biosynthesis of these amino acids by P. stutzeri.

A scheme, shown on page 69 (Figure 9), has been prepared in an attempt to summarize the pathways thought to function in P. stutzeri under both conditions of oxygen relationship. Possible precursors of various amino acids are indicated, but it is to be recognized that these sites are for clarification and have not been proven by data presented in this thesis. Likewise, sites are indicated where substrate hydrogen might theoretically be passed from reduced NAD to nitrate by the postulated scheme of Fewson and Nicholas (14, p. 335-349).

Finally, it is concluded that the following pathways are functioning in P. stutzeri both under aerobic conditions and during nitrate respiration: ED pathway, PP pathway, TCA cycle and glyoxalate cycle. There is no apparent change in pathways when nitrate respiration is used in lieu of free-molecular oxygen. That there is a



POSSIBLE BIOSYNTHETIC ORIGIN OF AMINO ACIDS:

1. PHENYLALANINE, TYROSINE, TRYPTOPHAN
2. HISTIDINE
3. VALINE, ALANINE, LEUCINE
4. GLYCINE, SERINE, CYSTEINE
5. ASPARTIC ACID, LYSINE, THREONINE, METHIONINE ISOLEUCINE
6. GLUTAMIC ACID, ARGININE, PROLINE

Ⓝ POSSIBLE SITES OF NAD REDUCTION AND INVOLVEMENT OF NITRATE RESPIRATION

FIGURE 9. INDICATED MAJOR METABOLIC ROUTES FOR P. STUTZERI

difference in efficiency with these pathways is quite evident in the slower growth of P. stutzeri during nitrate respiration. The reason for this is still a matter of conjecture which deserves further clarification.

SUMMARY

The gross metabolism of Pseudomonas stutzeri has been studied during aerobic and nitrate respiration. Significant results may be summarized as follows:

1. Radiorespirometric studies indicate that glucose is dissimilated via the ED and PP pathways when oxygen or nitrate-oxygen is the terminal hydrogen acceptor.

2. The primary pathways will not operate to any extent without nitrate under anaerobic conditions.

3. Intermediates of the TCA cycle serve as sole carbon sources for P. stutzeri grown in synthetic media.

4. Radiorespirometric studies with acetate, as well as cell-free enzyme studies, indicate that the glyoxalate cycle is operative under aerobic and anaerobic conditions.

5. Glutamic acid metabolism further implicates TCA cycle activity under both conditions of oxygen relationship.

6. Glutamic acid is not metabolized under anaerobic conditions in the absence of NO_3^- which substantiates the role of NO_3^- as a hydrogen acceptor in terminal respiration.

7. Carbon dioxide fixation studies implicates a biosynthetic role for the TCA cycle in amino acid synthesis and conversely, substantiates TCA cycle activity during aerobic and nitrate

respiration.

8. Carbon dioxide fixation by a $C_2 + C_1$ condensation is briefly discussed and warrants further study.

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